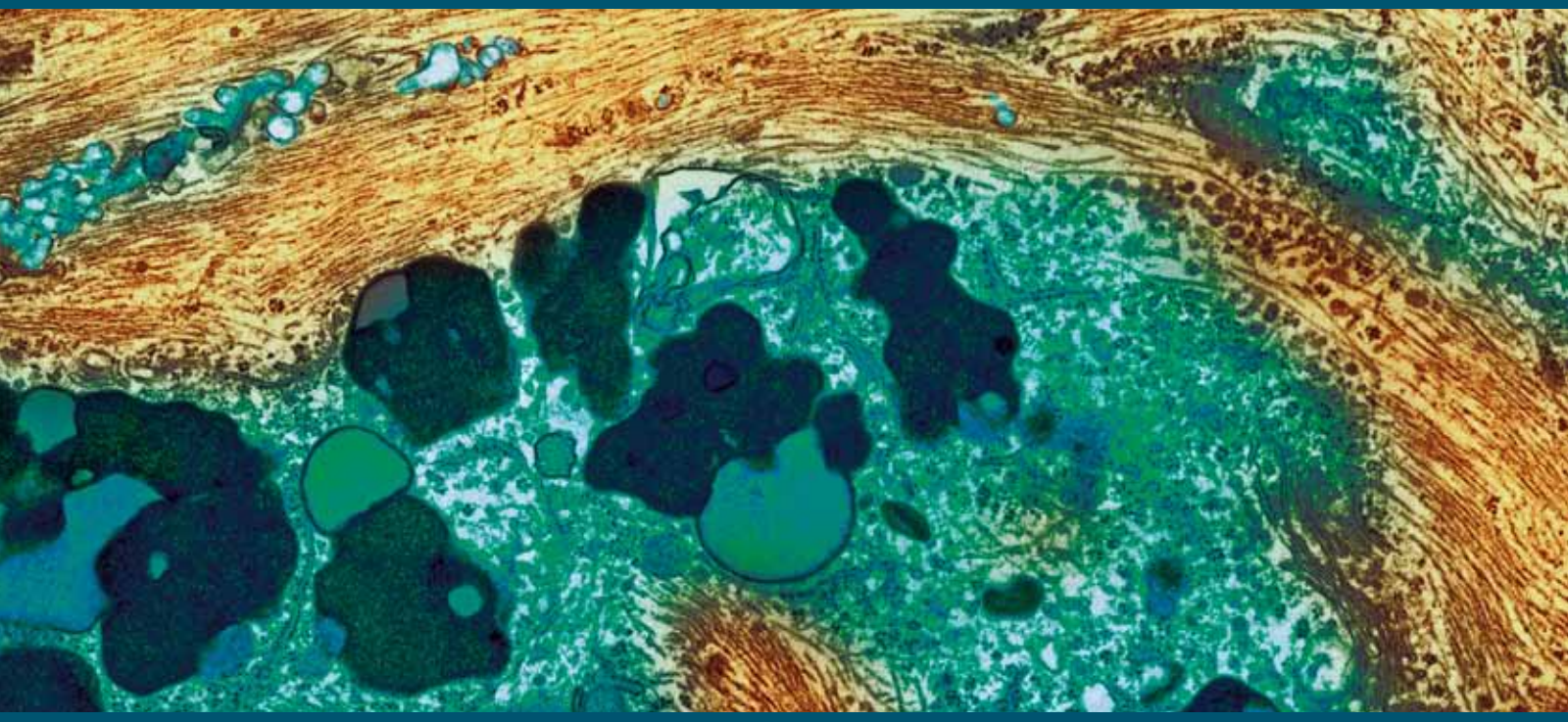
International Journal of Alzheimer's Disease

Genetics and Genomics of Late-Onset Alzheimer's Disease and Its Endophenotypes

Guest Editors: Christiane Reitz, Ekaterina Rogaeva, Tatiana Foroud, and Lindsay A. Farrer



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Editorial Board

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 284728, 2 pages
doi:10.4061/2011/284728

Editorial

Genetics and Genomics of Late-Onset Alzheimer's Disease and Its Endophenotypes

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Received 23 February 2011; Accepted 24 February 2011

Late-onset Alzheimer's disease is the most common cause of dementia in western societies. Despite remarkable achievements in human genetics over the years, in particular, techno-logical advances in gene mapping and in statistical methods, to date only a small proportion of the genetic contribution to Alzheimer's disease can be explained. One reason for the difficulty in gene identification is that Alzheimer's disease is a complex disorder in which multiple genes with small individual effects as well as environmental factors are likely to contribute to disease risk as well as the various quantitative traits associated with the disease such as age-of-onset, cognition, neuropsychiatric symptoms, amyloid/tau pathology, or structural brain changes. Identifying the genetic factors modulating changes in these individual endophenotypes would help elucidate disease pathogenesis. In this special issue on genetics and genomics of Alzheimer's disease and its endophenotypes, we have invited a few papers that address this issue.

The first two articles address the impact of APOE genotype, the best established Alzheimer's disease susceptibility gene, on neuropsychiatric endophenotypes associated with Alzheimer's disease including psychosis, late-life depression, anxiety, apathy, hallucinations, agitation, and aggressiveness. The third paper examines the effect of an intron 7 polymorphism in the amyloid precursor protein (APP) on the age of onset of Alzheimer's disease in persons with Down syndrome. The fourth and fifth papers explore mechanisms modulating components of the amyloid cascade pathway, focusing, in particular, on the APP intracellular domain (AICD), which is an end product of the proteolytic cleavage of APP by β- and γ-secretase. In the first of these two papers, Ansaloni et al. evaluated the effect of *NTRK2* which encodes the TrkB receptor on APP metabolism and AICD levels. TrkB is a member of the tyrosine kinase receptor family and binds specifically to brain-derived neurotrophic factor (BDNF). The paper reports on the effects of different TrkB isoforms on APP metabolism in the human neuroblastoma cell line. In the other paper, Raychaudhuri et al. examined the impact of overexpression of AICD on expression of proteins that are part of pathways involved in neurodegeneration and neuroregeneration. Finally, the sixth paper reviews the findings of genome-wide association studies (GWAS)

that tested whether SNPs in the top-ranked Alzheimer's disease-related candidate genes were associated with various brain imaging endophenotypes including the volumes of the temporal lobe, hippocampus, amygdale, and frontal lobe, as well as grey matter density, entorhinal cortex thickness, and white matter integrity. In addition, this article discusses multigene and more complex genetic models as a means to identify genetic contributions to Alzheimer's disease.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 721457, 14 pages
doi:10.4061/2011/721457

Review Article

Neuropsychiatric Symptoms, Endophenotypes, and Syndromes in Late-Onset Alzheimer's Disease: Focus on APOE Gene

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Received 11 October 2010; Revised 11 February 2011; Accepted 11 February 2011

Academic Editor: Christiane Reitz

Neuropsychiatric symptoms, previously denominated as behavioural and psychological symptoms of dementia, are common features of Alzheimer's disease (AD) and are one of the major risk factors for institutionalization. At present, the role of the apolipoprotein E (APOE) gene in the development of neuropsychiatric symptoms in AD patients is unclear. In this paper, we summarized the findings of the studies of neuropsychiatric symptoms and neuropsychiatric syndromes/endophenotypes in AD in relation to APOE genotypes, with special attention to the possible underlying mechanisms. While some studies failed to find a significant association between APOE and neuropsychiatric symptoms in late-onset AD, other studies reported a significant association between the APOE $\varepsilon 4$ allele and an increase in agitation/aggression, hallucinations, delusions, and late-life depression or anxiety. Furthermore, some negative studies that focused on the distribution of APOE genotypes between AD patients with or without neuropsychiatric symptoms further emphasized the importance of subgrouping neuropsychiatric symptoms in distinct neuropsychiatric syndromes. Explanations for the variable findings in the existing studies included differences in patient populations, differences in the assessment of neuropsychiatric symptomatology, and possible lack of statistical power to detect associations in the negative studies.

1. Introduction

Dementia and age-related cognitive disorders are reaching epidemic proportions, given the significant increase in the aging population. Alzheimer's disease (AD), the most prevalent form of dementia [1], is a complex brain disorder that has effects on multiple cerebral systems, and characterized by relatively slow but progressive neurodegeneration and impairment in cognition, behavior, and functionality. Accurate AD epidemiological data have been recently released for the USA. The 2010 figures suggested that 5.3 million Americans have AD [2], with >26 million patients with AD worldwide, and an expected increase to more than 106 million by 2050 [3]. Neuropsychiatric symptoms, previously denominated as behavioral and psychological symptoms of dementia, are common features of AD [4, 5] and are one of the major risk factors for institutionalization [6] as well as for increasing costs both in USA and Western countries [7, 8]. Neuropsychiatric symptoms in AD include psychosis (delusions and hallucinations) as well as affective and behavioral changes such as depressive mood, anxiety, irritability/lability, apathy, euphoria, disinhibition, agitation/aggression, aberrant motor activities, sleep disturbance, and eating disorder [9]. Neuropsychiatric symptoms associated with AD tend to follow a trajectory of increasing severity over time, a feature they have in common with cognitive and functional decline. Neuropsychiatric symptoms may be associated to AD irrespective of cognitive impairment severity and may

be the presenting complaint or may emerge in the course of the disease being important cause of a more rapid cognitive decline [9]. It has been estimated that up to 80% of patients with AD showed neuropsychiatric symptoms in the history of the disease [9, 10]. Recently, the Behavioural Subgroup of the European Alzheimer's Disease Consortium has performed a factor analysis of the neuropsychiatric inventory (NPI) [11] in a homogeneous sample of patients with AD, analyzing the largest AD population ever studied for this purpose [5]. The Behavioural Subgroup of the European Alzheimer's Disease Consortium identified 4 separate neuropsychiatric syndromes: hyperactive, psychotic, affective, and apathetic [5], providing also evidence of the relative consistency of neuropsychiatric syndromes across dementia subtypes, age and gender [12], and stressing the importance of thinking about neuropsychiatric syndromes instead of separate symptoms in AD patients.

The majority of AD cases are sporadic (i.e., without an apparent familial patterns of inheritance) compared with fewer than 5% of cases that are caused by autosomal dominant inheritance of mutations in presenilin 1, presenilin 2, or amyloid precursor protein [13]. Several genes have been identified as possible risk factors for the development of sporadic late-onset AD, particularly the gene dose of apolipoprotein E (APOE) $\varepsilon 4$ alleles [13]. In fact, the APOE gene is the only globally valid genetic determinant of sporadic AD to have been unambiguously identified in 15 years of intensive research [14]. However, as with other multifactorial diseases, this systematic inability to detect new genetic determinants has prompted more comprehensive investigations using genome-wide association studies. Three large genome-wide association studies in this field were performed and reported that the clusterin (CLU), phosphatidylinositol binding clathrin assembly protein (PICALM), complement component (3b/4b) receptor 1 (CR1), bridging integrator 1 (BIN1), and exocyst complex component 3-like 2 (EXOC3L2) loci were associated with AD [15–17], in addition to the established APOE relationship. The genetic origin of the three common variants of the human APOE, known as E2, E3, and E4, was understood in 1981 [18], and since the mid of the 80s these are probably the most studied protein variants in human races. A further study in 1982 proposed a nomenclature for these protein isoforms, to be identified as E2, E3, and E4 [19]. These important findings have led several researchers to the identification of the APOE encoding gene, that was recognized in 1982 [20], localized on chromosome 19 at locus q13.31 [21], and definitively sequenced [22]. Three years later, also the DNA encoding these isoforms was definitively sequenced [23] and named as $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$. The important implications of this study are the demonstration that these common gene variants were generated by the two single-nucleotide polymorphisms rs429358 and rs7412 in exon 4 of the APOE gene (AF261279) and that these three allelic forms of the APOE gene are indeed different haplotypes of the APOE gene, generated by the combination of the allele of these two single-nucleotide polymorphisms at the APOE locus [13].

Therefore, risk for late-onset AD is known to be associated with polymorphisms of the APOE gene; people with an $\varepsilon 4$ allele have an increased risk of both familial and sporadic forms, accounting for 20–50% of the attributable risk [13]. Nonetheless, APOE is neither necessary nor sufficient to cause AD, and this is the main reason why APOE is classified as a risk factor for AD and not a causative one. Although this is currently a nonmodifiable risk factor, it has potential for modifying the impact of other factors, in particular vascular and lifestyle-related factors [24], implying that some interventions may perhaps best be restricted to people at genetic risk. At present, the role of the APOE polymorphism in the development of neuropsychiatric symptoms [25–27] or neuropsychiatric syndromes/endophenotypes in AD patients is unclear [28–30]. It has been suggested that neuropsychiatric symptoms in AD may be influenced by the APOE polymorphism in various case-control studies [25, 26, 28, 29], but a recent longitudinal report showed a substantial lack of association between the APOE $\varepsilon 4$ allele and neuropsychiatric symptoms in AD after correction for multiple testing [27].

In this paper, we summarized the findings of the studies of neuropsychiatric symptoms and neuropsychiatric syndromes/endophenotypes in AD in relation to APOE genotypes from the English literature published before September 2010. We reviewed clinical and epidemiological studies from the international literature, including both cross-sectional and longitudinal studies that involved subjects aged 65 years and older. We searched through the PubMed database of NCBI (available at http://www.ncbi.nlm.nih.gov/) by author and the following keywords: neuropsychiatric symptoms, neuropsychiatric syndromes, neuropsychiatric endophenotypes, apolipoprotein E, APOE, dementia, Alzheimer's disease, late-life depression, late-life anxiety, affective syndromes, apathy, apathetic syndrome in dementia, agitation, aggressiveness, hyperactive syndrome in dementia, psychosis, psychotic syndrome in dementia, and behavioral and psychological symptoms of dementia. Special attention was paid to the possible mechanisms linked to the development of neuropsychiatric symptoms and neuropsychiatric syndromes/endophenotypes in AD in relation to APOE genotypes.

2. APOE Genotypes and Affective Syndromes/Endophenotypes in AD: Late-Life Depression and Anxiety

In adults older than 65 years, late-life depression refers to depressive syndromes encompassing both late-onset cases as well as early-onset cases that recur or continue into later years of life [31, 32]. Late-life depressive syndromes often arise in the context of medical and neurological disorders [33]. The prevalence of major depressive disorder is about 17% in patients with AD [34] and is even higher in those with subcortical dementias [31, 32]. Furthermore, emerging research implicates also a consistent reciprocal relationship between late life anxiety and cognition [35, 36]. In fact, anxiety disorders are the most common psychiatric diagnoses in

late life with an estimated lifetime prevalence of 15.3% in older adults, surpassing population estimates for depressive disorders [37], and severe cognitive impairment [38]. There is evidence of more prevalent anxiety in cognitively impaired older adults, elevated anxiety related to poorer cognitive performance, and more severe anxiety symptoms predicting future cognitive decline [35].

Early studies examining the relationship between APOE genotypes and depression showed that the APOE $\varepsilon 4$ allele may be a risk factor for depressive symptoms in patients with AD and dementia [39–41], and other investigators have demonstrated combined risks of developing AD among those with late-life depression and APOE $\varepsilon 4$ allele in nondemented geriatric populations [42–45] (Table 1). These findings were confirmed in two very recent longitudinal population-based studies [46, 47]. A recent study suggested that depression and APOE $\varepsilon 4$ genotype may be higher in women with AD but not in men [48]. Consistent with these previous studies demonstrating a relationship between APOE genotype and depression in AD [29–41, 48], a recent report found that depressed AD patients had a significantly higher frequency of APOE $\varepsilon 4$ allele than nondepressed AD patients [49]. Also in this study the association between APOE genotype and depression in AD was primarily seen in women and not men, so conforming the results of a study showing an increased rate of the APOE $\varepsilon 4$ genotype in late-onset depressed women with AD relative to men [43]. Finally, in a very recent study, when AD patients were subdivided according to the European Alzheimer's Disease Consortium classification of neuropsychiatric syndromes, in APO $\varepsilon 4$-carriers there was an increased risk of affective syndrome (i.e., AD patients with the NPI symptoms depression and anxiety) [30]. These results on increased risk of an affective syndrome in AD patients APOE $\varepsilon 4$-carriers confirmed recent findings indicating that neuropsychiatric symptoms in AD are not purely an epiphenomenon of cognitive impairment but could be attributed to specific biological brain dysfunction, suggesting the presence of specific AD endophenotypes [28, 29, 50–52].

Despite these findings supporting an association between APOE and depression, several other published studies have not supported the notion that APOE $\varepsilon 4$ genotype influences depression in AD [25, 58, 66–69, 71], also when specific AD endophenotypes were considered [28, 29] (Table 1). Other reports suggested that in AD the APOE $\varepsilon 4$ allele may be protective against depression [80] or that APOE $\varepsilon 2$-carriers may be at higher risk of depressive symptoms [53–55]. In addition, others have shown trends toward association between APOE genotype and depression but have rendered conclusions of no relationship [60, 77]. One possible explanation for these contrasting findings may be the different sample size, with several studies including few depressed AD patients [60, 67, 69], thus resulting in a different power to detect group differences. Also varying schemes for diagnosing depression in AD may be a source of variability [25, 68, 71]. Another factor may be that the use of limited screening measures for determining depression could be leading to poorer diagnostic accuracy and thus different prevalence rates of depression in AD (range 11–50%) [25, 49, 60]. Thus, the bulk of published studies reporting a lack of association between APOE $\varepsilon 4$ allele and depression in AD have suffered from small sample sizes, low reported prevalence rates for depression, very brief inventories of depressive symptoms, and/or low diagnostic rigor in the determination of the presence or absence of a Diagnostic and Statistical Manual of Mental Disorders-based diagnosis of depression.

Anxiety is most common among older subjects with mild cognitive impairment [81] and AD patients with a younger age at onset (under age 65) [82]. APOE e4 allele is a risk factor for developing AD at an earlier age [83] and might contribute to this effect [84]. Interestingly, APOE-null mice carrying the human $\varepsilon 4$ transgene had higher anxiety ratings than those expressing either wild-type murine APOE or the human $\varepsilon 3$ [73]. Very recently, these findings were also confirmed in female APOE TR mice expressing APOE under control of the mouse APOE promoter. APOE4 mice showed decreased activity levels and higher measures of anxiety than mice expressing APOE2 or APOE3 across all ages [85]. Notwithstanding the higher prevalence and symptom expression of anxiety disorders in late life, not all measures of neuropsychiatric symptoms in dementia reported in the literature include an assessment of anxiety [82]. Two studies using longitudinal assessments with the NPI found no association with anxiety [25, 65], and no association was found when assessed by the Behavioural Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD) in a cross-sectional study design [69] (Table 1). Consistent with the mouse studies [73, 85], APOE also has isoform-dependent effects on measures of anxiety in probable AD patients [73]. In particular, male $\varepsilon 4/\varepsilon 4$ subjects had higher anxiety scores than gender-matched $\varepsilon 3/\varepsilon 3$ subjects. In males, but not in females, subjects with $\varepsilon 4/\varepsilon 4$ had also higher anxiety scores than those with $\varepsilon 3/\varepsilon 4$ [73], suggesting that APOE3 can antagonize the effects of APOE4 on measures of anxiety in males but not in females. The anxiety scores did not correlate with the Mini-Mental State Examination scores [73]. Pritchard and colleagues did not support these findings using the same methods of analysis [27]. However, they found an association with the APOE $\varepsilon 3/\varepsilon 4$ genotype, but not with the APOE $\varepsilon 4/\varepsilon 4$ genotype or the e4 allele [27], so confirming a previous study in which anxiety appeared more frequently in APOE $\varepsilon 3/\varepsilon 4$ demented patients, although this difference was not statistically significant [56]. Finally, when AD patients were subdivided according to the European Alzheimer's Disease Consortium classification of neuropsychiatric syndromes, for the affective syndrome (i.e., AD patients with the NPI symptoms depression and anxiety), these previous findings in AD and demented patients [27, 73, 74] were confirmed suggesting that the presence of an APOE $\varepsilon 4$ allele may be a risk factor also for anxiety symptoms [56].

3. APOE and Apathy in AD

Apathy has been increasingly recognized as a distinct psychiatric syndrome, and defined as a lack of motivation, evidenced by diminished goal-directed overt behavior, diminished goal-directed cognition, and diminished emotional

Table 1: Principal studies on the association of neuropsychiatric symptoms, endophenotypes, and syndromes with the apolipoprotein E (APOE) polymorphism in Alzheimer's disease (AD) patients.

Reference	Study sample	Cognitive and neuropsychiatric assessment	Principal results
Ramachandran et al. [39]	46 AD patients; 135 controls, restricted to APOE ε3/3 and ε3/4 only	CDR, HAM-D, and SCID-DSM-III-R	Depression rating greater with APOE ε3/4 versus ε3/3 reference genotype. Psychosis greater with ε3/4 versus ε3/3 reference genotype
Holmes et al. [53–55]	164 AD patients 232 AD patients 210 AD patients	BDRS CAMDEX and MOUSEPAD	APOE ε2 allele significantly associated with depressive symptoms. APOE ε2 allele significantly associated also with persecutory delusions
Cacabelos et al. [40, 56]	207 demented patients	MMSE, GDS, ADAS, BCRS, FAST, BEHAVE-AD, HAM-D, HAM-A, and SDASDS	Disorientation, agitation, and motor disorders were slightly more frequent in demented patients with APOE ε4/e4, while anxiety and sleep disorders appeared more frequently in APOE ε3/ ε4. However, these differences were not statistically significant
Lehtovirta et al. [57]	58 AD patients and 16 controls	MMSE, BCRS, and HAM-D; the presence of rigidity, hypokinesia, tremor at rest, orofacial dyskinesia, myoclonus, hallucinations, delusions, and different kinds of paresis was recorded in the neurologic examination. The occurrence of epileptic seizures, hallucinations, and delusions was also inquired from the caregivers	Cognitive and neuropsychiatric symptoms and signs were not related to the APOE genotype
Murphy et al. [41]	77 AD patients	MMSE, TBDQ, and ADAS non-cog	APOE ε4 allele associated with higher scores on TBDQ
Cantillon et al. [58]	162 AD patients	MMSE and CSDD	The APOE e4 allele frequency was not increased in the late-onset depression group among these AD patients
Ballard et al. [80]	51 AD patients	CAMCOG, CSDD, Burns symptom checklist, and SCID-DSM-III-R	Protective effect of APOE ε4 allele against depression. APOE ε4-carriers more likely to have future psychotic episode
Forsell et al. [60, 61]	806 participants aging 78 years and over 668 participants aging 75 years and over	MMSE and CPRS Dementia was diagnosed using the DSM-III-R criteria Psychotic symptoms were defined according to DSM-IV criteria	Depressed and nondepressed subjects had similar APOE genotype distributions among the demented, and among the nondemented, subjects. There was also no statistical significant difference in APOE genotype between subjects with and without psychotic symptoms, stratified by dementia diagnosis
Lopez et al. [62]	194 AD patients	MMSE. BRS-CERAD, and semi-structured psychiatric interview	No evidence for an association of NPS with any specific APOE genotype in probable AD patients
Lyketsos et al. [63]	120 AD patients	Diagnoses for major and minor depression according to DSM-IV, and assessment of delusions or hallucinations according to DSM-IV glossary definitions	There was no association between APOE genotype and the presence of NPS or the neuropsychiatric syndromes examined. There was an interesting suggestion that the e4 allele may be protective against the development of major depression; however, this association did not reach statistical significance
Hirono et al. [64, 65]	228 AD patients 175 AD patients	MMSE, BEHAVE-AD, and NPI MMSE, CDR, and the Japanese version1of the NPI	The APOE ε4 allele had no effect on the manifestation of delusions, hallucinations, depression, or other NPS in AD
Levy et al. [25]	605 AD patients	MMSE and NPI	Among patients with comparable disease severity, the APOE ε4 allele does not confer additional psychiatric morbidity
Harwood et al. [66]	501 AD patients	MMSE, HAM-D, and structured interview for specific delusions and hallucinations	Increased risk for psychosis with APOE ε4 allele
Müller-Thomsen et al. [48]	137 AD patients	MMSE and MADRS	Overrepresentation of the APOE ε4 allele in female AD patients

Table 1: Continued.

Reference	Study sample	Cognitive and neuropsychiatric assessment	Principal results
Liu et al. [67]	149 AD patients	CASI, CDR, HAM-D, and SCID-DSM-III-R	No evidence of an association between depression in AD patients and presence or absence of the APOE $\varepsilon 4$ or $\varepsilon 2$ allele
Scarmeas et al. [68]	87 AD patients	MMSE, CUSPAD, BDRS, and SCID-DSM-III-R	APOE $\varepsilon 4$ alleles associated with risk for incident delusions. APOE $\varepsilon 4/\varepsilon 4$ predicted protective effect against hallucinations
Gabryelewicz et al. [69]	139 AD patients	MMSE, GDS, and BEHAVE-AD	The APOE e4 allele had no effect on the behavioural changes in AD
Sweet et al. [70]	316 AD patients	MMSE, BRS-CERAD, and SCID-DSM-III-R	There was no significant association of APOE genotype with time to psychosis onset and no significant interaction of this genotypes with time to psychosis onset
Craig et al. [26, 71]	400 AD patients 404 AD patients	NPI with caregiver distress MMSE and NPI with caregiver distress	Increase in agitation/aggression in patients with the APOE $\varepsilon 4$ allele, while APOE genotype created no additional risk for depressive symptoms in AD
Chang et al. [72]	135 AD patients	CASI, CDR, and SCID-DSM-III-R	APOE $\varepsilon 4$ significantly associated with hallucinations and delusions
Robertson et al. [73]	125 AD patients	CSDD and NPI	Greater level of anxiety in APOE $\varepsilon 4/\varepsilon 4$ versus $\varepsilon 3\varepsilon/3$ bearers in both genders and versus $\varepsilon 3/\varepsilon 4$ in males only
Borroni et al. [28]	232 AD patients	By Principal Component Analysis of NPI symptoms, four endophenotypes were identified, these were termed "psychosis," "moods," "apathy," and "frontal"	APOE genotype did not correlate with any neuropsychiatric endophenotype
Hollingworth et al. [29]	1,120 AD patients	By Principal Component Analysis of NPI symptoms, four interpretable components were identified: behavioral dyscontrol (euphoria, disinhibition, aberrant motor behavior, and sleep and appetite disturbances), psychosis (delusions and hallucinations), mood (depression, anxiety, and apathy), and agitation (aggression and irritability)	None of the neuropsychiatric endophenotypes identified were associated with age at assessment, years of education, or number of APOE $\varepsilon 4$ alleles
Monastero et al. [74]	197 AD patients	MMSE and NPI	The presence of apathy was significantly associated with the APOE $\varepsilon 4$ allele independently from age, education, sex, and duration of disease
Spalletta et al. [59]	171 AD patients	MMSE and NPI	The association between NEUROPSYCHIATRIC SYMPTOMS and APOE $\varepsilon 4$ allele in AD was confirmed for delusions only
Pritchard et al. [27]	388 AD patients	MMSE and NPI	Protective effect of the APOE $\varepsilon 3/\varepsilon 4$ genotype that was significantly associated with hallucinations symptoms. APOE $\varepsilon 3/\varepsilon 4$ genotype is significantly associated with anxiety
van der Flier et al. [75]	110 AD patients	MMSE and NPI	Delusions and agitation/aggression were more common and severer among homozygous APOE $\varepsilon 4$ carriers than among heterozygous or APOE-$\varepsilon 4$-negative patients
Zdanys et al. [76]	266 AD patients	MMSE, ADL, IADL, and NPI	APOE $\varepsilon 4$ was significantly associated with psychotic symptoms, adjusting for age, sex, education, and MMSE score
Delano-Wood et al. [49]	323 AD patients	MMSE and SCID-DSM-III-R	Higher prevalence rate of the APOE $\varepsilon 4$ genotype in the depressed group compared to the nondepressed AD patients. This effect was primarily accounted for by women

Table 1: Continued.

Reference	Study sample	Cognitive and neuropsychiatric assessment	Principal results
Chopra et al. [77]	175 cognitively impaired subjects, of which 92 AD and 80 MCI patients	MMSE, GDS-15, and NPI	The difference in the proportion of participant reporting "low energy" at GDS-15 between the three APOE ε4 allele frequency groups approached statistical significance
Del Prete et al. [78]	53 AD patients	MMSE and NPI	Patients with APOE 4 allele showed a wider range of NEUROPSYCHIATRIC SYMPTOMS when compared to noncarriers and higher scores for hallucinations and aberrant motor behaviors. Over time, ε4 carriers showed an increase/delayed onset in some symptoms and a parallel decrease in others, while noncarriers presented an undifferentiated worsening of symptomatology
Woods et al. [79]	36 demented patients	MMSE and mABRS	Patients with an APOE ε4 allele had a significant increase in their observed NEUROPSYCHIATRIC SYMPTOMS, including restlessness and vocalizations, compared to those who did not have an APOE ε4 allele present
D'Onofrio et al. [30]	201 AD patients and 121 controls	MMSE, ADL, IADL, CIRS, and NPI. Furthermore, AD patients with NEUROPSYCHIATRIC SYMPTOMS were further subdivided in four groups according to the EADC classification of neuropsychiatric syndromes in AD: hyperactive, psychotic, affective, and apathetic	No difference in the distribution of APOE genotypes was found between AD patients with and without NEUROPSYCHIATRIC SYMPTOMS. In AD patients APOE ε4-carriers, there was an increased risk of affective and apathetic syndromes

CDR: Clinical Dementia Rating scale; HAM-D: Hamilton rating scale for depression; SCID-DSM-III-R: Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders-III-revised; BDRS: Blessed Dementia Rating Scale; CAMDEX: The Cambridge examination for mental disorders of the elderly; MOUSEPAD: Manchester and Oxford universities scale for the psychopathological assessment of dementia; MMSE: Mini Mental State Examination; GDS: Global Deterioration Scale; ADAS: Alzheimer's Disease Assessment Scale; BCRS: Brief Cognitive Rating Scale; FAST: Functional Assessment Stages; BEHAVE-AD: Behavioural Pathology in Alzheimer's Disease Rating Scale; HAM-A: Hamilton rating scale for anxiety; SDASDS: Senile Dementia-Associated Sleep Disorders Scale; TBDQ: time-based behavioural disturbance questionnaire (does patient display any of following symptoms in 1 month prior to assessment (combativeness, agitation, wandering, incoherent speech, hallucinations, confusion, and disorientation); ADAS non-cog: Alzheimer's disease assessment scale noncognitive subscale; CSDD: Cornell Scale for Depression in Dementia; CAMCOG: Cambridge assessment for mental disorders in the elderly; CPRS: Comprehensive Psychopathological Rating Scale; BRS-CERAD: Behavior Rating Scale for dementia of the Consortium to Establish a Registry for Alzheimer's Disease; DSM-IV: Diagnostic and Statistical Manual of Mental Disorders-IV; NPI: Neuropsychiatry Inventory; MADRS: Montgomery-Asberg Depression rating Scale; CASI: Cognitive Abilities Screening Instrument; CUSPAD: Colombia University Scale for Psychopathology in AD; ADL: activities of daily living; IADL: instrumental activities of daily living; GDS-15: 15-item Geriatric Depression Scale; MCI: mild cognitive impairment; mABRS: modified Agitated Behavior Rating Scale; CIRS: Cumulative Illness Rating Scale; EADC: European Alzheimer's Disease Consortium.

concomitants of goal-directed behavior [86, 87]. To date, there is no clear consensus on the definition of apathy, and it is not included in the glossary of the Diagnostic and Statistical Manual of Mental Disorders-lV [88] and mentioned merely as a nonspecific symptom of several disorders. Anhedonia or loss of interest or pleasure can be used as a principal symptom to diagnose major depressive disorder instead of or along with depressed mood. Because other criteria for Diagnostic and Statistical Manual of Mental Disorders-IV diagnosis of major depressive disorder [88] such as fatigue, hypersomnia or insomnia, loss of appetite, weight loss, and diminished ability to concentrate are prevalent among demented patients, a demented patient with apathy may be misdiagnosed as having major depressive disorder even in the absence of dysphoria [89]. This diagnostic challenge stems from the apparent overlap between apathy and depression [90]. Apathy has been reported to be common in AD outpatients, and the reported prevalence for apathy using the NPI was between 32.1% and 93.2% [91, 92]. Also the relationship between the APOE e4 allele and the apathetic syndrome and/or the apathy symptom was studied [28–30, 36–38, 70, 74, 75, 93], although it has not been as thoroughly investigated as for depression or psychosis. In a sample of 197 subjects with probable AD, the presence of apathy was significantly associated with the APOE e4 allele independently from age, education, sex, duration of disease, Mini-Mental State Examination score, and other neuropsychiatric symptoms [74]. Furthermore, the recent results on an increased risk of the European Alzheimer's Disease Consortium apathetic syndrome (i.e., NPI symptoms apathy and eating abnormalities) in AD patients APOE ε4-carriers [30] confirmed these previous findings suggesting a relationship between the APOE ε4 allele and apathy in patients with AD [74], although other studies did not show

any correlation between apathy endophenotypes [28, 29] or NPI apathy symptom [75] and APOE genotypes (Table 1).

4. APOE and Psychotic Symptoms and Syndromes/Endophenotypes in AD

Among neuropsychiatric symptoms in AD, major attention has been dedicated to psychotic symptoms, that typically consist of delusions and/or hallucinations [94]. The prevalence of psychosis is quite substantial, with estimates for delusions in AD ranging from 9.3% to 63% (median 36%) and estimates for hallucinations ranging from 4% to 41% (median 18%) [95]. However, psychosis proved to be a coherent grouping of psychiatric symptoms in AD in studies using cluster and factor analysis [94, 96]. Therefore, it has been reported that AD patients with psychosis (AD + psychosis, AD + P) may be clinically different from AD [93, 94, 97]. Indeed, an increased familial risk for the AD + P endophenotype has been observed [70], suggesting heritability [88, 98] and thus a possible genetic component of this clinical feature. In fact, genes that have been implicated in psychosis of AD include those for dopamine-3 and serotonin-2A receptors [98]. Major attention has been dedicated to psychotic episodes in relation to APOE genotypes, and a number of investigations have examined whether the APOE $\varepsilon 4$ allele might contribute to the AD + P endophenotype [98]. Among initial studies conducted in unrelated AD + P and AD without psychosis subjects, one large [66] and two smaller studies [39, 80] have found an association of APOE $\varepsilon 4$ allele and the AD + P endophenotype. However, most successive reports have found no association of the APOE e4 allele with AD + P [25, 27, 40, 56, 61, 99, 100] (Table 1), suggesting that it may be necessary to operate a distinction among different psychotic symptoms in AD in relation to APOE polymorphism. However, also association studies with hallucinations have been contradictory, with two copies of $\varepsilon 4$ being reported as protective [68], $\varepsilon 4$ being associated with increased risk [72], and hallucinations appeared to increase with more $\varepsilon 4$ alleles [101] or also, in a small longitudinal study, to decrease 1 year after diagnosis in AD patients with one $\varepsilon 4$ allele [78]. The association of hallucinations in AD with the e3/$\varepsilon 4$ genotype [27] also added further inconsistencies to reported findings. Hallucinations are sometimes incorporated into a measure of psychosis, but also these studies have again been inconsistent, with significant associations being reported for the $\varepsilon 4$ allele [66] and for $\varepsilon 3/\varepsilon 4$ versus $\varepsilon 4/\varepsilon 4$ carriers [39]. Furthermore, when the outcome was the presence of delusions in AD patients, in a longitudinal study, one $\varepsilon 4$ allele carried 2.5-fold risk whereas the presence of two $\varepsilon 4$ alleles carried 5.6-fold risk for development of delusions during a 9.3-year followup [68]. These findings were confirmed in other two longitudinal studies in which the presence of the APOE $\varepsilon 4$ allele carried a 3.4-fold risk for developing delusions [72] and the presence of one e4 allele, 1 year after diagnosis, showed a moderate, but significant, increase in delusions [78]. Also in cross-sectional studies, APOE $\varepsilon 4$ allele possession was associated with increased levels of delusions within the last month from the first visit and with the presence of categorical delusions at the early stage until the first visit [59], delusions appeared to increase with more $\varepsilon 4$ alleles [101] and $\varepsilon 4/\varepsilon 4$ genotype [75], and $\varepsilon 4$ was preferentially associated with delusions also in a large sample of AD patients from the USA [76]. However, when AD patients were subdivided according to the European Alzheimer's Disease Consortium classification of neuropsychiatric syndromes, for psychotic syndrome, including AD patients with delusions, hallucinations, and night-time disturbances, no association was found between APOE polymorphism and this neuropsychiatric syndrome [30]. These findings were consistent with those of a larger number of studies in which no association was found between APOE genotypes and the psychotic endophenotype [28, 29] or also single psychotic symptoms or measures incorporating these symptoms, that is, hallucinations [26, 57, 62–65, 69] and delusions [62, 65] (Table 1).

5. APOE, Agitation/Aggressiveness, and Hyperactive Syndrome/Endophenotype in AD

Agitation/aggressiveness differs from psychosis and depression of AD in that it may be conceptualized as a single symptom or a symptom complex [94]. The prevalence of agitation in dementia ranges from 20% to 60%, depending on diagnostic definitions used and the population studied [102]. A formalized definition of agitation was proposed as "inappropriate verbal, vocal, or motor activity that is not judged by an outside observer to be an obvious outcome of the needs or confusion of the individual" [103]. Agitation/aggressiveness in dementia often co-occurs with psychosis and depression. There is substantial evidence that verbal agitation is associated with depression, and there may be some relationship to delusions [94, 104]. Psychosis, particularly delusions, and depression occur with increased frequency in aggressive patients and may be a causative factor [105]. Also the symptom complex agitation/aggressiveness has been linked to APOE genotypes. Agitation and disorientation were more common in demented patients $\varepsilon 4/\varepsilon 4$ carriers, whereas anxiety and sleep disorders were more common in $\varepsilon 3/\varepsilon 4$ carriers, although these differences were not statistically significant [40, 56]. In another report, it was noted that the presence of the $\varepsilon 4$ allele was associated with increased combativeness, agitation, wandering, and confusion [41]. These findings were confirmed in two other recent studies in which the APOE $\varepsilon 4$ allele has been associated with agitation/aggressiveness using the Neuropsychiatric Inventory Caregiver Distress Scale (NPI-D) [26, 75], and in a recent report in which the presence of the APOE e4+ genotype increased the risk for agitated behaviour, including restlessness and vocalizations, in nursing home residents with dementia [79] (Table 1). However, when AD patients were subdivided according to the European Alzheimer's Disease Consortium classification of neuropsychiatric syndromes, for hyperactive syndrome, including AD patients with agitation, euphoria, disinhibition, irritability, and aberrant motor behaviour, no association was found between APOE

genotypes and this neuropsychiatric syndrome, as also was found for psychotic syndrome [30]. These findings on the European Alzheimer's Disease Consortium hyperactive syndrome were similar to previous negative results in endophenotype [29], single symptom approach [25, 65], and in a study evaluating risk for incident neuropsychiatric symptoms in a prospectively followed cohort [68] (Table 1).

6. Mechanisms Linking Neuropsychiatric Symptoms and Syndromes/Endophenotypes with APOE Genotypes in AD

The present reviewed evidence on a possible role of the APOE polymorphism on the development of neuropsychiatric symptoms in AD patients confirmed that genetic factors may account for some of the neuropsychiatric heterogeneity associated with AD [106, 107]. The role of APOE in AD-related depression is still controversial, and additional research is needed [108]. However, recent evidence has been accumulating to suggest that APOE may be linked to vascular risk factors in late life and, in turn, may be associated with depression. Indeed, it has been posited that the $\varepsilon 4$ allele may be a predisposing genetic marker for ischemic cerebrovascular disease [109] given its association with hyperlipidemia [110], atherosclerosis [111], myocardial infarction [112], and subcortical white matter lesion pathology [113]. Some late-life depressive syndromes might predispose, precipitate, or perpetuate by cerebrovascular disease, the so-called "vascular depression" hypothesis [114, 115]. In fact, a history of stroke was associated with a 3-4-fold increased risk of apathy and depression in AD [116], and cerebrovascular disease has previously been linked to depression in several studies of individuals with dementia [117]. In addition, the APOE $\varepsilon 4$ allele may increase the odds that older depressed patients develop mild cognitive impairment [118]. Finally, it has been proposed that, for those carrying a copy of the $\varepsilon 4$ allele, the destructive effect of subtle, underlying vascular risk factors, may be enhanced [113]. Furthermore, the APOE $\varepsilon 4$ allele appears to be less efficient than other isoforms in inducing cholesterol transport [119], which may have an important role in maintaining the integrity of membranes, and in synaptic plasticity. Thus, it appears that the APOE $\varepsilon 4$ allele is associated with impaired response to cerebral damage and diminished capacity for neuronal repair [120, 121] and that this poorer neuronal reparative capacity may be implicated in the development of cognitive decline and depression in older adults with the APOE $\varepsilon 4$ genotype. Finally, for affective syndromes and/or symptoms in AD, also the mechanisms underlying the suggested association between late-life anxiety and APOE in AD are, at present, not completely understood [73, 84, 85]. Interestingly, higher measures of anxiety were reported in male mice lacking murine APOE ($APOE^{-/-}$) than in sex-matched wild-type controls [73, 84]. There were isoform-dependent effects of APOE on measures of anxiety in male $APOE^{-/-}$ and APOE3 and APOE4 mice expressing human APOE in neurons under control of the neuron-specific enolase (NSE) promoter or in astrocytes under control of the glial fibrillary acidic protein (GFAP) promoter [73, 84]. $APOE^{-/-}$ and APOE4 male mice showed increased measures of anxiety, whereas APOE3 mice behaved like wild-type controls [73, 84]. Very recently, these findings were also confirmed in female APOE TR mice expressing APOE under control of the mouse APOE promoter [85]. APOE4 mice showed decreased activity levels and higher measures of anxiety than mice expressing APOE2 or APOE3 across all ages [85].

The association of cerebrovascular disease with apathy may reflect stroke-related damage to areas of the prefrontal cortex or related neural pathways, involved in the planning and execution of goal-directed behavior [122]. In fact, apathy is associated with frontal and subcortical pathology [123, 124], and more severe apathy has been related with a severe impairment of frontal executive functions [125]. Functional imaging studies revealed that apathy in AD is related to dysfunction of the right temporoparietal and anterior cingulate cortices [122, 123], regions involved in frontal-subcortical networks. A recent neuropathological study reported a significant relationship between chronic apathy and anterior cingulated cortex tangle pathology [124]. Apathetic patients with AD treated with cholinesterase inhibitors showed a significant reduction in apathy [126]. This is probably related to loss of nucleus basalis of Meynert cholinergic input to prefrontal and subcortical regions [122]. Interestingly, the APOE $\varepsilon 4$ allele has been associated with a severe loss of cholinergic activity in the frontal cortex [127] and with enhanced β-amyloid deposition in the frontal region of nondemented presenile subjects [128].

Although the $\varepsilon 4$ allele has been shown to promote the neuropathological features of AD, including β-amyloid deposition [129, 130] and neurofibrillary tangle formation [129, 130], attempts to relate these features to psychotic symptoms in AD have yielded conflicting results [131, 132]. Therefore, neuropsychiatric symptomatology could be associated with more severe neuropathological changes, although there is no clear consensus on this. Furthermore, the $\varepsilon 4$ allele has been associated with more profound deficits in cholinergic neurons, in particular in the frontal cortex [127] and medial temporal lobe [133], whereas the development of neuropsychiatric symptoms in AD appears to be related to specific neurotransmitter imbalances, notably acetylcholine [134]. Psychotic manifestations in AD have been associated with pathology in the temporal lobe and hippocampus [131, 135]. One single photon emission computed tomography study has suggested that delusions in AD may be associated with hypoperfusion in the temporal lobes [136], and some [137] but not all [138] functional imaging studies have shown that AD patients who carry the $\varepsilon 4$ allele have reduced temporal lobe function. The structural magnetic resonance imaging literature is more unified in showing greater medial temporal lobe atrophy in association with the $\varepsilon 4$ allele in AD [139–141]. It is conceivable that the detected association between APOE genotype and psychotic symptom, particularly delusions, might reflect neuropathology more heavily concentrated in the temporal lobe. Finally, also the mechanisms of the possible association between APOE and aggressive/agitated behaviour in AD are unclear. APOE is associated with more rapid progression as

measured by single photon emission computed tomography and higher tangle burden in the brain [130, 138, 142, 143]. The accepted spread of neuropathological damage seen in AD, from the hippocampus to frontal-temporal-parietal regions, may encourage the development of those behavioural symptoms that not only localize regionally within the brain but are dependent on progressive neuronal loss and amyloid deposition away from the mesial temporal lobe. Frontal involvement is the best neuroanatomical correlate for aggression and agitation with secondary disruption of the serotonergic and dopaminergic systems [144–147]. High agitation scores correlate with bilateral orbitofrontal and left anterior cingulated tangle burden [144] and with left fronto-temporal hypoperfusion on single photon emission computed tomography scanning [145].

7. Conclusions

Environmental factors that have been associated with late-onset AD include depressive syndromes, various vascular risk factors, level of education, head trauma, and dietary factors. This complexity may help explain their high prevalence from an evolutionary perspective, but the etiologic complexity makes identification of disease-related genes much more difficult [148]. The "endophenotype" approach is an alternative method for measuring phenotypic variation that may facilitate the identification of susceptibility genes for complexly inherited traits [148]. The association of the APOE genotypes with neuropsychiatric symptoms or neuropsychiatric syndromes and endophenotypes in AD appeared to be still unclear. Neuropsychiatric symptoms in different times could coexist in a single patient showing a very complex psychological profile. The discrepancy in dementia syndromes between the occurrence of neuropsychiatric symptoms from rather linear cognitive decline implies independent pathophysiological pathways between these symptoms. In particular, contrasting findings existed on the possible association between affective symptoms or syndromes in AD and APOE genotypes, with studies with measures of late-life depressive syndromes and symptoms more frequent than studies that focused on late-life anxiety. At present, there is only limited evidence of an increased risk of apathy or agitated behaviour linked to the presence of APOE $\varepsilon 4$ allele in AD patients. Furthermore, some studies have found an association between APOE $\varepsilon 4$ allele and the AD plus psychosis endophenotype, with several studies suggesting a possible role of this polymorphism when the outcome was the presence of delusions in AD patients rather than of hallucinations. The fact that some studies assessing neuropsychiatric symptoms in relation to APOE status often analyzed the dichotomized APOE $\varepsilon 4$ status rather than the number of APOE e4 alleles may partially account for these contrasting findings, suggesting a methodological limit for the correct evaluation of predisposing factors and the pathogenetic basis of neuropsychiatric symptoms in AD. Discrepant findings may be due also to other factors including small sample sizes, differences in sample compositions (e.g., general "dementia" groups versus strictly diagnosed patients with "probable AD," with some of the studies including patients with Lewy body disease), the use of very brief, wide-ranging, or unstructured psychopathology inventories, and possible lack of statistical power to detect associations in the negative studies. Furthermore, some negative studies that focused on the distribution of APOE genotypes between AD patients with or without neuropsychiatric symptoms further emphasized the importance of subgrouping these symptoms in distinct neuropsychiatric syndromes, suggesting also genetic basis for individual neuropsychiatric symptoms. In addition, many of the above studies did not control for potential confounding variables. Most important, many reviewed studies were cross-sectional, whereas it would be of paramount importance to evaluate the risk for incident neuropsychiatric symptoms in relation to the APOE genotypes in prospectively followed cohorts of AD patients. In fact, cross-sectional studies have limitations in interpreting the causal relationship between APOE and neuropsychiatric symptoms or neuropsychiatric syndromes/endophenotypes. Further longitudinal studies on larger sample of patients are needed to clarify the possible role of this gene in neuropsychiatric symptoms or neuropsychiatric syndromes/endophenotypes in AD.

Acknowledgment

This work was fully supported by "Ministero della Salute," IRCCS Research Program, Ricerca Corrente 2009-2011, Linea no. 2 "Malattie complesse."

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 926597, 8 pages
doi:10.4061/2011/926597

Research Article

Psychosis in Alzheimer's Disease in the National Alzheimer's Disease Coordinating Center Uniform Data Set: Clinical Correlates and Association with Apolipoprotein E

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Received 18 August 2010; Accepted 3 January 2011

Academic Editor: Tatiana Foroud

Approximately 50% of late-onset Alzheimer's disease (AD) patients develop psychosis (AD+P), a heritable phenotype associated with more rapid cognitive decline. Prior studies conflict regarding whether apolipoprotein E (APOE) $\epsilon 4$ alleles are associated with AD+P, possibly due to small sample sizes, inconsistent diagnostic criteria, and different methodologies to assess psychosis. We used the National Alzheimer's Coordinating Center Uniform Data Set to evaluate the largest uniformly characterized sample of AD+P subjects studied to date for the association of APOE $\epsilon 4$ genotype, along with other demographic and clinical variables. Greater cognitive impairment and depressive symptoms were associated with AD+P, while the Caucasian race was protective. Neither APOE $\epsilon 4$ carrier status nor allele number was associated with psychosis. The AD+P phenotype is not associated with the APOE $\epsilon 4$ genotype. AD+P may represent a useful phenotype for the discovery of non-APOE $\epsilon 4$ genetic variation contributing to the risk of AD.

1. Introduction

Psychotic symptoms, delusions, and hallucinations are common in Alzheimer's disease (AD; AD+P), occurring in approximately 40% of individuals over the course of the illness [1]. AD+P causes significant distress for patients and family members [2]. AD+P is a predictor of worse cognitive and functional outcome, higher likelihood of institutionalization, and higher mortality rate [3]. Importantly, a number of studies indicate that the occurrence of psychosis in AD is familial [4–6], with an estimated heritability of 61% [7], indicating a distinct neurobiology of this phenotype [8].

Recently, Ropacki and Jeste [1] comprehensively reviewed the literature on psychosis in AD. They reviewed 55 studies comprised of 9,749 subjects. The most consistent correlate of AD+P was greater cognitive impairment than is found in AD without psychosis (AD−P). In twenty of thirty studies which assessed this relationship, the prevalence of psychosis increased as cognitive impairment worsened as determined by the Mini Mental State Exam (MMSE). Studies conducted more recently have continued to support the relationship between greater cognitive impairment and AD+P [9, 10]. In contrast, only inconsistent associations have been detected between AD+P and age, age at onset of AD, illness duration, gender, and education. AD+P may be associated with race, though it has only been examined in a limited number of studies to date [1]. It should be noted that the above conclusions are limited by the inconsistencies across the reviewed studies. Few variables are examined in all studies; sample sizes of individual studies were not always sufficient to detect small-to-moderate effects, and approaches to characterization of subjects, including identification of psychosis, also varied considerably.

Apolipoprotein E (APOE) is well documented as being an important genetic determinant for the development of late-onset AD [11]. However, its association with development of psychosis is less clear. We have identified 22 studies that examined the association of the APOE $\epsilon 4$ allele with AD+P, with nine reporting that $\epsilon 4$ increased the risk for AD+P, whereas 13 studies found no effect of $\epsilon 4$ [12]. As in the studies of clinical correlates of AD+P, these reports varied considerably in their subject populations, sample sizes, definitions of AD+P, and analytic approaches, precluding clear interpretation of the conflicting pattern of results. Moreover, as highlighted by recent genome-wide association studies, genes of small, but real, effects (e.g., RR 1.1-1.2) often show inconsistent results in studies with small sample sizes, with the currently recommended sample sizes numbering in the thousands. Although APOE $\epsilon 4$ confers greater relative risk for AD, it may have a smaller, if any, effect on the development of psychosis in AD.

To more reliably determine the correlates of the AD+P syndrome, it would be desirable to analyze a large cohort of individuals with late-onset AD who have been characterized in a standardized manner. To accomplish this goal we utilized the Uniform Data Set (UDS) collected by the National Alzheimer's Coordinating Center (NACC) to characterize the clinical correlates of AD+P. To date, this is the largest data set used to look at the association of APOE $\epsilon 4$ with AD+P. We hypothesized that the APOE $\epsilon 4$ genotype is not associated with the development of AD+P. We also hypothesized that AD+P is associated with greater cognitive impairment.

2. Methods

2.1. NACC Data Center. The NACC was developed in 1999 with the purpose of developing and maintaining a database that included data from NIH-funded Alzheimer's Disease Centers (ADC) across the country. The UDS was developed by NACC to provide the ADCs with standardized assessments thereby allowing uniformity amongst centers when diagnosing these subjects with mild cognitive impairment or Alzheimer's disease [13]. Individual centers may use additional assessments for their particular research protocols, but every center must complete the UDS assessments [14]. Since 2005, these have included ratings of psychosis on the Neuropsychiatric Inventory Questionnaire (NPI-Q) [15].

2.2. Eligibility Criteria. Subjects were selected for analysis on the basis of having a primary diagnosis of possible or probable AD with an age of onset ≥ 60. Subjects with comorbid Parkinson's disease or dementia with Lewy bodies were excluded. Additionally, subjects were required to have available psychosis ratings on the NPI-Q and an APOE genotype. Other variables requested, but not available for all subjects, included demographics (age at first visit, age of onset of dementia, sex, race, ethnicity, primary language, education) and scores on the Mini-Mental State Exam (MMSE) [16], global Clinical Dementia Rating Scale (CDR) [17], Hachinski Ischemic Scale [18], and the Geriatric Depression Scale (GDS) [19]. Because some subjects had multiple visits over time, for these individuals the last available scores for MMSE, CDR (global), Hachinski, and GDS were used for analysis. Regarding race and ethnicity, subjects reported their race to a clinician from the following choices: White, Black, or African-American, American-Indian, or Alaska Native, Native Hawaiian or other Pacific Islander, Asian, other, or Unknown. From this, subjects were grouped as Caucasian, African-American, or other for analysis. Subjects also reported as to whether they had Hispanic/Latino ethnicity. This study had Institutional Review Board approval through the University of Pittsburgh and Universities contributing their data to the NACC.

2.3. Classification of Psychosis. Each subject was assessed for psychosis at each visit using the NPIQ. Informants for the NPIQ ratings were most commonly a subject's spouse (2889, 57.7%) or child (1606, 32.1%) and rarely other informants (515, 10.3%). Subjects were rated positive for psychosis at any visit if they exhibited delusions (question 1) and/or hallucinations (question 2) within a one-month time frame prior to the interview. Subjects with neither item endorsed at any visit were categorized as Never Psychotic. Subjects with one item present at no more than one visit were characterized as having Single Psychosis. Subjects with both items present at any one visit, or one or more items present at multiple visits, were characterized as Multiple/Recurrent Psychosis.

2.4. Statistical Analysis. The association of AD+P with baseline clinical and demographic variables was analyzed using univariate (Chi-square and ANOVA, as appropriate) tests. We tested the associations of AD+P with APOE genotype and $\epsilon 4$ carrier status by Chi-square. Follow-up analyses of association used multinomial logistic regression models, including APOE genotype, and clinical and demographic variables. Because MMSE and CDR score are highly correlated, in these latter analyses we omitted the CDR score. Additional multinomial logistic regression analyses were conducted including a main effect of site (24 ADCs contributed to the data set) but were not reported as they yielded essentially identical results.

3. Results

We identified 2317 subjects in the NACC database who fulfilled all eligibility criteria for analysis. Of these subjects, 777 (33.5%) had one visit, 730 (31.5%) had two visits, 485 (20.9%) had three visits, 307 (13.2%) had 4 visits, and 18 (0.8%) had five visits for a total number of 5010 visits reported to NACC. The majority of subjects were diagnosed with probable AD (2117, 91.4%) and the remaining (200, 8.6%) with possible AD. The sample predominately consisted of Caucasian (1957, 84.5%) and female (1309, 56.5%) subjects (Table 1). The majority of patients were carriers of the APOE $\epsilon 4$ allele (1383, 59.7%) (Table 1).

Table 1: Demographic and clinical characteristics and their association with AD+P.

Variable	Psychosis Status			Total N (%) or mean (SD)	$\chi^{2\dagger}$ or $F^{\ddagger}$	df	P value
	Never N (%) or mean (SD)	Single N (%) or mean (SD)	Multiple/Recurrent N (%) or mean (SD)				
Age	78.2 (6.8)	79.0 (7.1)	78.3 (7.1)	78.4 (6.9)	2.197‡		.111
Age of onset	73.3 (6.9)	73.4 (7.1)	72.3 (6.9)	73.2 (6.9)	2.862‡		.057
Sex							
Male	704 (46.5)	188 (38.1)	116 (37.5)	1008 (43.5)	15.673†	2	<.001
Female	811 (53.5)	305 (61.9)	193 (62.5)	1309 (56.5)			
Race							
Caucasian	1347 (89.0)	394 (80.0)	216 (70.0)	1957 (84.5)	82.409†	4	<.001
African-American	132 (8.7)	76 (15.4)	69 (22.3)	277 (12.0)			
Other	35 (2.3)	23 (4.6)	24 (7.7)	82 (3.5)			
Hispanic							
No	1429 (94.3)	448 (90.9)	270 (87.4)	2147 (92.7)	21.161†	2	<.001
Yes	86 (5.7)	45 (9.1)	39 (12.6)	170 (7.3)			
Primary language							
English	1425 (94.1)	455 (92.3)	273 (88.3)	2153 (92.9)	13.100†	2	.001
Other	90 (5.9)	38 (7.7)	36 (11.7)	164 (7.1)			
Education	14.2 (3.6)	13.4 (3.6)	13.0 (4.2)	13.8 (3.7)	19.138‡		<.001
MMSE	18.7 (6.7)	16.8 (7.0)	14.2 (7.1)	17.7 (7.0)	59.922‡		<.001
CDR (global)							
0.0	12 (0.8)	0 (0.0)	0 (0.0)	12 (0.5)			
0.5	364 (24.0)	52 (10.5)	12 (3.9)	428 (18.5)	177.772†	8	<.001
1.0	583 (38.5)	192 (39.0)	83 (26.9)	858 (37.0)			
2.0	408 (26.9)	182 (36.9)	132 (42.7)	722 (31.2)			
3.0	148 (9.8)	67 (13.6)	82 (26.5)	297 (12.8)			
Hachinski	1.1 (1.4)	1.1 (1.3)	1.3 (1.5)	1.1 (1.4)	2.417‡		.089
GDS	2.2 (2.4)	2.5 (2.8)	2.7 (3.0)	2.3 (2.6)	6.849‡		.001
APOE $\epsilon 4$ allele carrier status					4.008†	2	.135
$-\epsilon 4$	632 (41.7)	190 (38.5)	112 (36.2)	934 (40.3)			
$+\epsilon 4$	883 (58.3)	303 (61.5)	197 (63.8)	1383 (59.7)			
APOE $\epsilon 4$ allele number							
0	632 (41.7)	190 (38.5)	112 (36.3)	934 (40.3)	5.097†	4	.277
1	712 (47.0)	236 (47.9)	158 (51.1)	1106 (47.7)			
2	171 (11.3)	67 (13.6)	39 (12.6)	277 (12.0)			

Abbreviations: AD+P, Alzheimer's disease plus psychosis; MMSE, Mini-mental state exam; CDR, Clinical dementia rating scale; GDS, Geriatric depression scale; APOE, Apolipoprotein E.
†Pearson's Chi-square test: χ^2 values are presented.
‡One-way analysis of variance: F values are presented.

Demographic and clinical variables were analyzed for association with AD+P. Univariate analyses revealed significant associations for psychosis with sex (female), race (non-Caucasian), Hispanic ethnicity, primary language (non-English), lower education, lower MMSE score, higher CDR score, and higher GDS score (Table 1). There were no significant associations of psychosis with age at presentation, age of onset of dementia, Hachinski score, APOE $\epsilon 4$ allele carrier status, or the number of APOE $\epsilon 4$ alleles (Table 1).

Because psychosis does not typically manifest in early stages of AD, classifying someone as a "true AD−P" requires individuals to have reached at least mild to moderate stages of disease [6]. Therefore, we conducted follow-up analyses in which individuals classified as AD−P were restricted to those who had at least reached a CDR score ≥ 1, $N = 1941$. In these univariate analyses, significant associations remained for sex, race, Hispanic ethnicity, primary language, education, MMSE, CDR, and GDS. Psychosis was now also significantly

Table 2: Demographic and clinical characteristics and their association with AD+P with Never Psychotic (AD−P) cases restricted to CDR ≥ 1.

Variable	Psychosis status			Total N (%) or mean (SD)	$\chi^{2\dagger}$ or $F^{\ddagger}$	df	P value
	Never N (%) or mean (SD)	Single N (%) or mean (SD)	Multiple/Recurrent N (%) or mean (SD)				
Age	78.8 (6.8)	79.0 (7.1)	78.3 (7.1)	78.7 (6.9)	0.886‡		.413
Age of onset	73.4 (6.9)	73.4 (7.1)	72.3 (6.9)	73.2 (7.0)	3.094‡		.046
Sex							
Male	529 (46.4)	188 (38.1)	116 (37.5)	833 (42.9)	14.035†	2	.001
Female	610 (53.6)	305 (61.9)	193 (62.5)	1108 (57.1)			
Race							
Caucasian	1024 (90.0)	394 (79.9)	216 (70.0)	1634 (84.2)	83.311†	4	<.001
African-American	87 (7.6)	76 (15.4)	69 (22.3)	232 (12.0)			
Other	27 (2.4)	23 (4.7)	24 (7.7)	74 (3.8)			
Hispanic							
No	1069 (93.9)	448 (90.9)	270 (87.4)	1787 (92.1)	15.243†	2	<.001
Yes	70 (6.1)	45 (9.1)	39 (12.6)	154 (7.9)			
Primary language							
English	1070 (93.9)	455 (92.3)	273 (88.3)	1798 (92.6)	11.252†	2	.004
Other	69 (6.1)	38 (7.7)	36 (11.7)	143 (7.4)			
Education	14.2 (3.6)	13.4 (3.6)	13.0 (4.2)	13.8 (3.7)	16.762‡		<.001
MMSE	16.9 (6.5)	16.8 (7.0)	14.2 (7.1)	16.4 (6.8)	19.386‡		<.001
CDR (global)							
0.0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)			
0.5	0 (0.0)	52 (10.5)	12 (3.9)	64 (3.3)	186.669†	6	<.001
1.0	583 (51.2)	192 (39.0)	83 (26.9)	858 (44.2)			
2.0	408 (35.8)	182 (36.9)	132 (42.7)	722 (37.2)			
3.0	148 (13.0)	67 (13.6)	82 (26.5)	297 (15.3)			
Hachinski	1.1 (1.4)	1.1 (1.3)	1.3 (1.5)	1.1 (1.4)	1.864‡		.155
GDS	2.1 (2.3)	2.5 (2.9)	2.7 (3.0)	2.3 (2.6)	6.947‡		.001
APOE $\epsilon 4$ allele carrier status					3.100†	2	.212
$-\epsilon 4$	471 (41.4)	190 (38.5)	112 (36.2)	773 (39.8)			
$+\epsilon 4$	668 (58.6)	303 (61.5)	197 (63.8)	1168 (60.2)			
APOE $\epsilon 4$ allele number							
0	471 (41.3)	190 (38.5)	112 (36.3)	773 (39.8)	3.502†	4	.478
1	527 (46.3)	236 (47.9)	158 (51.1)	921 (47.5)			
2	141 (12.4)	67 (13.6)	39 (12.6)	247 (12.7)			

Abbreviations: AD+P, Alzheimer's disease plus psychosis; AD−P, Alzheimer's disease minus psychosis; MMSE, Mini-mental state exam; CDR, Clinical dementia rating scale; GDS, Geriatric depression scale; APOE, Apolipoprotein E.
†Pearson's Chi-square test: χ^2 values are presented.
‡One-way analysis of variance: F values are presented.

associated with age of onset (younger). Age at presentation, Hachinski score, APOE $\epsilon 4$ allele carrier status, and APOE $\epsilon 4$ allele number remained insignificant for association with psychosis (Table 2).

Using multinomial regression, increased age at presentation, lower age of onset of dementia, being non-Caucasian, lower MMSE, and increased GDS were significantly associated with both Single and Multiple/Recurrent Psychosis (Table 3). Lower education was significantly associated with Single, but not Multiple/Recurrent Psychosis. When AD−P status was restricted to patients with a CDR ≥ 1, race and GDS remained significant predictors of both Single and Multiple/Recurrent Psychosis (Table 4). Age, age of onset, and MMSE were significantly associated with only Multiple/Recurrent Psychosis, while education was significantly associated with Single Psychosis. Sex, Hispanic origin,

Table 3: Multinomial logistic regression analysis of Single and Multiple/Recurrent Psychosis.

Psychosis category	Variable	OR	95% CI	Wald χ^2 (df = 1)	P value
Single	Age	1.053	(1.016, 1.092)	8.083	.004
	Age of onset	0.960	(0.926, 0.995)	5.047	.025
	Sex	0.799	(0.633, 1.010)	3.515	.061
	Race				
	Caucasian	0.457	(0.233, 0.898)	5.168	.023
	African-American	0.834	(0.398, 1.750)	0.230	.632
	Other[a]				
	Hispanic origin	0.520	(0.263, 1.029)	3.524	.061
	Primary language	1.925	(0.901, 4.110)	2.862	.091
	Education	0.965	(0.932, 0.999)	4.181	.041
	MMSE	0.969	(0.951, 0.987)	10.924	.001
	Hachinski	0.972	(0.895, 1.056)	0.437	.508
	GDS	1.051	(1.007, 1.096)	5.179	.023
	APOE ϵ4 allele number				
	0	0.778	(0.542, 1.115)	1.867	.172
	1	0.815	(0.575, 1.154)	1.329	.249
	2[a]				
Multiple/Recurrent	Age	1.078	(1.034, 1.123)	12.485	<.001
	Age of onset	0.914	(0.876, 0.953)	17.716	<.001
	Sex	0.847	(0.631, 1.136)	1.233	.267
	Race				
	Caucasian	0.311	(0.151, 0.642)	9.977	.002
	African-American	1.008	(0.455, 2.232)	0.000	.985
	Other[a]				
	Hispanic origin	0.457	(0.207, 1.007)	3.774	.052
	Primary language	1.157	(0.495, 2.703)	0.113	.736
	Education	0.974	(0.935, 1.014)	1.654	.198
	MMSE	0.911	(0.892, 0.931)	71.322	<.001
	Hachinski	1.039	(0.948, 1.140)	0.673	.412
	GDS	1.077	(1.025, 1.132)	8.489	.004
	APOE ϵ4 allele number				
	0	0.817	(0.518, 1.290)	0.751	.386
	1	0.999	(0.647, 1.541)	0.000	.996
	2[a]				

Abbreviations: MMSE, Mini-mental state exam; GDS, Geriatric depression scale; APOE, Apolipoprotein E.
[a]Reference group.

primary language, Hachinski score, and APOE ϵ4 allele number were not significantly associated with psychosis in either analysis.

The UDS also requires reporting on several vascular burden and comorbidity variables, such as hypertension. We did univariate analyses of 12 such variables (see Supplementary Table 1 in Supplementary Material available online at doi:10.4061/2011/926597). Only hypertension and diabetes were significantly associated with psychosis. When the analyses were restricted to patients who has at least reached a CDR score $\geq$1, significance for diabetes remained, but significance for hypertension was lost (Supplementary Table 2). In multinomial regression analyses in which hypertension and diabetes were entered as covariates, along with age, age of onset, sex, race, ethnicity, primary language, education, MMSE, GDS, Hachinski, and APOE, neither was significantly associated with any psychosis measure. The association of all other variables with psychosis remained unchanged (data not shown).

4. Discussion

This is the largest study to date to look at the association between APOE ϵ4 and AD+P. We were able to examine the associations of APOE and other variables with both any occurrence of psychotic symptoms and with progressively more heritable (and therefore perhaps more biologically relevant) psychotic phenotypes defined by the presence of multiple and/or recurrent psychotic symptoms and with a more stringently restrictive definition of nonpsychotic AD cases. Neither APOE ϵ4 carrier status nor APOE ϵ4 allele number was associated with psychosis in any analysis.

Table 4: Multinomial logistic regression analysis of Single and Multiple/Recurrent Psychosis when Never Psychotic (AD−P) cases were restricted to CDR ≥ 1.

Psychosis category	Variable	OR	95% CI	Wald χ^2 (df = 1)	*P* value
Single	Age	1.022	(0.985, 1.061)	1.326	.250
	Age of onset	0.980	(0.944, 1.017)	1.178	.278
	Sex	0.833	(0.653, 1.063)	2.151	.143
	Race				
	Caucasian	0.482	(0.236, 0.983)	4.028	.045
	African-American	1.046	(0.476, 2.297)	0.013	.911
	Other[a]				
	Hispanic origin	0.590	(0.295, 1.178)	2.234	.135
	Primary language	1.699	(0.787, 3.670)	1.822	.177
	Education	0.958	(0.924, 0.993)	5.583	.018
	MMSE	1.016	(0.995, 1.036)	2.221	.136
	Hachinski	0.966	(0.886, 1.053)	0.605	.437
	GDS	1.053	(1.007, 1.101)	5.160	.023
	APOE ϵ4 allele number				
	0	0.858	(0.592, 1.243)	0.655	.418
	1	0.917	(0.641, 1.313)	0.223	.637
	2[a]				
Multiple/Recurrent	Age	1.052	(1.009, 1.098)	5.543	.019
	Age of onset	0.929	(0.889, 0.969)	11.458	.001
	Sex	0.876	(0.649, 1.182)	0.750	.387
	Race				
	Caucasian	0.326	(0.153, 0.695)	8.438	.004
	African-American	1.204	(0.523, 2.769)	0.190	.663
	Other[a]				
	Hispanic origin	0.522	(0.237, 1.151)	2.597	.107
	Primary language	1.030	(0.440, 2.408)	0.005	.946
	Education	0.969	(0.930, 1.010)	2.274	.132
	MMSE	0.947	(0.925, 0.968)	22.376	<.001
	Hachinski	1.034	(0.942, 1.136)	0.499	.480
	GDS	1.080	(1.026, 1.137)	8.692	.003
	APOE ϵ4 allele number				
	0	0.883	(0.557, 1.398)	0.283	.595
	1	1.087	(0.702, 1.685)	0.140	.709
	2[a]				

Abbreviations: AD−P, Alzheimer's disease minus psychosis; MMSE, Mini-mental state exam; GDS, Geriatric depression scale; APOE, Apolipoprotein E.
[a]Reference group.

Similarly, the degree of vascular disease as rated on the Hachinski scale was not associated with AD+P in any analysis. Our analysis of these multiple definitions of AD+P also revealed other patterns. Greater cognitive impairment and greater depressive symptoms were associated with increased incidence of psychosis across multiple analyses, with the strongest associations observed with Multiple/Recurrent Psychosis. A similar pattern of association was seen for Caucasian race, although it was protective against AD+P. Other variables demonstrated less consistent associations, emerging (e.g., age and age of onset of AD), or disappearing (sex, Hispanic ethnicity, primary language), after controlling for the effects of other variables in multivariate analyses. Finally, years of education showed associations with AD+P in both univariate and multivariate analyses, but not in the latter with the Multiple/Recurrent phenotype.

Many studies have looked at the association of APOE ϵ4 with AD+P, with conflicting, albeit largely negative, results [12]. A number of possible reasons might have contributed to these contradictory findings, including the variability in sample size across studies, heterogeneity of AD subjects (with regard to early versus late stages of AD and early- or late-onset AD), and different methods of psychosis assessment and classification. Adding to the inconsistencies across studies, prior studies varied regarding whether they analyzed APOE genotype, ϵ4 allele number, or ϵ4 carrier

status and the extent to which they included relevant clinical and demographic information in multivariate models.

We were able to overcome many of these problems by utilizing the NACC UDS to evaluate the largest sample of subjects studied for the association of APOE with AD+P to date. Subjects from 24 ADCs across the country were assessed using a standardized battery allowing for multivariate analyses including a number of potentially relevant clinical and demographic measures in essentially all subjects. Further, guided by data indicating the stage-dependent emergence of psychotic symptoms during AD [6, 20] and the increased heritability of an AD+P phenotype defined by the presence of multiple and/or recurrent symptoms [6, 7], we were able to assess the association of APOE with a restrictive definition of AD+P likely to be enriched for association with causal genetic variants.

Using these approaches we found no evidence that APOE $\epsilon 4$ is associated with AD+P. It is possible from the small magnitude of the odds ratios observed in our study that a significant association would be observed in a much larger sample. However, the current results are consistent with the majority of the prior evidence, in which only nine of 22 prior studies, comprising more than 5,200 subjects with AD, found any evidence of significant association [12]. Furthermore, we showed previously that the time from entry into our clinic to the onset of psychosis was not associated with APOE genotypes, lending additional support to the current findings [21]. Thus, the most likely conclusion is that APOE $\epsilon 4$ carrier status and allele number are not associated with psychosis in a population of patients with late-onset Alzheimer's disease.

As with APOE, the current data set and analytic approach can also shed some light on the conflicting results of prior studies which have examined the association of AD+P with other clinical and demographic variables. As summarized in a recent review by Ropacki and Jeste [1], AD+P was significantly associated with older age in 12/25 studies, with age of onset in 5/12 studies (older in 4/5, younger in 1/5), with gender in 7/24 studies (male in 3/7, female in 4/7), with African-American race in 5/7 studies, and with lower education in 4/17 studies. More recent studies have found a significant association between AD+P and the severity of depressive symptoms [6, 22–24]. Our findings would suggest that the associations with AD+P of age, age of onset of dementia, non-Caucasian race, and depressive symptom severity represent "true positives" while those for sex, ethnicity, and primary language are explained by confounding with other variables.

One of the most consistent correlates of AD+P in prior studies is greater burden of cognitive impairment, whether measured as degree of cognitive impairment at time of psychosis or as more rapid cognitive decline [1, 10, 25]. Our current findings are congruent with the former of these prior observations; however, we did not measure the rate of cognitive decline in this study because of the limited number of subjects who have had follow-up assessments entered to date. The mechanisms underlying the association between greater cognitive impairment and AD+P are not known, but the current analysis suggests that one potential mediator, increased vascular burden [26], as measured by a global summary, the Hachinski, does not explain the association., We followed this up by analysis of direct measures of vascular risk factors and cerebrovascular disease. Of the 12 UDS variables, only history of hypertension and diabetes achieved marginal significant associations with AD+P in univariate analyses, In multivariate analysis the significance for both was lost. Therefore, the most conservative interpretation is that increased vascular burden is not associated with AD+P.

Amongst the other demographic variables that we tested, the finding that non-Caucasian (predominantly African-American) subjects are more likely to develop psychosis during the progression of AD deserves some comment. It is not clear if this association is due to differences between ethnic groups in allele frequencies of potential AD+P risk genes [27] or if being rated as positive for psychotic symptoms, such as persecutory delusions, represents culturally or socioeconomically biased measures of psychosis or referral bias within minority communities. Without having a representative sample of minorities from all socioeconomic backgrounds, it will be impossible to make this distinction. Regardless, the implication for studies of genetic association in AD+P is that non-Caucasian subjects should be evaluated separately from Caucasian populations and combined in analyses only if heterogeneity is not observed.

The presence of $\epsilon 4$ alleles of APOE is currently the strongest genetic determinant for late-onset AD, a fact underlined by the findings in most genome-wide association studies of late-onset AD, which have "rediscovered" the association with APOE (http://www.alzgene.org/). We provide evidence that a heritable subtype of AD, defined by the presence of multiple or recurrent psychotic symptoms, is not associated with APOE $\epsilon 4$. As the discovery of non-APOE genetic variation contributing to the risk of late-onset AD remains a high priority, one approach to enriching cohorts to enhance successful discovery may be to examine the AD+P phenotype.

Acknowledgments

This work was supported by the National Institute on Aging (NIA) Grant U01 AG016976 to the National Alzheimer's Coordinating Center and by AG 027224 and AG 05133. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Mental Health or the National Institutes of Health.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 929102, 5 pages
doi:10.4061/2011/929102

Research Article

An Intron 7 Polymorphism in *APP* Affects the Age of Onset of Dementia in Down Syndrome

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Received 23 August 2010; Revised 21 October 2010; Accepted 6 December 2010

Academic Editor: Lindsay A. Farrer

People with Down syndrome (DS) develop Alzheimer's disease (AD) with an early age of onset. A tetranucleotide repeat, $attt_{5-8}$, in intron 7 of the amyloid precursor protein has been associated with the age of onset of AD in DS in a preliminary study. The authors examine the impact of this polymorphism in a larger cohort of individuals with DS. Adults with DS were genotyped for $attt_{5-8}$ and *APOE*. The results were analysed with respect to the age of onset of dementia. The presence of three copies of the six-repeat allele resulted in onset of dementia seven years earlier than in the presence of other genotypes. Further study is essential to elucidate the mechanism by which this polymorphism functions, with an exciting opportunity to identify novel treatment targets relevant for people with DS and AD.

1. Introduction

Down syndrome (DS), defined cytogenetically by trisomy 21, is the most common chromosomal disorder associated with learning disability, occurring in approximately 1/1000 live births [1]. The prevalence of Alzheimer's disease (AD) in people with DS increases significantly with age. However, the nature of the early clinical presentation, course, and incidence rates of dementia are uncertain. Despite the nearly universal occurrence of AD pathology by age 40 [2, 3], there is wide variation in age of onset of clinical dementia. Most studies have indicated that the average age at onset of dementia is between 50 and 55 years of age, with a range from 38–70 years [4]. Many studies have now confirmed that age-related cognitive decline and dementia affecting people with DS occurs 30–40 years earlier than in the general population. Age-specific rates of dementia begin to increase in the patient's 30s from 1-2% to 40% in the 50s [5], hence dementia is becoming an increasingly important issue in people with DS as life expectancy continues to increase.

The neuropathological manifestations of AD in DS have been at least in part attributed to triplication and overexpression of the gene for amyloid precursor protein (*APP*) located on chromosome 21 [6]. In fact, an additional copy of *APP* can cause early onset AD with cerebral amyloid angiopathy, even when only small regions of chromosome 21 including only the *APP* gene are triplicated [7–10]. Triplication of chromosome 21 leads to an increase in expression levels of its genes. APP is expressed at levels which are four- to five-fold higher in DS than in the general population [11]. This is not only due to triplication of the gene, but is also caused by regulators of *APP* expression, for example *ETS2*, present on chromosome 21, which have increased expression levels [12]. Processing of APP can result in the production of beta-amyloid ($A\beta$) which is deposited extracellularly as a core disease feature in the brains of people with AD.

Along with overexpression, other APP changes are seen in AD, which include variations in the proportion of APP splice variants. Three of these isoforms are APP695, APP751, and APP770. APP695 is predominantly expressed in neurons

whilst APP751 and APP770 are ubiquitously expressed. A kunitz protease inhibitor domain (KPI) encoded by exon 7 is present in both APP751 and APP770, but not in APP695. The ratio of APP751 : APP695 mRNA is found to be increased in the brains of people with AD [13, 14], and there appears to be a relationship between this ratio and the density of plaques in the hippocampus and entorhinal cortex [15]. In fact, mice expressing human APP751, but not APP695, develop an AD-like pathology, which most closely resembles that found in the brains of young people with DS [16]. This pathology not only involves Aβ, but also abnormal tau isoforms. Therefore, it would seem that not only overexpression of APP in DS, but also variation in isoforms, may have a role in determining the onset of AD.

Intron 7 of *APP* has been sequenced previously to determine if polymorphic sites which may have the potential to regulate exon 7 splicing were present [17]. The tetranucleotide repeat ($attt_{5–8}$) was identified and the $attt_6$ allele was found to be the most common, present in 96% of the genotyped population. When this region was genotyped in a preliminary study of 105 people with DS, there was found to be an association between the number of $attt_6$ alleles and the age of onset of dementia [18]. Specifically, those individuals carrying three copies of the $attt_6$ allele developed dementia with an earlier age of onset than those with any other combination of the repeats.

In DS polymorphisms on chromosome 21 may have an amplified effect. As most people with DS develop AD with an early age of onset, these amplified genetic effects may allow us to more clearly detect the associated phenotypes, such as accelerated onset of dementia or severity. Such genetic effects may be too subtle to detect in the general population, but the mechanistic information provided will be valuable in drug design for all those at risk of developing AD. Following the preliminary study [18] we examine whether the tetranucleotide repeat, $attt_{5–8}$, in intron 7 impacts upon age of onset of dementia in a larger cohort of individuals with DS.

2. Materials and Methods

2.1. Samples and Clinical Assessments. Clinical evaluation was completed and DNA samples were obtained from 291 adults with DS (Newcastle-181, Birmingham-78, MEADOWS Clinical Trial-32), substantially extending a previous pilot study [18]. In the previous study the difference in age of onset between the genotypes was 13.4 years, with a standard deviation of 7.2 years [18]. Using these values, a power calculation shows that a study using a total of 18 participants (9 $attt_6$ homozygotes and 9 heterozygotes) has a 95 percent chance to detect a difference at a two-sided 0.05 significance level.

This study was approved by the appropriate local research ethics committee and consent was obtained from the participants. Detailed prospective clinical assessments were completed which included the DAMES battery (Newcastle, MEADOWS) and the Adaptive Behaviour Scale (ABS) [19] (Birmingham, MEADOWS), which enabled the operationalized diagnosis of dementia by an expert in the field (MH, VP) according to the International Classification of Disease [20]. Clinicians were blind to genotyping results.

2.2. Genotyping. Blood samples were collected into K_2-EDTA vacutainers (BD Diagnostics), and DNA was extracted using the FlexiGene DNA kit (Qiagen). Genotyping of the *APP* intron 7 tetranucleotide repeat was performed using a CEQ8000 Genetic Analyser (Beckman Coulter). Briefly, the region surrounding the repeat was amplified by PCR (Dye 4-labelled forward primer 5′CCATGTCGTTAACAGACTTCC3′, reverse primer 5′GAGTAGTTCATACTTCCTAC3′, both from Proligo). PCR enzymes and buffers were from Qiagen. Cycling was carried out as follows: 94°C for 2 minutes, 35 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute), 72°C for 10 minutes. This reaction produced a 105 bp product for the $attt_6$ allele. Labelled PCR products were combined with a 400 bp size standard and sample loading solution (Beckman Coulter) and processed on the CEQ8000 genetic analyser.

APOE genotyping was carried out using PCR (forward primer 5′TCCAAGGAGCTGCAGGCGGCGCA-3′ and reverse primer 5′-ACAGAATTCGCCCCGGCCTGGTACACTGCCA-3′, Sigma-Aldrich). AmpliTaq Gold enzyme and buffer (Applied Biosystems) and 10% DMSO (Sigma-Aldrich) were used, and cycling proceeded as follows: 94°C for 5 minutes, 45 cycles of 94°C for 30 seconds, 65°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes. The resulting product was digested with HaeII or AflIII (New England Biolabs). Fragments were separated on 1% standard agarose (Invitrogen Life Technologies) gels containing 3% Metaphor agarose (Cambrex).

2.3. Statistics. Statistical analysis of clinical and genotyping data was carried out using SPSS version 17. For the primary analysis we compared the age of onset of dementia in people with and without the $attt_6$ polymorphism using independent sample *t*-tests. The same evaluation was used to examine the impact of *APOE –ε4*.

3. Results

Two-hundred and ninety-one DNA samples were genotyped for the intron 7 repeat polymorphism $attt_{5–8}$ in *APP* and *APOE*, using fragment analysis with a CEQ8000 genetic analyser and restriction fragment length polymorphism, respectively. Clinical data for each participant was collected by the examining psychiatrists. The mean age of the participants was 52.20 years ±13.93 (range from 24 to 89 years), and 38% were female. Based upon the operationalized psychiatric evaluations, 103 participants had dementia (35%), with a mean age of onset of 50.46 years ±7.85 (range 34–74 years). A further 76 individuals were 45 or older, but did not meet operationalized criteria for a diagnosis of dementia. Thirty people (10.3%) were heterozygous for the $attt_{5–8}$ genotype, which is in agreement with frequencies found in previous genotyping studies [17, 18]. Of the 103 participants with dementia, 9 were heterozygous for the tetranucleotide repeat polymorphism, which is an appropriately sized sample when

considering the power calculation shown in Section 2. Due to variation in the peak sizes for the fragment analysis it is not possible to tell if the heterozygotes have one or two copies of the rarer alleles, so we have chosen to group all heterozygotes together based upon the presence of alleles $attt_5$, $attt_7$, or $attt_8$. This may be due to variations in amplification efficiencies between the different repeat alleles.

A significant difference was evident in the age of onset of dementia between individuals with 3 $attt_6$ alleles and those with any other allele combination ($n = 103$, independent samples t-test, $t = -2.65$, df $= 102$, $P = .009$) (a Kaplan-Meier plot is shown in Figure 1). The mean age of onset in individuals with 3 $attt_6$ alleles was 49.85 years ±7.81 whilst in people with a different repeat combination the mean age was 56.89 years ±5.09. None of the individuals with less than 3 $attt_6$ repeats developed dementia before 52 years.

We also examined the link between the $attt_{5–8}$ polymorphism and the risk of developing dementia before the age of 45. Only individuals who had dementia or had reached the age of 45 without developing dementia were included in this analysis, as individuals under the age of 45 and without dementia have not yet reached the high-risk period for dementia development. A trend was found between the risk of developing dementia before the age of 45 and the *APP* polymorphism ($n = 179$, $\chi^2 = 2.95$, df $= 1$, $P = .086$).

Gender was not related to the age of onset of dementia ($t = -1.19$, df $= 84$, $P = .24$). The presence of an *APOE-ε4* allele was not associated with the age of onset of dementia ($t = 0.49$, df $= 80$, $P = .62$). Neither gender nor the presence of an *APOE-ε4* allele were associated with $attt_{5–8}$ genotype ($\chi^2 = 1.22$, df $= 1$, $P = .27$ and $\chi^2 = 0.27$, df $= 1$, $P = .60$, resp.).

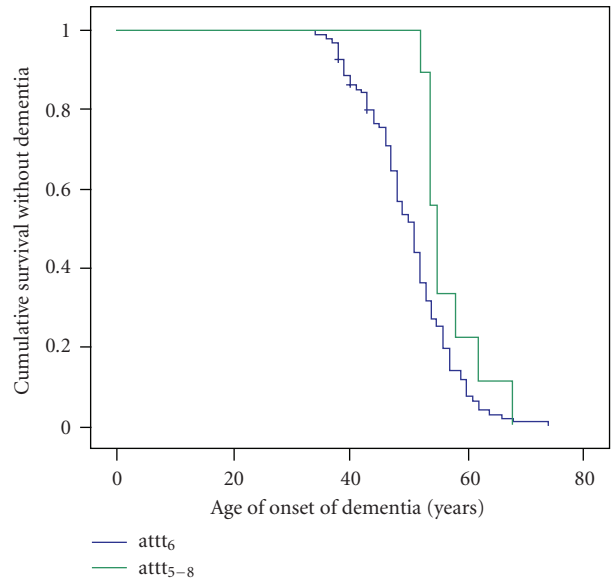

Figure 1: Kaplan-Meier survival plot showing the effect of the tetranucleotide $attt_{5–8}$ upon age of onset of dementia in 103 people with DS. Those people carrying three copies of the $attt_6$ allele of this polymorphism develop dementia with an earlier age of onset than those with any other combination of alleles ($P = .009$).

4. Discussion

These data highlight a striking association between 3 copies of the $attt_6$ allele in intron 7 of *APP* and a substantially earlier age of onset of dementia. People with 3 $attt_6$ alleles developed dementia 7 years earlier. The next step is to determine how the acceleration of dementia in DS is brought about in those people with 3 $attt_6$ alleles and to understand the implications for AD in the wider population, so that the related novel therapeutic opportunities can be fully utilized.

The tetranucleotide repeat region is located 1200 bp into the 2598 bp sequence of intron 7 and is 193 bp 3′ from an Alu sequence which is key for binding of splicing factors and splicing of exons 7 and 8 from APP [7, 21] (Figure 2). The tetranucleotide repeat lies in a 524 bp region between single nucleotide polymorphisms (SNPs) rs9982544 and rs2409162, but neither of these is polymorphic in the CEU population and so cannot be used for haplotype analysis of this region for our samples. The closest SNPs which are sufficiently polymorphic in the CEU population, rs3787637 and rs8132200, enclose a region of 2313 bp extending beyond intron 7, and further analysis of how the tetranucleotide repeat is associated with these will be required before we can understand the linkage of this location.

It is unclear why the 6-repeat allele is associated with risk when neither the 5- or 7-repeat alleles are, and why risk is not associated with increasing or decreasing repeat length although similar results have been found for other genes and disease risk: CYP19 and breast cancer risk, and MIF and gastric cancer risk [22, 23].

A further possibility is that the tetranucleotide repeat $attt_6$ is a marker for a functional polymorphism which is altering the expression, activity, or regulation of APP, which in turn alters the development of pathology and onset of dementia. Further examination of other SNPs in APP and how they are linked to $attt_6$ is required to understand fully the mechanism involved.

Levels of exon 7-containing isoforms of APP are found to be increased in AD brain [10] and associated with increased deposits of Aβ [24]. The protease inhibitor activity of the KPI domain has been shown to act on an extracellular trypsin-like serine protease which degrades secreted APP [25] and therefore, isoform variation may play an important role in regulating the activity of this protease. KPI-containing APP may also bind to low-density lipoprotein receptor-related protein (LRP), which is involved in the clearance of Aβ from the brain into peripheral tissues, and this has been shown to reduce Aβ clearance [26]. Receptors and other proteins involved in this clearance process may prove to be useful drug targets, and early intervention in this pathway may provide a method of preventing increased deposition of Aβ in the brain.

The role of this polymorphism, or other associated polymorphisms, in regulation of *APP* splicing is not known, and examining this will play an important role in determining how dementia risk is affected. It will also be important to

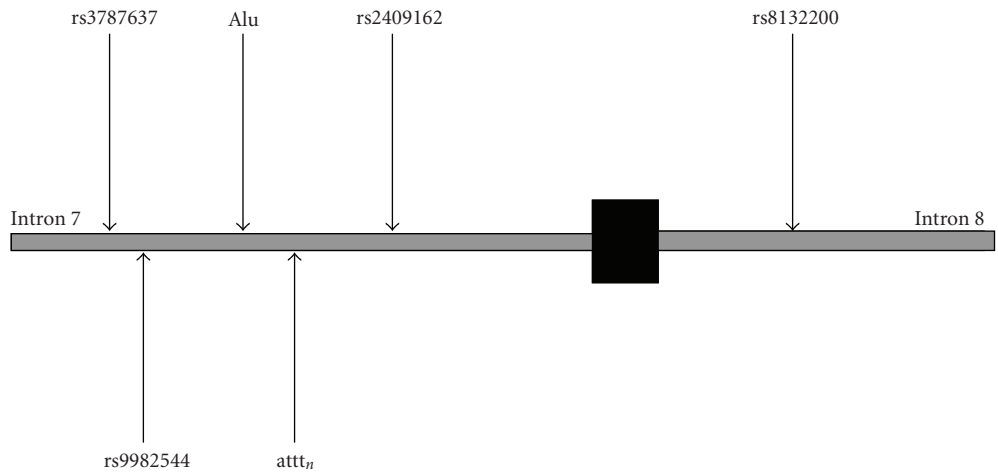

Figure 2: Diagrammatic representation of introns 7 to 8 of *APP* showing the locations of the tetranucleotide repeat, Alu sequence and closest SNPs. Grey bars indicate introns and black bar indicates exon 8. Figure is not to scale.

determine the impact of the tetranucleotide repeat upon Aβ deposition in the brain in postmortem studies. This will give an indication of how large a role this polymorphism plays in Aβ regulation.

The *APOE-ε4* allele had no effect upon the age of onset of dementia in this study. Previously it has been suggested that APOE does not affect the age of onset of dementia in cases of familial Alzheimer's disease (FAD) due to chromosome 14 mutations [27]. It may be that when APP regulation is strongly affected through triplication in DS or through particular mutations, APOE effects are masked by the presence of the more powerful APP effects. Triplication of *APP* in DS may have resulted in amplified effects of this polymorphism, but it may still have important potential implications for AD which have not yet been detected in the general population.

When this polymorphism was previously studied by Li et al. [17] the less common repeat sizes were only found in a small number of samples, which may result in low statistical power. Given that this polymorphism appears to take effect in a population with an increased risk of AD and increased expression of APP leading to increased Aβ levels, it would be valuable to examine the effects of this polymorphism in a second population with an increased incidence of AD and Aβ concentrations, namely those in the wider population over the age of 80. The incidence of AD in this age group is approximately 25%, which is similar to that for people with DS in their 40s, and those over the age of 80 also have increased brain Aβ 1–40 and 1–42 levels [28]. A large study in such a population would allow us to analyse the effects of this attt$_{5–8}$ in the general population at risk of developing AD.

In this study we have taken a unique opportunity to examine AD-type dementia in a population in which a high proportion of people will develop dementia with an early age of onset. This has allowed us to examine more closely the association of this polymorphism to the development and progression of dementia. The amplification of the effects of *APP* polymorphisms in DS allows us to detect differences which may be more subtle in the wider population.

Studies such as these not only provide the opportunity to discover therapies which may benefit those with DS, but also those in the older general population in which the incidence of AD is increasing. The benefits of studying the relatively small DS population must not be forgotten in the search for new therapies to tackle AD.

5. Conclusions

The attt$_{5–8}$ polymorphism in *APP* is associated with the age of onset of AD in DS, from which we conclude that it may function by accelerating the pathological process or is a marker for an, as yet unidentified, functional variant. The mechanism by which this is achieved has yet to be established, but the proteins involved may prove to be useful targets for future drug development.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

The authors would like to thank the individuals who participated in this study and their families, and the Alzheimer's Research Trust for funding the study. The authors would also like to acknowledge Dr. Chris Morris of the Institute for Ageing and Health, Newcastle General Hospital for his discussions.

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International Journal of Alzheimer's Disease
Volume 2011, Article ID 729382, 11 pages
doi:10.4061/2011/729382

Research Article

TrkB Isoforms Differentially Affect AICD Production through Their Intracellular Functional Domains

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Received 2 September 2010; Revised 12 November 2010; Accepted 16 November 2010

Academic Editor: E. A. Rogaeva

We report that *NTRK2*, the gene encoding for the TrkB receptor, can regulate APP metabolism, specifically AICD levels. Using the human neuroblastoma cell line SH-SY5Y, we characterized the effect of three TrkB isoforms (FL, SHC, T) on APP metabolism by knockdown and overexpression. We found that TrkB FL increases AICD-mediated transcription and APP levels while it decreases sAPP levels. These effects were mainly mediated by the tyrosine kinase activity of the receptor and partially by the PLC-γ- and SHC-binding sites. The TrkB T truncated isoform did not have significant effects on APP metabolism when transfected by itself, while the TrkB SHC decreased AICD-mediated transcription. TrkB T abolished TrkB FL effects on APP metabolism when cotransfected with it while TrkB SHC cotransfected with TrkB FL still showed increased APP levels. In conclusion, we demonstrated that TrkB isoforms have differential effects on APP metabolism.

1. Introduction

Alzheimer's Disease (AD) is a neurodegenerative disorder that will affect 15 million people in USA alone in the next ten years [1, 2]. The most common form of the disease is the late onset form (LOAD) that affects people older than 65. LOAD is caused by a complex interaction of risk factors including age, genetics, and environmental factors, such as level of education, diet, and physical activity [3–7].

The accumulation of Aβ, a neurotoxic product of amyloid precursor protein (APP) cleavage, is central to AD pathogenesis [8–10]. This accumulation causes synaptic dysfunction and eventually neuronal death [9, 10]. Therefore proteins that affect APP metabolism and synaptic function are likely to be important in AD pathogenesis.

TrkB is a member of the tyrosine kinase receptor family (Trk). TrkB specifically binds Brain-Derived Neurotrophic Factor (BDNF). The neurotrophin receptors (TrkA, TrkB, and TrkC) are important in neuronal development and synaptic function [11, 12]. Levels of TrkA, TrkB, and TrkC, but not $p75^{NTR}$, are downregulated in AD brain samples [13]. Trk downregulation has been proposed as a biomarker of AD progression since Trk mRNA levels correlate with the degree of cognitive impairment [13].

Further evidence for a role of TrkB in AD is the fact that TrkB can modulate APP levels and proteolysis. Expression of the longest TrkB isoform, full-length TrkB (TrkB FL), can increase APP promoter transcription and promotes accumulation of sAPP-α [14–17]. Conversely, Aβ has been found to reduce TrkB FL/BDNF levels and to impair TrkB-mediated signaling [18–21]. These results suggest a dynamic interaction between TrkB/BDNF signaling and APP metabolism.

Interestingly, knockdown of another splice variant of TrkB, truncated TrkB (TrkB T) in a mouse model of Down syndrome rescued neuronal death [22]. Conversely, mice overexpressing TrkB T display synaptic dysfunction and long-term potentiation defects [23].

The gene encoding TrkB, *NTRK2*, is located on chromosome 9, specifically 9q22. This region has been genetically linked to AD [24, 25]. Despite the experimental evidence functionally linking TrkB signaling to APP metabolism and synaptic function, case-control and genomewide association studies of *NTRK2* single nucleotide polymorphisms (SNPs)

found no significant association with AD [25–30]. One family-based study did observe genetic association of *NTRK2* haplotypes with AD [26].

Three major splice variants of TrkB are expressed in neurons, TrkB FL, TrkB SHC, and TrkB T. We hypothesized that these different TrkB isoforms differentially affect APP metabolism and could play a role in the pathogenesis of AD. The aim of this work was to test this hypothesis.

The three TrkB splicing isoforms we investigated share an extracellular BDNF binding domain and differ in their cytoplasmic-domain (Figure 1) [31]. Two splice variants encode full-length receptors, TrkB full-length (FL), that contain a tyrosine kinase domain, an SHC-binding domain and a PLC-γ-binding domain in the intracellular portion [32, 33]. Two isoforms encode shorter receptors, TrkB SHC, that contain only an SHC-binding domain, and the remaining isoform is a truncated receptor, TrkB T, that does not have any known intracellular functional domain (Figure 1). Recently, isoforms differing in the extracellular domain have also been identified [34].

We used a previously described cell-based functional screen [35] to identify putative APP metabolism regulators. We found that *NTRK2* knockdown altered both AICD-mediated luciferase activity and APP full-length levels. To characterize the role of TrkB FL and truncated isoforms we knocked down and overexpressed the isoforms in an SH-SY5Y neuroblastoma cell line overexpressing APP as a fusion protein with the yeast transcription factor Gal-4 [35]. We then measured APP FL levels and proteolytic products using Western blots and luciferase assays.

Our results demonstrate for the first time that TrkB isoforms differentially affect APP metabolism. Specifically, overexpression of TrkB FL increases AICD-Gal4-mediated luciferase activity. While overexpression of TrkB T does not alter the luciferase activity and TrkB SHC decreases the luciferase activity compared to control. We determined that the tyrosine kinase and PLC-γ functional domains contribute to the observed TrkB FL-mediated effects. We also found that the SHC-binding site contributed to the observed TrkB-SHC-mediated effects. BDNF stimulation of the exogenously expressed TrkB receptors amplified the APP metabolism effects and cotransfection of the TrkB-truncated isoforms with TrkB FL altered the effects on APP metabolism.

2. Materials and Methods

2.1. Constructs and Site-Directed-Mutagenesis. Four shRNA-containing plasmids specific for *NTRK2* were obtained from the pSM2 retroviral Library of the Drexel RNAi Resource Center purchased from Open Biosystems. The constructs ID numbers are NTRK2.1: 1920; NTRK2.2: 2295; NTRK2.3: 29734; NTRK2.4: 30795. We also used APP (ID 39147) and luciferase targeting shRNA (RHS1705) as positive controls and a scrambled shRNA sequence (non-silencing, NS, RHS1707) as a negative control. The TrkB full-length and truncated GFP fusion constructs and the GFP-F control overexpression plasmid were kindly donated

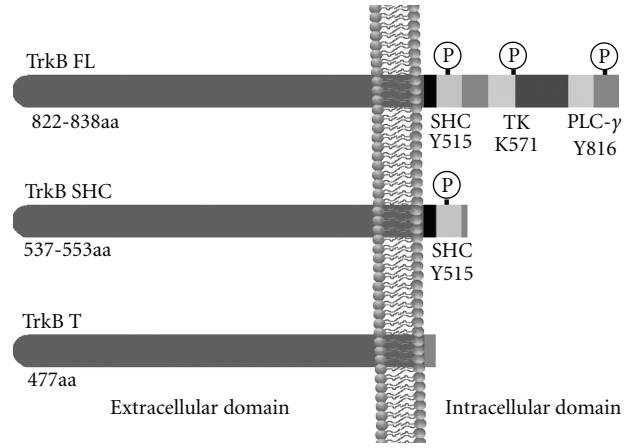

Figure 1: TrkB isoforms structure and functional domains. The extracellular portion of the receptor is conserved. The intracellular domain differs among the isoforms. TrkB FL contains a tyrosine kinase domain, a SHC-binding domain, and a PLC-γ-binding domain. TrkB SHC contains a SHC-binding domain and TrkB T does not have any functional domain.

by Dr. Eero Castren (University of Helsinki, Finland) and were previously described [36, 37]. Site-directed mutagenesis (Stratagene, Quikchange mutagenesis kit) was utilized to generate point mutants on the TrkB full-length receptor functional domains. Mutagenesis was carried out according to manufacturer's instructions and the primers employed are reported in Table 1 (the bolded sequences represent the mutations/insertion). The mutant amino acid indicated refers to the amino acidic sequence of TrkB. Therefore TrkB FL K571M indicates the tyrosine kinase dead receptor since it is mutated on the ATP-binding site; TrkB FL Y515F indicates the receptor mutated on the SHC-binding site; TrkB FL Y816F indicates the receptor mutated on the PLC-γ-binding site [38]. Note that in some literature the TrkB mutants are referred to with the numbering of the amino acidic sequence of TrkA, the NGF receptor, that has functional sites in common with TrkB and therefore are referred to as K560M, Y490F and Y785F respectively [32, 33]. TrkB SHC indicates the other human truncated isoform (isoform d and e, NCBI Gene NM_001018064.2 and NM_001018066.2). TrkB SHC was obtained by insertion of the exon 19 followed by a STOP codon after the SHC-binding site on the TrkB FL constructs. After obtaining the TrkB SHC isoform by insertion, the SHC-binding site was mutated on that isoforms using the same primer sequence employed for the TrkB FL mutant on the SHC-binding site (TrkB Y515F).

Successful mutations were identified by sequencing. One clone per construct was transformed in *E. coli* (DH5-α competent cells, InVitrogen). Transformed bacteria were selected on 100 μg/mL ampicillin LB-agar plates and liquid cultures were grown overnight at 37°C. Bacterial cultures were miniprepped (MiniPrep Kit, Quiagen) and used for transfection after DNA quantification.

2.2. Cell Culture and Transfection. SH-SY5Y cells stably transfected with UAS-Luciferase and APP-Gal4 described

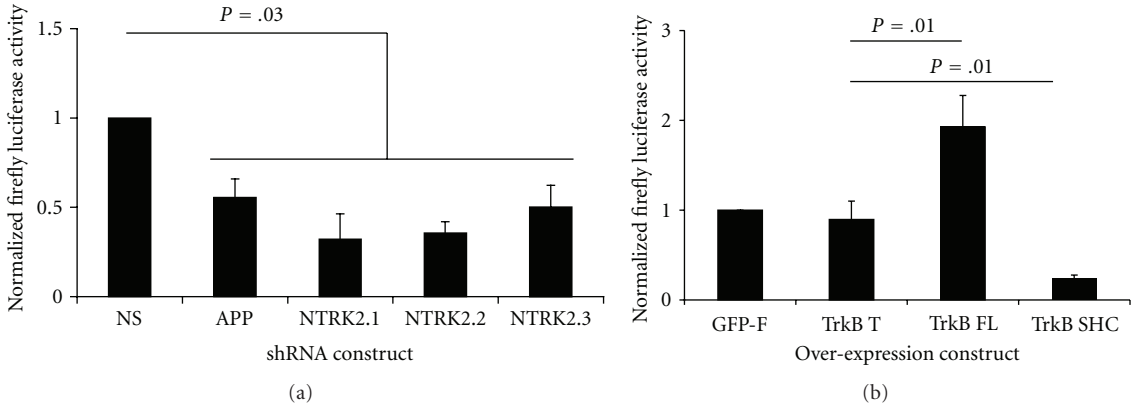

Figure 2: AICD-mediated Luciferase activity in SH-SY5Y-APP-Gal4 cells with TrkB knockdown or TrkB overexpression. (a) shRNA-mediated NTRK2 knock-down. Luciferase activity is decreased by *NTRK2* knockdown compared to nonsilencing control ($P = .03$). There is no significant difference between luciferase activity in NTRK2 knockdown and APP knockdown cells. shRNA constructs: negative control, NS, scrambled shRNA sequence; positive control, APP, shRNA targeting APP; experimental shRNA, NTRK2.1-3, shRNA constructs targeting all TrkB isoforms. The experiment was repeated four times independently and each time with six replicates. (b) TrkB isoform overexpression. Luciferase activity was not altered by TrkB T compared to GFP control. Luciferase activity was increased by TrkB FL ($P = .01$) and decreased by TrkB SHC ($P = .01$) compared to a GFP-control vector. The experiment was repeated 3 to 6 times independently with 6 replicates each time. The *y*-axis represents the arbitrary light units of firefly luciferase normalized to cell number (a) or transfection efficiency (b). Error bars = SEM. Statistical analysis: ANOVA and Students *t*-test with Bonferroni correction where applicable.

Table 1: Primer sequences used to obtain the point mutations and the sequence insertion on the TrkB FL receptor. The mutated bases are represented in bold.

Primers	Sequence 5′ to 3′
TrkB.Y490F.F	GTCATTGAAAACCCCCAGT**T**CTTCGGTATCACCAACAG
TrkB.Y490F.R	CTGTTGGTGATACCGAAG**A**ACTGGGGGTTTTCAATGAC
TrkB.Y816F.F	GCGTCGCCCGTCT**T**CCTGGACATCCTAG
TrkB.Y816F.R	CGCAGCGGGCAGA**A**GGACCTGTAGGATC
TrkB.Shc.F	CTCAAGCCGGACACA**TGGCCCAGAGGTTCCCCCAAGACCGCCTGATAGTAA**TTTGTTCAGCACATC
TrkB.Shc.R	GATGTGCTGAACAAA**TTACTATCAGGCGGTCTTGGGGGAACCTCTGGGCCA**TGTGTCCGGCTTGAG
TrkB K560M.F	GGTGGCCGTGA**T**GACGCTGAAGG
TrkB K560M.R	CCTTCAGCGTC**A**TCACGGCCACC
TrkB.SHC	GTCATTGAAAACCCCCAGT**T**CTTCGGTATCACCAACAG
TrkB.SHC	CTGTTGGTGATACCGAAG**A**ACTGGGGGTTTTCAATGAC

before [35] were maintained in DMEM (Gibco) supplemented with 10% FBS, penicillin streptomycin and 200 μg/mL G418 (Gibco).

To assess the effects of TrkB knockdown or overexpression on AICD-Gal4 mediated luciferase we used the following transfection protocol previously described [35]. Briefly, one day before transfection cells were plated in 96-wells plates at approximately 40–50% confluency. The day of transfection media was removed from the cells and replaced with transfection media: 100 μL of serum free DMEM media containing 2 μg/well Arrest-In (Open Biosystems) and 0.2 μg/well plasmid DNA. The pSM2 plasmids referred to as NTRK2.1 through 4 were transfected in the cells. Cells were also transfected with shRNA targeting APP, luciferase and a control shRNA that contains a scrambled sequence that does not target any human gene. In addition a mock transfection, containing only Arrest-In was performed to control for selection effectiveness. 6 replicate wells per shRNA constructs and mock control transfection were set up for each independent experiment. The transfection media was left on the cells for 8 hours and then replaced with complete media. 48 hours after transfection, transfected cells were selected with 4 μg/mL puromycin (Sigma) in 10% FBS DMEM with 200 μg/mL G418. Puromycin selection was used to preserve transiently transfected cells. The media was changed every 48 hours and cell death was monitored and compared to the mock-transfected control. Once all the cells in the mock control wells were dead, surviving cells in the shRNA transfected wells were split and transferred to another 96-well plate and a 24-well plate. Cell lysates were collected from 60–80% confluent 96-wells 11–13 days after transfection in 100 μL Glo Lysis Buffer per well (Promega). Lysates were used immediately after collection or frozen prior to performing Steady Glo luciferase assays (Promega). The luciferase assays were performed as per manufacturer's instructions. shRNA-mediated knockdown effectiveness was

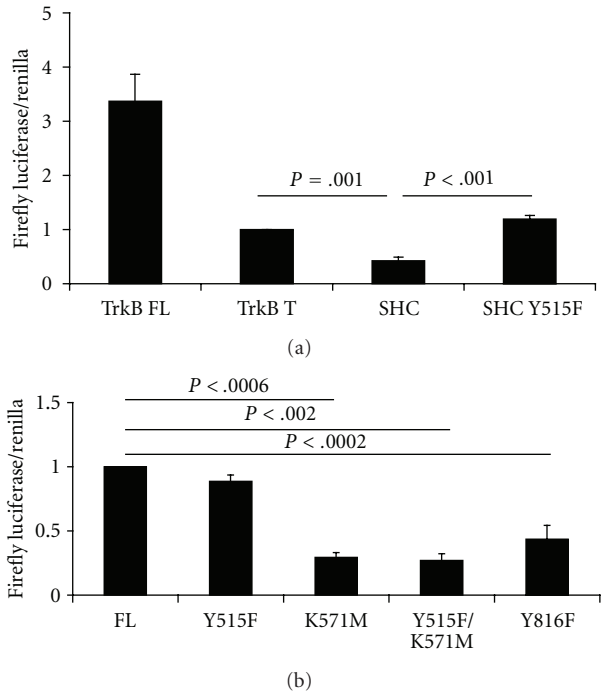

Figure 3: Mutation of TrkB FL intracellular functional domains changes the effect on AICD-mediated luciferase activity. (a) The TrkB SHC isoform significantly decreases luciferase activity when compared to TrkB T ($P = .001$). The TrkB SHC Y515F mutant, unable to bind SHC, increases luciferase activity compared to TrkB SHC ($P < .001$) and does not significantly alter luciferase activity compared to TrkB T ($P = .1$). (b) TrkB Y515F (no SHC-binding) does not significantly alter luciferase activity compared to TrkB FL. TrkB K571M with an inactive tyrosine kinase domain significantly decreases luciferase activity compared to TrkB FL ($P = .0006$); TrkB Y515F/K571M significantly decreases luciferase activity compared to TrkB FL ($P = .002$) but not to a greater extent of TrkB K571M. TrkB Y816F that does not bind PLC-γ also significantly decreases luciferase activity compared to TrkB FL ($P = .0002$). 4–8 independent experiments with 6 replicates each, error bars SEM. 4–6 independent experiments with 6 replicates each, error bars SEM. Statistical analysis: Student's t-test with Bonferroni correction.

monitored by comparing the luciferase signal of the non-silencing control shRNA with the APP targeting shRNA. After assessing successful knock-down, luciferase data for the experimental shRNA targeting NTRK2 was collected and analyzed. In parallel, 24-well plates and 12-well plates were seeded with the same cells that had been assayed for luciferase signal and collected for Western Blot analysis.

The same transfection procedure was followed for the overexpression experiments, but lysates were collected 48 hours after transfection and transfection efficiency was monitored by fluorescence microscopy, no antibiotic selection was performed in this case.

2.3. Western Blotting Procedures. Conditioned media was collected from the cells (48 hours after transfection) in eppendorf tubes and centrifuged at 14,000 rpm for 10 minutes at 4°C (Beckman Coulter, Microfuge 22R). The resulting supernatant was collected and 142 μL were mixed with 33 μL of 4X Reducing loading buffer (InVitrogen) supplemented with 0.4% β-mercaptoethanol (Sigma). These samples were heated at 70°C for 10 minutes. The remaining conditioned media was stored at −20°C for later Western Blot analysis.

Whole cell lysates were collected (48 hours after transfection) by lysing the cells with ice-cold radio immunoprecipitation (RIPA) buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 1% SDS, 50 mM Tris, pH 8.0) supplemented with Halt cocktail of protease and phosphatase inhibitors (ThermoScientific). Cell lysates were sonicated in an ice-cold water bath sonicator for 6 minutes then centrifuged 20 minutes at 4°C at 14,000 rpm. The resulting supernatants were collected and protein concentration measured with a BCA protein concentration kit (Pierce) according to manufacturer's instructions.

Western blot samples were prepared at a final concentration of 1-2 μg/μL in 4X reducing loading buffer (InVitrogen) and heated at 70°C for 10 minutes. 15–25 μg of total protein/well were separated on 4–12% Tris-Glycine midi gels (InVitrogen) in MES-SDS running buffer (InVitrogen) and run at 190 mVolts for 45 minutes. The separated proteins were transferred to PVDF FL membranes (Millipore) in a Semi-Dry transfer apparatus (AA Hoefer TE77X) for 3 hours at 125 milli Amp per gel.

Membranes were blocked one hour at room temperature using Licor blocking buffer then probed overnight with primary antibodies diluted in Licor blocking buffer at 4 or 25°C. Membranes were then washed for 5 minutes 4 times with 0.1% Tween (Sigma) in PBS. One last wash was performed with PBS to rinse off the detergent. After washing, membranes were incubated in the dark with the appropriate secondary antibody IRDye (Licor) diluted in Licor blocking buffer for one hour. Again membranes were washed as above and finally rinsed with PBS. Membranes were scanned on an Odyssey InfraRed scanner (Licor) at appropriate intensities and images acquired. Band intensities were quantified with the provided in-built software (Licor) and always normalized to the actin loading control. When conditioned media was analyzed the signals were normalized to the protein concentration of the corresponding lysates.

2.4. Antibodies. Detection of TrkB-GFP tagged constructs utilized mouse anti-GFP antibody (1 : 1000, Living Colors, Clontech); detection of APP full-length and C-terminal fragments utilized A8717 rabbit antibody (1 : 2000, Rb, Sigma); detection of sAPP 22C11 utilized mouse antibody (1 : 1000, Millipore); detection of sAPP-α 6E10 utilized mouse antibody (1 : 1000, Covance); detection of actin A5441 utilized mouse antibody (1 : 15,000, Sigma). The secondary antibodies: IRDye700 anti-mouse antibody (1 : 15,000) and IRDye800 anti-rabbit antibody (1 : 15,000) were obtained from by Licor.

3. Results

3.1. NTRK2 Knockdown Decreases AICD-Mediated Luciferase Activity. We applied our functional screening method [35]

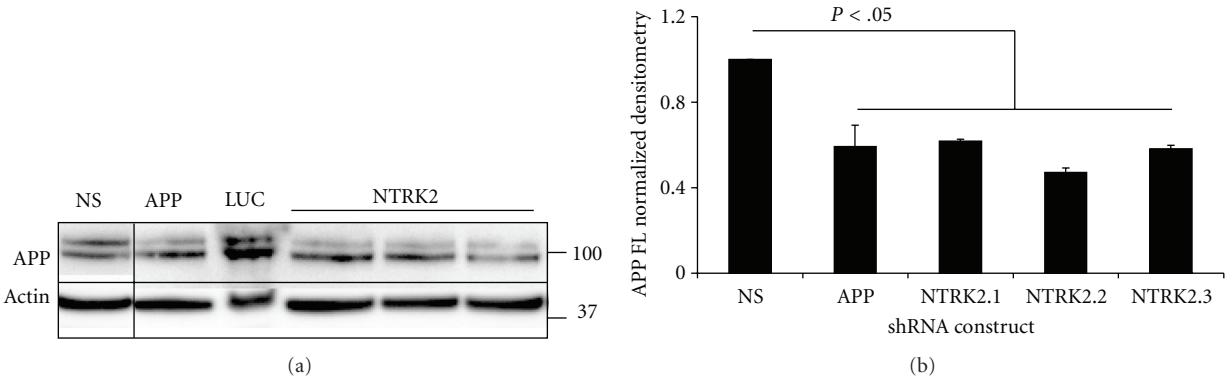

Figure 4: NTRK2 knockdown decreases APP full-length levels. (a) Representative Western Blot of APP full-length and actin as a loading control in lysates from cells transfected with shRNA constructs targeting *APP*, *luciferase* (LUC), *NTRK2* and a negative, nonsilencing (NS), control. (b) Average quantification of APP full-length levels from four independent Western Blot analysis conducted on independent transfections. Within each transfection APP FL densitometry was normalized to the APP FL densitometry of the cell lysate obtained from cells transfected with CTRL shRNA. A statistically significant difference was observed between CTRL and the APP or NTRK2 shRNA targeting constructs ($P < .05$). The experiment was repeated four times independently with three replicates each, error bars indicate SEM. Statistical analysis: ANOVA.

to all the genes in the linkage region on chromosome 9 that displays a high likelihood of disease score for AD [39]. This screening is conducted in SH-SY5Y cells stably transfected with a luciferase reporter driven by the yeast UAS promoter and APP fused to Gal4. When APP is cleaved by the secretases, the AICD-Gal4 domain is released and can activate the transcription of the luciferase reporter. Variations in AICD-mediated luciferase activity are measured. Since changes in AICD-mediated luciferase activity can occur through a variety of mechanisms affecting APP, this is an effective and general way of identifying regulators of APP metabolism [35]. We targeted *NTRK2* with 4 different shRNA constructs (see supplementary Figures 1 and 2 in the supplementary material available online at doi: 10.4061/2011/729382). Three shRNAs targeted all the TrkB isoforms (NTRK2.1-3) and one (NTRK2.4) targeted all the isoforms except the TrkB T. We also transfected a nonsilencing scrambled shRNA (NS) that does not target any human gene as a negative control and a shRNA targeting APP as a positive control. Of the four transfected constructs NTRK2.1-3 decreased AICD-mediated luciferase to the same extent of the APP targeting shRNA compared to the NS shRNA (Figure 2(a)). The fourth construct, NTRK2.4 targeting all TrkB isoforms except TrkB T, consistently caused cell death (data not shown). This result suggests that *NTRK2* can affect APP metabolism and that the isoforms have different roles since downregulation of all the isoforms except TrkB T was lethal. Therefore we investigated the effect of the single isoforms in the same experimental model.

We transiently transfected individual TrkB isoform overexpression constructs in the cells and measured AICD-mediated luciferase activity. We found that there was no difference in AICD-mediated luciferase activity between TrkB T and the GFP-control, while TrkB FL significantly increased luciferase activity ($P = .01$) and TrkB SHC significantly decreased it ($P = .01$) (Figure 2(b)). These results demonstrate that TrkB isoforms have different effects on APP metabolism. Moreover, we show that there is a difference between the isoforms TrkB SHC and TrkB T, even though both isoforms lack the tyrosine kinase domain.

3.2. The Tyrosine Kinase and PLC-γ-Binding Domains of TrkB FL and the SHC-Binding Domain on TrkB SHC Determine the Effect on AICD-Mediated Luciferase Activity . TrkB T did not alter AICD-mediated luciferase activity compared to the GFP-F control, while TrkB SHC decreased it and TrkB FL increased it. We hypothesized that the intracellular domains of the TrkB SHC and TrkB FL are responsible for the effects observed. To determine which domain was responsible for this effect, we mutated each cytoplasmic functional domain individually.

We generated a mutant of the TrkB SHC isoform that cannot bind SHC (Y515F). We transfected this mutant and the other TrkB wild-type isoforms, into SH-SY5Y-APP-Gal4 cells and measured AICD-mediated luciferase activity. We observed that TrkB SHC Y515F (SHC-binding site mutant) does not significantly alter luciferase activity compared to TrkB T but significantly increased it compared to the TrkB SHC wild-type isoform ($P < .001$) (Figure 3(a)). Therefore, disrupting the SHC-binding site on the TrkB SHC isoform impairs its ability to decrease AICD-mediated luciferase activity.

We then generated mutants in the three functional sites of the TrkB FL. We mutated the SHC-binding site (Y515F) to generate a mutant that cannot bind SHC [33]. Then we mutated the ATP-binding site (K571M) to generate a TrkB FL tyrosine kinase inactive receptor K571M [32, 33]. Similarly, we disrupted the PLC-γ-binding site by introducing the mutation Y816F. We also generated a double mutant that is both tyrosine kinase inactive and does not bind SHC (TrkB Y515F/K571M). We then transfected these TrkB FL mutant constructs in SH-SY5Y-APP-Gal4 cells. We measured the AICD-mediated luciferase activity and compared it to TrkB FL wild-type (Figure 3(b)).

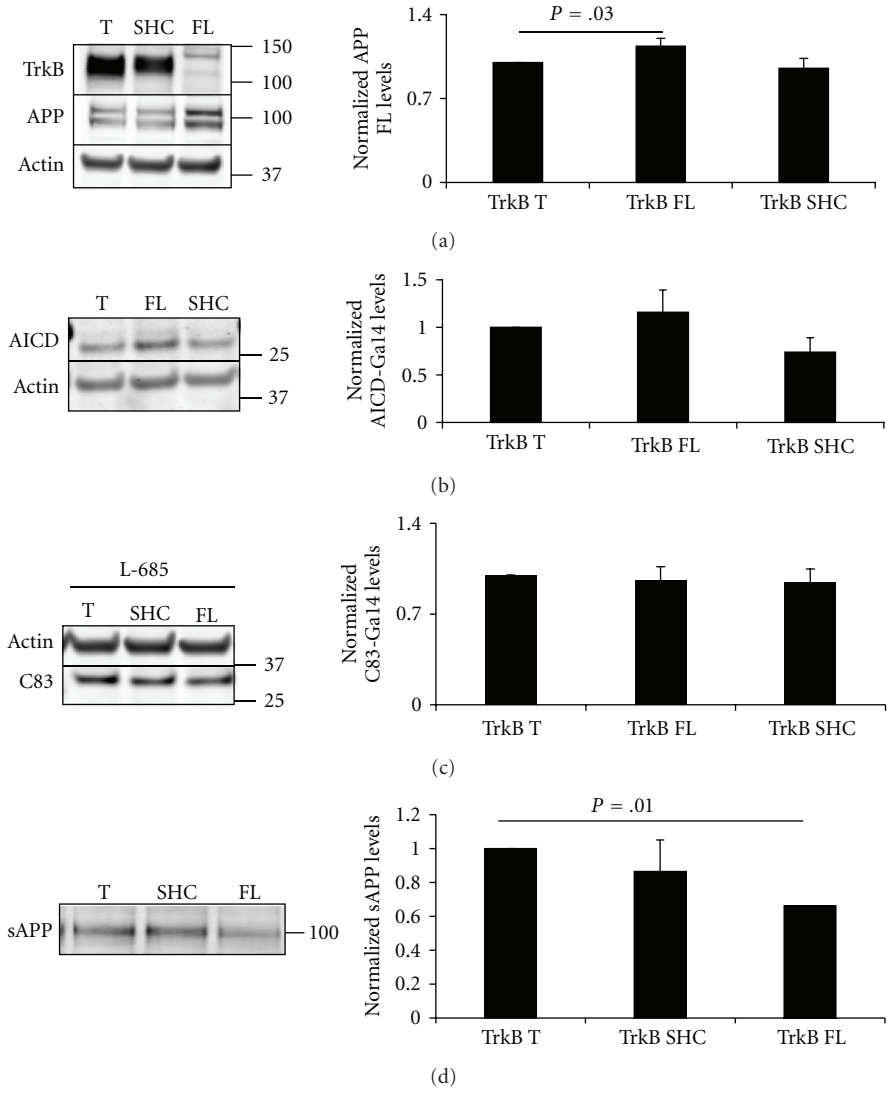

Figure 5: TrkB isoform overexpression affects APP FL levels and APP processing. (a) TrkB FL increases APP FL levels compared to TrkB T ($P = .03$) and TrkB SHC isoforms ($P = .008$). 5 independent experiments in duplicate, error bars indicate SEM. (b) TrkB FL increases AICD levels compared to TrkB T and TrkB SHC decreases AICD levels. (c) TrkB FL, TrkB T, and TrkB SHC do not alter C83 levels. Cells were treated with L-685,485 to aid in C83-Gal4 visulaization (d) sAPP levels in conditioned media. TrkB FL significantly decreases sAPP levels compared to TrkB T ($P = .003$). The experiment was repeated four times independently in duplicate. Error bars indicate SEM. Statistical analysis: Students t-test with Bonferroni correction.

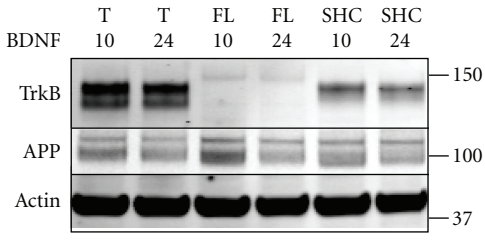

Figure 6: Short-term or long-term BDNF treatment of TrkB isoforms transfected cells. APP full-length (FL) levels are increased by a 10 minutes BDNF treatment of TrkB FL transfected cells while a 24 hours treatment shows lower APP FL levels. TrkB T and TrkB SHC transfected cells are not significantly affected by short term or long-term BDNF treatment.

The TrkB Y515F mutant (preventing SHC-binding) did not significantly alter AICD-mediated luciferase activity compared to TrkB FL (Figure 3(b)). The TrkB FL K571M (tyrosine kinase inactive) significantly decreased luciferase activity compared to TrkB FL ($P = .0006$). TrkB FL Y816F, (preventing PLC-γ-binding) also significantly decreased luciferase activity compared to TrkB FL ($P = .0002$). The double mutant TrkB Y515F/K571M (preventing SHC-binding and tyrosine kinase inactive) significantly decreased luciferase compared to TrkB FL ($P = .002$) but did not differ from the tyrosine kinase inactive TrkB K571M (Figure 2(a)).

In summary, TrkB FL overexpression increases AICD-Gal4 mediated luciferase activity two-fold compared to

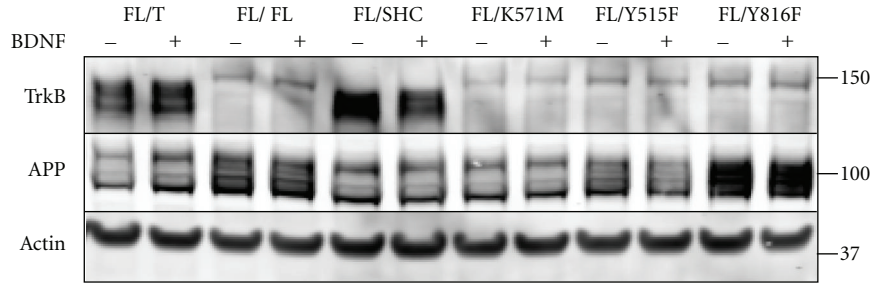

Figure 7: Cotransfection of TrkB FL with isoforms and mutants alters the TrkB FL effects on APP full-length. TrkB FL transfection increases APP FL levels compared to when TrkB FL is cotransfected with TrkB T, TrkB SHC or TrkB K571M. APP FL levels did not significantly differ between TrkB FL transfected cells and TrkB FL cotransfection with TrkB Y515F or TrkB Y816F. 24 hours BDNF treatment of cotransfected cells did not significantly alter APP FL levels compared to cotransfected but nontreated cells.

controls TrkB T (Figure 2(b)). The tyrosine kinase inactive mutant receptor, TrkB K571M, the PLC-γ-binding site mutant, and the TrkB SHC isoform mutated on the SHC-binding site also cause a 60–70% decrease in AICD-mediated luciferase activity compared to the TrkB FL (Figures 3(a) and 3(b)). The TrkB SHC wild-type isoform causes an AICD-mediated luciferase decrease of about 90% (Figure 2(b)).

3.3. NTRK2 Knockdown Decreases APP FL Levels. The effects we observe on AICD-mediated luciferase activity can occur through many different mechanisms: decreased APP transcription, increased APP degradation, decreased APP cleavage, destabilization of AICD, and trafficking that affects APP localization. Anything that decreases AICD levels will be reflected in a decrease in luciferase activity. The most immediate way of decreasing AICD levels is to decrease APP levels. To determine if *NTRK2* knockdown decreased APP levels, we tested if APP levels were altered. We transfected the *NTRK2* targeting shRNA, a CTRL shRNA, and an APP targeting shRNA as a positive control. As an additional control we used shRNA targeting the luciferase gene: this construct accounts for overexpression of shRNA that have to be processed by the endogenous RNAi machinery. We then measured APP protein levels by Western blot (Figure 4(a)). We found that knockdown of all the TrkB splice variants cause a significant decrease in APP FL levels ($P < .05$) (Figure 4(b)) and we concluded that decreased APP levels might be at least partially responsible for the observed reduction in luciferase activity.

3.4. TrkB FL Overexpression Increases APP FL Levels and AICD Gal4 Levels. Based on the previous knockdown results, we then hypothesized that overexpression of TrkB FL causes increased AICD-mediated luciferase activity by increasing APP FL levels. We transfected the TrkB isoforms in the cells, performed Western blot analysis and quantified APP FL levels in cell lysates. Overexpression of TrkB FL significantly increased APP FL levels compared to TrkB T ($P = .03$) and TrkB SHC ($P = .008$) (Figure 5(a)). There was no difference in APP levels between TrkB T- and TrkB SHC-transfected cells (Figure 5(a)).

We then verified that AICD-Gal4 levels in TrkB FL-transfected cells correlated to the observed increase in luciferase activity. AICD-Gal4 is the intracellular domain of APP that is, generated by γ-secretase cleavage, translocates to the nucleus and activates transcription. We found that, as expected, TrkB FL displayed increased AICD-Gal4 levels compared to TrkB T, but this difference was not statistically significant. Compared to TrkB T, TrkB SHC overexpression resulted in a decrease in AICD-Gal4 levels, as expected, but this difference was not statistically significant (Figure 5(b)). Interestingly, we consistently observed TrkB FL lower levels compared to TrkB T and TrkB SHC in our Western blot analysis (Figure 5(a)).

3.5. TrkB FL Overexpression Decreases sAPP Levels without Altering C83 Levels. To assess changes in APP proteolysis we measured APP C-terminal fragments (CTFs) and sAPP levels upon TrkB transfection. CTFs include both C83 and C99. C83 and C99 are generated by the cleavage of APP by α-secretase and β-secretase, respectively. In our cell line we measure C83-Gal4 and C99-Gal4 levels since APP overexpressed is a fusion protein with Gal4. These fragments are the precursors of AICD, that is, released in the cytoplasm by γ-secretase cleavage [40, 41]. While C83 and C99 are membrane-bound fragments of APP, the soluble N-terminal fragment of APP, sAPP, generated by α/β-secretase cleavage is released in the extracellular environment. In SH-SY5Y cells, the β-secretase cleavage occurs to a much lower extent than α-secretase cleavage. Therefore, the majority of the luciferase signal observed is due to AICD-Gal4 generated from γ-secretase cleavage of C83-Gal4. If the AICD-Gal4 levels are increased, as measured by luciferase and Western blot, then the levels of its precursor C83-Gal4 should also be increased.

We then tested the hypothesis that C83-Gal4 and sAPP levels are increased by TrkB FL overexpression and decreased by TrkB SHC. To aid detection of C83-Gal4 we treated cells with the γ-secretase inhibitor L-685,485. We did not detect a difference in C83-Gal4 levels among the cells transfected with the different TrkB isoforms (Figure 5(c)). Surprisingly, TrkB FL decreased sAPP levels compared to TrkB T ($P = .01$). TrkB SHC showed a nonsignificant difference in sAPP levels compared to TrkB T (Figure 5(d)).

3.6. BDNF Treatment of TrkB Isoforms Does Not Significantly Alter Their Effects on APP Metabolism. All three TrkB

isoforms studied here are capable of binding BDNF [12]. Moreover, it has been previously shown that TrkB FL BDNF-mediated intra-cellular signaling can alter APP metabolism [14–17]. We hypothesized that application of exogenous BDNF would stimulate the TrkB FL-mediated effects on APP FL and proteolytic products levels. We then tested this hypothesis applying BDNF to cells transfected with TrkB isoforms and measured the levels of APP FL by Western blot. We found that short term (10 minutes) BDNF application increases APP FL levels in cells transfected with the TrkB T or TrkB SHC isoforms and to a greater degree in cells that had been transfected with TrkB FL (Figure 6). Twenty-four hour BDNF treatment of TrkB FL transfected cells did not further increase APP FL levels compared to short-term BDNF treatment.

3.7. Cotransfection of the TrkB Isoforms Modulates TrkB FL-Mediated Effects on APP Metabolism. It has been previously shown that TrkB T has a dominant negative effect on TrkB FL [42]. We hypothesized that cotransfection of TrkB T with TrkB FL would eliminate the TrkB FL effects on APP metabolism observed when we transfect TrkB FL alone. Moreover we hypothesized that cotransfection of the TrkB SHC with TrkB FL would also have dominant negative effect on TrkB FL. Finally we hypothesized that cotransfection of TrkB FL with TrkB Y515F or TrkB Y816F would not significantly alter the effects seen on APP since they seem to be primarily mediated by the tyrosine kinase domain and not by the SHC-binding domain. For this reason we also hypothesized that cotransfection of TrkB FL with TrkB K571M (TrkB FL/K571M) would have the same effect as the cotransfection of TrkB FL and TrkB T (TrkB FL/T).

Consistent with our hypothesis, TrkB FL/T cotransfection did not increase APP FL levels, nor did cotransfection of TrkB FL/K571M, the tyrosine kinase inactive mutant (Figure 7). Also, as expected, there was very little difference between the APP FL levels in cells transfected with TrkB FL/Y515F, TrkB FL/Y816F, and TrkB FL/FL. Surprisingly, cotransfection of TrkB FL/SHC increased APP FL levels compared to TrkB FL/T cotransfection but not compared to TrkB FL/FL (Figure 7).

We also hypothesized that BDNF treatment of the cotransfected cells would affect the transfected isoforms mediated effects on APP. Surprisingly BDNF treatment did not significantly alter these effects of the cotransfected TrkB receptors.

In summary, both truncated isoforms were able to decrease APP FL levels compared to TrkB FL/FL transfection; TrkB T to a greater extent than TrkB SHC. The tyrosine kinase inactive receptor decreased APP FL levels to the same extent of TrkB FL/T cotransfection while TrkB FL/Y515F and TrkB FL/Y816F cotransfection did not alter APP FL levels compared to TrkB FL/FL.

4. Discussion

We investigated the role of the TrkB isoforms on APP metabolism in SH-SY5Y cells overexpressing an APP-Gal4 fusion protein that can transactivate a luciferase reporter gene [35]. This system monitors changes in APP metabolism that are reflected in altered AICD-mediated transcription of the luciferase gene [35].

We found that knockdown of all TrkB isoforms in SH-SY5Y-APP-Gal4 cells caused a decrease in AICD-mediated luciferase activity. This decrease is probably due to a decrease in APP levels observed in cells with *NTRK2* knockdown. We hypothesize that decreased APP levels in this system are mainly due to increased APP degradation caused by altered trafficking in absence of TrkB. Transcriptional downregulation of APP might be partially responsible for the decreased signal observed in the Western Blot but that is only possible for the endogenous APP. Because the endogenous *APP* gene is under the physiologic transcriptional regulation, while the APP-Gal4 overexpressed is under CMV promoter regulation.

Concomitant knockdown of the TrkB FL and SHC isoforms lead to cell death, and this is consistent with the finding that TrkB T is one of the causes of neuronal death in a mouse model of trisomy 21 [43].

To discriminate between the effects of the different isoforms, we overexpressed one isoform at a time and measured the resulting AICD-mediated luciferase activity. As a control we employed a GFP expression vector (GFP-F) that includes a farnesylation sequence that targets GFP to the cell membrane. This is a better control for a membrane-bound receptor than a cytoplasmatic GFP. Interestingly, we observed isoform specific effects. TrkB FL increased luciferase activity while no difference was observed between TrkB T and GFP-F control transfected cells. TrkB SHC induced a decrease in AICD-mediated luciferase activity. We hypothesize that the decrease in AICD-mediated luciferase activity induced by TrkB SHC might be mediated by binding of SHC adaptor proteins. Binding of adaptor proteins to TrkB and possibly to APP, might decrease the endocytosis of APP decreasing its β-secretase cleavage [44]. The luciferase assay described here has been found to be particularly sensitive in detecting decreased β-secretase processing [45] and that can be the cause of the decrease in luciferase activity that we observe, at least with cotransfection of the TrkB SHC isoform.

Our data demonstrates differential effects of the TrkB isoforms on AICD-mediated transcription showing that TrkB SHC behaves differently from both TrkB FL and TrkB T. It has been previously demonstrated that BDNF application does not improve the cognitive function in a trisomy 21 mouse model because TrkB T is upregulated [43, 46]. Therefore, a better understanding of the individual TrkB isoforms and their signaling role will improve the therapeutic potential of BDNF or BDNF agonists.

Experimentally, we found that the detected protein levels of TrkB FL were much lower than TrkB T and TrkB SHC levels. We can exclude effects due to plasmid copy number in the cells since we used equimolar amounts of plasmid DNA that account for differences in plasmid size. We can also exclude differences in transcription levels due to plasmid promoters since the TrkB SHC vector was generated by mutagenesis of the TrkB FL vector. The difference in expression levels of the TrkB isoforms is highly reproducible suggesting that there

might be a tight regulation of TrkB FL expression levels. TrkB FL is stored in intracellular vesicles that rapidly fuse to the cell membrane upon BDNF stimulation of the cells [37]. This causes a fast BDNF-mediated phosphorylation of the receptor and initiates intracellular signaling [47]. After this spike of activity TrkB/BDNF complexes are rapidly endocytosed and degraded [48]. High TrkB FL expression levels increase malignancy in neuroblastomas reinforcing the idea that regulatory mechanisms of TrkB expression and signaling are necessary to maintain homeostasis [49, 50]. TrkB FL expression is also decreased by chronic BDNF stimulation of H4 neuroblastoma cells while TrkB T levels remain constant [51]. We therefore hypothesize that in our model system, TrkB FL levels are controlled by mechanisms that cannot be overcome by TrkB FL overexpression and that BDNF expressed by the cell line might be one of the causes of this downregulation.

To determine which TrkB functional domain and signaling pathway was mediating the TrkB mediate effects, we overexpressed the mutant TrkB isoforms and monitored AICD-mediated luciferase activity. The observed TrkB FL-mediated increase in luciferase activity was suppressed by either inactivating the tyrosine kinase activity (K571M) or mutating the PLC-γ-binding site (Y816F). We hypothesize that the PLC-γ effect is due to lack of PLC-γ activation which produces DAG (Diacyl Glycerol), an activator of PKC, a protein that mediates ADAM10 activation [52]. The fact that there is a difference between the TrkB K571M mutant and the TrkB Y816F PLC-γ-binding site mutant suggests both of these functional domains and their associated pathways can regulate APP metabolism.

The SHC-binding site on the TrkB FL receptor did not seem to be involved in mediating increased AICD-mediated luciferase activity since the TrkB Y515F mutant did not differ from the TrkB FL isoform in increasing AICD-mediated luciferase activity. Also the AICD-mediated luciferase signal in cells transfected with the double mutant TrkB K571M/Y515F did not differ from the cells transfected with the TrKB K571M mutant suggesting that there is no additive effect in eliminating both signaling pathways. This does not completely exclude a role for the SHC-binding domain. In fact, the binding of SHC might occur more efficiently when the site is phosphorylated so that when phosphorylation is prevented the small change in luciferase signal is not detectable in our experimental system. Supporting this hypothesis is a nonsignificant decrease of AICD-mediated activity caused by the TrkB Y515F mutant compared to TrkB FL.

We observe a significant effect when the TrkB SHC isoform is mutated to eliminate SHC-binding. This mutant induces the same luciferase activity signal of the TrkB T. This finding suggests that binding of SHC to the TrkB SHC isoform might mediate signaling pathways independently of phosphorylation. Importantly, we demonstrate that there is a difference in signal transduction between the two truncated TrkB isoforms and that they act on APP-mediated transcription. Moreover, we identify the SHC-binding domain as responsible for the difference in signaling mechanism between TrkB T and SHC. Mutation of the binding site for SHC adaptor proteins on the truncated TrkB SHC isoform increases AICD-mediated luciferase signal, while the same SHC site on the TrkB FL is not responsible for the observed changes in luciferase activity. This contrasting result suggests that interaction between the same proteins and specific TrkB isoforms mediates different signaling pathways.

The cell line used, SH-SY5Y, expresses basal levels of TrkB receptors and BDNF [31], the endogenously expressed BDNF can promote dimerization and activation of the overexpressed receptors. Also, BDNF independent activation of TrkB FL receptors has been previously demonstrated [53] and we hypothesize that both BDNF independent and dependent activation coexist in our experimental system. Endogenous TrkB receptors might also be upregulated or downregulated in response to exogenous TrkB expression.

To assess the effect of BDNF-dependent activation we added exogenous BDNF to the transfected cells. BDNF is hypothesized to activate the receptors by mediating their dimerization [12]. In our experimental system BDNF treatment did not significantly alter the effects of the TrkB isoform overexpression on APP FL levels. It has been observed before that TrkB FL overexpression can cause receptor autoactivation [53]. Our data suggests that a similar mechanism occurs in our experimental system. The close proximity of the overexpressed receptors on the membrane probably allows dimerization and activation of the receptors independently from BDNF so that even when BDNF is added to the system any additional effect on TrkB activation is not detectable.

We mentioned above that SH-SY5Y cells express basal levels of the TrkB receptors. To investigate the role of TrkB isoforms interaction on TrkB FL-mediated signaling, we coexpressed exogenous TrkB FL with truncated isoforms and mutated variants. Cotransfection of the TrkB FL with the truncated T and SHC isoforms or the tyrosine kinase inactive mutant abrogated the increase in APP FL levels induced by TrkB FL. Interestingly TrkB FL/SHC cotransfection had higher APP FL levels than TrkB FL/T cotransfection. This points to a possible difference between the two TrkB truncated isoforms in the regulation of the TrkB FL catalytic receptor. The fact that in the cotransfection experiments TrkB FL/SHC showed increased APP FL levels compared to TrkB FL/T also suggests that SHC-binding to this isoform might occur more efficiently when TrkB FL and TrkB SHC interact, maybe causing phosphorylation on the SHC-binding site. Cotransfection of TrkB FL/Y515F had similar effects on APP FL to TrkB FL/FL but was less effective in inducing an increase in APP FL levels. TrkB FL/Y816F cotransfection was indistinguishable from TrkB FL single transfection suggesting that PLC-γ signaling is not involved in determining APP FL levels. BDNF treatment of the cotransfected cells seemed to accentuate the effect of TrkB FL on APP FL levels. For example, it increased APP FL in cells cotransfected with TrkB FL/T but not in cells cotransfected with TrkB FL/K571M. On the contrary, TrkB FL/Y515F cotransfection seemed to cause lower APP FL levels when BDNF was applied. It is intriguing to think that when all TrkB isoforms are expressed in the cells, as should be the case in our model, BDNF promotes homodimerization versus

heterodimerization. The issue of preferential homo- versus heterointeraction of TrkB isoforms has not been investigated so far and it would be important to address.

5. Conclusions

This work demonstrates that truncated TrkB isoforms affect APP processing and transcriptional signaling differently than full-length TrkB. Not only do the truncated isoforms have a different effects when transfected alone, they were also able to modify the TrkB FL effects when co-transected with it. These findings point to the possible roles of the TrkB isoforms in the pathogenesis of AD. In fact all the isoforms are present on neurons and other cell types of the CNS. The proportion of TrkB FL to TrkB T and TrkB SHC is then important in determining the effect on TrkB signal transduction and APP metabolism. Since all the isoforms bind BDNF in the extracellular domain, a therapeutic approach that uses BDNF biomimetic drugs might not be as effective as if only TrkB FL was expressed. In fact, expression of truncated isoforms could scavenge the drugs, decrease the benefit of engaging TrkB FL triggered pathways, and also inhibit the TrkB FL effects. Depending on the relative amounts of the TrkB receptors on the cells, BDNF-mimetic drugs could cause an overall worsening of the conditions [46] by, for example, increasing the inflammatory response. It will be important in the future to dissect the contributions of the TrkB isoforms to BDNF-dependent and -independent signaling pathways in the context of AD to better understand which isoforms and pathways are beneficial and which ones are detrimental.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 239453, 5 pages
doi:10.4061/2011/239453

Research Article

AICD Overexpression in Neuro 2A Cells Regulates Expression of PTCH1 and TRPC5

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Received 2 July 2010; Accepted 26 July 2010

Academic Editor: Christiane Reitz

Amyloid precursor protein (APP), implicated in Alzheimer's disease, is a transmembrane protein of undetermined function. APP is cleaved by gamma-secretase that releases the APP intracellular domain (AICD) in the cytoplasm. *In vitro* and *in vivo* studies have implicated the role of AICD in cell signaling and transcriptional regulation of Gsk3β, KAI1, BACE1, EGFR, and other proteins. In this study, by overexpressing AICD in mouse neuroblastoma cell lines, we have demonstrated the alteration in the expressions of two proteins, patched homolog 1 (PTCH1), a receptor for sonic hedgehog signaling, and transient receptor potential cation channel subfamily C member 5 (TRPC5), a component of receptor-activated nonselective calcium permeant cation channel. Our results indicate the possibility of regulation by AICD in developmental processes as well as in the maintenance of calcium homeostasis at the transcription level.

1. Introduction

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder that occurs gradually and results in memory loss, unusual behavior, personality changes, and a decline in thinking abilities. A fundamental abnormality that plays a pivotal role in the dysfunction and death of neurons in AD is altered proteolytic cleavage of APP. The function of the APP holoprotein is not yet established and mice lacking the APP gene show relatively minor neurological impairments. This subtle phenotype is probably due to compensatory effects mediated by two other members of the APP gene family: amyloid-precursor-like protein-1 and -2 (APLP1 and APLP2). This view is supported by evidence showing that the combined ablation of APP and APLP2, both APLP genes or all three family members together leads to early postnatal lethality [1]. Both the amyloidogenic and nonamyloidogenic pathways, that is, the cleavages of APP by β- and α-secretases, respectively, liberate the soluble ectodomain of APP (ectodomain shedding) and retain the C-terminal fragments (CTF) (CT99 and CT83, resp.). Subsequent cleavages by γ-secretase in the transmembrane domain generate the amyloidogenic Aβ peptide or the nonamyloidogenic p3 peptide along with the intracellular C-terminal domain of APP (AICD). Biochemical and genetic interaction screens have led to the identification of both extracellular and multiple intracellular binding partners, which seem to anchor the APP/APLP C-termini to a complex protein network at the cell surface, which may transduce various cellular responses [2, 3]. Notably, a highly conserved cytoplasmic—^{682}YENPTY^{687}—motif is present in all APP/APLP family members, which confers clathrin-mediated endocytosis and was shown to bind several multidomain adaptor proteins, including X11/Mints, Fe65 family proteins and mDab [4]. A number of type-I transmembrane proteins including Notch, p75NTR, CD44, ErbB4, neuregulin-1, and alcadein undergo a similar secretase mediated processing leading to ectodomain shedding and generation of intracellular domains (ICD's) [5]. Some of these ICD's are known to take part in cellular differentiation and development by nuclear signaling and transcriptional transactivation [6]. Like NICD (Notch intracellular domain), several recent studies have suggested that AICD has transactivation activity and can regulate transcription of multiple genes including APP, GSK-3β,

Table 1: Primer sequences and PCR conditions of real-time PCR.

Name of the genes	PCR condition	PCR Cycle	Primer sequences (5′-3′)	Size (bp)
PTCHI	95°C → 10 min [95°C 30 sec, 50°C 30 sec, 60°C 1 min] 72°C → 10 min	35	GAAAAATGAGCAGAACCATGG TGTCTTCCTTCTGAACCCCTG	102
TRPC5	95°C → 10 min [95°C 30 sec, 50°C 30 sec, 60°C 1 min] 72°C → 10 min	35	TTCCAGCTCTCTTCACTGTGC AAGTCACAAGCCTCTCCCCAA	102
Beta actin	95°C → 10 min [95°C 30 sec, 55°C 30 sec, 60°C 1 min] 72°C → 10 min	35	TCCTGTGGCATCCACGAAACT GAAGCATTTGCGGTGGACGAT	315

KAI1, neprilysin, BACE, and EGFR [7–11]. Recently, it has been shown that AICD-mediated transcriptional regulation of EGFR by directly binding to the EGFR promoter [11].

The role of APP in neuronal development and in calcium homeostasis is well established [1, 12, 13]. The expression of APP in brain is developmentally regulated and it is expressed ubiquitously in differentiated neurons. APP is axonally transported and secreted forms of APP (sAPPs) are released from neurons in an activity-driven manner. Secreted APPs modulate neuronal excitability, counteract effects of glutamate on growth cone behaviors, and increase synaptic complexity [14]. Moreover, aberrant processing of APP can also cause neurodegeneration by impairing a neuroprotective function sAPPs which normally regulate calcium homeostasis [12, 15].

But the role of AICD, if any, in both developmental processes and in maintenance of calcium ion homeostasis is yet to be elucidated. In the present study, we intended to look into the possibility of AICD having any role in the transcriptional regulation of the components of sonic hedgehog pathway and calcium channel forming proteins. Initially, microarray analysis was done to screen the genes whose expression would alter upon AICD overexpression (data not shown).

2. Materials and Methods

2.1. Cloning of AICD in pGFP C1 Vector. For the overexpression of AICD in mammalian cell line, it was cloned in pGFP vector. Specific primers for AICD (Forward: 5′ ACGCGTCGACAAGAAGAAACAGTACACATCC3′ and the Reverse: 5′CGGGATCCTAGTTCTGCATCTGCTCAAAGAA C3′) with adaptors (underlined), for the restriction enzymes (RE) SalI and BamH1, were synthesized (Integrated DNA Technologies) to amplify the domain using brain c-DNA library (Stratagen) as template. PCR products were digested with *SalI* and *BamH1* (New England Biolabs) and ligated to pGFP C1 vector (BD Biosciences). Construct was confirmed both by DNA sequencing and restriction enzyme digestion.

2.2. Cell Culture and Transfection. Neuro 2A cells were obtained from National Cell Science Centre, Pune, India and were cultured in DMEM (HiMedia) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2 atmosphere under humidified condition. Transfection of cells with empty vector (pGFP C1) or AICD-GFP was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen).

2.3. Protein Extraction. For extraction of proteins, PBS-washed pellets from cell lines were lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 15 mM EDTA, 0.5% Triton X-100) for 30 min in presence of Complete protease inhibitor (Roche Diagnostics) and centrifuged at 13,000 rpm for 15 minutes. Protein concentration was determined by Bradford protein estimation assay.

2.4. RNA Isolation, c-DNA Preparation and Real-Time PCR. RNA was isolated from Neuro 2A cells by RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturers protocol. RNA equivalent to 500 ng–1 μg was taken to synthesize the first strand cDNA using random hexamer primers and reverse transcriptase (Invitrogen). Real time RT-PCR reaction was carried out using Syber green 2X Universal PCR Master Mix (Applied Biosystems, USA) in ABI Prism 7500 sequence detection system. Each reaction was performed in triplicate using 100 ng of total RNA using corresponding primer sequences (primer sequences and PCR conditions were mentioned in the Table 1). For each gene, nontemplate control was used at the same condition to ascertain the baseline and threshold value for the analysis. The absolute quantification given by the software was in terms of Ct values. The relative quantification of a target gene in a sample compared to parental cell is expressed in terms of $2^{-\Delta\Delta Ct}$ values after normalization with respect to internal control (β-actin gene).

2.5. Western Blot. Proteins were separated on SDS-polyacrylamide gels and transferred onto PVDF membranes

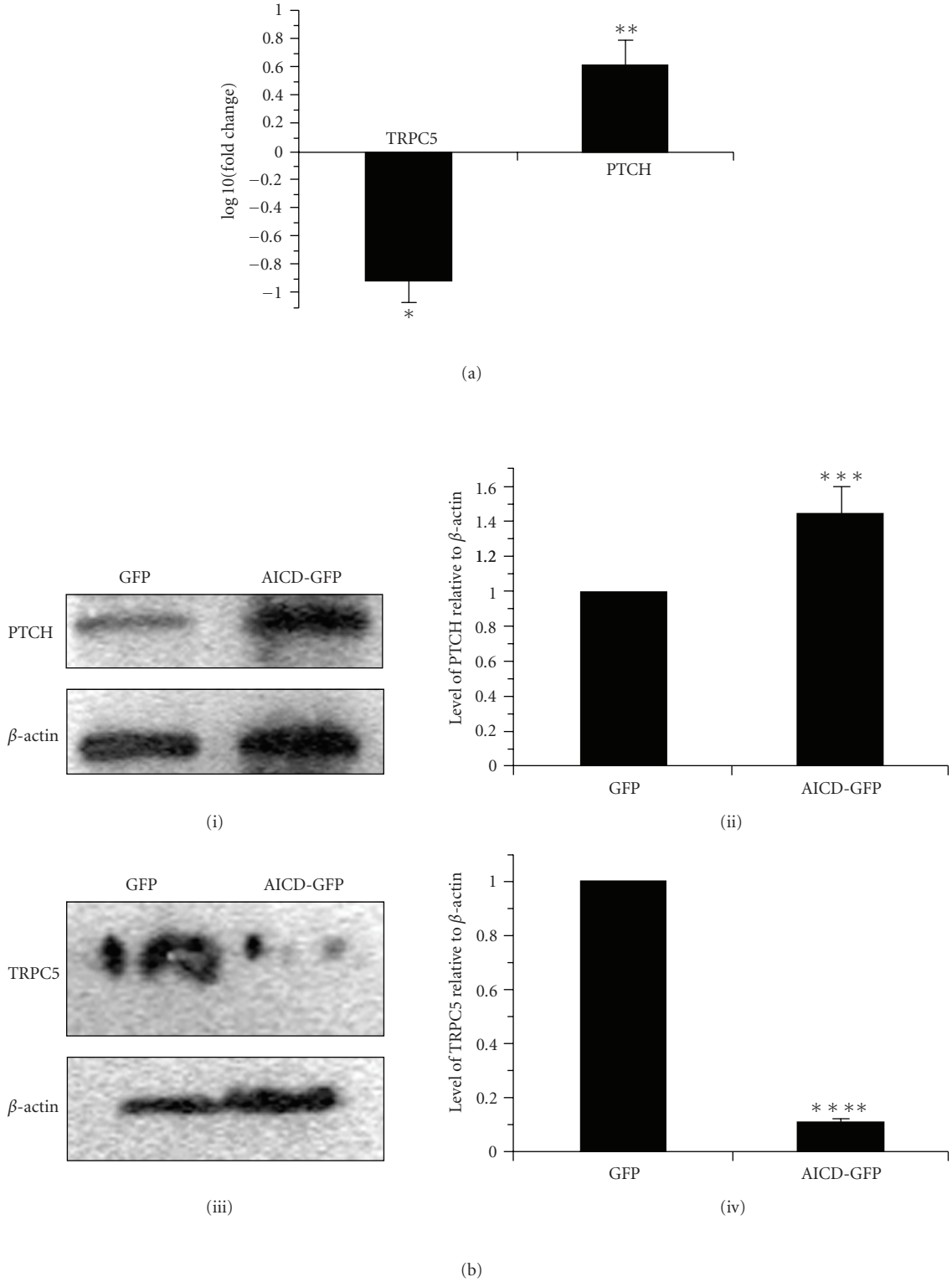

Figure 1: (a) RNA was isolated from Neuro 2A cells transfected with either GFP or AICD-GFP. The first strand cDNA was synthesized using random hexamer primers and reverse transcriptase. Using that c-DNA as template, expression of PTCH1 and TRPC5 were also checked by real-time PCR in both the cell lines. The relative quantification of both the genes in AICD-GFP transfected cell compared to only GFP transfected cell were expressed in terms of $2^{-\Delta\Delta Ct}$ values after normalization with respect to internal control (beta-actin gene) and plotted in log scale (log10(Fold change)) * indicated $P < .02$ and ** indicated $P < .05$. (b) Proteins were prepared from both GFP and AICD-GFP transfected cells after 24 hours of transfection, run on SDS-PAGE and western blot was done with antibody against PTCH1 (i) as well as TRPC5 (iii) and beta actin as loading control in both the cases. Fold change was calculated by densitometry analysis taking beta-actin as loading control (ii) and (iv). *** indicated $P < .05$ and **** indicated $P < .005$.

(Millipore Corporation), which were blocked by incubation in 5% dried milk in TBST (50 mM Tris-HCl, 150 mM NaCl, pH 7.5 containing 0.05% Tween 20). Membranes were probed with primary antibodies against PTCH (ab53715, Abcam plc, 1 : 1000); TRPC5 (ab58374, Abcam plc, 1 μg/mL); beta actin (loading control for whole cell extracts; Abcam plc, 1 : 5000). HRP-conjugated antibodies (Chemicon; 1 : 5000) were then added to the blots. Immunoreactive bands were detected with enhanced chemiluminescence reagent (Super Signal West Pico Substrate; Pierce) and signals were visualized by exposing the membranes to ECL Hyperfilm (Amersham Biosciences). Quantification of western blots was carried out using Quantity One software of Bio-Rad. At least three separate experiments were analyzed and band intensities were normalized to loading control. *P*-values were determined using unpaired *t*-tests.

Mean and standard deviation were calculated by Microsoft Office Excel 2007. The error bar represents standard error ((standard deviation/$\sqrt{n}$) n = sample size).

3. Results and Discussion

From microarray experiment we got the hint that AICD overexpression might alter the expression level of PTCH1 and TRPC5 (data not shown). To provide further evidence, the mRNA expression levels of both the proteins were checked by real-time PCR using total RNA from AICD-GFP overexpressed cells and compared those with the expression of only pGFP C1 transfected cells. About 2-fold increase and 3-fold decrease in the expressions of PTCH1 and TRPC5, respectively, was observed upon AICD overexpression (Figure 1(a)). The changes in the expressions of the genes at RNA level was further verified at protein levels also. By western blot analysis it was revealed that endogenous PTCH1 level was raised 1.5 fold (Figure 1(b), panels (i) and (ii)) and TRPC5 level was decreased 9 fold (Figure 1(b), panels (iii) and (iv)) in AICD-GFP overexpressing Neuro 2A cells compared to control.

PTCH1 acts as a receptor for sonic hedgehog (SHH) [16] and seem to have a tumor suppressor function, as inactivation of this protein is probably a necessary, if not sufficient step for tumorigenesis [17]. Defects in PTCH1 are known to be a cause of sporadic basal cell carcinoma (BCC) [18]. Hence overexpression of PTCH1 by AICD could be a protective measure of cells against tumorigenesis. This observation seems to be interesting because AICD is previously reported to modulate EGFR-mediated tumorigenesis by reducing the expression of EGFR [11].

On the other hand TRPC5 forms a receptor-activated nonselective calcium permeant cation channel. TRPC1 and TRPC5 are subunits of a heteromeric neuronal channel in mammalian brain [19]. TRPC5 is reported to colocalize with stathmin-like-2, a neuronal growth protein, within the vesicles and in the growth cone. A dominant-negative form of TRPC5 allowed significantly longer neurites and filopodia to form, suggesting that TRPC5 regulates neuronal growth [20]. It was also reported that influxes of calcium via voltage-gated channels play a role in neuronal outgrowth and suggested that TRPC5 is a candidate for the regulation of calcium waves [20]. A decrease in the expression of TRPC5 by AICD overexpression might affect neurite outgrowth.

4. Conclusion

In conclusion, our results suggest that overexpression of AICD can modulate both developmental and degenerative pathways in the cell. Whether the cell would take the survival or the degenerative route at the end depends on other regulatory parameters.

Acknowledgment

This work was supported by the SPGHGD (DAE) project, Government of India.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 490140, 15 pages
doi:10.4061/2011/490140

Review Article

Neuroimaging Measures as Endophenotypes in Alzheimer's Disease

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Received 2 September 2010; Revised 8 January 2011; Accepted 7 February 2011

Academic Editor: Lindsay A. Farrer

Late onset Alzheimer's disease (AD) is moderately to highly heritable. Apolipoprotein E allele ε4 (*APOE4*) has been replicated consistently as an AD risk factor over many studies, and recently confirmed variants in other genes such as *CLU, CR1*, and *PICALM* each increase the lifetime risk of AD. However, much of the heritability of AD remains unexplained. AD is a complex disease that is diagnosed largely through neuropsychological testing, though neuroimaging measures may be more sensitive for detecting the incipient disease stages. Difficulties in early diagnosis and variable environmental contributions to the disease can obscure genetic relationships in traditional case-control genetic studies. Neuroimaging measures may be used as endophenotypes for AD, offering a reliable, objective tool to search for possible genetic risk factors. Imaging measures might also clarify the specific mechanisms by which proposed risk factors influence the brain.

1. Introduction

Alzheimer's disease (AD) is thought to be at least 58–74% heritable [1–3]. However, much of that heritability has yet to be explained by variants in specific risk genes. Mutations in the amyloid precursor protein (*APP*) [4], presenilin 1 (*PSEN1*) [5], and presenilin 2 (*PSEN2*) [6, 7] genes are known to lead to early onset, familial AD. In familial AD, the disease typically follows an autosomal dominant, usually highly penetrant mode of inheritance. However, for many years only the ε4 allele of apolipoprotein E (*APOE*4) [8] was identified as a reliable genetic risk factor for late-onset AD. On average, 24% of control subjects carry at least one copy of *APOE*4 [9], and each risk allele carries more than threefold odds of developing AD [9], although these numbers vary across studies; this is a relatively large odds ratio for a highly prevalent risk gene. Recently, large sample genome-wide association (GWA) studies have successfully identified and replicated associations between several single nucleotide polymorphisms (SNPs) and AD [10] (Table 1), namely, in the *CLU* [11, 12], *PICALM* [11–13], and *CR1* [13] genes and near the *BIN1* and *EXOC3L2* genes [12]. Numerous other genetic polymorphisms also have been associated with a diagnosis of AD, but with less statistical evidence, and replication results are frequently inconsistent [14] (http://www.alzgene.org/). Much work yet remains in discovering the sources of AD heritability. As we note below, large-scale neuroimaging studies provide an approach to discover, replicate, and study new genetic risk factors.

AD is a complex disease whose onset and trajectory are influenced by (1) environmental factors and (2) many genetic polymorphisms having small effects and/or rare polymorphisms having larger effects. Because contributing genes have large effects in aggregate but small effects individually, association studies typically require large samples to reliably identify the individual contribution of any one polymorphism, especially since stringent corrections for multiple comparisons are required by GWA studies. Additionally, genes involved in either neurodevelopment or degeneration or both may contribute to AD risk. The onset of AD is

Table 1: Top AD risk genes.

	Gene	Protein	Population	Polymorphism
1	APOE_e2/3/4	apolipoprotein E	all	APOE_e2/3/4*
2	CLU	clusterin	all	rs11136000*
3	EXOC3L2	exocyst complex component 3-like 2	all	rs597668
4	BIN1	bridging integrator 1	all	rs744373
5	PICALM	phosphatidylinositol binding clathrin assembly protein	all	rs541458*
6	SORL1	sortilin-related receptor	Asian	rs2282649*
7	GWA_14q32.13	unknown	all	rs11622883
8	TNK1	tyrosine kinase non-receptor, 1	all	rs1554948
9	ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	Caucasian	rs1800764
10	IL8	interleukin 8	all	rs4073
11	LDLR	low density lipoprotein receptor	all	rs5930
12	CST3	cystatin C	Caucasian	rs1064039*
13	CR1	complement component (3b/4b) receptor 1 (Knops blood group)	all	rs6656401
14	hCG2039140	unknown	all	rs1903908
15	CHRNB2	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	all	rs4845378
16	SORCS1	sortilin-related VPS10 domain containing receptor 1	all	rs600879
17	TNF	tumor necrosis factor alpha	Asian	rs4647198
18	CCR2	chemokine (C-C motif) receptor 2	Caucasian	rs1799864

Genes listed represent those most highly associated with AD per alzgene.org [9] as of August 22, 2010. Only those with high or moderate epidemiological evidence are included above. Grading was based on HuGENet (Human Genome Epidemiology Network) interim criteria for the assessment of cumulative evidence of genetic associations [132].

*At least one neuroimaging study has investigated the effects of this polymorphism in the brain.

clinically detectable only when the pathological hallmarks of the disease such as amyloid plaques, neurofibrillary tangles, and neuronal loss have advanced to the point where memory impairment and other behavioral changes become evident. Therefore, symptoms may be manifest when abundant pathology overwhelms an otherwise healthy brain, or limited pathology occurs in a brain whose health and resilience is compromised by cortical thinning, reduced white matter integrity, or restricted blood flow.

It is difficult for case-control studies to identify genetic risk factors for AD based on clinical diagnosis alone. This is because AD diagnosis relies on evidence of cognitive deficits identified using standard cognitive tests. Performance on cognitive tests may be influenced by factors unrelated to disease, such as fatigue, anxiety, general test-taking ability, and practice effects. As such, well-educated people suffering from cognitive decline can appear normal in a clinical setting, while cognitively normal worriers may appear to be impaired. Other late-life dementias also may be clinically misdiagnosed as AD. Using brain endophenotypes that are objective and highly reproducible over time may make it easier to identify AD genetic risk factors and to understand their impact on the brain.

In recent multisite efforts, researchers have performed brain scans on and genotyped large numbers of cognitively intact and impaired older adults. These studies have improved the ability of researchers to identify AD-related genes. In this article, we review the results of neuroimaging studies that evaluate the effects on the brain of top AD-related candidate genes other than *APOE* as well as genetic contributions to brain vulnerability. We discuss the findings from GWA studies that have used neuroimaging measures as endophenotypes for AD, and we offer suggestions for future studies. Finally, we discuss multigene and more advanced genetic models as means to identify specific genetic contributions to AD. The main findings of the studies discussed here are summarized in Table 2 by imaging phenotype.

2. Candidate Gene Approach

There are two main ways to investigate effects of AD-relevant genes using brain imaging—the first is to study candidate genes already associated with AD, and the second is to use genome-wide scanning to perform an unbiased search of up to a million genetic polymorphisms. Both types of approach have been applied in neuroimaging studies of AD. The earliest studies have focused on the most widely studied candidate gene, *APOE*.

Although not without conflicting results, many studies have linked *APOE4* to neuroimaging measures such as regional hypometabolism assessed using fluorodeoxyglucose positron emission tomography (FDG-PET) (which measures brain glucose metabolism) [15–17], functional magnetic resonance imaging (fMRI) activity (which measures variations in regional levels of blood oxygenation and is thought to reflect both blood flow and neuronal activity) during memory tasks and at rest [18–26], regional brain volume

Table 2: SNPs with AD-relevant effects detected by neuroimaging measures.

Neuroimaging measure	SNP	Gene	Location[c]	Neuroimaging association
Hippocampal volume or gray matter density	rs429358/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	CG [47], GWA [104]
	rs10501927	*CNTN5*	11q22.1	CG [47]
	rs3851179[b]	*PICALM*	11q14.2	CG [47]
	rs4646994[b]	*ACE*	17q23.3	CG [68]
	rs2075650[b]	*TOMM40*	19q13.32	GWA [104]
	rs4692256	*LOC391642*	4p15.1	GWA [104]
	rs10074258[b]	*EFNA5*	5q21.3	GWA [108]
	rs12654281[b]	*EFNA5*	5q21.3	GWA [108]
	rs10781380	*PRUNE2*	9q21.2	GWA [108]
	rs1888414	*FDPSP*	21q21.1	GWA [108]
ERC thickness	rs429358/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	CG [47]
	rs3851179[b]	*PICALM*	11q14.2	CG [47]
	rs10501927	*CNTN5*	11q22.1	CG [47]
	rs1408077[b]	*CR1*	1q32.2	CG [47]
	rs7561528[b]	*BIN1*	2q14.3	CG [47]
PHG cortical thickness	rs429358/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	CG [47]
	rs10501927	*CNTN5*	11q22.1	CG [47]
Amygdala volume	rs429358/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	CG [47], GWA [104]
	rs2075650[b]	*TOMM40*	19q13.32	GWA [104]
	rs4646994[b]	*ACE*	17q23.3	CG [68]
MTL volume	rs4935775[b]	*SORL1*	11q24.1	CG [58]
Temporal pole cortical thickness	rs429358/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	CG [47]
	rs10501927	*CNTN5*	11q22.1	CG [47]
	rs7561528[b]	*BIN1*	2q14.3	CG [47]
Temporal lobe volume	rs429368/rs7412 (ε2/3/4)[b]	*APOE*	19q13.32	GWA [81]
	rs10845840	*GRIN2B*	12p13.1	GWA [81]
	rs2456930	chromosome 15 intergenic region	15q22.2	GWA [81]
Frontal lobe volume	rs3751812	*FTO*	16q12.2	CG[76]
GM density-precuneus	rs10932886	*EPHA4*	2q36.1	GWA [104]
GM density-frontal cortex	rs10932886	*EPHA4*	2q36.1	GWA [104]
	rs6463843	*NXPH1*	7p21.3	GWA [104]
Regional brain tissue volume in temporal lobe	rs2429582	*CADPS2*	7q31.32	vGWA [113]
Regional brain tissue volume in parietal lobe	rs476463	*CSMD2*	1p35.1	vGWA [113]
Whole brain volume	rs1468063[b]	*FAS*	10q23.31	CG [71]
Ventricular volume	rs1468063[b]	*FAS*	10q23.31	CG [71]

Table 2: Continued.

Neuroimaging measure	SNP	Gene	Location[c]	Neuroimaging association
WM lesion volume[a]	rs10501927	*CNTN5*	11q22.1	CG [47]
	rs560573[b]	*SORL1*	11q24.1	CG [58]
	rs668387[b]	*SORL1*	11q24.1	CG [58]
	rs689021[b]	*SORL1*	11q24.1	CG [58]
	rs641120[b]	*SORL1*	11q24.1	CG [58]
	rs2276346[b]	*SORL1*	11q24.1	CG [58]
	rs4646994[b]	*ACE*	17q23	CG [65]
WM integrity[a]	rs11136000[b]	*CLU*	8p21.1	CG [54]

This table summarizes the most promising single SNPs relevant to AD research and identified from associations with neuroimaging characteristics. These characteristics show correlations with the SNP alleles either specifically in AD-related regions (in healthy adults) or anywhere in the brain (in normal adults and those with AD and/or MCI).
Key: GM: gray matter; WM: white matter; MTL: medial temporal lobe; PHG: parahippocampal gyrus; ERC: entorhinal cortex; CG: candidate gene approach; GWA: genome-wide association scan approach; vGWA: voxelwise genome-wide association scan approach.
[a]White matter lesion volume is calculated from a structural MRI scan (usually a T2-weighted scan), while white matter integrity is measured using diffusion tensor imaging and reflects water diffusion directionality.
[b]Previously identified as an AD risk allele [9].
[c]Locations were determined using http://genome.ucsc.edu/ [133], using values from dbSNP build 131.

or cortical thickness (measures of structural gray matter integrity) [27–31], white matter integrity [32–35], cerebral blood flow [36–39], and AD-related pathology such as amyloid and neurofibrillary tangle load [40–44]. Results from such *APOE* neuroimaging studies have been reviewed previously [14, 45, 46].

Neuroimaging differences associated with the *APOE* genotype may result from incipient AD, or they may relate instead to differences specific to the genotype independent of AD pathology (e.g., developmental differences). If other AD risk genes were to resemble *APOE* in their effects on the brain, it would support the notion that those brain differences are related to the pathological processes of AD. Additionally, determining the effects on the brain of other AD risk gene variants would help to characterize the mechanisms of those risk alleles, enabling more targeted therapeutic treatments to be developed. Thus far, relatively few neuroimaging studies have examined the effect of AD candidate risk genes other than *APOE* on the brain (Table 1).

The most recent and comprehensive candidate gene study to date was performed by Biffi and colleagues (2010), who evaluated the effects of top AD risk polymorphisms on six measures shown to predict AD risk and measure disease progression [47]. The authors measured hippocampus, amygdala, and white matter lesion volumes and thickness of the entorhinal cortex, parahippocampal gyrus, and temporal pole cortex in AD patients, mild cognitively impaired (MCI) patients, and normal controls. People with MCI have some degree of demonstrable cognitive impairment not severe enough to warrant a diagnosis of dementia. Approximately 10–15% of those with amnestic MCI convert to probable AD each year compared with an estimated 1-2% of similarly aged cognitively intact individuals [48]. MCI therefore can be used as an indicator of early AD-related changes in the brain. The authors focused on confirmed risk polymorphisms and other potential risk variants identified in recent GWA studies. Among these were *APOE*, *CLU*, *PICALM*, *CR1*, *CNTN5,* and *BIN1*. *APOE,* which encodes apolipoprotein E—an apolipoprotein that interacts with β-amyloid [49]—was correlated with all brain measures except for white matter lesion volumes. *CNTN5,* which codes for contactin 5—a protein that may play a role in regional axonal development [50]—is not currently listed as a top AD risk gene [9]. However, it was associated with all measures except for amygdala volume. All the genetic variants except for *CLU* were statistically correlated with entorhinal cortex thickness. The *CLU* gene encodes clusterin (also known as apolipoprotein J)—another apolipoprotein that interacts with β-amyloid [51]. Additionally, a variant in the *PICALM* gene, which codes for phosphatidylinositol binding clathrin assembly protein—a protein involved in regulating the fusion of synaptic vesicles [52]—was correlated with hippocampal volume. Finally, *BIN1,* which encodes bridging integrator 1—a protein involved in neurite growth [53]—was correlated with temporal pole cortical thickness [47]. The authors suggested that although sample sizes affect the power to detect gene effects, the specificity of relationships with particular polymorphisms may reflect the function and expression patterns of the resulting proteins, possibly elucidating mechanisms that contribute to AD risk [47].

The *CLU* risk variant rs11136000 was not associated with any of the measures here, but our research group recently found that in young healthy adults, the risk allele of that SNP was associated with reduced integrity of broad white matter regions, observed with diffusion tensor imaging [54]. The lipid transport and membrane recycling performed by the clusterin protein [55] may be important to myelin development but not to medial temporal lobe gray matter. Choosing measures that reflect the purported protein function associated with risk genes in question might help to focus the search for gene effects in the brain.

Another AD gene with structural effects on the brain is *SORL1,* which encodes the sortilin-related receptor. The gene product is a low-density lipoprotein receptor that may be involved in processing the amyloid precursor protein [56]. *SORL1* may also play a role in cardiovascular health [57]. Cuenco and colleagues (2008) evaluated how 30 different polymorphisms in the *SORL1* gene related to general cerebral atrophy, hippocampal atrophy, white matter hyperintensities and cerebrovascular disease, which they measured semiquantitatively [58]. Among the variants tested in African-American and white AD-control sibships was rs2282649—a top AD genetic risk factor [9]. In whites, this variant was associated with cerebral and hippocampal atrophy as part of a 3 SNP haplotype [58]. SNPs within *SORL1* also were associated with white matter hyperintensities in two studies [58, 59]. The strongest relationship between rs2282649 and AD is in Asian populations (as determined in a large meta-analysis) [9]. Future comparisons of SNP effects on the brain in Asians versus Caucasians may clarify how this polymorphism relates to AD.

Babiloni and colleagues (2006) used electroencephalography (EEG) to examine how another AD risk gene, *CST3,* affects resting cortical rhythmicity (the frequency of repetitive spiking of neuronal activity) in subjects with AD and MCI. One haplotype evaluated contained an AD top risk SNP (rs1064039) [60]. *CST3* codes for cystatin C, a protein that colocalizes with β-amyloid [61] and may be involved in the proliferation of neural stem cells [62]. The amplitude decrease of alpha 1 sources (parietal, occipital, and temporal areas) was more pronounced in AD and MCI patients with the *CST3* risk haplotype, possibly indicating greater amyloid load or neuronal death [60]. Follow-up studies of this polymorphism that evaluate brain atrophy using MRI or amyloid load using PET imaging may be valuable.

Some additional neuroimaging studies of major AD risk genes examined the *ACE* gene, which codes for angiotensin converting enzyme—a protein that modulates the cardiovascular system by helping to regulate extracellular volume. *ACE* also affects the central nervous system by influencing neurons in the hippocampus and amygdala and helping to maintain the blood brain barrier [63, 64]. All these studies evaluated the commonly evaluated *ACE* insertion/deletion (I/D). The *ACE* D/D polymorphism was associated with increased severity of white matter hyperintensities or cerebral infarction in some [65, 66] but not all [67, 68] studies. One study found that the I/I genotype was associated with increased AD risk, and smaller hippocampi and amygdalae [68]. Another found that D carriers with MCI showed differences in resting state fMRI brain activity compared with I homozygotes [63]. The I/D variant examined in these studies is not one of the two currently listed by a large meta-analysis (http://www.alzgene.org/) [9] as being significantly associated with Alzheimer's disease overall, although some evidence links it with AD risk or unspecified cognitive decline [69, 70]. Regardless, since this variant in the *ACE* gene appears to modulate brain structure and function, it would be valuable to investigate the effects of other *ACE* polymorphisms having stronger relationships to AD: namely rs1800764 and rs4291 [9].

Recently, Erten-Lyons and colleagues (2010) evaluated the effects of a less studied AD risk gene, *FAS*, on the brain in 242 older adults who were cognitively intact or had MCI or AD [71]. *FAS* codes for the Fas (TNF receptor superfamily, member 6) protein, which may be involved in apoptosis in AD [72]. The authors evaluated 97 SNPs in or near the *FAS* gene that had been previously associated with AD. After adjustment for multiple testing, they found that rs1468063 was associated with faster AD progression. Carriers of the T allele of that SNP had greater ventricular volumes and smaller brain volumes in a subgroup of 56 subjects [71].

The candidate gene approach also may be used to evaluate the effects of genes predisposing subjects to characteristics (such as hypertension, obesity, high cholesterol, and diabetes) that increase the risk of AD [73–75] without necessarily being directly involved in the development of classic AD pathology such as amyloid plaques or neurofibrillary tangles. Examining the effects of these variants in healthy adults and focusing on brain areas susceptible to earliest AD processes may be productive in isolating polymorphisms that create a vulnerability that AD-related pathology later exploits. Recent work has already demonstrated that some such genes have an effect in the brain. For instance, Ho et al. (2010) recently demonstrated in 206 cognitively intact older adults from the ADNI study that risk allele carriers for rs3751812 in the fat mass and obesity associated gene (*FTO*) had smaller average brain volumes in frontal and occipital lobes relative to noncarriers (Figure 1) [76]. Those of European descent carrying two copies of the common adverse variant of *FTO* have increased risk for obesity, relative to those carrying no copies [77]. The connection between *FTO* and brain atrophy is important, as it suggests one mechanism whereby cardiovascular risk factors (including risk genes) may make the brain more vulnerable to the later effects of AD. The *FTO* gene may cause brain atrophy by promoting a craving for greater caloric intake resulting in higher body mass index (which is also associated with brain atrophy; [78, 79]). It is also possible that *FTO* affects the brain by direct gene action to promote tissue atrophy or insufficiency. Even so, a variety of lifestyle factors, including education, diet, and exercise, are associated with reduced brain atrophy. This underscores the value of controlling preventable risk factors for brain atrophy [80].

Other studies have focused on variants associated with genes important for blood pressure regulation and cholesterol levels such as the previously mentioned *ACE* [65, 66] and *SORL1* variant studies [58, 59]. Studies focusing on regions affected early in AD such as the hippocampus,

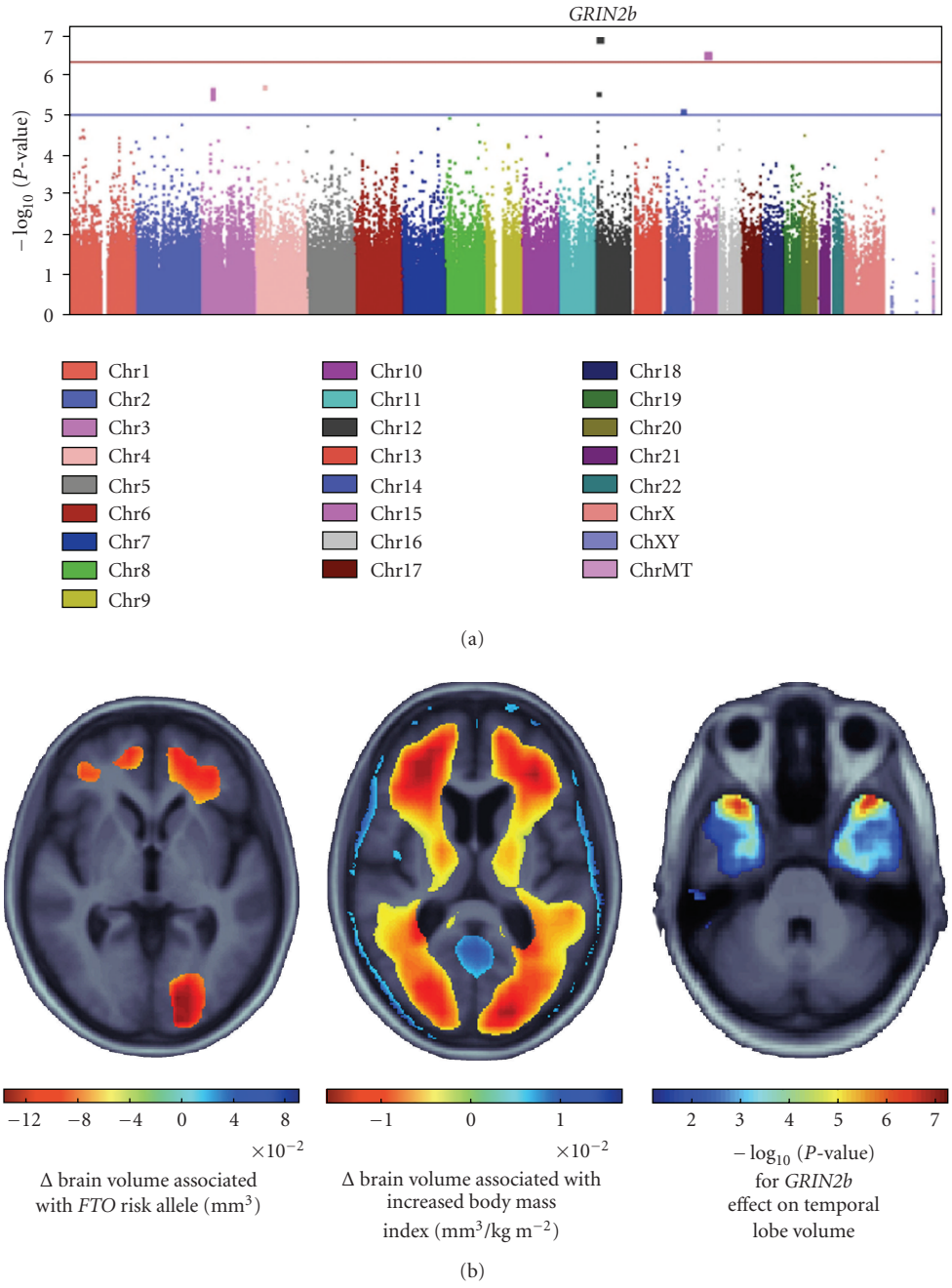

Figure 1: Common genetic variants (single nucleotide polymorphisms) associated with temporal lobe volume in a GWA study are shown in (a) along with an image showing the effects of the top hit, *GRIN2b*, on brain volume [81].The figure is adapted from Stein et al. (2010) with kind permission from the authors and publishers. (b) shows the effect (regression coefficients) of the candidate obesity gene, *FTO*, on brain atrophy in a cognitively normal adults and those with MCI and AD [76]. The figure is adapted from Ho et al. (2010) with kind permission from the authors and publishers.

entorhinal cortex [82], and posterior cingulate cortex [15] may be helpful in further elucidating the links between AD and cardiovascular health.

3. Genome-Wide Association Studies

Recently, a small number of studies have used genome-wide association (GWA) to search for novel genetic variants associated with AD endophenotypes. Discovering new risk genes would be extremely beneficial to the study of AD. Clinical trials could then selectively enroll, or perform sub-analyses on risk allele carriers, who are more likely to decline than noncarriers. Those at heightened genetic risk might also benefit the most from early treatment. Additionally, using AD risk genes as covariates would boost power in AD-related studies since modeling the identified genetic risk

factors reduces otherwise unexplained variance in the disease trajectory, making other influential factors easier to detect.

Several initiatives, such as Alzheimer's Disease Neuroimaging Initiative (ADNI) (www.loni.ucla.edu/ADNI), are now searching for new gene risk variants using neuroimaging traits that are highly heritable, easily measured in a reliable way, and associated with AD [83]. This may be a valuable way to overcome some of the obstacles inherent in diagnosis-based searches for risk polymorphisms. For instance, one might use as an endophenotype the baseline regional neuroimaging measures known to predict longitudinal cognitive decline in amnestic MCI or early AD. Such measures make specific diagnoses unnecessary because they focus on symptoms, namely the confluence of longitudinally decreased cognitive ability with specific functional or structural brain deficits that predict that decrease. Also, as continuous measures that vary across the continuum of normalcy from MCI to AD, neuroimaging measures may offer greater statistical power for genetic analysis than binary diagnostic categories. Suggested criteria for endophenotypes are that the measures are associated with illness, are heritable, are apparent in an individual regardless of whether the illness is active, and that they co-segregate with illness within families [84]. Some neuroimaging measures, such as hippocampal and ventricular volume largely meet these criteria as endophenotypes for AD. Both increased ventricular volume [85–88] and decreased volume of medial temporal lobe structures, especially the hippocampus [87–92] predict cognitive decline, are moderately to highly heritable [93–95], and are associated with AD and genetic risk for AD (Table 2). Other measures that show promise in predicting cognitive decline are brain amyloid burden as measured using Pittsburgh Compound B [96] and white matter integrity (in general and perhaps more specifically in the parietal lobe) as measured with diffusion tensor imaging [97] both of which are also highly heritable [98, 99]. Some neuroimaging measures may not yet be considered endophenotypes. For instance, glucose metabolism as measured with FDG-PET [100–102], and cerebral perfusion as measured with arterial spin labeling [103] also may predict cognitive decline, but large-scale heritability studies of these measures in healthy older adults are needed to ascertain their potential for identifying genetic influences. These guidelines may be useful when evaluating the utility of a measure as an endophenotype.

One recent GWA study by Shen and associates (2010) evaluated genetic associations with brain structure using a large number of nonspecific phenotypes. They studied 733 AD and MCI patients and normal controls from the ADNI cohort and controlled for age, sex, education, handedness, and baseline intracranial volume [104]. The authors examined 142 regions of interest and found that the well-known variants in *APOE* (rs429358/rs7412 a.k.a. ε2/3/4) and in a more newly identified gene, *TOMM40* (rs2075650), were strongly associated with bilateral hippocampus and amygdala volumes. Four additional SNPs were associated at the $P < 10^{-7}$ level with regional gray matter density. In the *EPHA4* gene, rs10932886 was correlated with gray matter density in the left precuneus and bilateral frontal regions—regions in which atrophy occurs in late AD [105]. *EPHA4* codes for the EPH receptor A4—a receptor tyrosine kinase that regulates dendritic spine morphology in pyramidal cells of the adult hippocampus. *EPHA4* also helps to control glial glutamate transport resulting in regulation of hippocampal function [106]. Its association with hippocampal structure and function makes this gene an intriguing target for future study. Likewise, rs6463843 in the *NXPH1* gene was associated with gray matter density in the left middle orbital frontal gyrus. *NXPH1* encodes the neurexophilin 1 protein, which is a physical ligand for α-neurexins—proteins that may participate in synaptic function [107]. Finally, rs4692256 (LOC391642) was associated with gray matter density in the right hippocampus, but the function of the genetic material containing that SNP is unknown. The authors also reported a number of other associations at the more liberal $P < 10^{-6}$ level [104].

Two other recent ADNI-based GWA studies focused their searches on temporal lobe structures; temporal lobe volume is highly heritable and is also a relatively good predictor of developing AD. Potkin et al. (2009) used a genome-wide search for polymorphisms affecting hippocampal gray matter density, and identified novel AD susceptibility genes in 381 subjects who had AD or were normal controls [108]. AD cases differed in genotype from controls at rs429358 (one of the two SNPs comprising the *APOE* 2/3/4 genotype), and at rs2075650 in the *TOMM40* gene. Using a significance threshold of $P < 10^{-7}$ and covarying for age, sex, and the number of *APOE*4 alleles, four SNPs were associated with right or left hippocampal gray matter density [108]. Two of these, rs10074258 and rs12654281, were in or near the *EFNA5* gene [108], which encodes the ephrin-A5 protein implicated in nervous system development including in the hippocampus [109]. The gene function and association with hippocampal structure across multiple SNPs makes it an alluring target for future study. Two other SNPs associated with hippocampal gray matter density at the $P < 10^{-7}$ level were rs10781380 in the *PRUNE2* gene and rs1888414 near the *FDPSP* gene [108]. These two SNPs have a less clear tie to AD-related symptoms compared with those in *EFNA5*. At the $P < 10^{-6}$ level, the authors also identified correlations of right or left hippocampal gray matter density with genotypes at an additional 11 SNPs.

In a larger study also using the ADNI dataset, Stein and colleagues (2010) used MRI and GWA to identify SNPs associated with temporal lobe and hippocampal volumes in 742 AD and MCI patients and healthy elderly adults, controlling for age and sex (Figure 1) [81]. The authors also evaluated the relationship between temporal lobe volume and the *APOE2/3/4* genotype, which was not part of the Illumina gene chip used in the GWA. As expected, *APOE*4 was associated with lower temporal lobe volume. Additionally, at a significance level of $P < 5 \times 10^{-7}$, the authors identified two SNPs that were associated with bilateral temporal lobe volume across diagnoses: rs10845840 in the *GRIN2B* gene (independent of an *APOE*4 effect), and rs2456930, located in an intergenic region of chromosome 15 [81]. The *GRIN2B* gene codes for a regulatory subunit 2B (NR2B) of the NMDA (N-methyl D-aspartate) glutamate receptor. NR2B is implicated in learning, memory, and

structural plasticity, and cognitive deficits in Alzheimer's disease [110, 111]. The same glutamate receptor is also the target of memantine [112], a drug designed to slow the progression of AD. This makes *GRIN2B* an attractive target for future AD investigations generally, and also specifically with respect to how it may modulate memantine drug effects.

Finally, in the first voxelwise GWA (vGWA) study, Stein and colleagues (2010) examined the effects of genetic variation on brain structure as determined using tensor-based morphometry, while controlling for age and sex [113]. Rather than testing for genetic associations with one or a small number of structural measures, associations were tested at each of hundreds of thousands of voxels in the image—leading to a whole-brain, whole-genome search. The authors evaluated 740 subjects from the ADNI study who had AD or MCI, or were normal controls, and identified only the most significant SNP association at each voxel. Top SNPs identified within known genes in this GWA search were rs476463 in the *CSMD2* gene and rs2429582 in the *CADPS2* gene [113]. *CSMD2* (CUB and Sushi multiple domains 2) maps to a chromosomal region that may contain a suppressor of oligodendrogliomas [114], although little is yet known about the protein function. *CADPS2* codes for Ca^{++}-dependent secretion activator 2, a protein that regulates synaptic vesicle and large dense core vesicle priming in neurons, and promotes monoamine uptake and storage in neurons [115]. Although no SNP survived a false discovery rate correction at $P < .05$ [113], this method remains promising when larger sample sizes become available. Stringent corrections are needed when searching an entire image for genomic effects, but the size of the search space can be greatly reduced by carrying forward promising voxels to later analyses. Because of this, the sample sizes needed to replicate a GWA finding, when searching an entire image, are typically much smaller than the discovery sample size (as low as 300-400 rather than 700 subjects [113]) as the voxels with no effects can be discarded in the replication analyses.

The sample size needed to detect statistical relationships between genetic risk factors and specific brain measures depends upon the measure being studied. Beckett and colleagues (2010) recently compared the ability of various MRI- and PET-derived attributes to track the progression of MCI and AD [116]. Regions of interest derived from specific brain voxels showing significant relationships to cognitive impairment in previous studies gave greater power to detect a slowing of the disease than measures related to whole structures such as the hippocampus. The increased power of statistical voxel selection was later reinforced by studies using both MRI [117] and FDG-PET [118]. Such statistically predefined regions of interest may be promising targets of genetic studies in which gene effects can be mapped using statistical mapping approaches. By focusing on regions with greatest statistical effects, the power to detect or replicate genetic effects in follow-up studies is vastly increased [119]. In that regard, imaging studies can avoid a general problem in large-scale genetics; by focusing on promising voxels, replication samples may in fact be smaller than the discovery samples, if the effects of the genes in the brain are somewhat localized. The selection of sets of voxels showing significant genetic associations is helpful to boost power, above and beyond focusing solely on regions that are clinically important to the disease of interest (which is also important). Such an approach has been advocated by Chen et al. (2010) and Wu et al. (2010) [118, 120]. There are at least three advantages in focusing on specific voxels over predefined anatomical regions of interest. First, although a given gene variant may affect a region that shows dramatic effects in a given disease, that whole region may not be equally affected. Using a voxelwise approach may help to identify subregions that would provide a more concentrated focus for future replication efforts. An example is a recent study of the brain derived neurotrophic factor (*BDNF*) genes, in which common variants were associated with brain fiber integrity on DTI, in 455 subjects [119]. When the sample was split into two, the same regions of the white matter showed associations in each subsample, but there would have been no *a priori* reason to select those regions as implicated. Limiting a search to significant voxels in follow-up studies boosts power by avoiding image wide corrections for statistical tests at voxels less likely to show an effect. Secondly, although the focus of a study may be AD, pathways altered by a specific gene variant may be relevant to multiple complex diseases and disorders. Data collection and analysis are costly in genetic neuroimaging studies. Therefore, reporting all significant results can provide information that may not otherwise be easily obtained but may be useful to researchers at large. Thirdly, image based tests for replication, such as conjunction tests, can be devised that allow specific sets of brain regions, not just specific genes, to be replicated as showing associations (see, e.g., Ho et al. 2010 [76]).

In GWA studies, it is conventional to enforce a significance cut-off of $P < 10^{-7}$ or 10^{-8}. This represents a Bonferroni-type correction for the false positives that could occur when 500,000 SNPs are searched for statistical effects. As adjacent SNPs are somewhat correlated (due to linkage disequilibrium effects), the effective number of tests is slightly fewer than the number of SNPs tested, but even SNPs falling below $P < 10^{-7}$ are considered to show "genome-wide evidence" requiring replication in subsequent studies or in meta-analyses of multiple independent datasets. So far, there is no universal agreement as to what statistical threshold for GWA studies is the best. The above ROI-based GWA studies reviewed here all used a threshold of at least 10^{-7} to report their top findings [81, 104, 108], which controls for multiple comparisons in the tests performed. Dudbridge and Gusnanto (2008) suggested that a genome-wide significance threshold should not account only for markers that have been tested in a study, but also for all possible genomic variation. This leads to a more conservative threshold of $P < 7.2 \times 10^{-8}$ [121]. Because of the required time and cost of collecting and analyzing neuroimaging data, the sample sizes here, although large for imaging studies, remain small for genetic studies. These smaller sample sizes may produce false positives unless independent replication is performed. Still, functionally promising SNPs have been identified in these studies, highlighting numerous replication targets for future work.

All four of the above GWA studies were performed using scans from the ADNI dataset with a high degree

of overlap of subjects. Even so, the top SNPs were not replicated across studies. This may be due to a number of methodological factors. First, the sample sizes needed to detect a genetic association depend on the minor allele frequency and effect size, and are typically between a few hundred and several thousand subjects. With this limitation, measures that show association in one study may be missing in another. Even different software used to measure the same structure do not give perfectly correlated measures. Also, many associations will be missed due to imprecision in the measures—single gene effects are typically only detectable for measures with the highest precision and reproducibility. Additionally, across studies, the initial genetic searches did not adjust for the same covariates in addition to age and sex. For instance, Potkin and colleagues covaried for *APOE* genotype [108], but Shen and colleagues covaried for education, handedness, and baseline intracranial volume [104], and the Stein et al. studies did not use additional covariates [81, 113]. Finally, the choices of ROI and methods of delineating those regions varied across studies. The ENIGMA (Enhancing Neuro Imaging Genetics through Meta-Analysis) project (http://enigma.loni.ucla.edu/) [122] is one of several multicenter initiatives to standardize genetic and imaging methods. Its goal is to empower future replication efforts and make it easier to perform meta-analyses. Because different SNP sets are genotyped in different studies, imputation methods are employed to allow the same set of genomic variations to be queried across every dataset.

Using GWA to evaluate how genetic variance affects AD endophenotypes in cognitively intact younger and older adults may also aid in identifying AD genetic risk factors. Genetic variants associated with brain measures in young cognitively normal adults are less likely to be associated with molecular pathology. More likely, they support early vulnerabilities in the brain that AD pathology later exploits. The polymorphisms may, for instance, relate to health factors that increase the risk of AD, such as obesity and diabetes, or may relate to neural development in regions affected in early AD, such as the hippocampus and entorhinal cortex. Variants identified in cognitively intact older adults may relate to both AD molecular processes and vulnerabilities in the brain. Using amyloid imaging measures in these subjects may be helpful in identifying genetic risk factors for earliest AD changes.

An imaging measure may be associated with a particular polymorphism during development but may also be related to other gene polymorphisms with respect to degeneration later in life. Therefore, it is not the measurement, but rather its context and other demographic factors that determine whether gene effects relate to neurodevelopment or degeneration. This should be borne in mind when replicating gene effects across cohorts. For instance, in Stein et al. (2011), caudate volume was associated with commonly carried variants in dopamine-related genes, and the effects were found in an large elderly cohort scanned in North America, and replicated in a young adult cohort scanned in Australia [123]. Such replications of SNPs may indicate gene effects that persist throughout life. The use of two very different samples is likely to identify genes of enduring relevance across the lifespan, but may miss or fail to replicate effects that exist or are more dominant only in late or early life. Naturally, there is a greater preponderance of apoptotic events in an elderly sample and more developmental or synaptogenic processes in the younger samples. For this reason, genome-wide meta-analyses must not regard failure to replicate as a sign that gene is not influential in a given part of the lifespan, or in a given cohort or continent.

In a study of normal brain aging, Seshadri and colleagues (2007) investigated genetic associations with measures of total cerebral brain volume, lobar, ventricular and white matter hyperintensity volumes, and scores on six cognitive tests. They identified three SNPs (located in *ERBB4*, *PDLIM5*, and *RFX4*) that were associated both with measures of frontal or parietal brain volumes and with tests of executive function and abstract reasoning. These results did not survive testing for multiple comparisons, but they may be used to generate future hypotheses or to offer support to findings in future GWA studies [124]. As this study was one of brain aging rather than of AD, cognitively normal adults were studied and not all measures examined were specific to AD risk. Therefore, some of the SNPs generated may relate more to brain aging or normal development than to AD risk.

Two GWA studies that we know of have examined endophenotypes in healthy young adults—a GWA study of caudate volume in 1198 young and old adults [123] and the first voxelwise GWA study of diffusion tensor images [125]. Further studies that focus on brain measurements specific to AD would be useful additions to the field. Since the brain differences that are likely to occur in normal adults are subtle compared to those in studies of a brain disease, very large numbers of subjects are needed to perform GWA in healthy young adults and to show that the results are reliable and reproducible across independent samples. The ENIGMA network brings together researchers in imaging and genetics, and current analyses are probing structural and functional neuroimaging and GWA data from over 10,000 subjects. This type of effort will prove invaluable in replication studies. ENIGMA also allows for the identification of "slow climbers"—genetic variants that may not be significant in all studies or in any one study alone, but may become highly significant when data is aggregated across studies.

GWA and vGWA involve huge numbers of comparisons, which may result in false positives if not properly controlled. It is therefore incumbent upon readers of such studies to critically evaluate the significance levels of the studies before basing potentially costly experiments upon their results. However, such exploratory studies may provide information that would not otherwise be easily obtained and can be extremely useful in focusing future work. For instance, one might not collect thousands of MRI scans to test the effect of one SNP previously found to be marginally significant. However, it may make sense to test the effects of that SNP in conjunction with other more established ones when GWA data has already been collected and the MRI scans have been physically analyzed. In this way, it is possible to build easily on previous results until they are strong enough to warrant independent exploration.

In addition, the large number of statistical tests involved in a genome-wide and/or image wide search requires special methods to boost power, including gene-based tests [126], ridge regression models [127], multilocus modeling, and meta-analysis. In the first voxelwise GWA studies of MRI and DTI [113, 125], no single SNP passed the conventional threshold for genome-wide significance; even so, the top SNPs can be prioritized when screening new imaging datasets for replications of these hits. Efforts such as the ENIGMA consortium have found that some SNPs identified by GWA are robustly associated with hippocampal volume and total brain volume. Although no single contributing site was able to find results that were genome-wide significant, the effects of several SNPs were robustly replicated when meta-analyzed across imaging datasets of more than 6400 subjects from 16 imaging sites [128].

4. Multiple Genetic Risk Factors

A statistical test of association between a set of SNPs and a disease can offer far greater power and success in determining genetic risk than tests of single SNPs [129]. This is in part because the risk conferred by different SNPs may depend on the context and on several demographic and environmental factors—the age of the cohort, their educational level, and even their socioeconomic status [130]. Because of this, more complex models of gene action in AD are likely to include not only multiple SNPs, but also environmental and other risk factors that affect whether those variants are relevant or innocuous.

Multilocus genetic modeling refers to a large class of methods that assesses the effects of sets of SNPs—within the same or different genes—in predicting clinical diagnosis, prognosis, or disease risk. Looking at the additive or epistatic (interactive) effects of multiple risk gene variants may be useful, especially when the genes in question have similar effects. For instance, Szolnoki and colleagues (2003) found in 961 subjects that carriers of *APOE*2 or *APOE4* had increased risk of white matter hyperintensities in their brains only if they also carried risk variants in the *ACE* or *MTHFR* (methylenetetrahydrofolate reductase (NAD(P)H)) genes [131]. All three are listed as top AD risk genes [9] and also affect the cardiovascular system, so it makes sense to examine their additive effects on the brain. Multilocus genetic models can assess the combined effects of multiple gene sets acting together.

Because adjacent SNPs in a genome-wide association study may be highly correlated due to linkage disequilibrium, it is not possible to use standard statistical methods, such as multiple regression, to identify which SNPs exert an influence on a trait. Machine learning methods that can cope with high-dimensional sets of predictors include such techniques as penalized regression, adaptive boosting, and the "Bayesian lasso". All of these methods have been used widely in quantitative genetics, and show substantial promise for analyzing brain imaging phenotypes.

Multilocus models are conceptually attractive as they allow the testing of the aggregate effect of several SNPs in the same gene, which individually may have effects too weak to detect on their own. In one study [126], we applied a novel method, multivariate principal components regression (PCReg) to test whole genes for associations with imaging data, not just single SNPs within them. When multiple partial-*F* tests were used to test the joint effect of all SNPs in a gene on regional brain volume differences, we identified several genes associated with brain-related disorders that are highly relevant to brain structure. GRB-associated binding protein 2 gene, *GAB2*—the most significantly associated gene in our analysis—has previously been linked to late-onset AD, and *GAB2* associations showed a symmetric signal in the white matter superior to the lateral ventricles. As a caveat, other methods that include multiple SNPs can sacrifice power as increasingly stringent corrections are applied to guard against finding spurious associations using high-dimensional regression models with many parameters. Even so, efficient gene-based association tests across the whole brain can drastically reduce the number of independent tests performed, detecting known genes highly relevant to brain structure that may be missed by univariate methods alone.

5. Conclusion

In summary, using neuroimaging endophenotypes to identify AD risk factors is a new and promising enterprise. Future studies of the combined effects of multiple candidate risk factors, and an expansion of genome-wide studies to a wide variety of imaging modalities may help generate new endophenotypes that predict AD. Additionally, a focus on particular contributions to AD risk, such as deposition of AD-related pathology, or developmental vulnerabilities might prove productive in unraveling disease complexity. For instance, searching for gene variants of an AD endophenotype in a large sample of healthy young adults would be most likely to uncover genes affecting developmental vulnerabilities to the disease. In contrast, examining a given endophenotype in AD and MCI patients while controlling for gene variants known to affect that measure in younger adults would boost the power to identify polymorphisms related to AD processes and cumulative environmental risk factors, while excluding some developmental effects. Careful selection of endophenotype, data pooling across studies and analysis of multiple different aspects of AD pathology and vulnerabilities may prove invaluable in the quest to explain the genetic risk for AD.

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