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Characterization of LR11/SorLA in Mild Cognitive Impairment

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Characterization of LR11/SorLA in Mild Cognitive Impairment

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ABSTRACT

Characterization of LR11/SorLA in Mild Cognitive Impairment

By Kristen Sager Cincotta

Alzheimer's disease (AD) is the leading cause of dementia in the elderly. We now recognize that the slow, progressive onset of cognitive impairment associated with AD is a lagging reflection of a decade or more of insidious pathological insults that are initiated by the abnormal accumulation of the Aβ peptide, suggesting that factors that can regulate Aβ levels could have potential value as disease-modifying therapeutic targets.

We recently identified LR11/SorLA as having markedly reduced expression in AD brain. *In vitro* and *in vivo* evidence has shown that LR11 may play a critical role in modulating Aβ production in healthy brain. Therefore, we hypothesized that the loss of LR11 protein expression is a primary event in the AD pathogenic cascade that directly contributes to the abnormal accumulation of Aβ in the earliest stages of the disease. As such, we predicted that LR11 expression would be similarly low in cases with mild cognitive impairment (MCI), a condition that largely constitutes prodromal AD.

To test this hypothesis, LR11 expression was measured in two unique cohorts comprised of cases with a clinical diagnosis of no cognitive impairment (NCI), MCI or AD, using a novel quantitative immunohistochemical technique. Here, we show that frontal cortex LR11 expression is low in at least a subset of cases in all of the diagnostic groups examined with the notable exception of the NCI group in the first experimental cohort, which was also the only group examined lacking amyloid pathology. We also show that low LR11 expression is not a universal pathological change in AD. Results from additional brain regions further show that LR11 expression is either consistently high or consistently low throughout the brain. Finally, to better understand the nature of the low LR11 cases, we performed an extensive series of statistical analyses designed to identify correlates of LR11 expression from a wide range of demographic, genetic, cognitive and pathological variables. No correlates of LR11 expression consistently emerged within the limits of this study, suggesting that the relationship between LR11 expression and the development of AD may be more complicated than previously believed.

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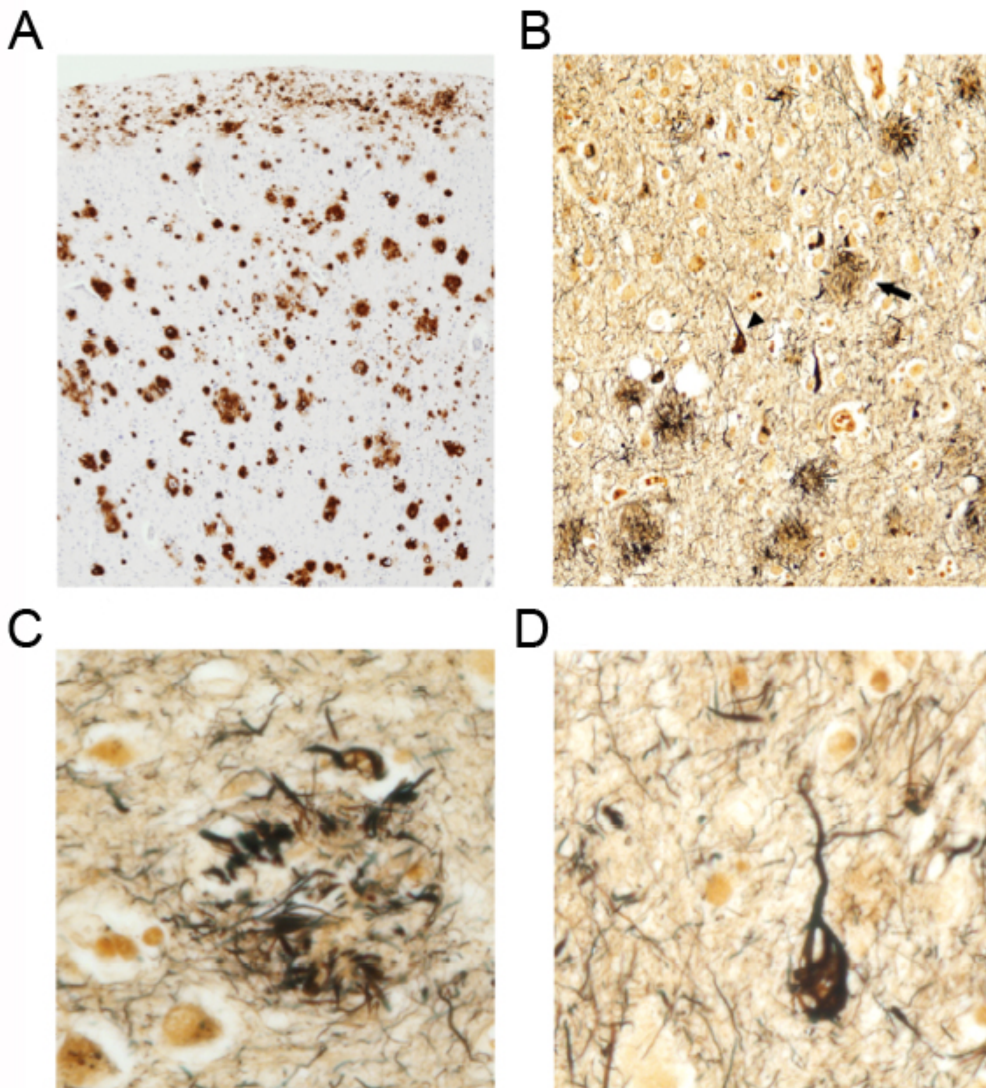
CHAPTER I. INTRODUCTION

1.1 Alzheimer's Disease: Initial Report and Prevalence of Disease

In 1901, Dr. Alöis Alzheimer was working as a senior physician at the Hospital for the Mentally Ill and Epileptics in Frankfurt am Main when he first saw and admitted the 51 year old woman who would become his most famous patient, Auguste D. Auguste presented with a host of symptoms suggesting significant cognitive impairment, including reduced comprehension and memory, aphasia, disorientation, paranoia, auditory hallucinations and psycho-social incompetence. As Auguste herself succinctly put it during a writing exercise to evaluate the degree of her amnestic memory loss, "I have lost myself."(Maurer et al 1997)

Dr. Alzheimer continued to follow Auguste's case until her death in 1906. Dr. Alzheimer had a strong interest in the neuropathology of dementing disorders and aided by recent technological advances by Franz Nissl and Max Bielchowsky, performed a post-mortem pathological evaluation on Auguste's brain. At the time, Dr. Alzheimer noted arteriosclerotic changes throughout the brain as well as the presence of two types of lesions: plaques, which Dr. Alzheimer speculated were determined by the storage of a peculiar material in the cortex and neurofibrillary tangles, which were noted for their characteristic thickness and peculiar pregnability (Figure 1.1). Dr. Alzheimer presented Auguste's case and his subsequent pathological observations on November 4th, 1906 at the 37th Conference

Figure 1.1 – Hallmark Lesions of Alzheimer’s Disease



(A) Low magnification image showing numerous Aβ immuno-positive plaques. (B) Higher magnification image of cerebral cortex showing argyrophilic amyloid plaques (arrow) and neurofibrillary tangles (arrowhead). (C) High magnification image of a single neuritic plaque. (D) High magnification image of a single neurofibrillary tangle.

Images courtesy of the Levey-Lah laboratory, Emory University.

of South-West German Psychiatrists in Tübingen in a lecture titled "A Peculiar Disease of the Cerebral Cortex" (Alzheimer 1906). Despite the rarity of this "presenile" dementia, it was included as a diagnostic entity in the 8th Edition of Dr. Emil Kraepelin's Handbook of Psychiatry under the name "Alzheimer's Disease", which was published in 1910 (Kraepelin 1910). While our knowledge and understanding of AD has increased enormously in the interceding decades, the clinical symptoms and pathological lesions that Dr. Alzheimer first described are still considered to be the primary hallmarks of Alzheimer's disease today.

At the time of his lecture in 1906 and the subsequent publication of his observations in 1907 (Alzheimer 1907), Dr. Alzheimer and his contemporaries believed that AD was very rare, only striking patients that were considered too young to be developing dementia as a result of the aging process. This belief persisted in large part due to the ongoing confusion about the relationship between the presence of the pathological lesions that Dr. Alzheimer had first identified and dementia. In particular, it was noted by a number of scientists that both plaques and NFTs were commonly found in the brains of non-demented patients (Gellerstedt 1933; Grünthal 1927; Rothschild 1942; 1956; Rothschild & Trainor 1937). While some debate around this issue still persists, it was mostly put to rest in the late 1960s and early 1970s following the publication of a series of seminal papers by Martin Roth, Bernard Tomlinson and Gary Blessed. Through painstaking work in which they developed methods to quantify both the extent of pathological lesions and the degree of cognitive impairment in the same patients, Roth, Bernard and Blessed were able to clearly demonstrate a strong relationship between lesions and

dementia, both in presenile AD patients and in senile dementia patients alike (Blessed et al 1968; Roth et al 1967; Roth et al 1966; Tomlinson et al 1968; 1970).

In light of this important breakthrough, a movement emerged to unite the presenile AD diagnosis and senile dementia under the same nomenclature. As Robert Katzman stated in his persuasive editorial in the *Archives of Neurology* in 1976, “The fact remains that neither the clinician, the neuropathologist nor the electon microscopist can distinguish between the two disorders, except by the age of the patient.” Katzman further noted that far from being a rare disorder, AD was in fact one of the leading causes of death among the elderly that deserved far more research attention than it was currently getting (Katzman 1976). The two diseases were finally united under the name “Alzheimer’s disease” following a consensus conference in 1977 (Katzman et al 1978), an event that marked an explosion in research funding for AD and the unofficial beginning of the modern age of AD research (Fox 1989).

While it took over 70 years to recognize the true impact of AD, we now recognize that it is the leading cause of dementia in the elderly, affecting 10% of all people over the age of 65 and nearly half of all individuals over the age of 85 (Evans et al 1989). Moreover, with modern medical advances leading to an increasingly long life expectancy, it is predicted that by the year 2050, there will be 13.2 million persons in the US with AD, an almost three-fold increase from the number of affected persons in 2000 (Hebert et al 2003). With communities and families bearing the brunt of the estimated $18 billion dollars spent on treating dementia

every year (Ferri et al 2005), it is clear that AD continues to be a major problem for not just the elderly and those afflicted with the early onset form of the disease, but for the population as a whole.

1.2 Seminal Advances in the Understanding of AD Pathophysiology

With the recognition of the true prevalence of AD, an increase in both federal and private research funds quickly followed, resulting in a relative explosion of new discoveries. On the heels of the groundbreaking work in the early 1960s by Hornykiewicz and colleagues identifying a specific dopamine deficit in Parkinson's disease (PD) as well as the benefits of dopamine replacement therapies for treating the disease (Birkmayer & Hornykiewicz 1961; Ehringer & Hornykiewicz 1960), research efforts on AD focused on identifying similar specific cell vulnerabilities and neurotransmitter deficits that could lead to dementia of the Alzheimer's type. Moreover, with the work of Blessed, Tomlinson and Roth refocusing researchers' attentions on the importance of amyloid plaques and neurofibrillary tangles, an intense effort was begun to identify the protein components of each lesion, the causative factors that could lead to the formation of each lesion and the mechanisms by which each lesion could evoke neuronal cell death, with different research groups generally choosing to focus on one lesion type to the exclusion of the other. As more information about the complete pathogenesis of AD has emerged, however, it has become increasingly clear that the true "cause" of AD is not one individual event, but rather a number of interrelated pathogenic mechanisms and factors that

influence each other both directly and indirectly in a prescribed cascade of events that results in the synaptic dysfunction and cell death that leads to the clinical disorder that Dr. Alzheimer first described in 1906 and that we recognize today as Alzheimer's disease. In this section, I will describe some of the seminal findings from the last 35 years, our current understanding about how each of the primary features may fit together to cause AD, and some of the factors that may influence the risk of developing this disease. I will also discuss the current state of AD therapeutics and the challenges facing scientists and clinicians going forward.

The Cholinergic Hypothesis

As noted above, in the early 1960s, the Hornykiewicz research group reported for the first time that a major underlying cause of PD was the specific loss of dopamine neurotransmission (Ehringer & Hornykiewicz 1960). Shortly thereafter, the group also showed that many of the symptoms of the PD could be alleviated through the administration of levadopa, a dopamine precursor that served to mitigate dopamine signaling deficits (Birkmayer & Hornykiewicz 1961). In light of this important breakthrough, researchers studying neurodegenerative diseases turned their attention to identifying similarly vulnerable neuronal populations and/or neurotransmitter signaling systems that might be lost in AD. In the mid-1970s, a number of groups reported deficits in the activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in the hippocampus and cerebral cortex in AD patients compared to healthy controls, with Davies and Maloney specifically observing that the brain regions with the greatest deficits in

ChAT and AChE activity also had the greatest density of NFTs (Bowen et al 1976; Davies & Maloney 1976; Perry et al 1977). A series of important findings followed quickly thereafter, supporting the hypothesis that decreased cholinergic neurotransmission plays a major role in the expression of the clinical symptoms of AD: (1) that there is significant neuronal degeneration in the basal forebrain (Whitehouse et al 1981; Whitehouse et al 1982), a major source of cholinergic projections to the cerebral cortex and hippocampus (Wenk et al 1980); (2) that acetylcholine plays an important role in learning and memory (Bartus 1979; Davis et al 1978; Drachman & Leavitt 1974; Hasselmo 2006); and finally, (3) that there was a positive correlation between the degree of cognitive impairment observed in AD and cholinergic cell loss (Bierer et al 1995a; Perry et al 1981; Perry et al 1978; Wilcock et al 1982).

This cholinergic hypothesis, which was formalized by Bartus et al in 1982 (Bartus et al 1982), led quickly to the use of AChE inhibitors as a means of ameliorating the cholinergic signaling deficit believed to underlie the cognitive symptoms of AD (Ibach & Haen 2004; Lleó et al 2006; Pepeu & Giovannini 2009; Summers et al 1986). After a series of clinical trials, the first acetylcholine replacement therapeutic drug, tacrine, was approved by the FDA for use in treating AD dementia in 1995 (Lleó et al 2006). Although AChE inhibitors result in statistically significant improvements in cognitive ability, they are often very modest clinically. Moreover, these treatments merely alleviate the symptoms of AD but do nothing to alter the actual progression of the disease. Nonetheless, in the

absence of other disease-modifiying therapies, AChE inhibitors remain the standard of care for treating AD today.

Since the first reports of cholinergic deficits in AD, impairments in other neurotransmitter systems have also been recognized, including changes in brain catecholamine neurotransmission and a loss of both glutamatergic and GABAergic innervation (Adolfsson et al 1979; Gómez-Isla et al 1996a; Hardy et al 1987a; Hardy et al 1987b; Kordower et al 2001). While the relative contribution of neurodegeneration in each of these various cell systems to the ultimate manifestation of AD are still under debate, it is increasingly clear that the degree of cortical atrophy and especially synaptic density correlates far more strongly with the severity of cognitive impairment experienced by the individual than either the density of amyloid plaques or NFTs, suggesting that it is the overall degree of neurodegeneration that most directly contributes to cognitive decline in AD (DeKosky & Scheff 1990; Terry et al 1991). However, the question of what events actually cause this neurodegeneration still remains. To answer that question, the majority of research efforts on AD have focused on the two primary pathological lesions that Dr. Alzheimer first described: the amyloid plaque and the NFT.

Two Primary Hallmark Lesions in AD: Amyloid Plaques and Neurofibrillary Tangles

The recognition that amyloid plaques are a common feature in the brains of cognitively intact individuals dates back almost as far as the earliest descriptions of

senile plaques in AD (Gellerstedt 1933; Grünthal 1927; Rothschild 1942; 1956; Rothschild & Trainor 1937). Because of this, it was widely believed that amyloid plaques were merely a feature of the aging process, rather than a pathological feature of AD and senile dementia. In order to clarify some of this confusion, Blessed, Roth and Tomlinson set out to characterize the true relationship between amyloid plaque pathology (and to a lesser extent, neurofibrillary changes) and various states of mental deterioration (including senile dementia, delirium and functional psychoses) as well as normal aging. In a series of seminal papers published in the late 1960s, they clearly demonstrated a specific relationship between the presence and extent of AD–associated pathological lesions and senile dementia, while also acknowledging that some degree of lesion formation could be tolerated without mental decline, at least for some period of years (Blessed et al 1968; Roth et al 1967; Roth et al 1966; Tomlinson et al 1968; 1970). This important work reasserted the importance of these hallmark lesions in the development of AD and led to an increased effort to better understand the true nature of both amyloid plaques and NFTs.

In his initial description of the pathological hallmarks of AD, Dr. Alzheimer notes that the amyloid plaques were likely to be determined by the accumulation of a peculiar material in the cortex (Alzheimer 1906; 1907). What this specific amyloid material was, however, remained a mystery until 1984, when Glenner and Wong first isolated and sequenced the protein component of cerebrovascular amyloid that accumulated in the meningeal blood vessels in AD, the Aβ peptide (Glenner & Wong 1984b). Subsequent work by this same group also identified Aβ as the primary

component of similar cerebrovascular amyloid deposits that accumulated in adult Down's syndrome brain (Glenner & Wong 1984a). Because Down's syndrome patients invariably develop a neurodegenerative disorder indistinguishable from AD in their 40s and 50s, including the accumulation of amyloid plaques, Glenner and Wong presciently predicted that the gene encoding the Aβ peptide would be located on chromosome 21. Once confirmed, this revelation led to a series of important discoveries regarding the production of Aβ and its role in initiating the AD pathogenic cascade, as will be discussed in more detail below. Confirmation that Aβ is the primary component in cerebral amyloid plaques in AD and DS brain came just a year after Glenner and Wong's seminal publications (Masters et al 1985).

The Aβ peptide can range in length from 38 to 42 amino acids, with the different lengths of the peptide having different propensities towards aggregation into dimers, trimers, tetramers and larger molecular weight fibrils (Holtzman et al 2011; Morgan et al 2004). While 90% of the amyloid in the brain is $A\beta_{40}$, the primary Aβ species found in plaques is the longer and more fibrillogenic $A\beta_{42}$ (Holtzman et al 2011; Iwatsubo et al 1994; Jarrett et al 1993; Mann et al 1996; Thinakaran & Koo 2008). There are three types of Aβ deposits that are found in the brain: neuritic plaques, diffuse plaques and cerebrovascular amyloid. Neuritic plaques (NPs) are extracellular spherical structures composed primarily of an amyloid core surrounded by dystrophic neurites, astrocytes, and activated microglia (Holtzman et al 2011; Selkoe 2001). Both $A\beta_{40}$ and $A\beta_{42}$ are found as part of NPs, although $A\beta_{42}$ is the predominant species. NPs are commonly found in large

numbers in the limbic and association cortices. The existence of diffuse plaques (DPs) was first discovered when specific antibodies to the Aβ peptide revealed far more amyloid deposits in the brain than were labeled by classical agryophillic stains (Selkoe 2001). DPs are generally more amorphous and less dense than NPs and are not associated with dystrophic neurites. DPs also differ from NPs in that they are composed solely of $A\beta_{42}$. DPs are more abundant and widespread in AD brain than NPs and are also frequently found in the brains of healthy, elderly people (Morgan et al 2004). As such, DPs are widely believed to be the immature form of amyloid plaques that will develop into mature NPs with the degeneration of surrounding neurites and increased inflammatory microglial activation (Selkoe 2001). Interestingly, DPs can be found in the brains of individuals with DS as young as teenagers, while NPs do not develop until the late twenties or early thirties. Cognitive impairment associated with AD doesn't begin until DS individuals are in their forties and fifties, suggesting that a long asymptomatic stage of amyloidosis precedes the onset of clinical symptoms in AD (Lemere et al 1996a), an idea that will be discussed at greater length below. Finally, Aβ can also be deposited in blood vessel walls in the form of cerebrovascular plaques. This form of Aβ accumulation is known as cerebral amyloid angiopathy and often contributes to vascular dementia, a form of dementia closely related to AD (Bell & Zlokovic 2009; Holtzman et al 2011).

The other primary pathological lesions that were first identified by Dr. Alzheimer are the intraneuronal neurofibrillary tangles (NFTs), which are found in the cell bodies and apical dendrites of degenerating neurons. In addition to NFTs, there are two other types of neurofibrillary lesions that are found in the brains of

AD patients: neuropil threads, which form in the distal dendrites and dystrophic neurites, which are associated with neuritic plaques (Goedert et al 1995; Holtzman et al 2011). All three types of neurofibrillary lesions are composed of aggregated protein in the form of paired helical filaments (PHFs), a structure that was first identified and described in 1963 (Crowther & Wischik 1985; Kidd 1963). The protein component of PHFs is a hyperphosphorylated form of tau, which was identified by a number of groups around the same time in 1986 (Goedert et al 1988; Grundke-Iqbal et al 1986a; Grundke-Iqbal et al 1986b; Ihara et al 1986; Kosik et al 1986; Wood et al 1986). There are six tau isoforms of varying lengths that are all commonly found in the adult brain (Goedert et al 1995). In healthy brain, tau binds to and stabilizes microtubules, allowing for neurite extension (Avila 2006; Holtzman et al 2011; Weingarten et al 1975). However, in AD and other neurodegenerative diseases featuring similar neurofibrillary lesions (collectively called tauopathies), tau becomes hyperphosphorylated (Lee et al 1991), likely due to an imbalance between the phosphorylating kinases and the dephosphorylating protein phosphatase PP2A. Hyperphosphorylated tau has a significantly impaired ability to bind microtubules, with the resulting “free” tau self-aggregating into insoluble PHFs. This dissociation likely results in the destabilization of microtubules and the interruption of critical cellular processes that ultimately leads to neuronal dysfunction and death (Goedert et al 1995).

The appearance of NFTs in AD follows a well-described route through the brain, starting in the entorhinal cortex and hippocampus and eventually progressing through the cerebral cortex with advancing disease (Braak & Braak 1991). This

progression is mirrored a few years later by a similar progression of cortical atrophy (Frisoni et al 2009). While amyloid plaques are often found in the brains of healthy, aged individuals, NFTs are found in these cases much less often and only very rarely outside of the entorhinal cortex and hippocampus (Braak & Braak 1997a). As noted above, neurofibrillary lesions are not unique to AD and are in fact associated with a number of neurodegenerative disorders, including fronto-temporal dementia with parkinsonism (FTDP) (Avila 2006; Goedert et al 1998; Selkoe 2001). Given these observations and the fact that the frequency of NFTs in the brain is more strongly correlated with the degree of dementia severity in AD than the frequency of either diffuse or neuritic plaques (Bierer et al 1995b), it is widely believed that the clinical symptomology of AD and the other tauopathies arises from the neurodegenerative processes that begin with the formation of NFTs and other neurofibrillary lesions.

While there has been a general acceptance regarding the primary role of neurofibrillary lesion formation in the development of memory impairment and dementia, an ongoing debate still persisted through the 1990s over the relationship between amyloid plaques and NFTs, with one faction (the "baptists") maintaining that amyloid plaque formation preceded NFT formation and was therefore the fate-determining lesion in AD. Conversely, the other faction (the "tauists") believed that NFT formation alone was causative of the disease and that amyloid plaques were formed simply as a secondary, downstream effect of neurodegeneration in the brain (Mudher & Lovestone 2002; Trojanowski 2002). However, by the time the amino acid sequence for tau was published by Goedert et al in 1988, genetic linkage of

autosomal dominant, early onset AD to chromosome 21 had already been reported (Goate et al 1989; Goedert et al 1988; St George-Hyslop et al 1987). Because the tau gene, *MAPT*, is located on chromosome 17, Goedert and colleagues conceded that it was unlikely that defects in tau were the true cause of AD. The subsequent identification of a series of causative mutations in genes all relating to the production and/or accumulation of Aβ coupled with the recognition that mutations in the tau gene lead to the development of FTDP (and not AD) largely established the formation of amyloid plaques as the primary, upstream trigger event in AD (Goedert et al 1998; Hutton et al 1998; Poorkaj et al 1998; Spillantini et al 1998).

The Genetics of Familial Alzheimer's Disease and the Production of Aβ

As mentioned, one of the earliest clues about the genetic causes of AD came from the recognition that nearly all adult individuals with Down's syndrome develop AD in their late 40s and early 50s (Olson & Shaw 1969; Rumble et al 1989). The finding by Glenner and Wong that the amyloid in both AD and in Down's syndrome was predominantly composed of Aβ strongly suggested that the gene encoding the Aβ peptide was likely to be on chromosome 21 (Glenner & Wong 1984a). Around the same time that Aβ was first identified, it was also reported that a predisposing gene locus for AD mapped to chromosome 21 as well (Goate et al 1989; St George-Hyslop et al 1987). As predicted by Glenner and Wong in their 1984 paper, it was confirmed shortly thereafter that the gene including the Aβ sequence, which encoded the amyloid precursor protein (APP), was indeed found on

chromosome 21 (Goldgaber et al 1987; Kang et al 1987). Identification of specific gene mutations in *APP* that led to the development of an autosomal dominant, familial form of early onset AD (FAD) quickly followed (Goate et al 1991; Murrell et al 1991).

We now know that APP is a type 1, single transmembrane protein that undergoes a series of proteolytic cleavages in order to generate, or preclude the generation of, the Aβ peptide, as will be described in more detail below. There are three different primary forms of APP that are found in humans that vary in length due to alternative splicing. The two longer forms of APP, APP_{751} and APP_{770}, are preferentially expressed in non-neuronal cells throughout the body. The third, and, due to lack of the Kunitz protease inhibitor (KPI) domain, shortest of the primary APP isoforms, APP_{695}, is highly expressed in neurons. All three forms of APP contain the Aβ sequence which is partially in the large APP ectodomain and partially within the transmembrane region of the precursor protein (Holtzman et al 2011). APP is part of a larger gene family, the amyloid-precursor like proteins (APLPs), along with two other related proteins, APLP1 and APLP2. However, unlike APP, neither of the human APLPs contain the Aβ peptide domain. APP homologs have also been identified in other species as well, including *Drosophila* (*Appl*), *C elegans* (*apl-1*) and other mammals (Holtzman et al 2011; Selkoe 2001). While the gene encoding APP appears to be well-conserved across species, it is worth noting that the Aβ sequence itself is not, suggesting that the primary function of APP is not related to the production of this peptide (Holtzman et al 2011).

As mentioned above, the first FAD mutations in *APP* were identified in 1991 (Goate et al 1991; Murrell et al 1991). Currently, there are at least 27 *APP* mutations that are known to cause FAD (Ertekin-Taner 2007). As will be described in more detail below, all of the identified *APP* mutations to date result in an increase in Aβ production and/or deposition, an important finding regarding the driving pathological causes of AD (Ertekin-Taner 2007; Holtzman et al 2011; Scheuner et al 1996; Selkoe 2001; Wisniewski et al 1991).

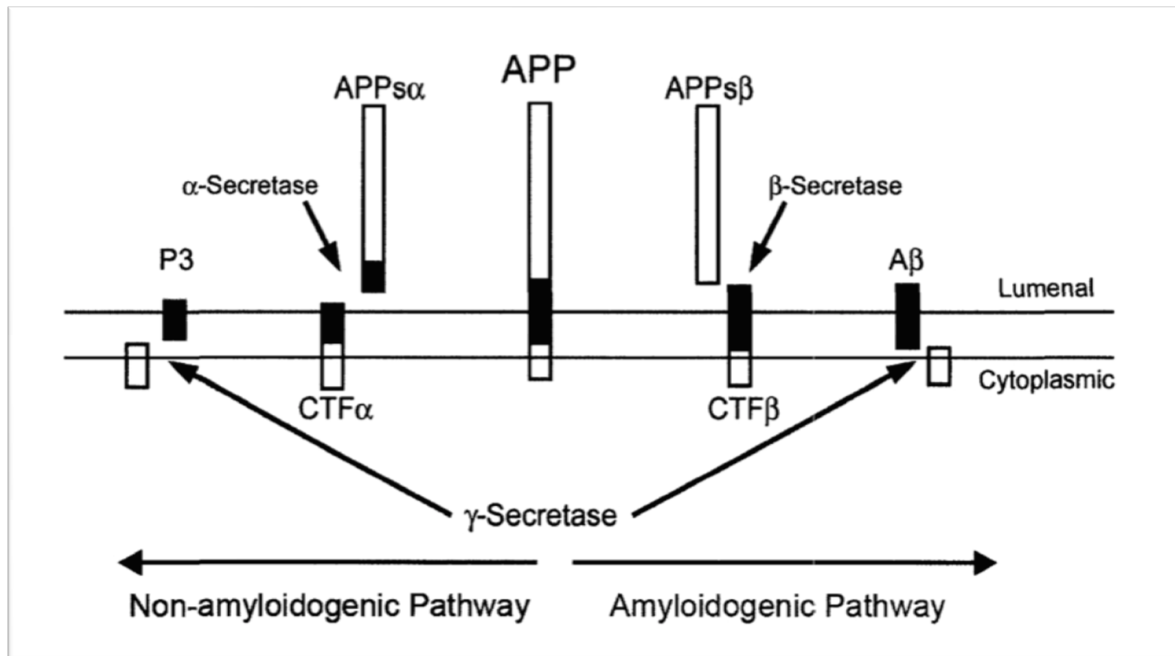
Shortly after the first APP mutations were published, an additional FAD gene locus was also identified on chromosome 14 via genetic linkage analysis (Schellenberg et al 1992). Sherrington et al subsequently reported the cloning of the presenilin 1 gene, *PSEN1*, from this site, as well as the identification of a series of missense mutations within this gene that were specifically associated with FAD (Sherrington et al 1995). Additional work by Levy-Lahad et al and others also identified similar missense mutations in the presenilin 2 gene, *PSEN2*, on chromosome 1 later that same year (Levy-Lahad et al 1995; Rogaev et al 1995). Presenilin 1 and 2 are highly homologous integral membrane proteins that are critical for the proper proteolytic processing of a number of transmembrane proteins within cells, including APP, as was later discovered. *PSEN* mutations are reported to account for up to 70% of all cases of FAD. There are at least 157 known mutations in *PSEN1* and 11 known mutations in *PSEN2* that cause FAD (Ertekin-Taner 2007). Because mutations in the presenilins almost all cause a specific increase in the more fibrillogenic and more toxic $A\beta_{42}$ peptide (Borchelt et al 1996; Duff et al 1996; Scheuner et al 1996), these mutations are associated with the most

aggressive forms of FAD, with individuals harboring these mutations sometimes showing the first signs of disease as early as in their 40s (Selkoe 2001).

While the mutations in *APP*, *PSEN1*, and *PSEN2* contribute to less than 1% of all cases of AD (Ertekin-Taner 2007; Holtzman et al 2011), understanding how mutations in these genes contribute the development of AD has been enlightening as to how Aβ is produced from APP and the causative role this plays in the disease process overall. The proteolytic processing of APP requires two sequential secretase cleavages (Figure 1.2). While the final cleavage is always made at the c-terminal end of the Aβ peptide fragment, the first cleavage event can occur at one of two sites within the APP lumenal domain and determines the fate of the Aβ peptide fragment. The first of these two potential cleavage sites is within the Aβ peptide fragment, at what is known as the α-secretase cleavage site. Cleavage at this site results in the release of the soluble APPα (sAPPα) fragment, precluding the formation of Aβ and committing the protein to the non-amyloidogenic processing pathway (Anderson et al 1991). The alternative cleavage site is 16 residues n-terminal to the α-secretase cleavage site, at the n-terminal of the Aβ peptide. Cleavage at this site, which is known as the β-secretase cleavage site, commits the protein to the amyloidogenic processing pathway and ultimately results in the liberation of the Aβ fragment from its full length precursor. β-secretase cleavage also releases the sAPPβ fragment (Haass et al 1992). Regardless of where APP is first cleaved, the second cleavage event always occurs at the γ-secretase cleavage site, which is within the transmembrane domain of the remaining membrane-bound portion of APP, at the c-

terminal of the Aβ fragment (Seubert et al 1992). In non-amyloidogenic APP processing, γ-secretase cleavage results in the release of the p3 fragment, while in amyloidogenic processing, this same cleavage event releases the Aβ fragment into the lumenal space (Selkoe 2001; Thinakaran & Koo 2008). There is some flexibility in the specific position where γ-secretase cleavage occurs, resulting in Aβ fragments of varying lengths ranging from 38 to 43 amino acid residues (Iwatsubo 2004). In both processing pathways, γ-secretase cleavage also results in the creation of the APP intracellular cytoplasmic domain (AICD) fragment, which may participate in intracellar signaling and/or transcriptional regulation (Thinakaran & Koo 2008). Each processing step occurs at a different point along the itinerant pathway that APP follows as it trafficks through the cell, with non-amyloidogenic processing occurring predominantly in the secretory pathway as newly synthesized full-length APP is trafficked to the cell surface (Sisodia 1992). Amyloidogenic processing, however, occurs predominantly in the endocytic and recycling pathways, as uncleaved APP is reinternalized to the endosomes (Koo & Squazzo 1994; Small & Gandy 2006). This differential distribution of secretase activity within the cell has important implications for determining the level of Aβ production in a given cell. Finally, it is important to recognize that both amyloidogenic and non-amyloidogenic processing of APP are generally believed to be normal metabolic events, as Aβ is found in the CSF and plasma throughout life (Selkoe 2001; Thinakaran & Koo 2008). However, in AD, various genetic and other causes result in an imbalance between the production and clearance of Aβ that results in a significant increase in the amount of Aβ (and especially $Aβ_{42}$) that is found in the brain.

Figure 1.2 APP Processing



Proteolytic processing of APP can occur along one of two pathways. In the amyloidogenic pathway (right), APP is first cleaved by β-secretase within the extracellular domain at the n-terminal of the Aβ peptide sequence, releasing the APPsβ fragment and leaving the CTFβ fragment remaining within the membrane. This fragment is then cleaved at the c-terminal of the Aβ peptide sequence, releasing Aβ from the membrane and generating the APP intracellular domain fragment (AICD). Alternatively, APP can be processed along the non-amyloidogenic pathway (left). In non-amyloidogenic processing, full length APP is first cleaved by α-secretase within the Aβ fragment itself, releasing the APPsα fragment and precluding the formation of Aβ. The remaining CTFα fragment is then cleaved by γ-secretase to generate the p3 fragment and the APP AICD.

Image courtesy of the Levey-Lah lab, Emory University.

All of the identified *APP* FAD mutations identified to date cluster around one of the three secretase cleavage sites (Ertekin-Taner 2007; Hardy 2006; Selkoe 2001). Mutations near either the α- or β-secretase cleavage sites (such as the APP_{SWE} mutations that occur in the two amino acids immediately preceding the β-secretase cleavage site) generally result in an increase in total Aβ production, similar to the effect of Trisomy 21 in Down's syndrome (Scheuner et al 1996; Selkoe 2001). Mutations in *APP* that are near the γ-secretase site, however, tend to result in a more specific increase in $Aβ_{42}$ levels while the amount of total Aβ being produced generally remains the same, resulting in a shift in the ratio of $Aβ_{42}$ to $Aβ_{40}$ (Hardy 2006; Holtzman et al 2011). $Aβ_{42}$ is the more fibrillogenic Aβ species and the deposition of $Aβ_{42}$ into diffuse plaques is thought to be the initiating event in amyloid plaque formation in the brain (Iwatsubo et al 1994; Jarrett et al 1993; Mann et al 1996; Selkoe 2001). As such, increasing the ratio of $Aβ_{42}$ to $Aβ_{40}$ has important ramifications for establishing the onset of AD pathogenesis.

It is worth noting here that the normal function of APP outside of its role as the Aβ precursor protein has not been well established. Among other proposed functions, the soluble APP fragments (particularly sAPPα) have been reported to stimulate cell growth and increase synaptic density, suggesting that the production of these particular fragments may have autocrine and/or paracrine neurotrophic effects (Holtzman et al 2011; Thinakaran & Koo 2008). Full length APP has also been reported to play a role in cell-cell adhesion, potentially acting as an integrin (Selkoe 2001; Thinakaran & Koo 2008). However, while it is likely that APP performs a

fundamental role (or roles) in cells under normal conditions (especially in light of its evolutionary conservation), none of the AD-associated *APP* mutations identified to date appear to interfere with any of the putative functions of APP (Holtzman et al 2011; Selkoe 2001). Moreover, AD-associated *APP* mutations are also known to increase the production of Aβ in CAA, suggesting that these mutations lead to the development of AD through a toxic gain-of-function effect relating to the increased production of Aβ (Thinakaran & Koo 2008).

Similar to the mutations in *APP*, mutations in the presenilin genes have almost all been associated with a specific increase in the production of $A\beta_{42}$ (Borchelt et al 1996; Duff et al 1996; Lemere et al 1996b; Mann et al 1996; Scheuner et al 1996; Xia et al 1997a). A large body of evidence has now demonstrated that the presenilins are a critical component of the heterogeneous multi-protein complex that mediates γ-secretase cleavage of APP and that this specific effect of presenilin mutations on $A\beta_{42}$ levels is due to changes in this cleavage event (Edbauer et al 2003; Iwatsubo 2004; Martoglio & Golde 2003). This evidence is covered in depth elsewhere (see, for example, (Selkoe 2001)) and will only be summarized briefly here. Of particular note is the observation that presenilin knock out mice have normal levels of the APP holoprotein and the sAPP fragments generated by α- and β-secretase cleavage, but have decreased levels of Aβ and increased levels of the membrane-bound γ-secretase substrates C99 (generated by α-secretase) and C83 (generated by β-secretase) (De Strooper et al 1998). Moreover, the phenotype of these presenilin knock out mice is highly similar to that associated with interrupting

the Notch signaling cascade. Given that Notch is also proteolytically cleaved by γ-secretase, this observation further confirms that the presenilins play a role in mediating γ-secretase cleavage (De Strooper et al 1999; De Strooper et al 1998; Selkoe 2001). Additional research has also shown that the presenilin proteins can bind to and be co-immunoprecipitated with APP (Weidemann et al 1997; Xia et al 1997b), that the mutation of two aspartates within the presenilin transmembrane domain results in markedly decreased levels of Aβ production (Wolfe et al 1999), and that pharmacological compounds that are known to inhibit γ-secretase cleavage bind specifically and selectively to presenilins (Esler et al 2000; Li et al 2000). Together, these findings have all conclusively demonstrated that the presenilins are the critical enzymatic component of the γ-secretase machinery, which we now recognize also includes nicastrin, APH-1 and PEN2 (Edbauer et al 2003; Iwatsubo 2004; Serneels et al 2009; Yu et al 2000).

The identity of the other secretases is also now known. α-secretase cleavage appears to be mediated by one of a host metalloproteases, including TACE/ADAM17, ADAM9, ADAM10, MDC-9 and BACE-2 (Allinson et al 2003). These proteases are generally found at the cell surface, which is in agreement with the reported site of α-secretase activity during the life cycle of APP (Sisodia 1992). β-secretase cleavage appears to be mediated exclusively by BACE-1, a transmembrane aspartyl protease (Cai et al 2001; Vassar 2004). BACE-1 predominantly localizes to the late Golgi/TGN and endosomes, which is also in line with reports that β-secretase cleavage primarily occurs during the endocytosis and recycling of APP

(Koo & Squazzo 1994; Small & Gandy 2006). High neuronal expression of BACE-1 is believed to channel APP preferentially through the amyloidogenic processing pathway (Koo et al 1990).

A key step in understanding the regulation of APP processing and Aβ generation came with this noted recognition that the different cleavage steps occurred at distinct intracellular locations. Based on this observation, it has been hypothesized that altering the trafficking of APP could have important ramifications for the production of Aβ. This hypothesis is supported by the finding that mutations in the putative YENPTY internalization sequence in the APP cytoplasmic tail decreases both the internalization of APP and the production of Aβ (Perez et al 1999). Ultimately, this suggests that factors that can influence the trafficking of APP, including APP cytosolic adaptors like X11 and Fe65, could prove to be important targets for disease-modifying interventions (Miller et al 2006). Finally, recent evidence has shown that secretase-mediated cleavage of APP takes place within cholesterol-rich lipid raft microdomains within the plasma membrane or the membranes of intracellular organelles (Ehehalt et al 2003; Riddell et al 2001; Vetrivel et al 2005; Vetrivel et al 2004), further emphasizing that the production of Aβ is likely to be influenced by factors that can enhance or abrogate the exposure of APP to the various secretases within the cell.

The Amyloid Cascade Hypothesis – A turning point in understanding the disease

In addition to providing insights into the production of Aβ, the identification of causative mutations in the *APP* and *PSEN* genes led quickly to the generation of animal models harboring mutations in these genes as well as in the gene encoding the tau protein, *MAPT*. By studying the progressive development of AD-related pathological changes in these animal models, as well as in human populations destined to develop AD as a result of trisomy 21 or FAD gene mutations, scientists have gleaned a more thorough understanding of the relationships between, and the temporal ordering of, the various pathological events in the development of AD.

One of the earliest and most important theories to come out of this work was the amyloid cascade hypothesis, which was first proposed almost simultaneously in the early 1990s in two separate papers by Dennis Selkoe (Selkoe 1991) and by John Hardy and Gerald Higgins (Hardy & Higgins 1992), the latter of which gave the theory its name. This hypothesis posited that it is the accumulation of Aβ in the brain that is the triggering event for the remainder of the AD pathogenic cascade. Or, as Hardy and Higgins wrote in their paper, “Our hypothesis is that deposition of amyloid β protein (Aβ), the main component of the plaques, is the causative agent of Alzheimer’s pathology and that the NFTs, cell loss, vascular damage, and dementia follow as a direct result of this deposition.” (Hardy & Higgins 1992) While the evidence for this hypothesis at the time was limited to the development of AD in individuals with Down’s syndrome with trisomy of *APP* and a handful of causative *APP* mutations, additional evidence accumulated over the past two decades has strengthened this hypothesis considerably. While the original hypothesis has been modified somewhat since it was first proposed in order to emphasize the

importance of toxic soluble Aβ oligomers and protofibrils, with a particular focus on the $Aβ_{42}$ species (Hardy 2006), the amyloid cascade hypothesis has generally stood the test of time to become one of the primary tenets in our understanding of how AD develops pathologically in the brain.

As noted, there is now significant evidence to support the amyloid cascade hypothesis. The earliest suggestion that Aβ may play an initiating role in AD came from Down's syndrome patients, as has been acknowledged elsewhere in this dissertation already [88, 89, 140]. The report by Prasher et al describing unique cases of Down's syndrome that failed to develop AD due to the trisomy of chromosome 21 occurring distally to the *APP* gene led considerable depth to this evidence (Prasher et al 1998). In addition, all of the known mutations to date in the FAD genes *APP*, *PSEN1*, and *PSEN2* have been shown to affect either the production or, through enhanced fibrillogenesis, the deposition of Aβ, with the majority of known mutations leading to a specific increase in $Aβ_{42}$, as described above (Ertekin-Taner 2007; Hardy 1997). Together, these findings show that altering Aβ production is sufficient to drive the development of AD.

The amyloid cascade hypothesis gained considerable strength with the finding that mutations in *MAPT*, the gene encoding tau cause frontotemporal dementia with parkinsonism (FTDP), and not AD (Goedert et al 1998; Hutton et al 1998; Poorkaj et al 1998; Spillantini et al 1998). The brains of patients with FTDP feature significant NFT pathology, but do not develop amyloid plaques. Thus, as Hardy and Selkoe put it in their ten year retrospective review of the amyloid

cascade hypothesis, "even the most severe consequences of tau alteration – profound NFT formation leading to fatal neurodegeneration – are not sufficient to induce the amyloid plaques characteristic of AD" (Hardy & Selkoe 2002). In addition, while mice expressing only *APP* or *PSEN* mutations fail to develop plaques due to the absence of human tau, in mice genetically engineered to express all three mutant genes (*APP*, *PSEN*, and *MAPT*), Aβ deposition consistently develops prior to the tangle pathology, as predicted by the amyloid cascade hypothesis (Oddo et al 2003). Moreover, transgenic mice expressing both mutant human *APP* and mutant *MAPT* show enhanced formation of NFTs (as compared to mice expressing mutant *MAPT* alone) while the structure and number of amyloid plaques are essentially the same as in *APP* single transgenic mice (Lewis et al 2001; Lewis et al 2000). Together, this evidence convincingly demonstrates that upstream Aβ production can accelerate and enhance neurofibrillary degeneration.

Additional evidence has also shown that both active and passive immunization against Aβ in transgenic mice results in decreased Aβ pathology, improved memory performance and may even promote the recovery and/or clearance of early neurofibrillary lesions, thus demonstrating that enhanced clearance of Aβ from the brain also improves downstream pathological changes associated with enhanced Aβ production (Bard et al 2000; Brendza et al 2005; DeMattos et al 2001; Dodart et al 2002; Ferrer et al 2004; Janus et al 2000; Morgan et al 2000; Oddo et al 2004; Schenk et al 1999; Weiner et al 2000). Moreover, novel means of monitoring pathological changes in the brain through cerebrospinal fluid (CSF) biomarkers and live imaging has definitively shown that biomarkers relating

to amyloid pathology become abnormal long before any disease-related changes in tau, synaptic function or cortical cell loss are apparent (Jack Jr et al 2010; Sperling et al 2011). Ultimately, the emergence of additional genetic, biochemical, histological and imaging evidence over the years has only served to strengthen the amyloid cascade hypothesis.

One of the primary arguments against the amyloid cascade hypothesis is that the amyloid plaque burden in one's brain does not correlate with the degree of cognitive impairment exhibited by that individual. This argument arose almost immediately after the amyloid cascade hypothesis was first published and continues to be made by detractors today. However, it is important to recognize that the degree of pathology in the brain does not need to correlate with the degree of disease severity in order for that pathology to have triggered the development of the disease, as is posited by the amyloid cascade hypothesis. Rather, it may be that a variety of genetic and environmental causes combine to establish a threshold level of Aβ that can be tolerated by each individual and that it is only when Aβ levels surpass this threshold that the downstream disease processes are initiated. Moreover, changes in Aβ levels in the brain often occur far in advance of the first clinical symptoms of the disease are generally maximally abnormal by the time cognitive impairment becomes apparent, as has been shown convincingly by work studying Aβ biomarkers in preclinical AD (Jack Jr et al 2010; Sperling et al 2011). This timeline is also in agreement with the early histological work of Blessed, Roth and Tomlinson that showed that the strongest correlations between amyloid plaque counts and cognitive impairment were in the mildest stages of the disease and that

while all end stage AD patients have some degree of plaque pathology, there is no longer a direct relationship between disease severity and plaque burden in the latest stages of the disease (Blessed et al 1968; Roth et al 1967; Roth et al 1966). Finally, as John Hardy bluntly put it in an early review of the amyloid cascade hypothesis in 1997, expecting the degree of plaque pathology to correlate with disease severity presumes that deposited plaques are permanent and that they stay around long enough for neuropathologists to count them, a presumption that is, to date, still unproven (Hardy 1997).

The other predominant argument that was levied against the amyloid cascade hypothesis when it was first proposed was the lack of a known mediating neurotoxic species of Aβ. Today, it is widely recognized that while the amyloid plaques themselves do not appear to be neurotoxic, smaller soluble oligomers of Aβ are likely to be the primary mediators of numerous downstream, disease-propagating effects (Walsh & Selkoe 2007). Unlike with the amyloid plaques, the concentration of soluble Aβ oligomers (which are also known as AD diffusible ligands/ADDLs or Aβ protofibrils) has been shown to correlate well with cognitive impairment, especially, again, in the earlier stages of the disease (Lue et al 1999; McLean et al 1999; Näslund et al 2000). Moreover, a considerable body of work has now shown that these soluble Aβ oligomers are highly neurotoxic, both to cultured neurons and *in vivo* following injection into animal models (Hartley et al 1999; Klein et al 2001; Lambert et al 1998; Mucke et al 2000; Shankar et al 2007; Walsh et al 2005; Yankner et al 1989; Yankner et al 1990). A host of downstream effects have

now been attributed to soluble Aβ oligomers, which may be related through a continuous cascade of events that is initiated by Aβ or that may occur in parallel to promote the neurodegenerative processes of the disease. In particular, soluble Aβ oligomers have been shown to increase both the hyperphosphorylation of tau and the activity of kinases known to phosphorylate tau, such as GSK3β (Alvarez et al 1999; Takashima et al 1998; Takashima et al 1993). Oligomers of Aβ also appear to block long term potentiation (LTP) in cultured hippocampal neurons, an important mechanism underlying learning and memory (Hartley et al 1999; Hsia et al 1999; Lambert et al 1998; Walsh et al 2002). Soluble Aβ has also been shown to act as a "pro-oxidant", causing disruption of the plasma membrane and Ca^{2+} homeostasis within the cell, which could have a number of important consequences for cellular function (Lau et al 2006; Masters & Beyreuther 2006; Mattson et al 1992). Finally, Aβ can initiate an inflammatory response through the activation of microglia and the classic complement system, resulting in the release of neuroinflammatory mediators and the recruitment of astrocytes to the site of Aβ accumulation (Akiyama et al 2000; Barger & Harmon 1997; Paresce et al 1996; Rogers et al 1992; Snyder et al 1994; Tan et al 1999; Yan et al 1998). While the specific neurotoxic mechanism (or mechanisms) of soluble Aβ is still under debate, it is increasingly clear that an increase in the production of soluble Aβ oligomers, as appears to occur in the earliest stages of AD, can have significant downstream effects that all work towards promoting the neurodegeneration and cognitive deficits that define AD.

The elucidation of the APP processing pathways and the downstream cascade of pathological events that is triggered by the abnormal accumulation of Aβ in the brain has suggested a number of potential targets for disease modifying (or disease preventing) therapeutic intervention. Given that the downstream disease processes are initiated by an increase in Aβ production, a large focus of therapeutic research has been on preventing the generation of Aβ from APP through the pharmacological inhibition of the amyloidogenic processing pathway (Citron 2004; Leung et al 2000; Lleó et al 2006). In particular, a number of γ-secretase inhibitors have been identified that result in a marked decrease in Aβ production (Dovey et al 2001). However, because γ-secretase is required for the proper cleavage of a number of other transmembrane proteins, including the Notch receptor (De Strooper et al 1999), inhibiting the normal function of γ-secretase results in a number of significant side effects that make this strategy generally untenable for therapeutic use (Siemers et al 2005; Wong et al 2004). However, mice lacking BACE-1, the enzyme responsible for β-secretase cleavage of APP (Cai et al 2001), appear to be phenotypically normal while producing significantly less Aβ, suggesting that β-secretase inhibitors may be particularly valuable as therapeutic targets (Citron 2002; Luo et al 2001; Roberds et al 2001).

An alternative strategy to blocking the production of Aβ via secretase inhibitors has been to promote the clearance of Aβ after it is has been produced, thus maintaining low, biologically safe levels of Aβ in the brain (Brody & Holtzman 2008; Lleó et al 2006). This strategy was initiated following the surprising finding

by Schenk et al that immunization with Aβ ameliorated APP pathology in a mouse model of AD (Schenk et al 1999). Additional studies of both active and passive Aβ immunotherapy confirmed this finding and further demonstrated an improvement in memory performance following Aβ immunization as well (Bard et al 2000; DeMattos et al 2001; Dodart et al 2002; Ferrer et al 2004; Janus et al 2000; Morgan et al 2000; Weiner et al 2000). These encouraging findings led quickly to a clinical trial of a similar approach in humans that was halted early on after several participants developed meningoencephalitis (Gilman et al 2005; Orgogozo et al 2003). Despite the early termination of the clinical trial, follow up studies on the participants from the trial have been encouraging, with post mortem analyses showing definitive clearance of Aβ pathology from the patients receiving $A\beta_{42}$ immunizations (Holmes et al 2008; Nicoll et al 2003). Moreover, the patients that were found to have the highest titer of anti-Aβ antibodies in their bloodstream were also found to have least cognitive decline over time (Hock et al 2003). Additional work to perfect this anti-Aβ therapeutic strategy is still ongoing.

Finally, while not specifically targeting Aβ itself, a number of AD therapies are being pursued that are focused on blocking or ameliorating the downstream effects of Aβ toxicity instead. For example, because Aβ is known to trigger a potentially destructive inflammatory response, non-steroidal anti-inflammatory drugs (or NSAIDs) have been increasingly proposed for use in treating AD patients (Akiyama et al 2000; Heneka & O'Banion 2007; McGeer & McGeer 1995; O'Banion & Finch 2006; Rogers et al 2006). Long term NSAID therapy has been shown to delay

the onset of cognitive symptoms, reduce symptomatic severity and slow the rate of cognitive decline in AD through the inhibition of microglial activation and astrocytic recruitment (Alafuzoff et al 2000; Lim et al 2000; Lim et al 2001; Mackenzie 2001; Mackenzie & Munoz 1998; Rich et al 1995). Interestingly, NSAIDs also appear to decrease the levels of $A\beta_{42}$ in the brain (Eriksen et al 2003; Lim et al 2000; Sung et al 2004; Yan et al 2003), potentially by altering the confirmation of the presenilin proteins and γ-secretase cleavage (Eriksen et al 2003; Lleó et al 2004; Weggen et al 2001), suggesting that these drugs may counteract the development of AD on multiple fronts. Because oxidative stress is known to be involved in the disease processes of AD and because Aβ itself has been proposed to act as a "pro-oxidant", traditional anti-oxidant therapies may also be of some benefit in blocking the development of AD (Masters & Beyreuther 2006; Selkoe 2001). Moreover, because the binding of metal ions like Zn^{2+} and Cu^{2+} to Aβ can promote Aβ aggregation (Bush et al 1994), and because this aggregation is known to produce H_2O_2 and hydroxyl radicals (Tabner et al 2005), metal chelators have also been proposed as potential disease-modifying treatments for AD (Cherny et al 2001). However, while many of these therapies are currently being pursued, none of these approaches have been shown to prevent the development of AD nor to slow the progression of the disease once dementia develops, suggesting that there is still much work to be done.

ApoE, Cardiovascular Risk Factors and Susceptibility to Disease

As mentioned above, mutations in *APP*, *PSEN1* and *PSEN2* account for less than 1% of all AD cases (Ertekin-Taner 2007; Holtzman et al 2011). While the true cause of the remaining 99% of AD cases has yet to be determined, there are a number of genetic and lifestyle factors that have been known to increase, or in rare instances, decrease the likelihood that an individual will develop AD.

To date, the best established and most widely accepted genetic risk factor for late onset, sporadic AD is apoE genotype (Kim et al 2009). ApoE is a 299 amino acid apolipoprotein that mediates the internalization of lipids via receptor-mediated endocytosis (Pfrieger 2003; Rensen et al 2000; Wernette-Hammond et al 1989). ApoE is found in several organs, with the highest expression levels being found in the liver and in the brain, where it is preferentially produced by non-neuronal cells such as astrocytes and microglia (Grehan et al 2001). While other apolipoproteins can be found in the brain (most notably apoA-1 and apoJ), apoE is the predominant apolipoprotein in the central nervous system (Pitas et al 1987). There are three common isoforms that arise from missense mutations at amino acids 112 and 158. The apoE ε2 isoform has cysteines at both positions 112 and 158, while the ε3 isoform, which is the most common isoform, has a cysteine at position 112 and an arginine at position 158. Finally, the apoE ε4 isoform has arginines at both amino acid 112 and 158 which results in conformational changes that likely result in functional deficiencies (Dong & Weisgraber 1996; Dong et al 1994; Mahley et al 2006).

An association between apoE and AD was first suspected in 1991, when, in the course of studying changes in lipid biology in AD, Namba and colleagues discovered that apoE immunoreactivity colocalized with amyloid plaques in the brain (Namba et al 1991), an observation that was later confirmed by others as well (Näslund et al 1995; Wisniewski & Frangione 1992). Around this same time, an AD genetic linkage study reported the presence of an AD susceptibility locus on chromosome 19, close to the site of the apoE gene (Pericak-Vance et al 1991). In short order, it was then discovered that the apoE ε4 genotype is over-represented in late onset AD (St Clair et al 1995; Strittmatter et al 1993) and that the presence of an apoE ε4 allele causes a dose-dependent increase in the risk of developing late onset AD (Corder et al 1993; Saunders et al 1993), as well as a related dose-dependent effect on lowering the average age of disease onset (Corder et al 1993; Gómez-Isla et al 1996b; Murphy et al 1997; Norrman et al 1995; Roses 1996). It was also found that having an apoE ε2 allele was protective against the development of AD (Corder et al 1994; Farrer et al 1997). We now know that individuals with one ε4 allele have a 2-3 fold increase in lifetime risk of developing AD, while individuals who are homozygous for apoE ε4 have up to a 12 fold increase in lifetime risk (Bertram et al 2011; Roses 1996). It has been estimated that 55% of apoE ε4/4 individuals will develop AD by age 80, while only 3.1% of apoE ε3/3 individuals will develop AD by the same age (Myers et al 1996). While apoE genotype clearly has a strong effect on the likelihood that an individual will develop AD in the future, it is important to note that unlike the causative FAD gene mutations, apoE genotype is a susceptibility

factor for AD. Although possession of an apoE ε4 allele has a marked increase in risk, it does not guarantee that an individual will develop AD.

While the effect of apoE genotype on AD risk and mean age of disease onset is now well established, the mechanism of action through which apoE exerts these effects is not yet well established and, as one would expect, is a matter of intense debate. The most prominent effect of apoE genotype on AD pathology that is thought to underlie the effect of apoE genotype on the average age of disease onset is the apoE ε4 dose-dependent increase in amyloid plaque burden that is seen in the brains of ε4 carriers, even in the absence of cognitive impairment or dementia (Polvikoski et al 1995; Rebeck et al 1993; Schmechel et al 1993). Because Aβ production does not seem to be altered by the different apoE isoforms, it has been widely hypothesized that this increase in amyloid burden is the result of decreased Aβ clearance (Gearing et al 1996). While all three isoforms of apoE are capable of binding Aβ, binding affinity of Aβ to apoE ε4 is the weakest (LaDu et al 1994; Strittmatter et al 1993; Yang et al 1997). Because apoE-bound Aβ can be cleared from the extracellular space by microglia and astrocytes via receptor-mediated endocytosis, this weak association between apoE ε4 and Aβ is believed to result in impaired clearance of Aβ in apoE ε4 positive individuals (Beffert et al 1999; Beffert et al 1998; Cole & Ard 2000; Nielsen et al 2009; Yamauchi et al 2000; Yang et al 1999). ApoE-mediated clearance of Aβ across the blood brain barrier is also likely to be impaired in apoE ε4 positive individuals for similar reasons (Zlokovic 2008). Ultimately, this decreased clearance contributes to the abnormal accumulation of Aβ

in the brain that is known to initiate the downstream AD pathogenic cascade, as described by the amyloid cascade hypothesis.

To date, decreased clearance of Aβ in apoE ε4 carriers is the best supported hypothesis to explain the effect of apoE genotype on AD risk and age of disease onset. However, a number of other theories have been proposed that may explain the effects of the apoE ε4 isoform on AD pathology, either independently or in concert with the reported effects of the ε4 isoform on Aβ clearance. One hypothesis that has been put forth proposes that the binding of apoE may induce structural changes in Aβ that promotes fibrillogenesis (Castaño et al 1995; Ma et al 1994; Wisniewski & Frangione 1992). This effect of apoE binding seems to be particularly strong on the $A\beta_{40}$ species, which is found in much higher levels in neuritic plaques in apoE ε4 positive individuals (Gearing et al 1996; Mann et al 1997). Moreover, it has been observed that compared to the apoE ε3 isoform, apoE ε4 is particularly ineffective at delivering lipids and cholesterol to neurons to aid in neurite outgrowth, synaptogenesis and membrane maintenance. Based on this finding, is has been suggested that the presence of apoE ε4 may promote neurodegeneration due to deficient membrane repair following an insult such as that exerted by the neurotoxic soluble Aβ oligomers (Bellosta et al 1995; DeMattos et al 1998; Narita et al 1997; Nathan et al 1994; Puttfarcken et al 1997; Teter et al 1999). Finally, while the apoE ε3 isoform has been reported to limit Aβ-driven neuroinflammation, the apoE ε4 isoform appears to be less effective in this regard and may even have pro-inflammatory effects in the brain (Barger & Harmon 1997; Colton et al 2004; Guo et

al 2004; LaDu et al 2001; Lynch et al 2003; Vitek et al 2009). Additional work is ongoing to determine the true impact of each of these observed effects of the apoE ε4 protein on the development of AD.

Although apoE genotype appears to be a contributing factor to the development of a large percentage of late onset AD cases, the large number of individuals who develop AD even in the absence of an apoE ε4 allele suggests that there are likely to be other AD susceptibility genes that have yet to be discovered (Gatz et al 2006). To date, over 500 genes have been proposed as putative AD genetic risk factors, as catalogued by the AlzGene database (Bertram et al 2011; Bertram & Tanzi 2008). Meta-analyses of AD genetic studies have identified a number of leading candidates, many of which appear to be related to systems that have been implicated in AD pathogenesis (Bertram et al 2007; Laumet et al 2010; Schjeide et al 2009). One of the most promising putative susceptibility genes (as determined by AlzGene) is *CLU*, the gene encoding apoJ/clusterin, an apolipoprotein that has been implicated in promoting Aβ fibrillogenesis and clearance, similar to apoE (DeMattos et al 2002; Ladu et al 2000). Mutations in the genes encoding the apoE receptors LDLR and LR11/SorLA have also been reported to increase risk of AD (L√§ms√§ et al 2008; Rogaeva et al 2007; Zou et al 2008). Other leading candidates for AD genetic risk factors include a number of inflammatory-related genes (*CR1*, *CCR2* and a number of interleukin genes), as well as the genes encoding ADAM10 (which may mediate α-secretase cleavage of APP) and the β2 subunit of the nicotinic acetylcholine receptor (Bertram et al 2011). However, while mutations in these top genes and others have been shown to nominally increase risk for

developing AD, few of these have been validated, in large part due to study populations that were too small to detect the relatively modest effects conveyed by these mutations (Bertram et al 2010). Ongoing work to validate these reported AD susceptibility genes and to identify others will likely prove fruitful in the future, both for enhancing our current knowledge of AD pathology and for suggesting potential risk-modifying therapeutic interventions.

While genetic mutations are likely to play a role in determining the risk of developing late onset AD, it is important to recognize that certain lifestyle factors can also have a strong effect on one's personal risk of developing AD. For example, it has been reported that conditions such as depression and head injury may increase the lifetime risk of developing AD (Caraci et al 2010; Geerlings et al 2000; Jordan et al 1997; O'Meara et al 1997; Ownby et al 2006; Tang et al 1996), while education may be protective against developing AD (Bennett et al 2003; Stern 2006). Perhaps the best established lifestyle factors that can influence AD risk are vascular-related risk factors and diseases. Risk factors that have traditionally been associated with an increased risk of heart disease, such as hypertension, high cholesterol levels and obesity, as well as having a history of one or more cardiovascular diseases, have also been shown to increase the risk for developing AD and other neurodegenerative dementias (de la Torre 2004; Honig et al 2003; Ivan et al 2004; Panza et al 2006; Solfrizzi et al 2004; Viswanathan et al 2009; Waldstein & Wendell 2010). For example, severe atherosclerosis has been associated with a 3-fold increase in the risk of developing dementia while approximately 30% of stroke patients are reported to develop dementia within three years of a stroke event, far beyond the

normal occurrence of disease related cognitive impairment (de la Torre 2004; Hénon et al 2001). A growing body of evidence now suggests that cardiovascular risk factors and diseases may increase the risk of developing AD through direct effects on the AD pathogenic cascade (de la Torre 2004; Hall et al 1995; Panza et al 2006; Snowdon et al 1997; Solfrizzi et al 2004). Of particular note, it has been observed that in animal models of AD, a high cholesterol diet increases the production of Aβ (Refolo et al 2000; Sparks et al 2000), possibly due to effects on the membrane lipid raft microdomains where APP processing is reported to occur (Ehehalt et al 2003; Riddell et al 2001; Vetrivel et al 2005; Vetrivel et al 2004). Because these vascular-related risk factors are often modifiable through diet, exercise and pharmacological intervention, controlling these factors may be of added benefit for delaying or preventing the development of dementia in general and AD in particular. For example, cholesterol lowering drugs have been shown to decrease Aβ pathology in AD transgenic mice while patients taking statins to control cholesterol levels have been reported to have a lower risk for developing AD (Forette et al 1998; Jick et al 2000; Refolo et al 2001; Wolozin et al 2000).

Current Conception of Alzheimer's Disease

Our current understanding of Alzheimer's disease holds that genetic and/or lifestyle risk factors combine to cause an abnormal increase in Aβ levels in the brain, with a particular increase in the more fibrillogenic $A\beta_{42}$ peptide, resulting in the ultimate aggregation of Aβ into first diffuse plaques and then later neuritic plaques.

This increase in toxic Aβ species initiates a host of downstream effects including membrane and synapse dysfunction, oxidative stress, tau hyperphosphorylation, and inflammation, which may occur individually or in series. Tau hyperphosphorylation then leads to the formation of paired helical filaments, which is thought to signal incipient cell death. This loss of first, synaptic contacts and later, whole cells, is reflected in a loss of signaling through acetylcholine and other neurotransmitters, memory dysfunction and ultimately dementia. This entire process, starting from the initial increase in Aβ levels, can take decades, with the development of cognitive impairment serving as a lagging clinical indicator for the presence of underlying disease. By the time that dementia develops, irreversible cell loss has already taken place in the brain.

While our current symptomatic treatments have some efficacy in dementia patients by replacing the functionality of cells that have already been lost, they do little to nothing to halt the disease's insidious march towards greater stages of dementia severity and eventually death. Therefore, in order for the disease modifying therapies that have been discussed in the previous sections to be the most effective, it is clear that intervention must occur prior to the first clinical signs of disease. In short, we must be able to predict the future for seemingly healthy patients if we are to prevent the development of AD. To that end, much recent work has focused on characterizing patients that have been diagnosed with mild cognitive impairment (MCI), a clinical disease stage that presages the development of AD. Often, by the time a neurologist first sees a patient that is destined to develop AD, they have already progressed to a stage of MCI. While many of the AD pathological

processes have likewise been long underway in MCI patients, understanding the underlying pathology in MCI has proven beneficial for establishing and confirming the earlier events in the AD pathological cascade and for identifying therapeutic targets that may prove beneficial at this stage for preventing or delaying the eventual conversion to dementia as a result of AD.

1.3 Mild Cognitive Impairment

Mild cognitive impairment is a clinical stage in which individuals have detectable levels of cognitive impairment that have not yet reached the severity of dementia. This stage of cognitive decline often presages the development of dementia due to AD, with MCI patients progressing to AD at a rate of approximately 10 - 15% per year, far above the 1 – 2% annual conversion rate of the normal, elderly population (Gauthier et al 2006; Maruyama et al 2001; Petersen 2004; Petersen et al 2001; Petersen et al 1999). It is important to recognize that due to differing underlying pathology, not all individuals who are diagnosed with MCI will ultimately go on to develop AD. Some individuals with MCI will develop other non-AD forms of dementia, some individuals will remain at a stable level of MCI for the remainder of life and some individuals with MCI will eventually revert to normal (Bennett et al 2002; Davis & Rockwood 2004; Hsiung et al 2004). However, while not all individuals with MCI will go on to develop AD, all individuals who are eventually diagnosed with AD will pass through a transient stage of MCI prior to the

development of full blown dementia, making MCI the first clinically detectable stage in the development of AD. As such, this diagnostic group has been widely used to identify and characterize early pathological events that may underlie the ultimate development of AD.

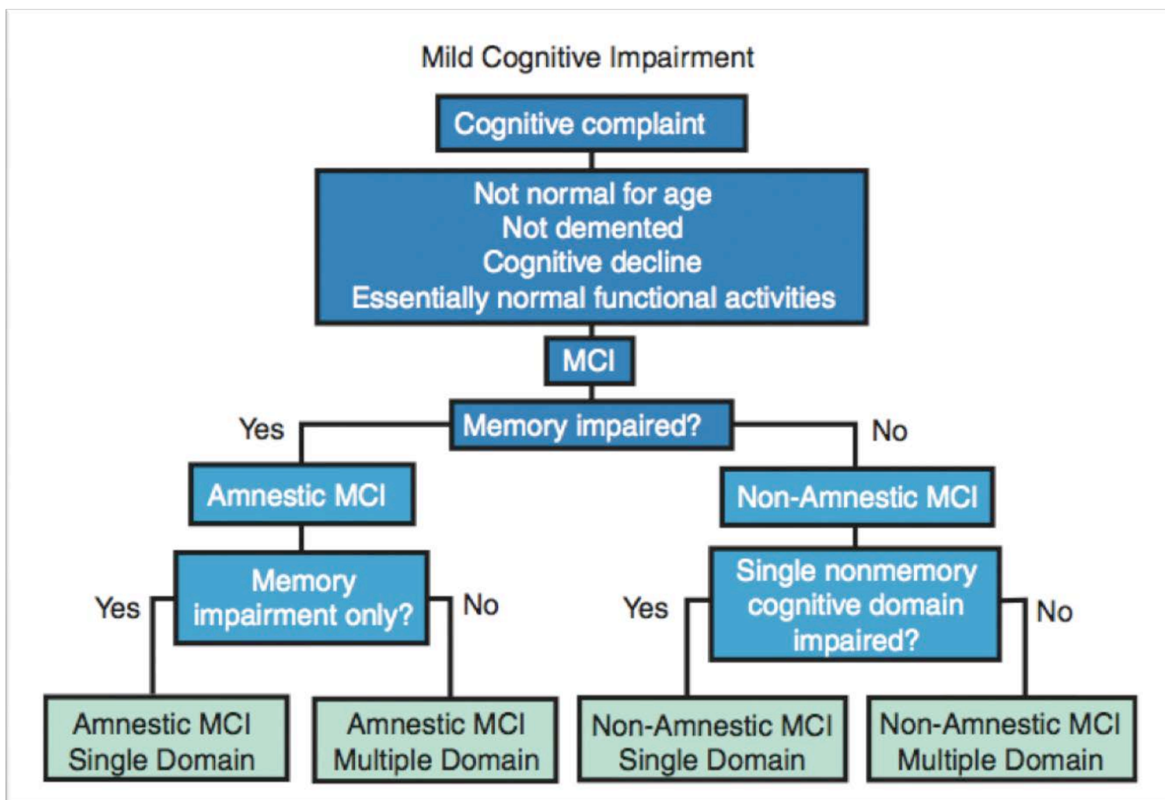
Reisberg and colleagues first used the terminology “mild cognitive impairment” in 1988 (Reisberg et al 1988), with this descriptor being used informally throughout the decade thereafter to describe individuals with a degree of cognitive impairment that was not normal for age but that was not severe enough to qualify for a diagnosis of dementia. Generally, this condition was considered to correspond to a Clinical Dementia Rating (CDR) of 0.5 or to a score of 3 on the Global Dementia Scale (Petersen et al 2001). Other terms that were used during this time to describe this same clinical phenotype include isolated memory impairment, cognitive impairment – no dementia (CIND), and incipient AD, with the latter term being preferred by those who focused on this stage purely in the context of the future development of AD at the exclusion of other possible underlying causes of MCI (Petersen et al 2009; Petersen et al 1999).

With the growing recognition that individuals with MCI were at a higher risk for developing AD than the general aging population, Petersen et al formally proposed the first clinical criteria for the diagnosis of MCI in 1999 (Petersen et al 1999). In particular, Petersen and colleagues noted that the primary clinical distinction between MCI and healthy control individuals was impaired memory performance, which did not distinguish between MCI and AD dementia. Rather, the

primary distinction between MCI and AD was the development of impairment in additional cognitive domains in the AD cases. As a result, a specific memory complaint in the absence of other cognitive difficulties was an important component of the original diagnostic criteria that were established for MCI.

Ultimately, this initial conception of MCI was found to be too limited, as it failed to acknowledge that while individuals diagnosed with amnestic MCI are at the greatest risk for progressing to AD, other subtypes of MCI, including those with limited impairment in other domains as well as those lacking specific memory difficulties were also at an increased risk for developing AD compared to the general population (Bozoki et al 2001; Panza et al 2007). As a result, an international working group came together in Stockholm, Sweden in 2003 in order to establish a consensus hierarchal scheme for the diagnosis of MCI and its various subtypes (Petersen 2004; Winblad et al 2004). This scheme lays out a two step process wherein the diagnosis of general MCI is made first using specific clinical criteria, with the subsequent assignment of a specific MCI subtype being made following the identification of the particular cognitive impairments that are present (Figure 1.3). The primary criteria for the diagnosis of MCI as laid out by this working group are: (1) That an individual is not cognitively normal for their age and education, but that the individual does not meet the criteria for dementia syndrome as laid out in the DSM IV manual. (2) There is evidence of cognitive decline. This can be determined either via self and/or informant report and impairment on objective cognitive tasks or through evidence of decline over time on objective cognitive tasks. And finally,

Figure 1.3 MCI Diagnostic Criteria



Criteria for the diagnosis of MCI, as established by the 2003 International Working Group on Mild Cognitive Impairment. MCI is first diagnosed using the following criteria: (1) Cognitive ability is not normal for age but is not severe enough for a diagnosis of dementia; (2) Evidence of progressive cognitive decline; (3) Essentially normal functional activities. Once a diagnosis of MCI is made, the patient is evaluated for the presence of a specific memory impairment and the presence of impairment in other cognitive domains, allowing for the subclassification of one of four MCI subtypes, as illustrated.

Image reproduced with permission from (Winblad et al 2004).

(3) that the degree of impairment present generally does not interfere with the normal activities of daily living. Following the diagnosis of MCI using these criteria, individuals can then be sub-classified as one of three MCI subtypes: (1) amnestic MCI (aMCI), wherein a subject's memory is significantly worse than would be expected for age with no additional impairment in non-memory domains; (2) multi-domain MCI (md-MCI), wherein mild deficits are noted in a number of different cognitive domains, which may or may not include memory impairment; or (3) single, non-memory MCI, which is exactly what it sounds like and features cognitive impairment in a single cognitive domain that is not memory-related. The criteria established at this conference continue to be widely used in both clinical and research settings today. In particular, these criteria serve as the basis for the Core Clinical Criteria as laid out by the 2011 National Institute of Aging – Alzheimer's Association Working Group (Albert et al 2011).

Because of the high rate of conversion to AD of individuals diagnosed with MCI in general, and aMCI in particular, a major research focus in the field has been on defining the pathological profile of MCI, especially with regard to pathological changes known to be related to the development of AD. It was initially thought that individuals with MCI were likely to have intermediate levels of pathological change compared to control or AD that would correspond to the intermediate level of cognitive impairment seen in these individuals. Alternatively, given the proposed linear cascade of pathological events that leads to AD, it was thought that MCI patients would have some of the pathological features of AD, but that the full complement of AD pathology would not develop until the dementia stage of the

disease, with this final pathological change underlying the final transition into dementia. However, contrary to either of these hypotheses, we now know that all of the major hallmarks of full-blown AD can be found in MCI brain, including extensive Aβ deposition (Guillozet et al 2003; Morris & Price 2001; Mufson et al 1999; Price & Morris 1999), NFTs (Guillozet et al 2003; Markesbery et al 2006; Mitchell et al 2000; Price & Morris 1999), synaptic dysfunction (Rombouts et al 2005; Scheff et al 2006) and some degree of cortical atrophy (Bozzali et al 2006; Carlson et al 2008; Kordower et al 2001; Price et al 2001). The recognition that the majority of MCI cases harbor a pathological profile that is highly similar to that found in AD brain, coupled with the longstanding finding that aMCI patients were at a particularly high risk of developing dementia in the near future led to the claim by Morris et al in 2001 that MCI was not a separate cognitive syndrome in and of itself, but was rather a very early stage of AD (Morris et al 2001).

Accumulating evidence from biomarker and imaging studies has now shown that while not every case of MCI is due to incipient AD, this is true for a large proportion of the MCI population. As a result, one of the primary goals of very recent work on MCI has been to distingush those individuals within the MCI group that have cognitive impairments due to underlying AD pathology from those that have MCI due to other, non-AD etiologies. In particular, this work has centered on the use of CSF protein biomarkers and live imaging of pathological changes in the brain in longitudinal, prospective studies of high risk cases in order to identify AD-related pathological changes in living patients with MCI (Blennow & Hampel 2003; Borroni et al 2006a; Borroni et al 2006b; Herukka et al 2007; Huang et al 2003; Jack Jr. et al

2010; Klunk et al 2004; Matsuda 2007; Shaw et al 2009; Vemuri et al 2009b). Through these studies, we now have a much clearer understanding of how the events of the AD pathological cascade relate temporally to the development of first MCI and later, dementia (Jack Jr et al 2010). Biomarkers for Aβ accumulation, including PET imaging of amyloid deposits in the brain and a corresponding decrease in the level of CSF $A\beta_{42}$, have demonstrated that Aβ accumulation in the brain occurs far in advance of the onset of MCI and has largely reached a plateau of maximal abnormality by this clinical stage (Engler et al 2006; Morris et al 2001; Perrin et al 2009). Biomarkers for tau and synaptic dysfunction (including increased levels of total and phospho-tau in the CSF, decreased brain glucose metabolism as detected by fluoro-deoxyglucose (FDG) PET imaging and others) generally become abnormal three to four years before a diagnosis of MCI (Craig-Schapiro et al 2010; Fagan et al 2007; Li et al 2007). Finally, MRI imaging suggests that neuronal degeneration begins just prior to the transition to MCI and becomes more extensive as throughout the MCI stage, as cognitive ability declines and the individual approaches the dementia stage of the disease (Carlson et al 2008; Jack Jr. et al 2010; Vemuri et al 2009a). In light of these findings, it is now recognized that MCI patients who have positive biomarkers for both Aβ accumulation and neuronal injury are at the greatest risk for developing AD in the near future, distinguishing them from the general MCI population as individuals most likely to benefit from the disease-modifying treatments that are now under development (Albert et al 2011).

Given our growing understanding of the relationship between MCI and AD, studying this unique population continues to have a number of important clinical

and experimental advantages. As described above, comprehensive work in this population to establish the value of known biomarkers and behavioral endophenotypes for predicting the conversion of MCI to AD is increasingly leading to the ability to detect incipient AD at earlier and earlier stages. This work provides hope that some day we can identify those people on the path towards developing AD before any degree of cognitive impairment is detectable (Howieson et al 2008; Reitz & Mayeux 2009; Storandt et al 2006). Moreover, because cortical atrophy has generally not yet become widespread in MCI, synaptic dysfunction and not overt cell loss is thought to be the main substrate contributing to cognitive impairment in MCI (Scheff et al 2006; Schliebs & Arendt 2011). This suggests that an individual's remaining cognitive faculties could be preserved at this stage by halting or reversing the pathological processes that have led to this synaptic dysfunction. Elucidating the active processes that are ongoing during this stage of disease progression could provide important insight for the development of treatments likely to be the most efficacious for preventing further cognitive decline and the development of dementia. Finally, while our understanding of the pathological processes leading to AD has grown immensely in recent decades, there are still many open questions yet to be resolved, with new contributing factors being identified all the time. By characterizing these factors in MCI as well as in full-blown AD, we can more clearly distinguish events that are likely to play a primary, contributing role to the development of AD from those changes that are likely to be secondary, end stage effects resulting from the ultimate degeneration of brain function towards the end of the disease.

1.4 LR11/SorLA

LR11: A multifunctional member of both the VPS10p and LDLR protein families

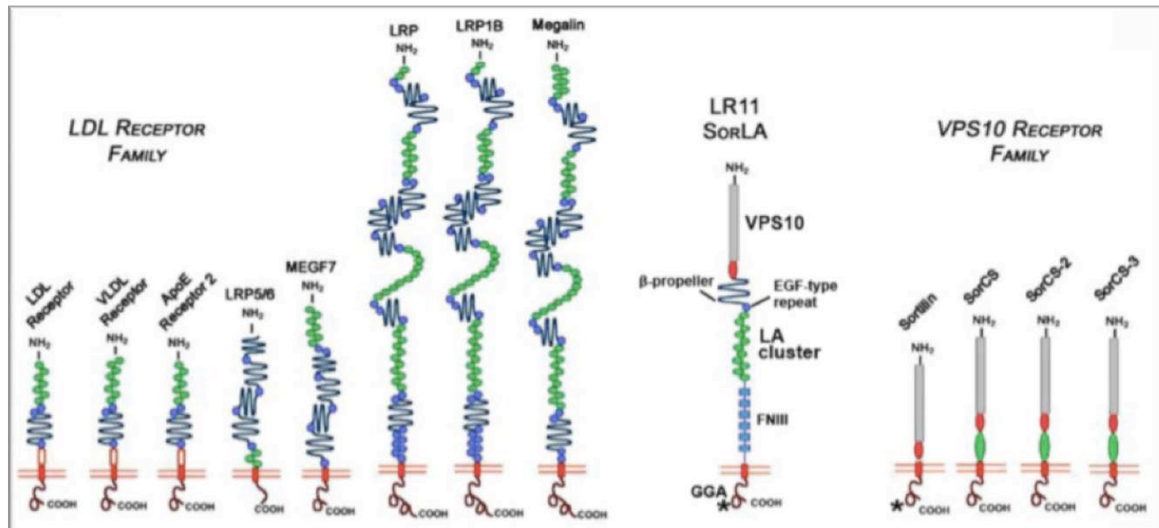
LR11 is a 250 kDa Type 1 transmembrane receptor that is found in a number of organs, including the liver, adrenal glands and testis (Hermans-Borgmeyer et al 1998; Yamazaki et al 1996). LR11 expression is particularly robust in the brain, where it is predominantly expressed in neurons in the cerebral cortex, hippocampus and in the Purkinje cells of the cerebellum (Hermans-Borgmeyer et al 1998; Motoi et al 1999). LR11 is a mosaic receptor that is composed of a series of functional domains in both the large, extracellular domain and in the shorter, 54 amino acid cytoplasmic tail (Jacobsen et al 1996). The extracellular functional domains include a short, N-terminal propeptide sequence, a larger vacuolar protein sorting 10 protein (VPS10p) homology domain, a β-propellor domain, 5 tandem LDLR consensus sequences found in an EGF precursor domain (also known as the EGF-type repeat), 11 low density lipoprotein receptor (LDLR) type A ligand binding repeats (also known as the LA cluster) and six copies of fibronectin type II repeats {Yamazaki, 1996 #7}. The primary functional domain in the cytoplasmic tail is an intracellular adaptor protein binding domain that facilitates the interactions between LR11 and the Golgi-localizing, γ-adaptin ear homology domain, ARF interacting proteins (GGAs) (Jacobsen et al 2002). The presence of both a VPS10p domain and the LDLR type A ligand binding repeats place LR11 in two separate

functional families: the VPS10p family of sorting receptors and the LDLR family of multifunctional receptors (Figure 1.4).

The VPS10p family-defining functional domain, the VPS10p domain was first identified in the *Saccharomyces cerevisiae* protein VPS10P, a sorting receptor that directs trafficking of lysosomal enzymes from the Golgi to the vacuole (Willnow et al 2008). In addition to this yeast protein, there are four known vertebrate VPS10p family members, in addition to LR11: Sortilin (the smallest of the VPS10p proteins) and three slightly larger and highly homologous proteins known as SorCS1, SorCS2, and SorCS3 (Hampe et al 2001). While VPS10p proteins have not yet been identified in *Drosphophila* or *C. elegans*, an additional VPS10p family member has been identified in *Chlorohydra viridissima* that facilitates head-specific differentiation of cells in response to the binding of the small ligand head activator (HA) and is therefore known as HAB (Christians et al 1993; Franke et al 1997). In addition to the extracellular VPS10p domain, Sortilin also contains a GGA-binding domain in the cytoplasmic tail, similar to that seen in LR11 (Nielsen et al 2001). To date, the best established function of VPS10p family members is the regulation of intracellular vesicular sorting from the Trans Golgi Network (TGN) to endosomal and/or lysosomal compartments (Willnow et al 2008). Because of its membership in this important sorting family, LR11 is also commonly referred to as SorLA.

The LDLR family is a group of transmembrane receptors that all harbor a series of Type A ligand binding repeats in their extracellular domains that vary in number and distribution in the assorted family members. Members of the LDLR

Figure 1.4 LR11 is a member of the LDLR and VPS10P protein families



The domain structure of LR11/SorLA places it both the LDL receptor family and the VPS10P family of sorting receptors. The large n-terminal ectodomain of LR11 contains a VPS10p homology domain, a β-propellor domain, five tandem LDLR consensus sequences found in the EGF precursor (labeled EGF-type repeat in the image), 11 LDLR type A ligand binding repeats (labeled LA cluster in the image) and six fibronectin type III repeats (labeled FNIII). The short cytoplasmic tail of LR11 also harbors a GGA-binding domain, which can also be found in sortilin.

family include the low-density lipoprotein receptor (LDLR) from which the family derives its name, the very low density lipoprotein receptor (VLDLR), ApoER2, the low density lipoprotein receptor related protein 1 (LRP1) and its homologues LRP1B, LRP5, LRP6 and megalin (LRP2), MegF7, and, of course, LR11 (Herz & Bock 2002; Wagner & Pietrzik 2011). All of the LDLR family members are capable of binding and internalizing low-density lipoproteins, including apoE, and they typically play an important role in regulating cholesterol homeostasis (Beffert et al 1998; Brown & Goldstein 1986; Nilsson et al 2007). To date, the best established general function of the LDLR family members is in clathrin-mediated endocytosis of extracellular or membrane-bound ligands (Jaeger & Pietrzik 2008). However, recent work has uncovered a growing number of extracellular ligands and intracellular adaptor proteins that are known to interact with one or more LDLR family members, resulting in a sizable array of putative functions that may be mediated by these receptors. These proposed functions include regulation of cell surface protease activity, transport and activation of steroid hormones, regulation of Ca^{2+} homeostasis, and the activation of a number of important intracellular signaling pathways both during development and in mature cells (Herz 2001). Indeed, the many functions that have been attributed to this family of receptors led Nykjaer and Willnow to dub them "cellular swiss army knives" in their 2002 review (Nykjaer & Willnow 2002).

As a particularly complex mosaic receptor itself, LR11 has also been implicated in a number of important cellular and developmental processes. LR11 was initially identified as a human orthologue of the *Hydra* protein HAB, a protein

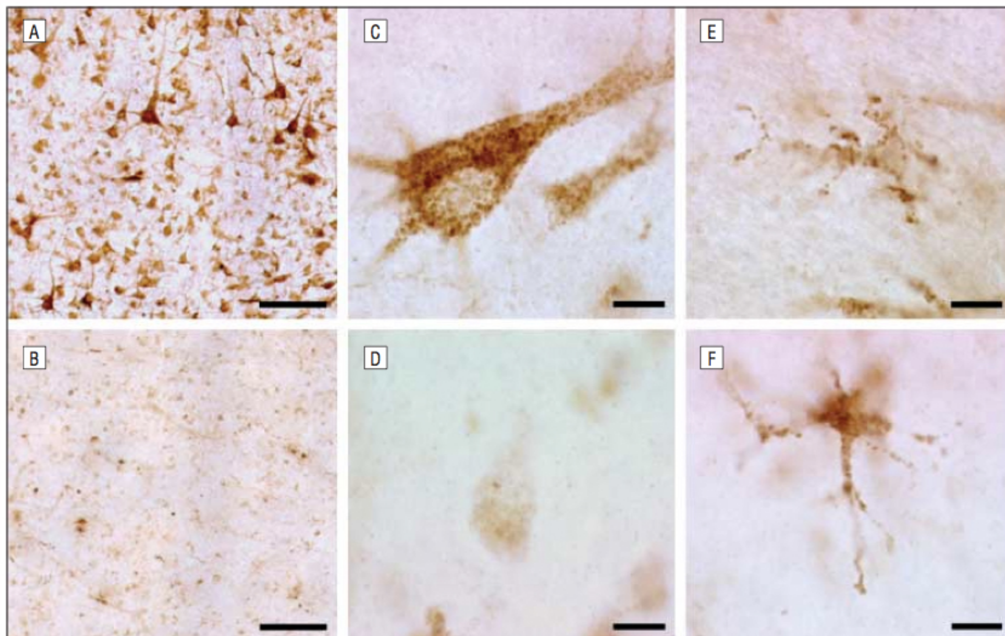
responsible for mediating head-specific differentiation in response to the binding of the undecapeptide head activator, as noted above (Christians et al 1993; Franke et al 1997; Hampe et al 2000). Given the particularly robust expression of LR11 in human cells during development, it has widely been believed to play a role in cellular morphogenesis, possibly through the γ-secretase mediated release of the LR11 cytoplasmic tail (Böhm et al 2006; Hermans-Borgmeyer et al 1998; Hirayama et al 2000; Nyborg et al 2006). Following this cleavage event, the LR11 intracellular c-terminal fragment can translocate to the nucleus, where it is known to act as a transcriptional factor (Fenger et al 1994; Galliot et al 1995; Hampe et al 2000). An increase in LR11 expression has also been reported in vascular smooth muscle cells in response to the presence of platelet derived growth factor – BB (PDGF-BB)(Kanaki et al 1999; Zhu et al 2002; Zhu et al 2004). Upregulation of LR11 in these cells has been associated with enhanced smooth muscle cell migration and invasion during atherosclerotic plaque formation. This likely results from an LR11-dependent increase in urokinase receptor (uPAR) presence at the cell surface (Bujo & Saito 2006; Zhu et al 2002), possibly due to competitive inhibition of the binding of uPAR to LRP1 and the subsequent rapid endocytosis of the uPAR/LRP1 complex (Gliemann et al 2004). In addition to these specific functions, LR11, like all LDLRs, is capable of binding and internalizing lipoproteins, including apoE, suggesting that LR11 may function at least in part as a regulated endocytic receptor (Jacobsen et al 2001; Nilsson et al 2008; Taira et al 2001). Finally, as might be predicted by the presence of the VPS10p domain in the LR11 extracellular domain and the GGA-binding domain in the LR11 cytoplasmic tail, LR11 plays a critical role in mediating

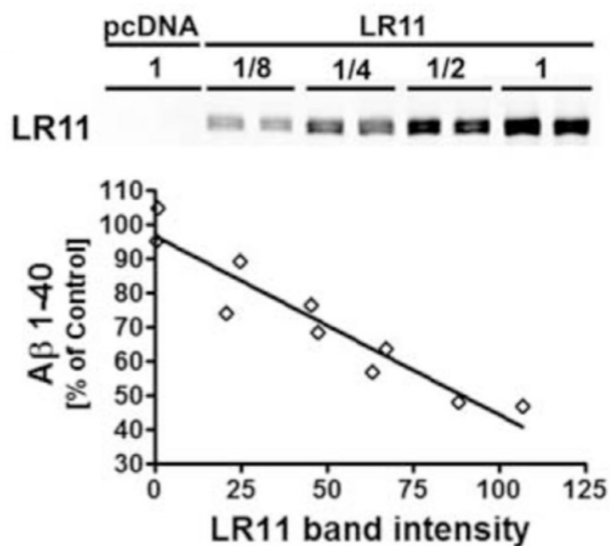
intracellular vesicular sorting of internalized cell surface proteins, including APP, a function that has important implications for regulating the production of Aβ from APP in healthy brain as well as in AD, as will be discussed in the next section (Schmidt et al 2007).

LR11 in Alzheimer's Disease

LR11 first came to the attention of AD researchers in 2004 when our research group reported it as a down-regulated transcript in lymphoblasts harvested from AD patients on an mRNA microarray (Scherzer et al 2004). It was subsequently shown by our group and others that LR11 protein expression is markedly reduced in otherwise healthy-appearing neurons in AD brain compared to healthy, non-demented control brain (Andersen et al 2005; Offe et al 2006) (Figure 1.5A). The loss of LR11 protein expression appears to be neuron-specific, as LR11 expression in glial cells is preserved in AD. LR11 expression in AD brain was found to be particularly low in the hippocampus and the cerebral cortex, two brain regions that are known to be especially vulnerable to the pathogenic processes of AD, while LR11 expression in the basal ganglia and the cerebellum remained robust even late into the disease (Offe et al 2006). This loss of LR11 also appears to be specifically associated with late onset, sporadic AD, as cases of familial AD that are driven by known mutations in *APP*, *PSEN1* or *PSEN2* were found to have robust, control like LR11 expression (Dodson et al 2006).

Figure 1.5. LR11 and Alzheimer's Disease





(A) LR11 immunostaining in healthy control brain (top panels) and in AD brain (bottom panels). Compared to control brain, LR11 expression is marked reduced in neurons in the frontal cortex (panels a-d) but is preserved in glia. (B)

Overexpression of LR11 *in vitro* results in a decrease in Aβ secretion into the culture media that is linearly related to the intensity LR11 expression.

Top panel reproduced from Scherzer CR et al (2004) (Scherzer et al 2004). Bottom panel reproduced from Offe K et al (2006) (Offe et al 2006).

Following the discovery of this intriguing LR11 phenotype in sporadic AD, a series of *in vitro* and *in vivo* studies were conducted to better elucidate the potential pathogenic impact of this loss of LR11 expression. *In vitro* experiments first showed that LR11 over-expression resulted in markedly reduced Aβ production, and that the amount Aβ secreted into the culture media was linearly correlated with the level of LR11 expression in the system (Andersen et al 2005; Offe et al 2006) (Figure 1.5B). Because LR11 over-expression had no effect on the level of total APP expressed by the cells, this strongly suggested that LR11 likely exerts its effect on Aβ levels through altered APP processing. In order to better replicate the LR11 phenotype that was identified in AD patients, transgenic mice were generated that were genetically engineered to express highly deficient levels of LR11. These $LR11^{-/-}$ mice are generally viable, with no discernable health problems. LR11 deficient mice produce normal levels of total APP, but have increased production of both soluble $A\beta_{40}$ and $A\beta_{42}$. These mice also show an increase in neuron-associated Aβ-immunoreactivity (Andersen et al 2005). Because murine Aβ fails to aggregate into amyloid plaques, the $LR11^{-/-}$ mice were then crossed with a well established AD mouse model expressing the genes for human APP and presenilin 1 that contain FAD mutations known to promote amyloidosis. Compared to their $LR11^{+/+}$ littermates, the LR11 deficient mice showed accelerated early amyloid pathology in the brain, resulting in an early age of amyloidosis onset. The $LR11^{+/-}$ mice had an intermediate phenotype, suggesting this effect of LR11 on Aβ deposition is dose dependent (Dodson et al 2008). Together, these results clearly show that LR11 loss

like that seen in AD can have an important impact on promoting Aβ accumulation in the brain.

Detailed immunocytochemistry and molecular biology studies have now shown that: (1) LR11 colocalizes with APP at the cell surface and, to a much larger extent, within intracellular vesicular compartments (Andersen et al 2005; Offe et al 2006); (2) LR11 binds to APP (Andersen et al 2006; Spoelgen et al 2006); (3) LR11 over-expression promotes APP accumulation in early endosomes and the TGN, thereby protecting APP from exposure to β-secretase and reducing the production of Aβ (Andersen et al 2005; Offe et al 2006; Schmidt et al 2007); and (4) in the absence of LR11, as seen in AD brain, APP missorts into alternative intracellular compartments, resulting in increased exposure to β- and γ-secretase and increased Aβ production overall (Andersen et al 2005; Offe et al 2006; Schmidt et al 2007). This now well-established mechanism clearly demonstrates that LR11 is a potentially important regulator of APP processing and Aβ production in the brain.

The importance of LR11 in maintaining low levels of Aβ production in the brain took on added significance in 2007 when Rogaeva and colleagues reported that single nucleotide polymorphisms (SNPs) within *SORL1*, the gene encoding LR11, were associated with an increased risk for developing late onset AD (Rogaeva et al 2007). The authors speculated that because all of the identified SNPs were in the intronic regions of *SORL1*, these genetic mutations were likely to have important consequences for LR11 expression levels. Since that initial report, a host of studies have confirmed that *SORL1* SNPs and SNP haplotypes are positively associated with

an increased risk for late onset AD, as well as an earlier age of disease onset (Bettens et al 2008; Kölsch et al 2009; Lee et al 2008a; Lee et al 2008b; Lee et al 2007a; Meng et al 2007). Moreover, *SORL1* variants have also been reported to be associated with cognitive ability and MRI measures of cortical atrophy (Cuenco et al 2008; Houlihan et al 2009; Seshadri et al 2007). It is important to acknowledge that there remains considerable debate at this time around which *SORL1* mutations convey increased risk and in which populations, with some groups maintaining that there is no association between genetic mutations in this gene and AD at all (Kauwe et al 2010; Li et al 2008; Liu et al 2009; Minster et al 2008; Schjeide et al 2009). Nonetheless, these reported genetic connections between *SORL1* and the risk for the development of late onset AD remain promising, with additional work underway to more clearly define the LR11 genotype or genotypes that may convey this increased risk.

Based on the reported upstream effects of LR11 on regulating APP processing and the production of Aβ, together with the reported genetic association between *SORL1* gene mutations and increased risk of AD, we therefore hypothesize that the loss of LR11 protein expression is a primary event in the AD pathogenic cascade that directly contributes to the abnormal accumulation of Aβ in the earliest stages of the disease. This central hypothesis will be tested through the work laid out in following section.

1.5 Proposed Research

Dr. Alöis Alzheimer characterized the first case of what would come to be known as Alzheimer's disease in 1906, at the time noting the peculiar presence of two types of lesions that appeared to be associated with the disease: the amyloid plaque and the neurofibrillary tangle. While advancements beyond this early understanding of AD were hindered due to confusion over the differences between the presenile and late onset forms of the disease as well as over the association between these pathological lesions and the clinical symptoms of the disease, breakthroughs in the 1970s and 80s ushered in a wave of new understanding about the pathogenesis of AD. It is now widely recognized that the pathological events underlying AD begin to develop far in advance of the onset of cognitive impairment, starting with the abnormal accumulation of both soluble and insoluble Aβ in the brain and culminating with the progressive loss of synaptic function and cortical atrophy that produces the symptoms that Dr. Alzheimer first described (Jack Jr et al 2010). Given this important role for Aβ as the triggering event of the AD pathogenic cascade, factors that can regulate the processing of APP into this neurotoxic peptide have significant potential therapeutic value. To date, none of the Aβ-focused therapies that seem so promising in the research lab have succeeded at the clinical trial level, in large part because we are essentially testing what would be preventative therapies at the latest of stages of the disease, far beyond the potential window for efficacy (Holtzman et al 2011). As such, research efforts have now shifted from characterizing those individuals with dementia onto those individuals with mild cognitive impairment, a condition that is increasingly recognized as a

prodromal form of AD. By studying the pathological underpinnings of cognitive impairment in this population, we have gained a more clear understanding of the early events that lead to the development of AD, including those involving factors that may play a role regulating Aβ production from APP.

The multifunctional receptor LR11/SorLA has recently emerged as an exciting candidate that may promote the non-amyloidogenic processing of APP in healthy brain. Neuronal expression of LR11 is markedly downregulated in AD brain, a condition that has been shown to accelerate amyloidosis in an AD mouse model (Dodson et al 2008; Offe et al 2006). Given the seeming importance of LR11 in the upstream regulation of APP trafficking and the production of Aβ, we hypothesize here that low LR11 expression will be apparent even in the earliest stages of AD, including in at least a subset of individuals with MCI. Moreover, we further hypothesize that LR11 expression levels will be closely related to other early events in the progression of AD, including amyloid plaque frequency and episodic memory impairment.

Specific Aim 1: To test the hypothesis that the level of LR11 protein expression in the frontal cortex of MCI brain is similar to that seen in AD brain and markedly less than that seen in control brain, in at least a subset of cases. To test this hypothesis, LR11 expression was measured in two distinct cohorts that were obtained through our long time collaboration with the Religious Orders Study using a novel quantitative immunohistochemical approach. In the first cohort, which

was comprised of individuals with pathologically confirmed final diagnoses of AD, MCI or no cognitive impairment (NCI), we found low LR11 expression in all ten AD cases examined and robust LR11 expression in nearly all of the NCI cases. LR11 expression in the MCI group was highly variable in this cohort, with five cases having robust, control-like LR11 expression and ten cases having low, AD-like LR11 expression. In the second cohort, which was comprised of individuals chosen on the basis of their final cognitive diagnoses at the time of death with no selection criteria based on underlying pathology, we found low LR11 expression in approximately 30% of the AD cases examined, far less than originally expected. Moreover, we also found low LR11 expression in a similar proportion of cases in both the MCI and NCI groups. Together, these results suggest that LR11 expression is low in at least a subset of cases diagnosed with MCI, similar to what was observed in AD.

Specific Aim 2: To test the hypothesis that low LR11 expression would be detectable earlier in the progression of AD in areas of the brain that are known to develop amyloid plaques very early and that LR11 expression would be persistently robust until very late in the disease in brain areas that are generally spared in AD. To test this hypothesis, LR11 expression was measured in the second cohort described above in two additional brain areas: the precuneus, a known predilection site for amyloid accumulation and the primary visual cortex, an area of the brain that is generally spared in AD. In both brain regions examined, we found reduced LR11 expression in a similar proportion of cases as in the frontal cortex in all three diagnostic groups. Moreover, of the 14 cases that were found to

have low LR11 expression in at least one brain region, ten of them had low LR11 expression in two or more brain regions, suggesting that LR11 expression is either consistently high or consistently low throughout the brain.

Specific Aim 3: To identify cognitive, pathological and/or genetic correlates of LR11 expression in order to identify other early changes in the progression of AD that may be related to LR11 expression. From the results of the previous two Aims, it became clear that low LR11 expression was not a universal element of the pathology present in MCI brain, despite the strong AD-like pathology present in nearly all of these cases. Therefore, in order to better understand the nature of the cases in both cohorts that featured low LR11 expression and to determine if low LR11 was related to other known early events in these cases, we performed an extensive series of statistical analyses designed to identify correlates of LR11 expression from a wide range of demographic, genetic, cognitive and pathological variables. Due to the relatively small size of the MCI groups in both cohorts, these analyses were performed on each cohort in full. While we found a strong relationship between LR11 expression in the frontal cortex and global cognitive score in the first experimental cohort, no correlates of LR11 expression consistently emerged in both cohorts within the limits of this study.

Chapter 2. MATERIALS AND METHODS

2.1 Case Materials

Religious Orders Study

All of the case materials that were used for the studies presented in this dissertation were acquired through our ongoing collaboration with the Religious Orders Study at Rush University. The Religious Orders Study is a longitudinal study of memory and aging that began in July 1993. There are currently more than 1100 religious clergy members (nuns, priests and brothers) from over 40 sites in 12 states enrolled in the study. Subjects with pre-existing dementia are precluded from enrollment. All participants have agreed to annual clinical evaluation and brain donation at the time of death. Since the study began, more than 450 participants have come to autopsy, an autopsy rate of greater than 90% (Schneider et al 2009).

As noted, each participant in the Religious Orders Study undergoes an annual uniform structured cognitive evaluation that includes procedures recommended by The Consortium to Establish a Registry for Alzheimer's Disease (CERAD)(Fillenbaum et al 2008; Mirra et al 1991; Morris et al 1989) for each year that they remain enrolled in the study. This evaluation includes a review of the individual's medical history, a complete neurologic examination, neuropsychological performance tests and a review of a brain scan when available. The evaluation

procedure, which is described in more detail elsewhere (Bennett et al 2005; Bennett et al 2002; Schneider et al 2009) is done in three stages. During Stage 1, an observer blinded to the individual's cognitive and medical histories administers the Mini-Mental State Exam (MMSE) (Folstein et al 1975) and a battery of 19 tests of cognitive ability, including seven tests of episodic memory, four tests of semantic memory, four tests of working memory, two tests of perceptual speed and two tests of visual-spatial ability. Test results are scored by a computer and are adjusted as necessary to account for the education level of the individual being evaluated. Summary z-scores for global cognition (Global Cognitive Score, GCS) and for each cognitive ability are calculated by a statistician following each evaluation. In Stage 2, a board certified clinical neuropsychologist (blinded to age, sex and race) reviews the results of the cognitive exam and determines whether there is evidence of cognitive impairment. Finally, in Stage 3, an experienced neurologist or geriatrician evaluates the individual in person and determines whether the subject meets the clinical criteria for dementia and AD recommended by the joint working group of the National Institute of Neurologic and Communicative Disorders and Stroke/AD and Related Disorders Association (NINCDS/ADRDA)(McKhann et al 1984). Because there are no consensus criteria for the diagnosis of MCI, that designation is given to individuals that are judged by the neuropsychologist to have cognitive impairment in Stage 2 but are not found to reach the accepted criteria for dementia in Stage 3. For the final cognitive diagnosis following death, a neurologist blinded to all post-mortem data reviews all available clinical data from the years in which the

individual was enrolled in the Religious Orders Study and a summary opinion of the most likely clinical diagnosis at the time of death is rendered.

The average post mortem interval (PMI) for the Religious Orders Study is approximately 8.4 hours. Following death, the brain is removed and weighed before being processed as previously described (Bennett et al 2005; Schneider et al 2009). Briefly, each hemisphere is then cut into 1cm coronal slabs. Slabs are examined for visible pathology before being either frozen or immersion fixed in 4% paraformaldehyde for 3 to 21 days. In some instances, whole hemispheres were immersion fixed in 4% paraformaldehyde for 30 days or longer. Following fixation, diagnostic blocks are dissected from nine brain regions and cut into sections. Alzheimer's disease pathological lesions (neuritic plaques, diffuse plaques and neurofibrillary tangles) are visualized by Bielschowsky silver stain. Hematoxylin and eosin stains are used to document chronic microscopic infarcts. The total numbers of each lesion present in a one mm^2 area viewed at 100X are counted in five brain regions (frontal, temporal, parietal and entorhinal cortices as well as the hippocampus). Using these counts, CERAD diagnoses (Fillenbaum et al 2008; Mirra et al 1991; Morris et al 1989), Braak stages of tangle pathology (Braak & Braak 1991) and National Institute on Aging (NIA)/Reagan Consensus diagnoses (1997; Cochran et al 1998) are determined for each case. ApoE genotyping is performed as previously described (Chow et al 1998; Gilmor et al 1999).

For the purposes of our examination of LR11 in MCI, two unique study cohorts comprised of cases from the Religious Orders Study were used. For

organizational purposes, these cohorts are designated “ROS 1.0” and “ROS 2.0” throughout the remainder of this dissertation and are described individually below.

ROS 1.0 Study Population

The case demographics for the ROS 1.0 study cohort are given in Table 2.1. This cohort consisted of fifteen MCI cases, ten AD cases and nine cases with a final diagnosis of no cognitive impairment (NCI). Cases were chosen from the larger Religious Orders Study cohort based on gender, education and PMI. Only cases with a final clinical diagnosis of NCI, MCI or AD with no other cause of cognitive impairment were considered. In order to ensure that the NCI group did not include cases at preclinical stages of AD, only control cases lacking significant amyloid pathology were included. A final diagnosis of AD was also confirmed on autopsy. Every attempt was made to match for age; however, the exclusion of control cases with significant amyloid pathology resulted in a younger NCI group.

ROS 2.0 Study Population

The case demographics for the ROS 2.0 study cohort are given in Table 2.2. This cohort consisted of fourteen NCI cases, fifteen MCI cases and fourteen AD cases chosen from the Religious Orders Study cohort using the following criteria: age at death between 75 and 95 years of age, final MMSE score greater than 10, PMI of 12 hours or less and a final cognitive evaluation less than 24 months prior to death.

Table 2.1 – ROS 1.0 Cohort Demographics*

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group
Age at death, years**	75.4 ± 5.2 (67-82)	83.6 ± 5.1 (75-97)	82.6 ± 4.8 (80-94)	82.6 ± 6.9 (67-97)	$p = 0.0034$[a]
Number (%) of males	6 (67%)	7 (47%)	4 (40%)	17 (50%)	$p = 0.72$[b]
Years of education	19.2 ± 4.2 (12-26)	17.4 ± 5.6 (8-30)	16.3 ± 3.9 (6-20)	17.9 ± 4.4 (6-30)	$p = 0.20$[a]
Post-mortem interval, hours	11.3 ± 9.7 (2.2-33.5)	7.5 ± 4.3 (3.5-16)	6.4 ± 3.0 (3-10.7)	8.3 ± 6.4 (2.2-33.5)	$p = 0.51$[a]
Subjects with *APOE* ε4 allele (%)	0 (0%)	5 (33%)	5 (50%)	10 (29%)	$p = 0.086$[b]

[a]Kruskal-Wallis test

[b]Fisher's Exact test

*Unless otherwise noted, data are presented as Mean ± SD (range).

**The preclusion of control cases with significant amyloid pathology entailed a younger NCI group.

Table 2.2 – ROS 2.0 Cohort Demographics*

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group
Age at death, years	84.6 ± 4.5 (78 – 93)	86.2 ± 4.4 (79 – 94)	89.0 ± 4.8 (76 – 95)	86.6 ± 4.8 (76 – 95)	$p = 0.031$[a]
Number (%) of males	5 (36%)	7 (47%)	4 (29%)	16 (37%)	$p = 0.60$[b]
Years of education	17.6 ± 4.0 (10 – 25)	17.8 ± 3.6 (10 – 25)	18.2 ± 3.4 (14 – 26)	17.9 ± 3.6 (10 – 26)	$p = 0.99$[a]
Post-mortem interval, hours	5.4 ± 2.4 (1.0 – 9.8)	6.2 ± 2.6 (2.0 – 11.5)	4.9 ± 2.0 (1.5 – 8.2)	5.5 ± 2.4 (1.0 – 11.5)	$p = 0.49$[a]
Subjects with *APOE* ε4 allele (%)	1 (7%)	6 (40%)	6 (43%)	13 (30%)	$p = 0.072$[b]

[a]Kruskal-Wallis test

[b]Chi-square test

*Unless otherwise noted, data are presented as Mean ± SD (range).

During work on the ROS 1.0 cohort, a potential confounding relationship between a history of stroke and LR11 expression was observed. As a result, cases with a clinical history of stroke and the presence of gross cerebral infarcts noted during autopsy were specifically excluded. This observation is discussed in further detail in Chapter 6. Only cases with a clinical diagnosis of NCI, MCI or AD with no other cause of cognitive impairment were considered. While a post-mortem evaluation of AD-related lesions was performed on these cases, pathological observations were not considered in case selection for this cohort, a notable change from the ROS 1.0 selection criteria. Cases in the ROS 2.0 cohort were matched for gender, education and PMI to the best of our ability.

2.2 Immunohistochemistry

Free-floating, frozen cut 40μm thick cortical sections from each brain region of interest were labeled with a polyclonal anti-sera to the LR11 C-terminus generated against the peptide CEDAPMITGFSDDVPMVIA (Covance Research Products, Inc, Denver, PA) (Herskowitz et al 2011). Sections were blocked with 8% normal goat serum, 0.1% Triton X-100 (Sigma Labs, St. Louis, MO) and 10μg/ml avidin in Tris-buffered saline and then incubated for either 24 hours (ROS 1.0) or 45 hours (ROS 2.0) with anti-LR11. Following primary antibody incubation, sections were incubated for 1 hour with biotinylated goat anti-rabbit antibody (Vector

Laboratories, Burlingame, CA) followed by avidin-biotinylated horseradish peroxidase (ABC reagent; Vector Laboratories) for 1 hour. Finally, sections were developed in 3,3'-diaminobenzidine for approximately eight minutes.

For ROS 1.0, brain sections were used from the superior frontal cortex (BA 9). Sections were stained in three successive runs with staining occurring on Days 1 and 2. Stained sections were mounted on slides on Day 3 and coverslipped on Day 4 after drying overnight. All cases stained together were imaged and analyzed as one set as described below. For ROS 2.0, brain sections were used from the frontal cortex (BA 10), precuneus (BA 7) and the primary visual cortex (BA 17). Sections were processed in batches of 36 cases at a time, with staining performed on one subset of 18 cases on Days 1 and 3 and the remaining 18 cases stained on Days 2 and 4. All tissues were mounted on slides on Day 5 and coverslipped on Day 6 after drying overnight. Following staining, all 36 cases were imaged and analyzed as one set, as described below. A total of 10 staining runs and 6 imaging and analysis runs were required to process all of the brain sections in the ROS 2.0 cohort.

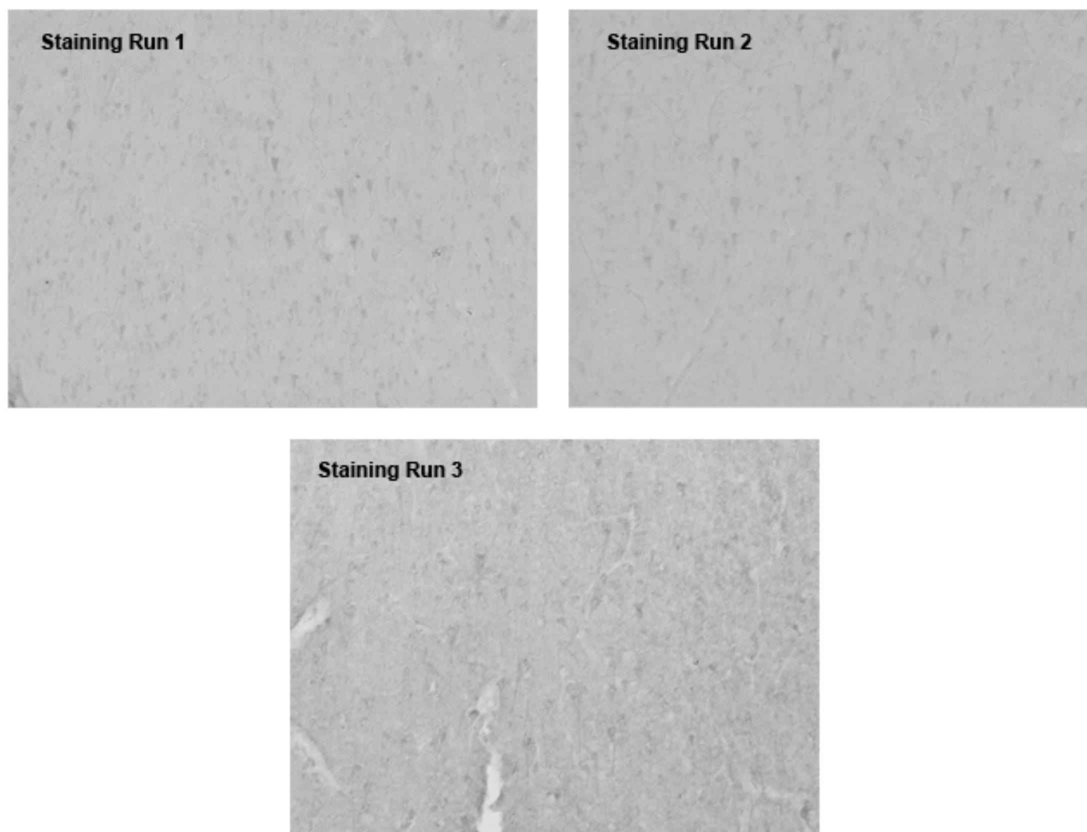
Three sections of frontal cortex tissue from a common case were included in each staining run to ensure that the staining procedure worked correctly and consistently across multiple staining runs. One section of common tissue per run served as a positive control and was labeled with an unrelated polyclonal antibody to an epitope that is known to be highly expressed in frontal cortex. The antibodies used for the positive control were anti-Calnexin (SPA-860; Assay Designs, Ann Arbor, MI) or anti-EEA1 (ab2900; Abcam, Cambridge, MA). Robust staining was

detected on all positive control sections. One section of common tissue per run served as a no primary control to detect any non-specific label of the tissue by the other reagents. No staining was detected in any of the negative control sections. Finally, one section of common tissue was stained with the LR11 CT anti-sera in parallel with the experimental sections in order to ensure consistent staining across multiple staining runs. LR11 label of the internal control sections was found to be highly consistent across staining runs. Representative images from the internal control sections from each staining run can be seen in Figure 2.1 (ROS 1.0) and Figure 2.2 (ROS 2.0).

2.3 Image Capture and Quantification of LR11 Immunostaining

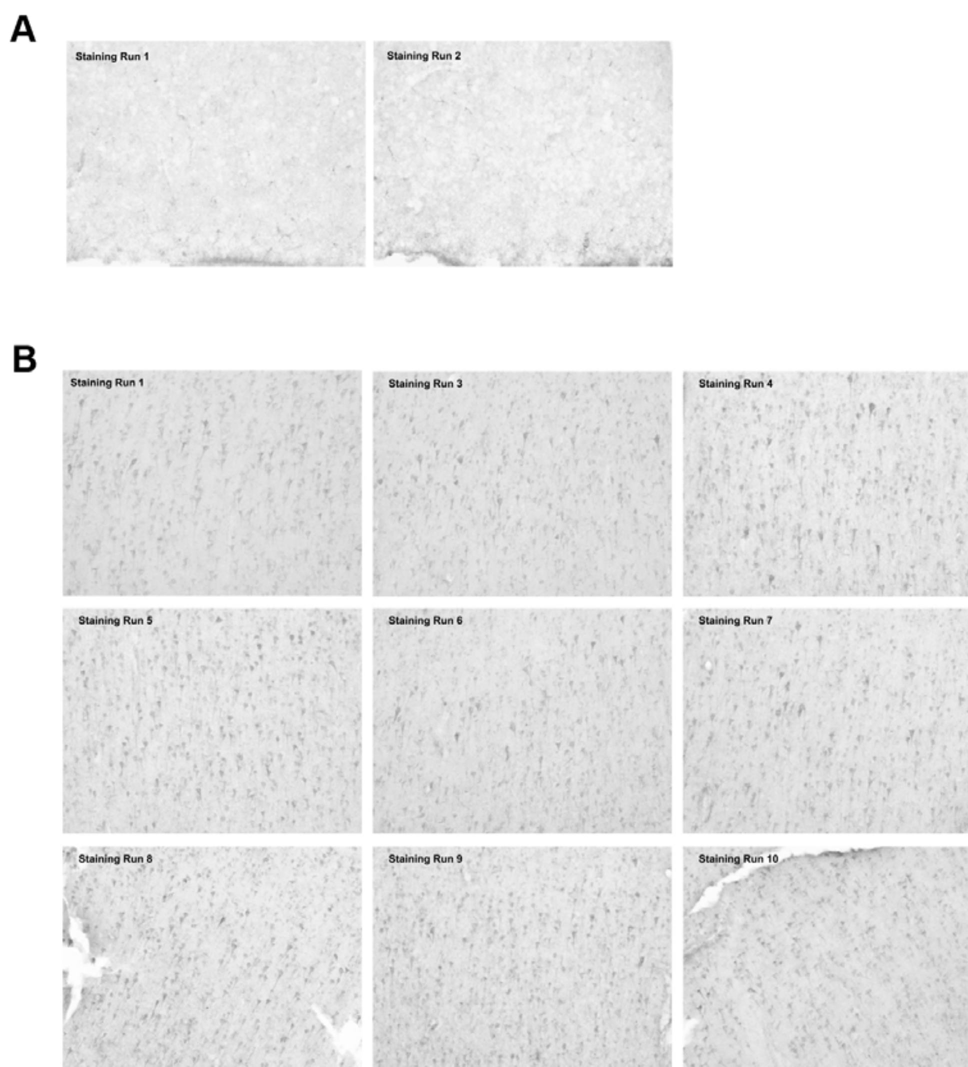
LR11 neuronal immunostaining was measured using a novel quantitative approach that we developed in order to overcome observer bias (Cregger et al 2006) and to allow for more powerful statistical analyses than traditional qualitative or semi-quantitative methods. This quantitative technique consists of two stages. In the first stage, distinct areas of each stained brain section are selected for analysis and imaging of individual cells is performed. In the second stage, the intensity of LR11 staining is measured in each imaged cell and a mean LR11 measure is calculated for each case using a technique adapted from a method that

Figure 2.1 – ROS 1.0 Cohort Internal Control



Representative images from the internal control slide from each of the three staining runs performed on the cases from the ROS 1.0 cohort showing a consistent level of staining across subsequent staining runs. Images are at 10X magnification.

Figure 2.2 – ROS 2.0 Cohort Internal Control



Representative images from the internal control slide from each of the ten staining runs performed on the cases from the ROS 2.0 cohort showing a consistent level of staining across subsequent staining runs. Staining Runs 1 and 2 both included tissue from the first case in the cohort as an internal control (A). Due to the very low LR11 expression in the internal control case used in the first two staining runs, the next sequentially numbered case in the cohort was used as the internal control for

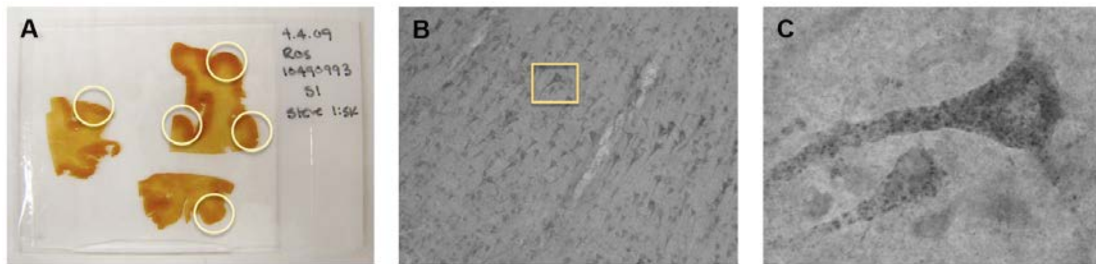
staining runs 3 through 10. (B) This case was also included in the first staining run allowing for comparisons between the two. All images are at 10X magnification.

was previously developed in our lab to measure antigen co-localization (Volpicelli et al 2001). The protocol used for both stages is described below.

Image Capture

Prior to viewing each slide under the microscope five separate sampling regions of each of the sections stained from each case and brain region were pre-selected for imaging. When multiple brain sections were used from one case/brain region, sampling regions were chosen from all sections to ensure sampling from all stained tissue. Each sampling region is then viewed at 10X magnification. An individual cell (or cells, if in the same plane of focus) was selected from pyramidal cell layer V of the gray matter to serve as the starting point for imaging. The selected cell was then viewed using a 100X oil immersion lens and imaged using an attached digital camera. Images of twenty successive cells per region were taken, with an average of one to two and a maximum of eight cells per image for a total of approximately 100 cells imaged per case and brain region. In the instance where more cells were captured in the final image taken of a given sampling region than needed to reach 20 total imaged cells for that sampling region, all of the cells in that image were analyzed. As a result, slightly more than (but never less than) 100 cells were imaged and analyzed for some cases. The selection and imaging of cells was performed by a single researcher blinded to clinical diagnosis. ROS 1.0 brain sections were viewed using a Leica Leitz DMRB fluorescence microscope (Leica Microsystems, Buffalo Grove, IL) and cells were imaged using a Hamamatsu C4742-

Figure 2.3 – Illustration of Sampling Methodology



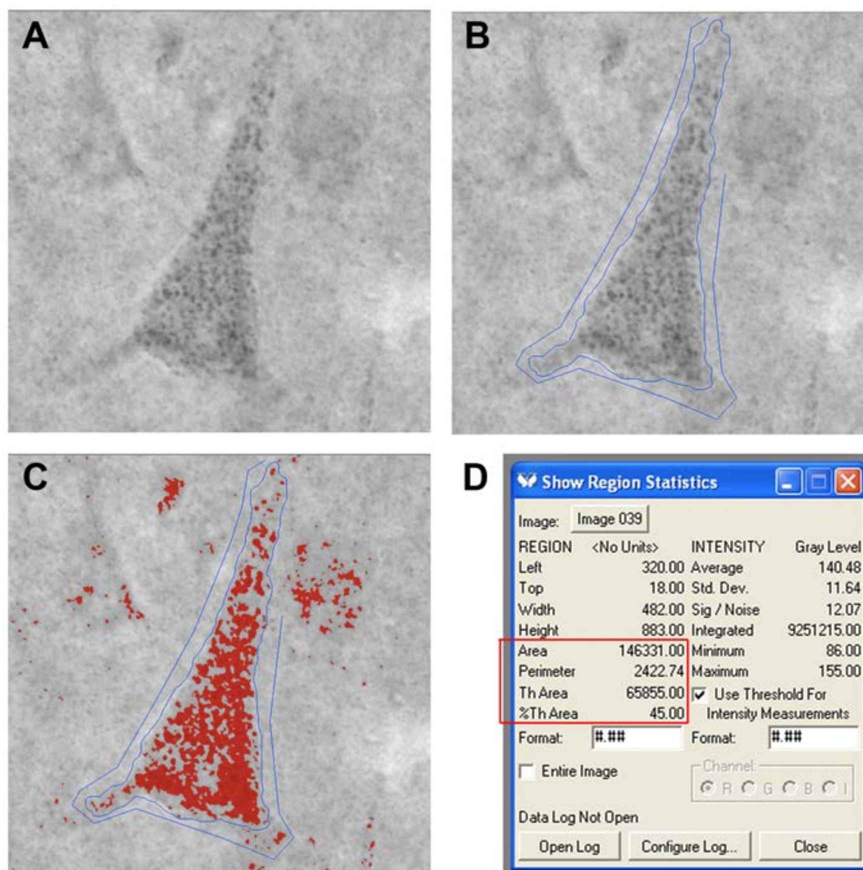
(A) Following the mounting of the sections on a slide, five separate regions are pre-selected for imaging before viewing the slide under the microscope. When multiple smaller brain sections are used, sampling regions are chosen from all sections to ensure sampling from all stained tissue. (B) For each region selected, the section is first viewed at 10X magnification. A representative image is taken and an individual cell from the pyramidal cell layer of the gray matter is selected as the starting point for imaging, as noted by the yellow box. (C) Cells are imaged at 100X, with each image containing anywhere from one to eight cells. Twenty consecutive neurons within the pyramidal cell layer are imaged from each region for a total of 100 cells per brain region per case.

95 digital camera (Hamamatsu Photonics, Bridgewater, NJ). For ROS 2.0, brain sections were viewed using an Olympus BX51 microscope and images were captured using an Olympus DP70 digital camera (both from Olympus America, Inc, Center Valley, PA). An illustration of this sampling and imaging approach can be seen in Figure 2.3.

Quantification of LR11 Immunostaining

Captured images were converted to black and white before viewing the files using the Metamorph Image Analysis software program (Meta Imaging Series, Molecular Devices, Sunnyvale, CA). The border of each cell within an image was first traced by hand using the Trace Outline tool in order to define the region within which the staining intensity was measured. A second outline was then drawn in the background immediately surrounding each cell using the Multi-Line tool. This tool measures the intensity of only those pixels that fall directly under this outline, allowing us to measure the intensity of the background label around each cell to be analyzed. A threshold was set for each cell at the level of the most intense staining in the local background around that cell. All pixels within the cell being analyzed that were stained more intensely than this threshold level were considered positively stained for LR11. The percentage of pixels stained positive for LR11 was calculated for each cell and a mean value was calculated from all 100+ cells imaged per case and brain region. (Figure 2.4) Data is presented as the mean percent surface area stained positive for LR11 ± SEM for both ROS 1.0 and ROS 2.0, unless otherwise

Figure 2.4 – Quantitative Immunohistochemistry Technique



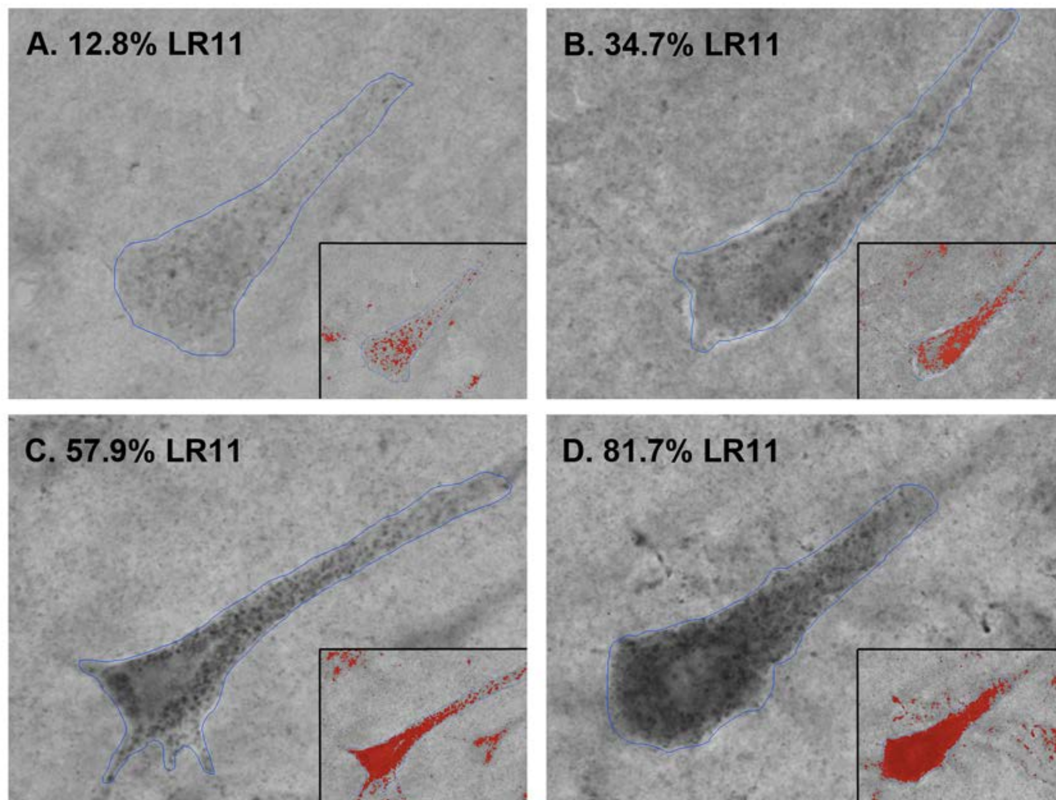
(A) Cells were imaged at 100X, as shown in Figure 2.3. Images were converted to black and white before being analyzed in the MetaMorph image analysis program. (B) An outline was drawn by hand around each cell within an image using the Trace Outline tool. This tool defines the region within which the image intensity was measured. A second outline was then drawn in the background immediately surrounding each cell using the Multi-Line tool. This tool measures the intensity of only those pixels that fall directly under this line, which allowed us to measure the intensity of the background around each cell to be analyzed. (C) The threshold level was set equivalent to the most intensely stained pixels in the background and an red overlay was applied to demarcate all pixels in the image that are stained more intensely than the threshold level. These pixels are considered to be stained

positively for LR11. (D) The Metamorph program then calculated the percentage of pixels within each cell that are stained positively for LR11.

noted. Representative images showing the wide range of LR11 expression present in the cases from both ROS cohorts and the quantitative measures of LR11 in those cells can be seen in Figure 2.5.

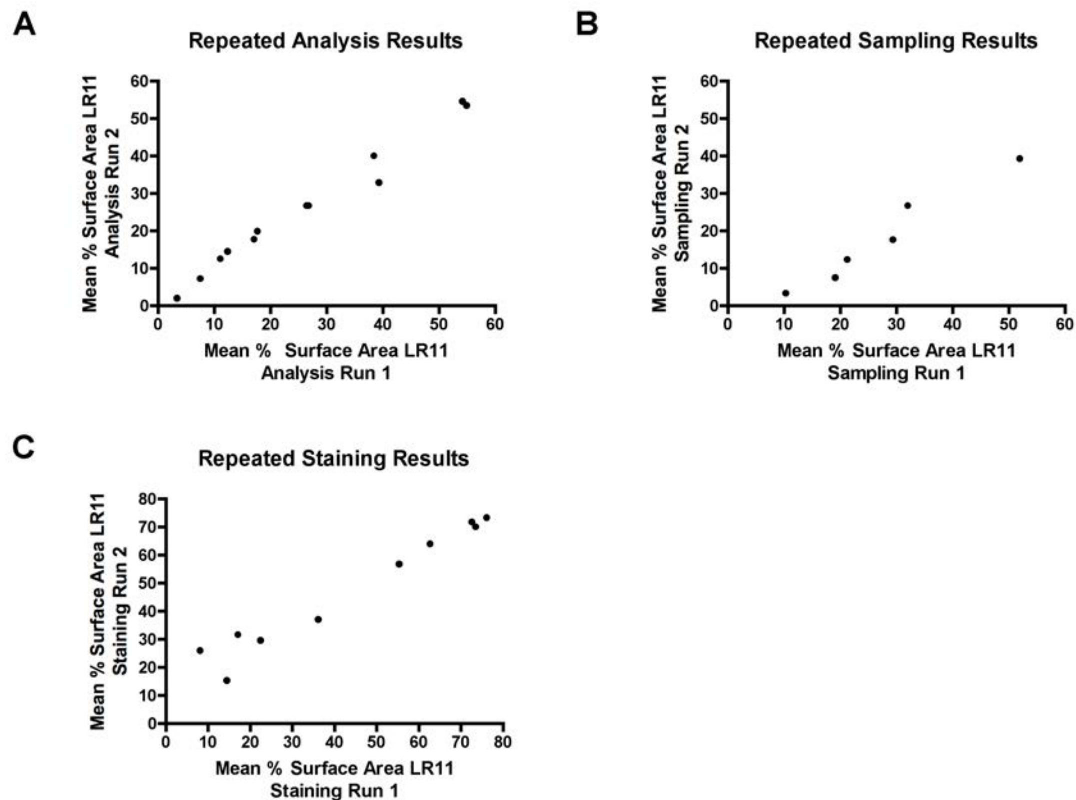
Because of the novelty of this quantitative immunohistochemical technique, it was critical to demonstrate the reproducibility of LR11 measures in repeated experiments. First, we evaluated the repeatability of the Metamorph analysis protocol itself by reanalyzing previously captured images from twelve cases known to have a range of LR11 expression. This approach ensured that the staining and sampling was identical between analysis runs. The new LR11 measures were directly compared to the previous measures from the same images and were found to be highly significantly correlated when analyzed by Pearson correlation (r^2 = 0.98, $p < 0.0001$***, Figure 2.6A), suggesting that very little run to run variability occurs during the analysis stage of this quantitative approach. To evaluate the degree of variability introduced in the sampling and imaging stage, previously stained sections from six cases were re-imaged and LR11 expression was measured in the new images as before. The new LR11 measures were then compared to the previous measures from the same stained sections. In this experiment, the repeated LR11 measures were again found to be significantly correlated (r^2 = 0.96, p = 0.0006***, Figure 2.6B). It should be noted that the LR11 measures did not replicate quite as well in this experiment as in the previous experiment, suggesting that a minor but important degree of variability occurs during the sampling and image capture stage. Finally, to determine the consistency of the full quantification protocol, additional sections from ten previously analyzed cases were stained,

Figure 2.5 – Quantitative Immunohistochemistry Can Distinguish Different Levels of LR11 Expression



The quantitative immunohistochemistry technique used for both of the studies presented in this dissertation can readily distinguish between a wide range of LR11 protein expression in neurons, as shown in these representative images from the ROS 2.0 cohort. Panel A shows a cell with low LR11 expression, Panels B and C show cells with medium low and medium high LR11 expression, respectively, and Panel D shows a cell with very high LR11 expression. The red overlay shown in the inset of each image represents the pixels determined to be stained positive for LR11 for each cell. The number of pixels stained positively for LR11 is expressed as a percentage of the total number of pixels present within the outlined cell in the image.

Figure 2.6 – Quantitative Immunohistochemistry Measurements are Highly Reproducible



To ensure the reproducibility of the LR11 measurements generated using this novel quantitative immunohistochemistry approach, the variability between repeated experiments was evaluated at each step of the protocol. (A) Previously captured and analyzed images from twelve cases were reanalyzed to evaluate the repeatability of the Metamorph analysis protocol when both the staining and sampling/imaging were kept consistent. The new LR11 measures were compared to the previously generated LR11 measures and were found to be highly significantly correlated when analyzed by Pearson correlation (r^2 = 0.98, p <0.0001***). (B) To evaluate the degree of variability introduced at the sampling and imaging stage, previously stained sections from six cases were reimaged and LR11 was measured in the new images. Repeated LR11 measures were found to be significantly correlated (r^2 =

0.96, $p = 0.0006$**, Pearson correlation), although slightly less than observed in the experiment in Panel A. (C) Finally, to determine the consistency of the full quantification protocol, additional sections from ten previously analyzed cases were stained, imaged and analyzed independently from the initial staining and analysis run. Repeated LR11 measures were found to be highly significantly correlated, with a nearly identical correlation coefficient as in the experiment shown in Panel B ($r^2 = 0.96$, $p < 0.0001$***, Pearson correlation). All repeated measures experiments were performed blinded to the original results.

imaged and analyzed independently from the initial staining and analysis run. LR11 measures in these cases were found to replicate as well as in the re-sampling experiment ($r^2 = 0.96$, $p < 0.0001$***, Figure 2.6C). Based on this series of experiments, we are confident that the quantitative immunohistochemical approach used here is highly consistent across repeated staining runs and that the majority of the variability seen in repeated staining runs of the same brain sections can most likely be attributed to differences in the sampling of the cells to be analyzed.

Finally, to confirm the validity of the reported results (that is, to show that the quantitative LR11 values are representative of a qualitative assessment of the same staining), three independent blinded raters scored LR11 immunostaining in the frontal cortex of selected cases from each cohort on a semi-quantitative scale. The correlation between those scores and the quantitative LR11 measures was evaluated, as well as the degree of agreement between raters. The results of these analyses are presented alongside the LR11 measures in the frontal cortex for each cohort in Chapter 3.

2.4 Statistical Analyses

Clinical, demographic and neuropathological characteristics were summarized and compared by either the Kruskal-Wallis test, Fisher's exact test with

Bonferroni corrections for pairwise comparisons (ROS 1.0) or a chi square test with Dunn's corrections for pairwise comparisons (ROS 2.0).

For the results from the ROS 1.0 cohort, the difference in LR11 among diagnostic groups was analyzed using mixed models with random intercept, fixed effect for diagnosis, Kenward-Roger denominator degrees of freedom and unstructured covariance structure. Mixed models take into account the correlation among observations from the same subject and give appropriate weighting to between-subject *vs.* within-subject variation. Levene's test was employed to test for the homogeneity of variances among the three diagnostic groups (Levene 1960). To evaluate the clustering of values within the MCI group in this cohort, a single-linkage agglomerative hierarchal cluster analysis was performed (Johnson & Wichern 2002). The distance matrix showing distances between each pair of individuals was derived from the Mann-Whitney U-statistic (the absolute value of U – ½).

For the results from the ROS 2.0 cohort, the difference in LR11 among diagnostic groups was analyzed by Kruskal-Wallis test for comparison of means. Bartlett's test was employed to test for the homogeneity of variances among the three diagnostic groups. A chi-square test was used to evaluate the distribution of cases designated as having "low" LR11 expression in this cohort. LR11 measurements for both cohorts are shown in the original scale for summary statistics, with square-root transformations applied in statistical testing to correct for skewed (non-normal) distribution.

In both cohorts, the inter-rater reliability in the semi-quantitative scorings of LR11 was examined by generalized weighted κ. The consistency of the semi-quantitative scores with the quantitative LR11 measurements was assessed by Spearman's rank correlation.

The associations between LR11 measures and clinicopathological variables in ROS 1.0 were assessed by similar mixed model analyses as described above. For ROS 2.0, these associations were analyzed by either Spearman's rank correlation or Kruskal-Wallis test.

Statistical analyses were performed using Graphpad Prism 4.0 (Graphpad Software, San Diego, CA) and SAS 9.1.3 (SAS Institute Inc, Cary, NC).

To account for the large number of statistical analyses performed in both the ROS 1.0 and ROS 2.0 studies, the level of statistical significance was set at 0.01 (two sided).

Chapter 3. FRONTAL CORTEX LR11 EXPRESSION IS REDUCED IN A SUBSET OF MCI CASES

3.1 Introduction

Alzheimer's disease (AD) is the leading cause of dementia among the elderly, affecting one in eight individuals over the age of 65 (Hebert et al 2003). AD is a complex disease, with a wide range of genetic and environmental causes and a dense puzzle of underlying neuropathological changes. While the first clinical signs of disease typically emerge late in life, the pathological abnormalities that lead to AD often appear in the brain decades prior to the onset of the cognitive impairment (Jack Jr et al 2010; Sperling et al 2011). A burgeoning area of AD research has therefore focused on identifying genetic risk factors, early molecular changes and behavioral endophenotypes in order to better identify those patients at the greatest risk for developing AD. Moreover, defining these early changes in the disease process can provide critical clues about potential therapeutic targets.

LR11, or SorLA as it is also known, is a multifunctional member of the lipoprotein receptor family that has recently emerged as a protein of interest in the neuropathology of AD. LR11 has been shown to play a critical regulatory role in the processing of the amyloid precursor protein (APP) and may help to maintain low levels of the pathological Aβ peptide (Andersen et al 2006; Dodson et al 2008; Offe et al 2006; Spoelgen et al 2006). While LR11 protein levels in healthy brain are generally robust, LR11 protein expression in AD brain is strikingly reduced

(Andersen et al 2005; Dodson et al 2006; Scherzer et al 2004). Moreover, an increasing number of studies report that single nucleotide polymorphisms (SNPs) in the LR11 gene (*SORL1*) are associated with an increased risk for developing AD (Bettens et al 2008; Kölsch et al 2009; Laumet et al 2010; Lee et al 2007a; Lee et al 2007b; Meng et al 2007; Rogaeva et al 2007). Together, this makes LR11 an exciting potential target for use as both a diagnostic tool and as a site of therapeutic intervention.

Given the important role that LR11 plays in the regulation of APP processing in healthy brain, we believe that the loss of LR11 that has been reported in end-stage AD brain is a primary, precipitating event in the AD pathogenic cascade that contributes to the accumulation of Aβ at the onset of disease development. It is therefore likely that a pathogenic reduction in LR11 protein expression occurs in the earliest stages of the disease process and should be detectable in the brains of patients with preclinical AD, including those diagnosed with mild cognitive impairment (MCI).

While the concept of pre-dementia cognitive decline has been recognized for many decades, the use of MCI as a diagnostic entity has only come into regular use in the last ten to fifteen years (Petersen et al 2001; Petersen et al 1999; Zaudig 1992). MCI is a clinical diagnosis that is given to patients whose cognitive ability is not normal but whose declines in cognitive and/or functional abilities are not sufficiently severe to meet the criteria for dementia (Morris et al 2001). While some MCI patients will maintain a stable level of cognitive impairment throughout their

lives (and some will even revert to normal cognitive function) (Gauthier et al 2006; Levey et al 2006), most individuals with MCI progress to greater stages of dementia, with a 12% annual conversion rate to AD (Petersen 2004). Moreover, people with MCI often have some degree of AD neuropathology in their brains, particularly those patients with the amnestic subtype of MCI (Bennett et al 2005; Markesbery et al 2006; Morris et al 2001; Schneider et al 2009). Together, this strongly suggests that at least a portion of MCI cases are actually prodromal AD. Therefore, we hypothesize that the level of LR11 protein expression in the frontal cortex of MCI brain will be similar to that seen in AD brain and markedly less than that seen in control brain, in at least of subset of cases.

In order to test this hypothesis, we quantified LR11 protein expression in the brains of individuals with MCI as well as in brains from both AD patients and individuals with no cognitive impairment (NCI), which served as our control group. Two cohorts of cases were used for this study, both of which were derived from the larger cohort of cases available through the Religious Orders Study. The first cohort, referred to herein as ROS 1.0 consisted of 34 cases whose final clinical diagnosis of NCI, MCI or AD was confirmed pathologically at autopsy. The second cohort, referred to herein as ROS 2.0, consisted of 43 cases that were selected on the basis of their final antemortem clinical diagnosis regardless of underlying pathology. Measurement of LR11 protein expression was performed by quantitative immunohistochemistry on frontal cortex brain slices. This brain region was chosen because the LR11 expression profile in both NCI and AD was well established in the literature (Offe et al 2006; Scherzer et al 2004). Using this approach, we found low

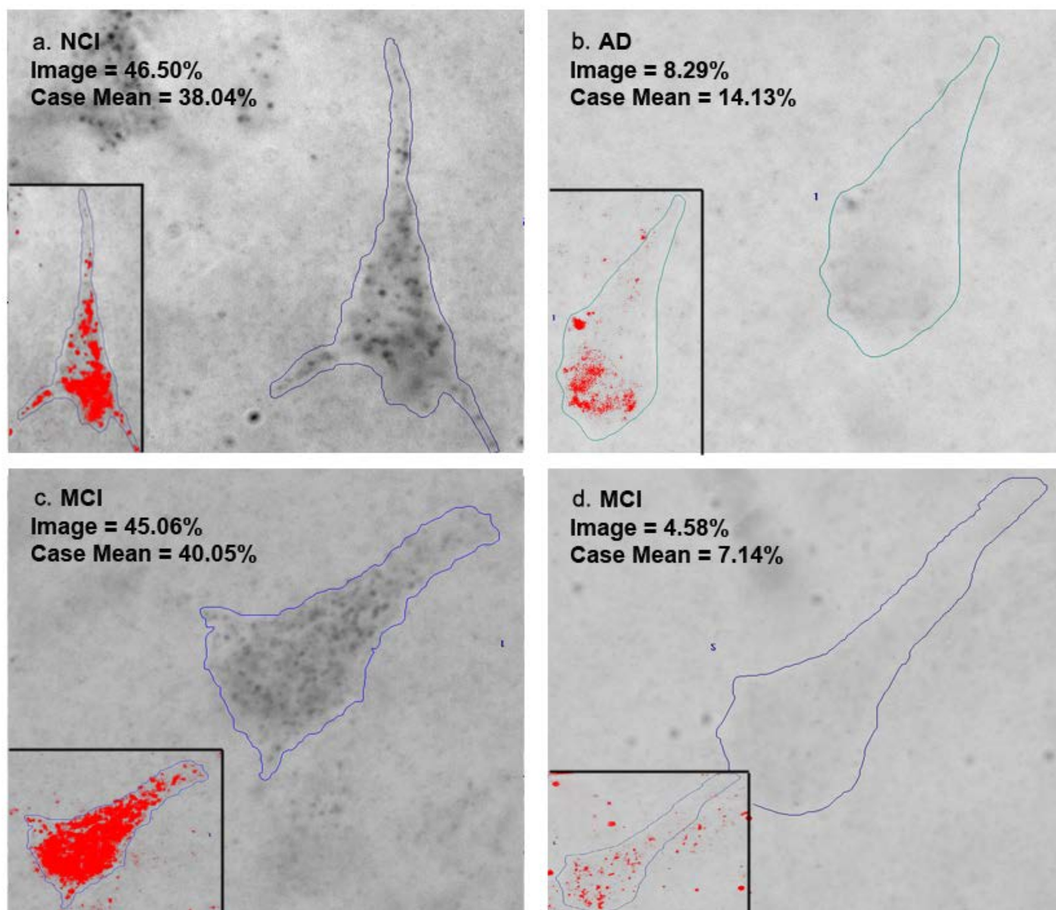
LR11 protein expression in at least a subset of cases in all of the diagnostic groups examined in both cohorts, with notable exception of the pathologically clean NCI group in ROS 1.0.

3.2 Results

ROS 1.0 Results

LR11 protein expression was measured in the frontal cortex of 34 cases that were selected based on a clinical diagnosis of NCI, MCI or AD following their final antemortem clinical diagnosis that was confirmed pathologically at autopsy. Neuronal LR11 immunolabeling appeared punctate, with protein expression predominantly localized to the soma and proximal dendrites of pyramidal neurons. Representative images of the staining seen in the ROS 1.0 cases can be seen in Figure 3.1. LR11 staining was generally strong in the NCI cases, ranging from 16.1% to 50.5% surface area stained positive for LR11, with a mean staining level of 28.6% ± 3.4. In the AD group, LR11 staining was markedly reduced, with staining levels ranging from 5.0% to 20.1% surface area stained and mean staining level of 13.0% ± 1.9. Finally, while the mean LR11 staining level in the MCI group was intermediate between NCI and AD (22.8% ± 4.7), the difference between MCI and either of the other two diagnostic groups failed to reach the level of statistical significance set for

Figure 3.1 – Representative Images of LR11 Immunostaining in ROS 1.0



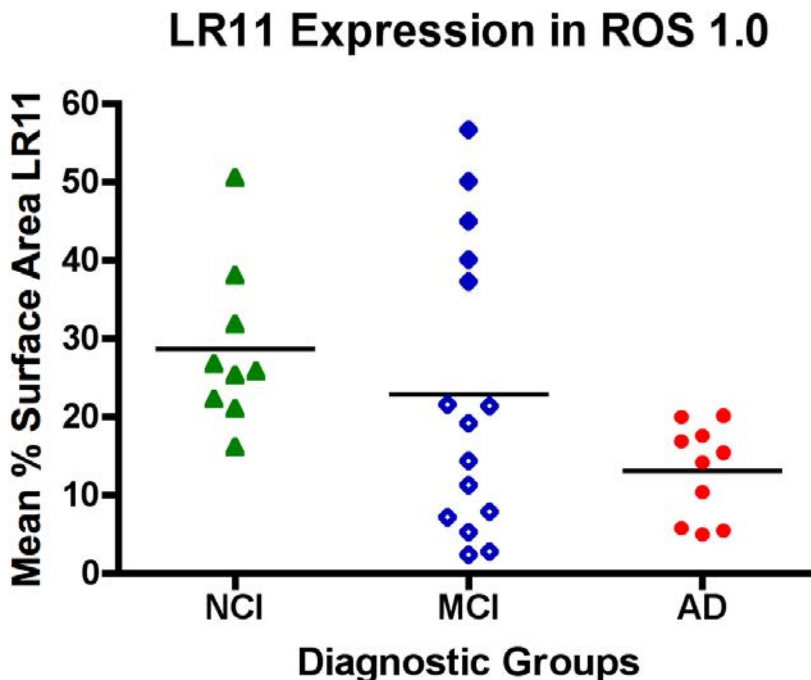
In the ROS 1.0 cohort, LR11 expression in mild cognitive impairment (MCI) is variable relative to no cognitive impairment (NCI) and Alzheimer's disease (AD). Shown here are representative images from an NCI case (A) showing robust LR11 expression (46.5% cell-surface area stained positive for LR11); an AD case (B) showing weak LR11 expression (8.3% LR11); and two MCI cases, one showing NCI-like LR11 expression (C, 45.1% LR11) and one showing AD-like LR11 expression (D, 4.6% LR11). In all images, the red overlay shown in the inset represents the pixels determined to be stained positive for LR11 for each cell.

this study ($p = 0.02$, Kruskal-Wallis test). Staining in the MCI cases was significantly more variable in MCI compared to NCI and AD ($p = 0.003$, Levene's test for equal variances), ranging from 2.3% to 56.6% surface area stained positive for LR11 (Figure 3.2).

To confirm the validity of these results, three independent raters blinded to diagnosis scored LR11 expression in the frontal cortex of each case on a semi-quantitative four point scale, with a score of 1 denoting no discernable LR11 staining and a score of 4 representing strong immunostaining. Spearman rank correlation confirmed a significant correlation between the semi-quantitative scorings from all three raters (as well as the mean rater score) and quantitative measures of LR11, indicating that the two approaches are generally consistent (Spearman r =0.72 – 0.79 for the individual raters, $r = 0.86$ for the mean rater score; $p < 0.0001$*** for all four comparisons). The three raters showed only moderate agreement (generalized weighted kappa, $\kappa = 0.46$), reflecting possible observer bias and demonstrating the benefits of quantitative approaches over semi-quantitative rating scales (Figure 3.3).

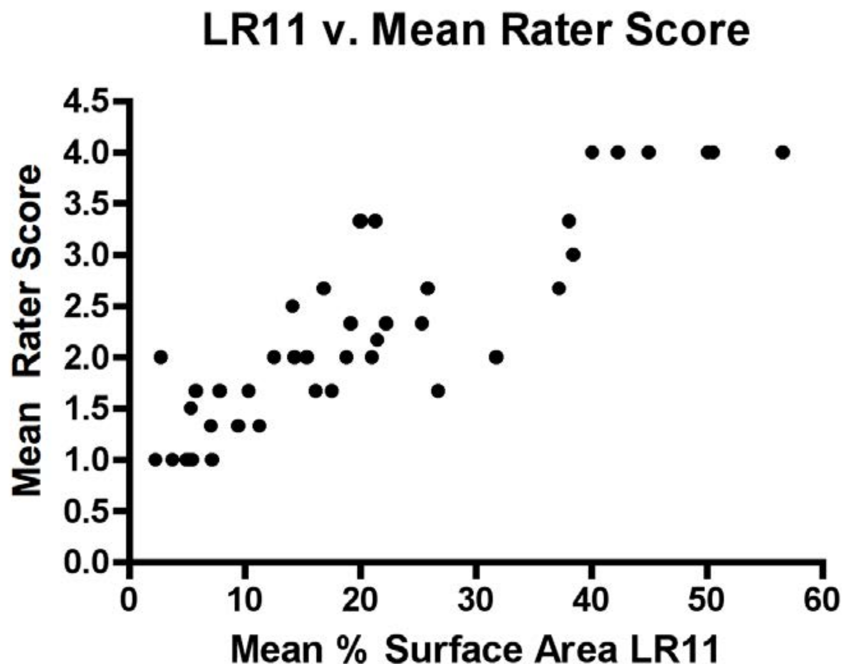
As noted above, LR11 immunostaining in the MCI cases was significantly more variable than in either the NCI or AD groups. Further examination of the distribution of case means within the MCI group revealed a bimodal distribution, suggesting a subdivision within the MCI group based on LR11 expression levels. A subsequent hierarchal cluster analysis of the distance matrix between pairs of MCI subjects confirmed this observation, revealing high LR11 expression (MCI-H) and

<u>Figure 3.2 - LR11 expression in mild cognitive impairment (MCI) is variable relative to no cognitive impairment (NCI) and Alzheimer's disease (AD)</u>



The distribution of the case means for each diagnostic group demonstrates that although there is an intermediate level of LR11 expression in the MCI group (22.8% ± 4.7) relative to NCI (28.6% ± 3.4) and AD (13.0% ± 1.9), the MCI group is significantly more variable (p = 0.003, Levene's test for equal variances) as a result of the bimodal distribution of LR11 expression in the MCI group. Cases in the MCI-H subgroup are indicated by the closed diamonds and cases in the MCI-L subgroup are indicated by the open diamonds.

Figure 3.3 – Verification of ROS 1.0 LR11 Quantitative Measures by Semi-quantitative Analysis



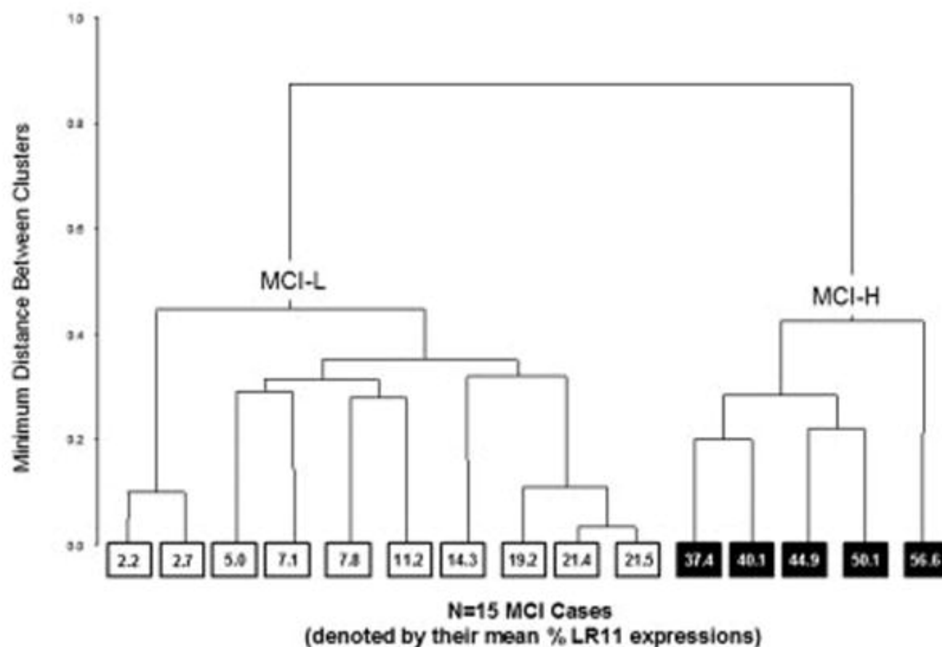
Three independent raters scored LR11 staining in each case on a scale of 1 to 4, with a score of 1 denoting no discernible LR11 staining and a score of 4 representing strong immunostaining. The quantitative LR11 measures and the semi-quantitative scores from each rater, as well as the mean rater score for each case, were found to be highly correlated indicating that the two approaches are generally consistent (Spearman $r = 0.72 – 0.79$ for the three raters, $r = 0.86$ for the mean rater score; $p < 0.0001$*** four all four comparisons). The correlation graph for the mean rater score is shown.

low LR11 expression (MCI-L) subgroups (Figure 3.4). A series of pair-wise comparisons were performed to test our hypothesis that LR11 expression in the MCI-H subgroup was similar to that seen in NCI and that LR11 expression in the MCI-L subgroup was similar to that seen in AD. As predicted, there was no significant difference between MCI-L (11.3% ± 2.3) and AD ($p = 0.43$). LR11 expression was significantly higher in the MCI-H subgroup (45.8% ± 3.5) than in NCI ($p = 0.0078$**). This can be attributed to the lack of cases with lower LR11 expression in the MCI-H group as a consequence of splitting the MCI group into two subgroups. Finally, this analysis confirmed a significant difference between LR11 expression in MCI-L and MCI-H ($p < 0.0001$***). MCI-H cases are indicated in Figure 3.2 with closed diamonds while open diamonds indicate the MCI-L cases.

ROS 2.0 Results

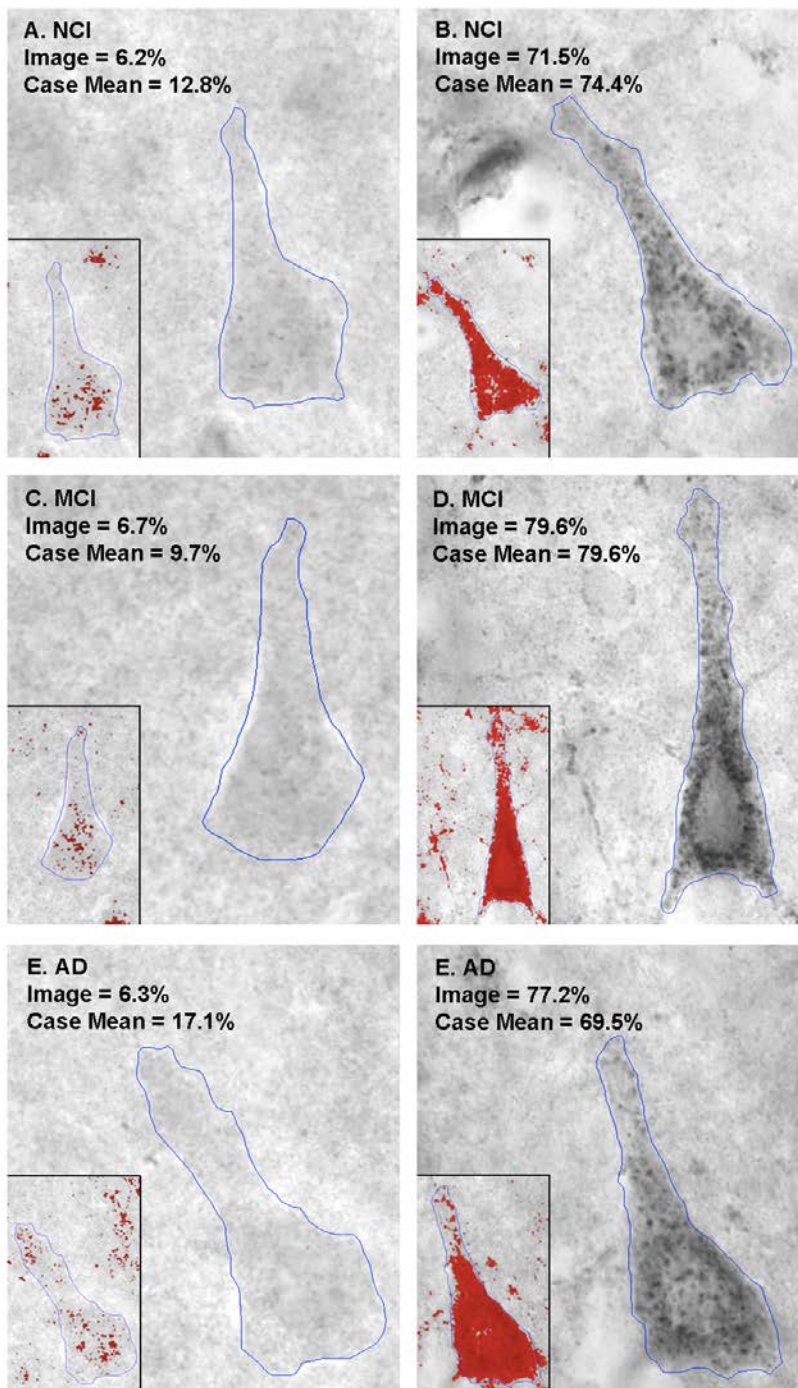
LR11 expression was measured in brain sections from the frontal cortex of 43 cases that were selected based on a clinical diagnosis of NCI, MCI or AD following their final clinical evaluation prior to death. Post-mortem pathological information was not considered in selecting the cases for this cohort. LR11 expression across these three diagnostic groups was highly varied, ranging from robust punctate LR11 staining in the majority of all cases observed to reduced LR11 expression in a handful of cases (Figure 3.5). LR11 expression in the NCI group ranged from 12.8%

Figure 3.4 – LR11 expression shows two distinct subgroups of mild cognitive impairment (MCI) cases



Independent statistical analysis using hierarchal clustering of 105 test statistics generated from the distribution-free, two-sample Wilcoxon rank-sum test demonstrated that MCI with high (MCI-H) and low LR11 expression (MCI-L) form two distinct clusters.

Figure 3.5 – Representative Images of LR11 Immunostaining in ROS 2.0

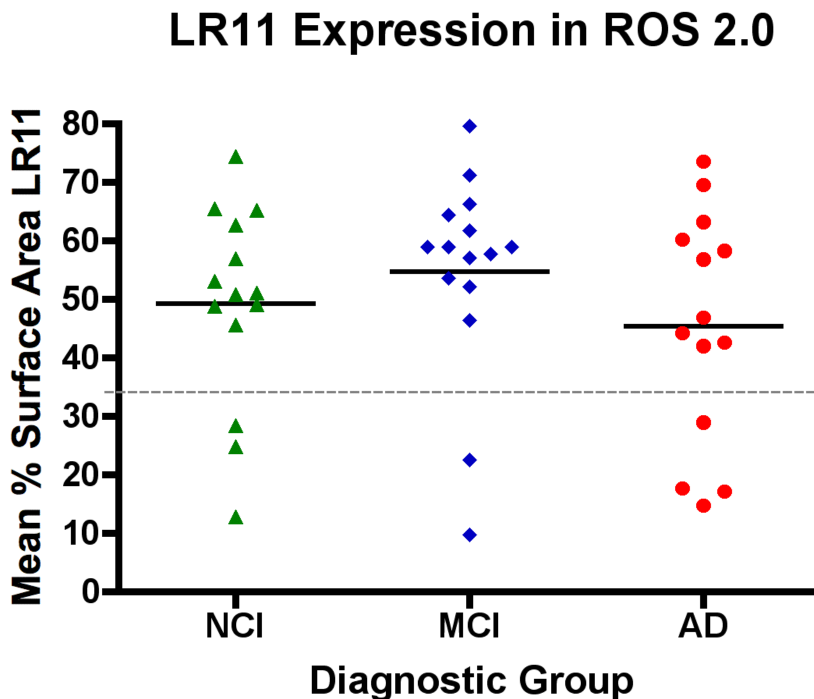


In the ROS 2.0 cohort, LR11 expression was highly variable in all three diagnostic groups. Shown here are representative images demonstrating the range of staining in each diagnostic group. Panels A and B are both from NCI cases, with the case in (A) having weak LR11 expression (12.8% mean surface area stained positive for LR11) and the case in panel B having robust LR11 expression (74.4% LR11). Panels C and D are from MCI cases with low LR11 expression (C, 9.7% LR11) and high LR11 expression (D, 79.6% LR11) and panels E and F are from a low LR11 AD case (E, 17.1.% LR11) and a high LR11 AD case (F, 69.5%). In all of the panels, the red overlay shown in the inset represents the pixels determined to be stained positive for LR11 for each pictured cell.

surface area to 74.4% surface area, with a group mean of 49.2% ± 4.6 surface area stained positive for LR11. LR11 expression in the MCI group was similarly varied, ranging from 9.7% surface area to 79.6% surface area, with a group mean of 54.6% ± 4.6 surface area stained positive for LR11. LR11 expression was surprisingly robust in the majority of AD cases examined, ranging from 14.7% to 73.5%, with a group mean of 45.4% ± 5.2 surface area stained positive for LR11 (Figure 3.6). There was no significant difference between the mean percent surface area for each diagnostic group ($p = 0.29$, Kruskal-Wallis test). Note that a protocol change from a 24-hour primary incubation time in ROS 1.0 to a 45-hour primary incubation time in ROS 2.0 resulted in more intense staining across all levels of LR11 protein expression in ROS 2.0.

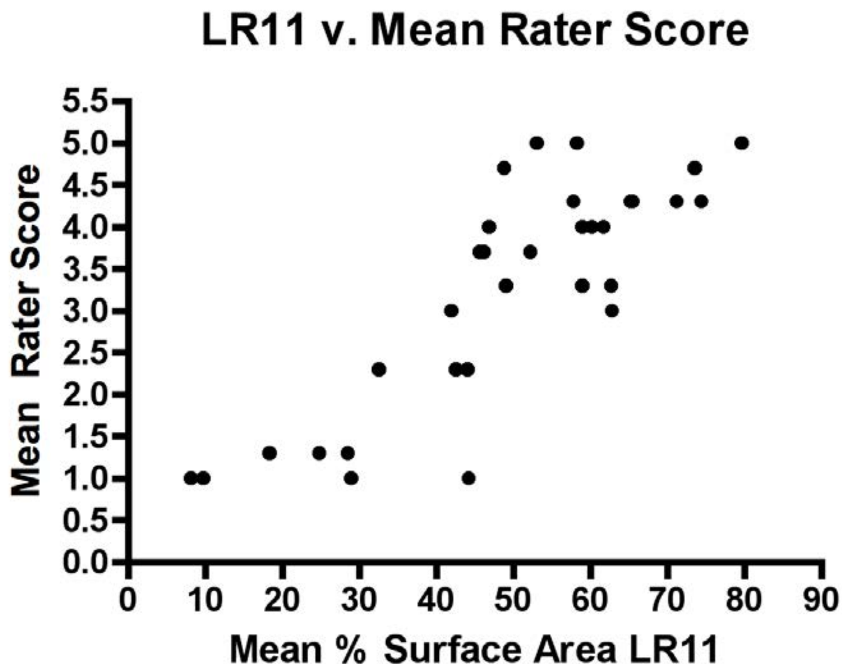
To confirm the validity of these results, three independent raters blinded to diagnosis scored LR11 expression in the frontal cortex of each case on a semi-quantitative five point scale, with a score of 1 denoting no discernible cellular LR11 staining above background and a score of 5 denoting strong, consistent cellular LR11 label across the brain section. Spearman's rank correlation between the scores from each individual rater as well as the mean rater score and the quantitative LR11 measures for each case indicated strong agreement between the two measures (Spearman's $r = 0.68 – 0.79$ for the individual raters, $r = 0.99$ for the mean rater score; $p < 0.0001$*** for all comparisons). The three raters showed moderate agreement, similar to the level of agreement seen in ROS 1.0 (Fleiss' kappa, $\kappa = 0.38$) (Figure 3.7).

<u>Figure 3.6 – LR11 Expression is highly variable in frontal cortex in all three diagnostic groups</u>



LR11 expression is highly variable in frontal cortex. No significant difference in mean LR11 expression between the NCI, MCI and AD groups was observed ($p = 0.29$). Further examination of the distribution of case means within each group revealed highly variable LR11 expression in all three diagnostic groups, with a handful of cases in each group having much lower LR11 expression than the majority of cases. Cases were classified as having low LR11 if the mean percent surface area stained positive for that case was in the lowest tertile of LR11 expression observed across all cases. This cut off (34.7%) is indicated by the dotted line.

Figure 3.7 - Verification of ROS 2.0 LR11 Quantitative Measures by Semi-quantitative Analysis



Three independent raters scored LR11 staining in each case on a scale of 1 to 5, with a score of 1 indicating little to no LR11 staining above background and a score of 5 indicating strong LR11 immunostaining. The quantitative LR11 measures and the semi-quantitative scores from each rater, as well as the mean rater score for each case, were found to be highly correlated suggesting strong agreement between the two methods (Spearman r = 0.68 – 0.78 for the three raters, r = 0.78 for the mean rater score; $p < 0.0001$*** four all four comparisons). The correlation graph for the mean rater score is shown.

To better understand the distribution of LR11 expression profiles within each diagnostic group, we characterized all subjects having LR11 expression levels within the lowest tertile of LR11 expression observed across all cases as “low” LR11 cases. This cut off (which is indicated by the gray dotted line in Figure 3.6) revealed that 3 of 14 NCI cases, 2 of 15 MCI cases and 4 of 14 AD cases had low LR11 expression. There was no significant difference in the number of cases with low LR11 expression between diagnostic groups ($p = 0.60$, chi-square test).

3.3 Discussion

A growing body of evidence suggests that LR11 is intricately involved in the pathogenesis of AD (Andersen et al 2006; Offe et al 2006; Rogaeva et al 2007; Scherzer et al 2004). The experiments decribed here characterized the expression of LR11 in MCI using a novel quantitative immunohistochemical procedure, which avoids the limitations of semi-quantitative methods. This approach also allowed for more powerful statistical analyses, the results of which will be presented in Chapter 5. In the ROS 1.0 cohort, we confirmed an earlier finding from our lab group that LR11 expression is reduced in AD compared with control cases in an independent and more mildly affected cohort (Offe et al 2006; Scherzer et al 2004). Moreover, we found that LR11 expression is highly variable in the MCI group, showing two distinct MCI subgroups. The MCI-H subgroup is characterized by robust, control-like LR11

neuronal immunostaining, whereas the MCI-L subgroup exhibited a marked reduction in LR11, similar to that seen in AD.

In contrast to our findings in the ROS 1.0 cohort, no significant difference in LR11 expression among the NCI, MCI and AD diagnostic groups was found in the ROS 2.0 cohort. Rather, LR11 expression in all groups was found to be highly variable, with all three diagnostic groups containing a small number of cases with low LR11 expression relative to that seen across the full set of cases. Taken together with our findings from the ROS 1.0 cohort, it is clear that the only diagnostic group in both cohorts to show robust LR11 expression in all cases was the NCI group in ROS 1.0. Interestingly, this is also the only group in both cohorts to be completely free of AD-related lesions, including amyloid plaques. The relationship between LR11 protein expression and the level of AD-associated lesions present in the brain will be examined in further detail in Chapter 5. However, the results presented here clearly indicate that LR11 protein deficits may precede the onset of cognitive impairment in the progression of the AD pathological cascade.

Notably, in the ROS 2.0 cohort, only about a third of the AD brains examined were found to have low LR11 expression relative to the full set of cases. This is far less than the near universal absence of LR11 in AD cases that has previously been reported (Andersen et al 2005; Offe et al 2006; Scherzer et al 2004). While this finding is unexpected in sporadic AD cases, we have previously observed persistent neuronal LR11 expression in familial AD brains (Dodson et al 2006), demonstrating that LR11 loss is not a universal element of AD pathology. Moreover, genetic studies

have shown that certain SNPs in the *SORL1* gene may confer a modest increased risk for developing AD, but that this relationship may be population-specific (Kauwe et al 2010; Li et al 2008; Minster et al 2008). Despite several lines of evidence linking it to AD pathogenesis, evidence is emerging that reduced LR11 expression is not required for the development of AD. Rather, it is increasingly likely that LR11 loss may be a susceptibility factor for the development of AD rather than a required causative event.

In this chapter, work has focused specifically on characterizing LR11 protein expression in MCI in the frontal cortex. However, the original reports linking a loss of LR11 expression with AD reported that while LR11 expression is markedly reduced in AD-vulnerable brain regions (like the frontal cortex and hippocampus), LR11 expression is preserved in other areas of the brain that are generally spared in AD (such as the cerebellum) (Offe et al 2006). Moreover, the appearance of pathological lesions in the brain is known to progress in an ordered fashion, often beginning in the entorhinal cortex and hippocampal formation before progressing into higher cortical areas. We hypothesize, then, that LR11 expression in MCI will be reduced specifically in brain areas affected early in AD, like the precuneus and that LR11 expression will be robust in brain regions that are only affected at the very end of the AD pathogenic cascade, such as the primary visual cortex. This hypothesis will be tested in the next chapter.

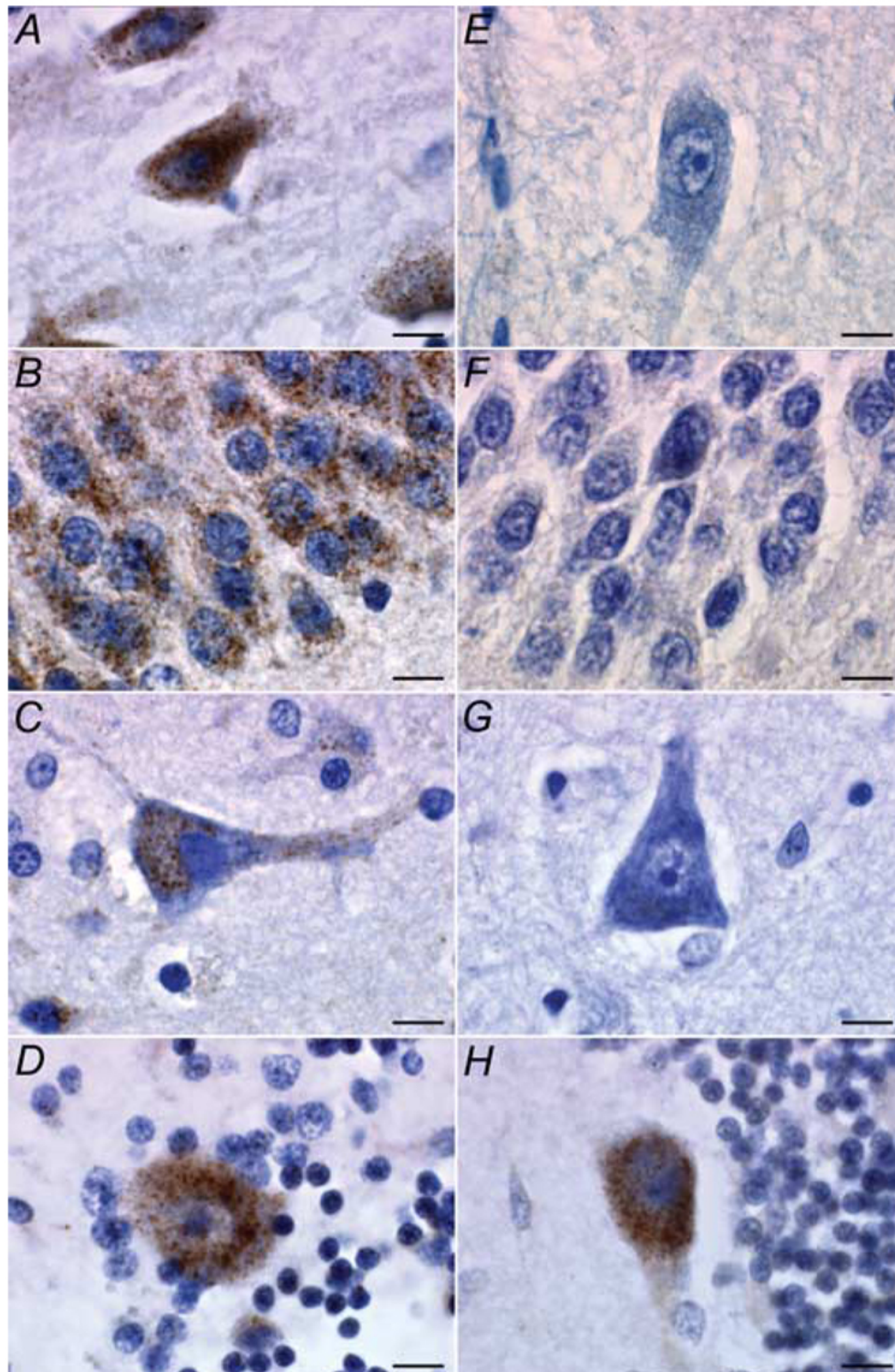
CHAPTER 4. REDUCED LR11 EXPRESSION IS NOT LIMITED TO AD-VULNERABLE BRAIN REGIONS

4.1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that initially presents clinically as specific memory complaints and ultimately progresses to full blown dementia. The primary pathological hallmarks of AD include the accumulation of the Aβ peptide into amyloid plaques in the isocortex and the appearance of neurofibrillary tangles (NFTs) coupled with extensive neuronal atrophy in the hippocampus and entorhinal cortex. Recent evidence has shown that the low density lipoprotein receptor LR11 plays an important role in maintaining low Aβ levels in the brain by regulating APP processing. LR11 protein expression is markedly reduced in at least a subset of AD brains, suggesting that the loss of this regulatory control of amyloidogenesis is an important early event in the AD pathogenic cascade.

An early publication focusing on the role of LR11 in AD observed that while LR11 expression was markedly reduced in AD-vulnerable brain regions such as the frontal cortex and the hippocampus, LR11 expression remained robust in areas of the brain that are traditionally spared in AD, including the cerebellum and basal ganglia (Figure 4.1) (Offe et al 2006). In the study reported in Chapter 3 of this

Figure 4.1 – LR11 is selectively lost in vulnerable brain regions in AD



In control brains, strong punctate immunolabeling was found in CA1-CA3 pyramidal neurons (A), dentate granule cells (B) and frontal cortex pyramidal neurons (C).

LR11 immunoreactivity was also detected in Purkinje cells of the cerebellum (D). In AD brains, LR11 immunoreactivity is absent in CA1-CA3 pyramidal neurons (E), dentate granule cells (F) and frontal cortex pyramidal neurons (G). Hematoxylin counterstain shows otherwise healthy appearing neurons in each brain region shown. LR11 is preserved in Purkinje cells of the cerebellum in AD patients (H). Scale bars, 10μm. Reproduced from Offe et al, 2006 (Offe et al 2006).

dissertation, we confirmed that LR11 expression in the frontal cortex is reduced in at least a subset of AD cases. Moreover, we showed that frontal cortex LR11 expression is also lower in a similar proportion of MCI and no cognitive impairment (NCI) control cases. In this chapter, we questioned whether reduced LR11 expression in MCI was also restricted to only AD-vulnerable brain regions.

It has long been recognized that the appearance and subsequent accumulation of both NFTs and amyloid plaques does not occur uniformly or haphazardly in the brain with the progressive development of AD (Arnold et al 1991; Arriagada et al 1992; Corder et al 2000; Duyckaerts & Hauw 1997; Gertz et al 1998; Markesbery et al 2006; Nagy et al 1999; Schönheit et al 2004). Rather, both lesions first appear in specific and distinct predilection sites before progressively spreading to other areas of the brain in a well established order. Because of this predictable sequence of pathological events in the brain, different schemes for the staging of AD have been established based on which brain areas are affected by specific types of lesions at any given time throughout the development and progression of the disease (1997; Khachaturian 1985; Markesbery 1997). Perhaps the best established of these staging schemes is that of Braak and Braak (Braak & Braak 1991), who in 1991 described six stages of AD based on the progressive appearance of NFTs in the brain. In the earliest stages, NFTs are seen exclusively in the transentorhinal cortex before spreading to the hippocampus and entorhinal cortex in subsequent stages. It is only in the latest stages of the disease that NFTs begin to appear and become numerous in the neocortical regions of the brain (Braak et al 2006; Braak & Braak 1995; Braak & Braak 1997b; c). While the progression of

amyloid plaques is less regimented than that of NFTs, they nonetheless follow a predictable pattern of spread. Amyloid plaques first appear in neocortical association areas such as the precuneus and the posterior cingulate gyrus and gradually increase in frequency and distribution throughout the cerebral cortex (Braak & Braak 1991; Lewis et al 1987; Rogers & Morrison 1985; Thal et al 2006). Unlike with NFTs, the hippocampus and entorhinal cortex are generally free of amyloid deposition in general and neuritic plaques specifically until relatively late in the disease. Likewise, the primary sensory and motor areas of the brain also harbor very low levels of amyloid deposition until very late in the disease. Similar patterns of progression with advancing disease stages have also been established based on neuronal atrophy (Brun & Englund 1981; Hyman et al 1990; Hyman et al 1984), alterations in functional imaging (Karas et al 2003; Shiino et al 2006; Whitwell et al 2007; Zakzanis et al 2003) and even gene transcriptional changes (Haroutunian et al 2009). With an increasing understanding of the temporal and topographic relationship between these different pathological events over the course of AD development, new profiles have begun to emerge that more clearly define both the earliest brain regions affected in AD and the pathological changes that can be detected in those brain regions at these earliest disease stages (Jack Jr et al 2010; Sperling et al 2011).

Given LR11's role in maintaining low Aβ levels in the brain and the proposed timeline for LR11 loss in AD, we hypothesized that LR11 expression would be reduced first in the areas of the brain known to accumulate amyloid plaques in the earliest stages of the disease and would be persistently robust until very late in the

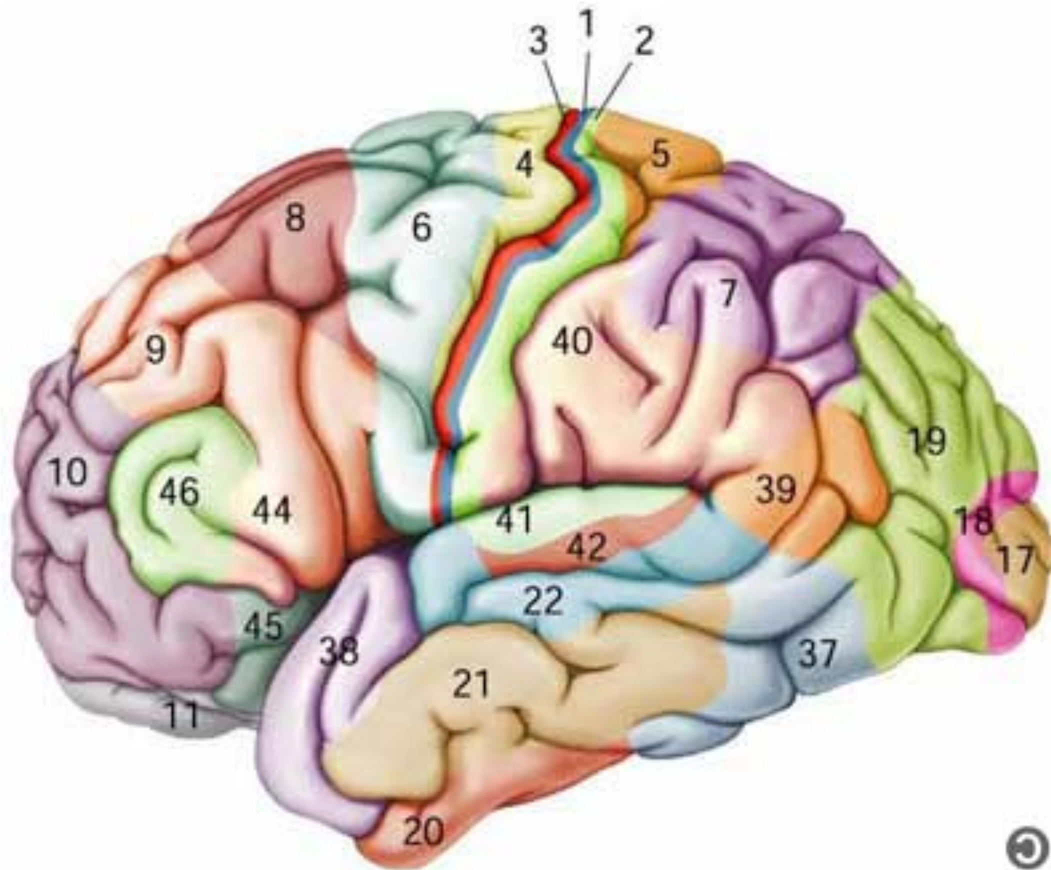
disease in brain areas that are generally spared in AD. To test this hypothesis, we quantified LR11 expression in NCI, MCI and AD cases in one brain region that is a known predilection site for amyloid accumulation (the precuneus) and in one brain region that is spared in AD (primary visual cortex) using the same quantitative immunohistochemistry approach that was used to measure LR11 expression in the frontal cortex from the same set of cases. Due to the availability of tissues, this study was limited to the ROS 2.0 cohort of cases.

The precuneus, one of the cerebral association cortices, is located in the posterior region of the medial parietal cortex and corresponds to Brodmann's area 7 (Figure 4.2). The precuneus is strongly and reciprocally interconnected with the hippocampus and the entorhinal cortex and together with the posterior cingulate gyrus forms an important part of the brain's default memory network. The primary function of the precuneus is to integrate external and self-generated information. In particular, the precuneus plays an important role in episodic and autobiographical memory retrieval (Cavanna 2007; Cavanna & Trimble 2006). In healthy brain, the precuneus shows high metabolic activity during conscious rest and selectively deactivates during non-self-directed cognitive tasks (Sperling et al 2010). However, in AD brain, there is a marked reduction in brain glucose metabolism at rest that corresponds to the severity of autobiographical memory impairment (Eustache et al 2004). Numerous functional imaging studies have corroborated this finding and have also shown a significant impairment in the precuneus in the ability to inactivate during cognitive tasks, even at the earliest stages of the disease before overt cognitive impairment is evident (Borroni et al 2006a; Greicius et al 2004;

Herholz et al 2007; Herholz et al 2002; Huang et al 2003; Karas et al 2007; Kogure et al 2000; Lustig et al 2003; Matsuda 2001; 2007; Matsuda et al 2007; Okamura et al 2002; Rombouts et al 2005). The precuneus has long been known to harbor a heavy amyloid plaque burden in AD brain and recent PiB binding studies in living patients has revealed that this brain region is particularly vulnerable to amyloid deposition in the earliest, pre-clinical stages of the disease (Buckner et al 2005; Ikonomovic et al 2011; Sheline et al 2010; Sperling et al 2009). This converging evidence highlights this brain region as one of the first sites in the brain to become impaired in AD. As such, we predict that LR11 expression in the precuneus will be low in at least a comparable proportion of NCI and MCI cases to that which had low LR11 expression in the frontal cortex.

The primary visual cortex is located on the medial brain surface of the occipital cortex and corresponds to Brodmann's area 17 (Figure 4.2) (Martin 1996). It is generally spared pathologically in AD. While some visual dysfunction has been associated with AD (Kirby et al 2010), these impairments are primarily associated with pathology affecting the surrounding visual association areas rather than the primary visual cortex (Jackson & Owsley 2003; Nobili & Sannita 1997). Notably, a very early study of pathogenic lesions observed that while there were almost no NFTs in the primary visual cortex, there was a 20-fold increase in NFTs in the primary visual association area (Brodmann's area 18) and a further doubling of NFTs in a higher order visual association area (Brodmann's area 20) (Lewis et al 1987). A more recent study has also shown that visual task performance correlates with reduced regional glucose metabolism in secondary visual cortex, but not in

Figure 4.2 – Brodmann's Area Map



Brain sections from the precuneus correspond to Brodmann's Area 7, which is colored bright purple and indicated by the digit 7 in the above brain map. Brain sections from the primary visual cortex correspond to Brodmann's Area 17, which is colored light orange and indicated by the number 17 in the above brain map. For reference, the brain sections from the frontal cortex analyzed in Chapter 3 correspond to Brodmann's Area 9 (ROS 1.0 cases, colored peach) and Brodmann's Area 10 (ROS 2.0 cases, darker purple).

Image was reproduced from the website "The Brain from Top to Bottom" (http://thebrain.mcgill.ca/flash/capsules/outil_jaune05.html) and was shared freely by the authors under the principles of "Copyleft" sharing.

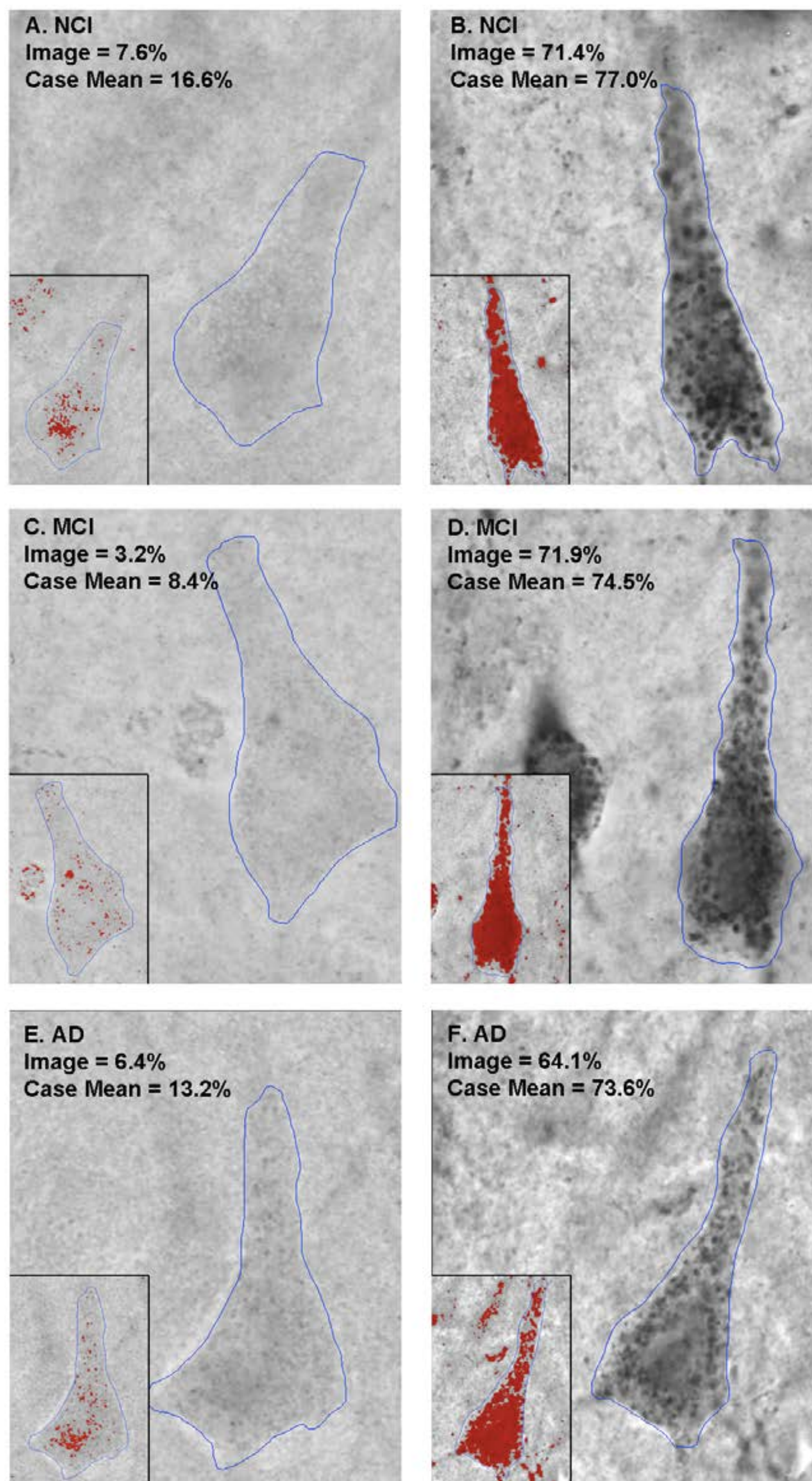
primary visual cortex (Mielke et al 1995). While it is possible (and even likely) that there is some pathogenic damage to the primary visual cortex in end stage AD, it is clear that this brain area is generally spared until very late in the disease process (Arnold et al 1991; Cui et al 2007; Duyckaerts & Hauw 1997; Herholz et al 2007; Karas et al 2003; Metsaars et al 2003). As such, we predict that LR11 expression will be persistently robust in the primary visual cortex in almost all of the NCI and MCI cases examined and will only be low in a very small subset of AD cases, if it is altered at all.

4.2 Results

Precuneus Results

LR11 protein expression in the precuneus is highly similar to LR11 expression in the frontal cortex. Intracellular LR11 expression was generally punctate and restricted to the cell soma and proximal dendrites of pyramidal neurons throughout layers III and V. LR11 expression was measured in the precuneus in 43 cases in the ROS 2.0 cohort using the same quantitative immunohistochemistry technique that was used to measure LR11 expression in the frontal cortex in these same cases. LR11 expression in the precuneus was highly variable across all three diagnostic groups (NCI, MCI and AD), much like in the frontal cortex (Figure 4.3). LR11 expression in the NCI group ranged from 15.9%

Figure 4.3 – Representative Images of LR11 Immunostaining in the Precuneus

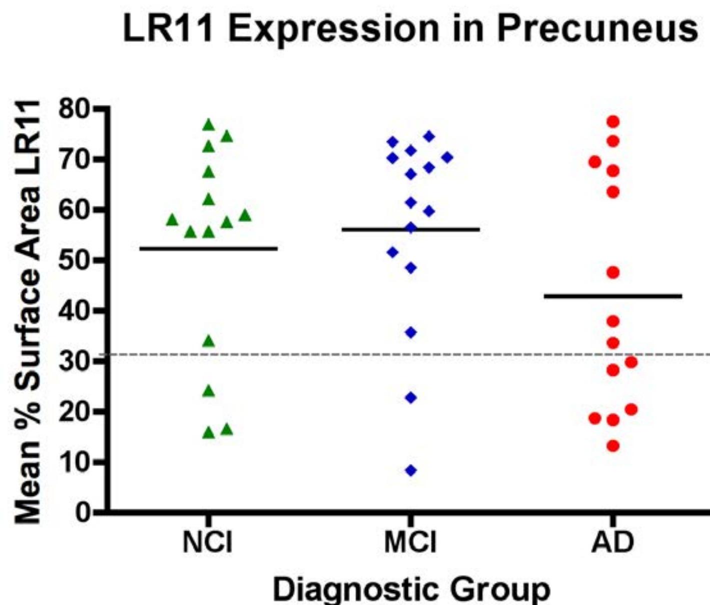


LR11 immunostaining in precuneus neurons was generally somatodendritic and punctate in distribution, similar to that seen in the frontal cortex. LR11 expression was highly variable from case to case in the precuneus, with some cases in each diagnostic group having robust LR11 expression and some cases having very little LR11 expression as shown in here in representative images. Panels A and B are both from NCI cases, with the case in panel A having weak LR11 expression (16.6% mean surface area stained positive for LR11) and the case in panel B having robust LR11 expression (77.0% LR11). Panels C and D are from MCI cases with low LR11 expression (C, 8.4% LR11) and high LR11 (D, 74.5% LR11) and panels E and F are from a low LR11 AD case (E, 13.2% LR11) and a high LR11 case (F, 73.6%). In all of the panels, the red overlay shown in the inset represents the pixels that are stained positive for LR11 in each pictured cell.

surface area to 76.9% surface area, with a group mean of 52.2% ± 5.6 surface area stained positive for LR11. LR11 expression in the MCI group was similarly varied, ranging from 8.4% surface area to 74.5% surface area, with a group mean of 56.1% ± 5.1 surface area stained positive for LR11. As in the frontal cortex, LR11 expression was surprisingly robust in the AD group as well, ranging from 13.2% surface area to 77.4% surface area, with a group mean of 42.8% ± 6.2 surface area stained positive for LR11. There was no significant difference in mean LR11 expression between the three diagnostic groups ($p = 0.37$, Kruskal-Wallis test) (Figure 4.4).

We again dichotomized the cases in all three diagnostic groups into "high" LR11 and "low" LR11 subgroups using a cut off score based on the lowest tertile of LR11 expression measured in the precuneus across the full cohort of cases. Using this cut off, which was set at 31.4% for the precuneus (as indicated by the gray dotted line in Figure 4.4), we determined that 3 of the 14 NCI cases, 2 of the 15 MCI cases and 6 of the 14 AD cases had low LR11 expression in the precuneus relative to the rest of the cases in the cohort. There was no significant difference in the number of cases with low LR11 expression between diagnostic groups ($p = 0.17$, chi square test). This was highly similar to the proportion of cases that were found to have low LR11 expression in the frontal cortex, with only the AD group having slightly more low LR11 cases in the precuneus.

<u>Figure 4.4 – LR11 Expression is highly variable in precuneus in all three diagnostic groups</u>



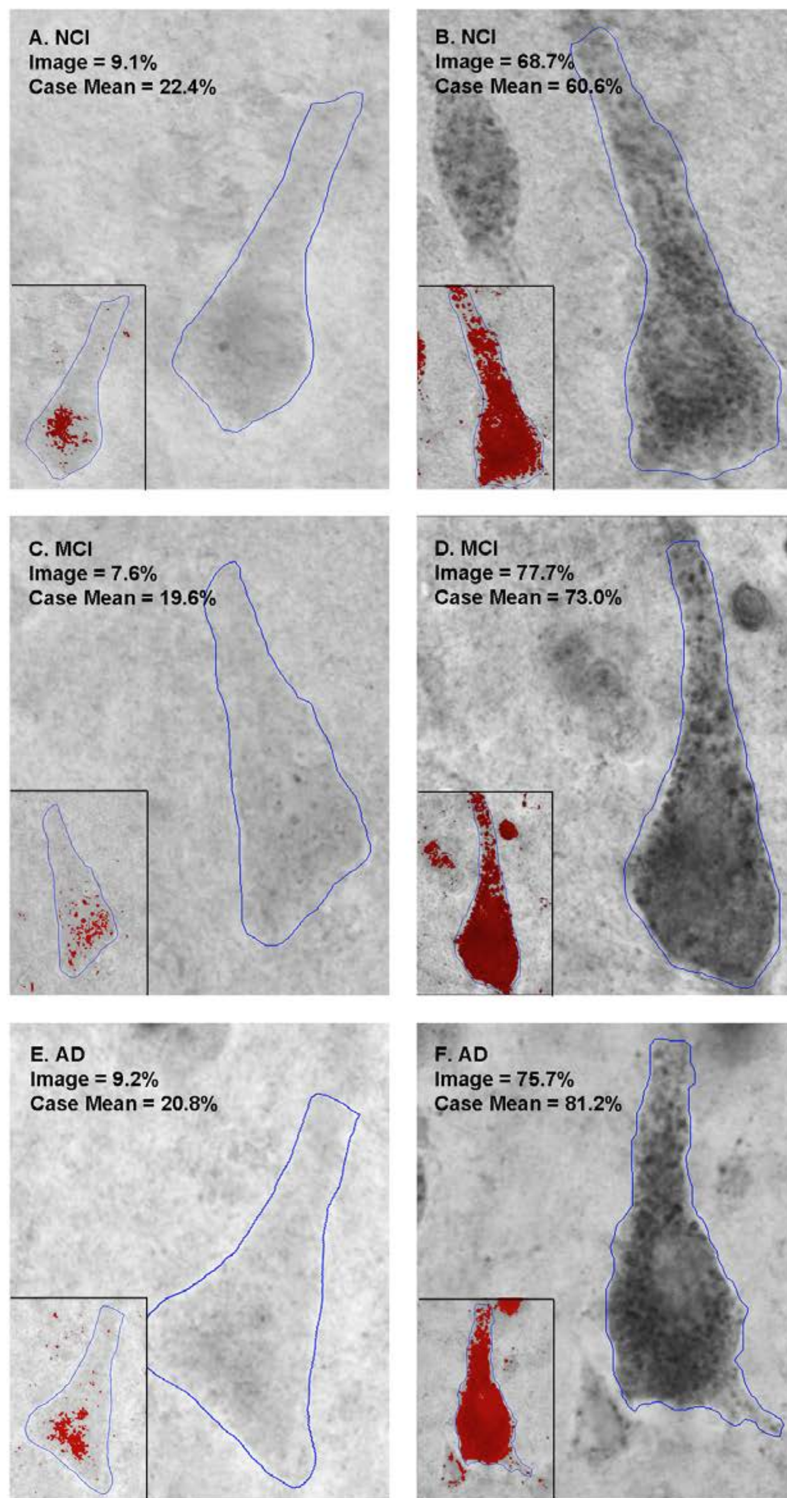
LR11 expression is highly variable in precuneus. No significant difference in mean LR11 expression (indicated by the short black bars) between NCI, MCI and AD groups was observed (p = 0.37). As in the frontal cortex, further examination of the distribution of case means within each group revealed highly variable LR11 expression in all three diagnostic groups, with a small subset of cases in each group having lower LR11 expression than the majority of the cases. Cases were classified as having low LR11 if the mean percent surface area stained positive for that case was in the lowest tertile of LR11 expression observed across all cases. This cut off (31.4%) is indicated by the dotted line.

Primary Visual Cortex Results

LR11 expression in the neurons of the primary visual cortex was robust and punctate in the majority of cases examined. While the cells were slightly smaller than in the precuneus and in the frontal cortex, the immunostained cells in layers III and V were pyramidal in shape and a comparable percent surface area of those cells stained positive for LR11. LR11 expression was highly variable in the primary visual cortex, again independent of clinical diagnosis (Figure 4.5). LR11 expression in the NCI group ranged from 16.0% to 77.1% surface area, with a group mean of 48.4% $\pm$ 5.7 surface area stained positive for LR11. LR11 expression in the MCI group ranged from 11.0% to 76.7% surface area, with a group mean of 58.1% $\pm$ 4.9 surface area stained positive for LR11 (Figure 4.6). Finally, in the AD group, LR11 expression ranged from 20.4% to 81.2% surface area with a group mean of 43.5% $\pm$ 5.2 surface area stained positive for LR11. While there appears to be a trend towards lower LR11 in the primary visual cortex in AD, there was no significant difference between the mean LR11 expression in the diagnostic groups ($p = 0.15$, Kruskal-Wallis).

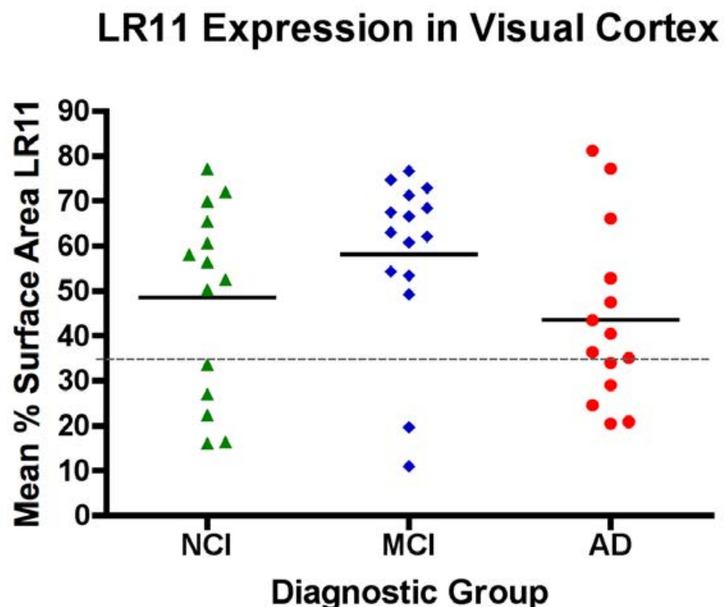
Cases with LR11 expression in the lowest tertile of LR11 expression measured in the primary visual cortex were again considered to have low LR11 expression. In this brain region, this cut off was set at 34.4%, as indicated by the gray dotted line in Figure 4.6. Using this threshold, we determined that 5 of 14 NCI cases, 2 of 15 MCI cases and 5 of 14 AD cases had low LR11 expression. There was no significant difference in the number of cases with low expression between the three diagnostic groups ($p = 0.30$, chi square test). The proportion of cases in each diagnostic group with low LR11 expression in the primary visual cortex was highly

Figure 4.5 – Representative Images of LR11 Immunostaining in the Primary Visual Cortex



LR11 expression in the primary visual cortex was very similar to that seen in both the frontal cortex and precuneus. As in the other two brain regions, LR11 expression was highly variable in the primary visual cortex, with some cases in all three diagnostic groups having robust LR11 expression and some cases having very little LR11 expression. Panels A and B are both from NCI cases, with the case in panel A having weak LR11 expression (22.4% mean surface area stained positive for LR11) and the case in panel B having robust LR11 expression (60.6% LR11). Panels C and D are from MCI cases with low LR11 expression (C, 19.6% LR11) and high LR11 (D, 73.0% LR11) and panels E and F are from a low LR11 AD case (E, 20.8% LR11) and a high LR11 case (F, 81.2%). In all of the panels, the red overlay shown in the inset represents the pixels stained positive for LR11 in each pictured cell.

Figure 4.6 – LR11 Expression is highly variable in primary visual cortex in all three diagnostic groups



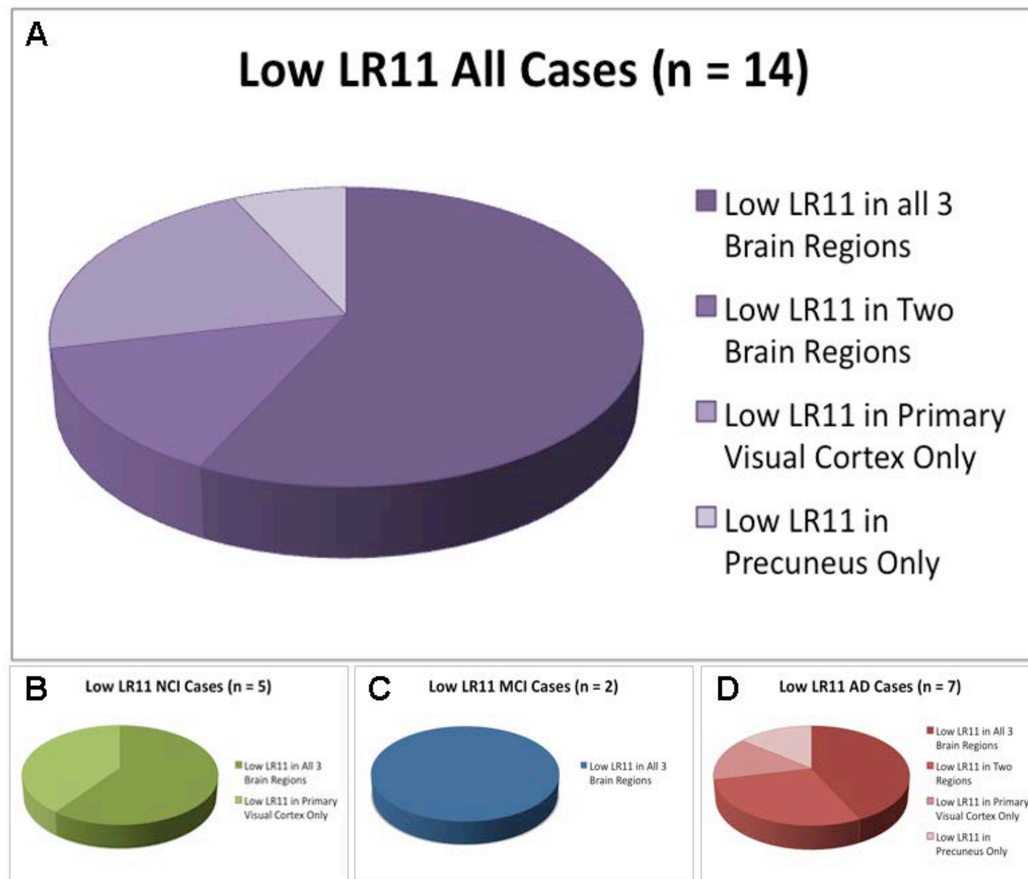
LR11 expression is highly variable in primary visual cortex. No significant difference in mean LR11 expression (indicated by the short black bars) between NCI, MCI and AD groups was observed (p = 0.15). As in the frontal cortex and precuneus, further examination of the distribution of case means within each group revealed highly variable LR11 expression in all three diagnostic groups, with a small subset of cases in each group having lower LR11 expression than the majority of the cases. Cases were classified as having low LR11 if the mean percent surface area stained positive for that case was in the lowest tertile of LR11 expression observed across all cases. This cut off (34.4%) is indicated by the dotted line.

similar to the proportion of cases with low LR11 expression in each diagnostic group in both the frontal cortex and precuneus.

Uniformity of LR11 loss across brain regions

Within the ROS 2.0 cohort, 14 cases were found to have low LR11 expression in at least one of the brain regions examined. Five of these cases were in the NCI group, two of these cases had a clinical diagnosis of MCI and seven of these cases had a clinical diagnosis of AD. There was no statistical difference in the number of cases in each diagnostic group that were found to have low LR11 in at least one brain region ($p = 0.10$, chi square test). More than half of the cases with low LR11 in at least one brain region actually had low LR11 expression in all three brain regions examined (8 of 14 cases, 57%). Three of these cases were in the NCI group, two were in the MCI group and three were in the AD group. Of the remaining six cases with low LR11 in at least one brain region, two of those cases (both AD) had low LR11 in two brain regions. One of these cases had low LR11 expression in the frontal cortex and precuneus but not the primary visual cortex and the other case had low LR11 expression in the precuneus and primary visual cortex but not the frontal cortex. Finally, four cases had low LR11 expression in only one of the brain regions examined. In three of these cases, only the primary visual cortex had low LR11 expression (2 NCI cases and 1 AD case). The remaining case only had low LR11 expression in the precuneus (AD) (Figure 4.7). Low LR11 expression was never seen exclusively in the frontal cortex. Based on these observations, we

Figure 4.7 – Low LR11 Expression is Generally Widespread in the Brain



Fourteen of the cases in the ROS 2.0 cohort were found to have low LR11 expression in at least one of the three brain areas examined. There was no significant difference in the proportion of low LR11 cases in each diagnostic group (5 NCI cases, 2 MCI cases, 7 AD cases; p = 0.10, chi square test). (A) More than half of the cases with low LR11 in at least one brain region actually had low LR11 expression in all three brain regions (8 of 14 cases, 57%). Two cases had low LR11 in two brain regions (one with low LR11 in precuneus and frontal cortex and one with low LR11 in precuneus and primary visual cortex) and the remaining four cases had low LR11 in only brain region (3 in primary visual cortex and one in precuneus). (B) Three of five NCI cases with low LR11 in at least one brain region were found to have low LR11 in all three brain regions and the other two cases were found to have low LR11 in primary visual cortex only. (C) Both MCI cases that had low LR11 in at least one brain region

had low LR11 in all three brain regions. (D) Three of the AD cases with low LR11 in at least one brain region had low LR11 in all three brain regions. Two of the AD cases had low LR11 in two brain regions and two of the AD cases had low LR11 in only one brain region (one in precuneus and one in primary visual cortex).

conclude that low LR11 expression in the brain is generally widespread, much more so than originally believed.

4.3 Discussion

Based on the role of LR11 in regulating Aβ production and the proposed timeline of LR11 loss in the AD pathogenic cascade, we hypothesized that LR11 expression would be reduced first in the areas of the brain known to accumulate amyloid plaques in the earliest stages of the disease (like the precuneus) and would be persistently robust until very late in the disease in brain areas that are generally spared in AD (like the primary visual cortex). To test this hypothesis, LR11 expression was measured in the precuneus and the primary visual cortex in 43 cases from the ROS 2.0 cohort using quantitative immunohistochemistry. These results were then compared to the measurements of LR11 expression in the frontal cortex from the same cases to determine the pattern of LR11 loss in the brain over three stages of the disease as represented by the NCI, MCI and AD diagnostic groups. We found that LR11 expression in both the precuneus and the primary visual cortex was highly variable in all three diagnostic groups, with a small subset of cases in each group having low LR11 expression, similar to what was seen in the frontal cortex. Moreover, the majority of cases with low LR11 expression in at least one brain area also had low LR11 expression in the other two brain areas examined,

suggesting that a reduction in LR11 expression related to the development of AD is more widespread than previously believed.

As noted in the Introduction to this section, original reports suggested that the loss of LR11 expression in AD was restricted to only AD-vulnerable brain regions like the frontal cortex and hippocampus (Offe et al 2006). However, we have shown here that LR11 loss is more widespread, with reduced LR11 expression apparent even the primary visual cortex in the earliest stages of the disease (NCI, MCI). This observation would make the loss of LR11 unique among AD pathogenic events, which traditionally occur in the brain in a predictable and progressive order. In light of this finding, it is tempting to speculate that reduced LR11 expression may not be a specific disease-triggering event that occurs only in specific brain regions but rather may be a universal change in the brain that further enhances the likelihood and/or degree of amyloid deposition seen in certain regions with a pre-existing predilection for developing amyloid plaques.

Our observations here in the precuneus and in the primary visual cortex have also added further support to our conclusion in Chapter 3 that a reduction in LR11 expression (at least in the brain areas examined) is not a prerequisite for the development of AD. Rather, as noted above, a loss of LR11 expression throughout the brain is likely to enhance the susceptibility of certain brain regions to developing AD-related lesions and ultimately to increase the likelihood that an individual will develop AD in their lifetime.

It is worth noting the conclusions stated here are very preliminary due to the limited nature of this study. Due to time and tissue availability constraints, LR11 expression was only measured in three brain regions. A more thorough survey of LR11 expression throughout the brain at these various stages of the disease would have added considerable depth to our conclusions. In particular, the inclusion of brain regions whose roles in AD are better established, including the hippocampus and entorhinal cortex would have been particularly valuable. Likewise, while the primary visual cortex is generally spared until very late in the disease process, it would have been beneficial to include other "uninvolved" areas as well, such as the cerebellum or basal ganglia, which were both used in the original LR11 study.

Moreover, because low LR11 expression was seen in so few cases, any conclusions about an ordered progression of LR11 loss are nearly impossible to make. With only about a third of the total cases examined showing any reduction in LR11 expression, the statistical power of the study is far too weak to make any concrete conclusions about which (if any) brain areas are affected before others. However, given the large number of cases with low LR11 expression in all of the brain regions examined, we feel confident in our conclusion that LR11 loss in the AD brain is more widespread that initially reported.

Finally, the progression of other AD pathologic changes in the brain relative to LR11 loss is very difficult to address in this cohort of cases. Because this work was done exclusively in the ROS 2.0 cases, the vast majority of cases in both the NCI and MCI groups had already progressed to later stages of pathology accumulation.

Therefore, if LR11 loss was in fact progressing through the brain in a similarly ordered pattern, it is unlikely that it would be detectable in this particular cohort. This relationship between LR11 expression and AD lesions in all three brain regions will be examined in more detail in Chapter 5.

CHAPTER 5. LR11 EXPRESSION LEVELS DO NOT CORRELATE WITH OTHER EARLY CHANGES IN THE DEVELOPMENT OF AD

4.1 Introduction

Dr. Alöis Alzheimer first described the neurodegenerative disease that bears his name over 100 years ago. As part of that case report, Dr. Alzheimer identified two primary lesions that were associated with the disease: amyloid plaques and neurofibrillary tangles (Alzheimer 1906; 1907; Maurer et al 1997). Over the intervening decades, an intense debate raged over which was the causative lesion resulting in cognitive impairment and dementia (Mudher & Lovestone 2002; Trojanowski 2002). Due to the abundance of post-mortem "healthy" brains that harbored significant amounts of amyloid plaques (Gellerstedt 1933; Grünthal 1927; Tomlinson et al 1968) and the poor correlation between plaque burden and the degree of cognitive impairment (Bierer et al 1995b; Fukumoto et al 2003; Näslund et al 2000; Rothschild & Trainor 1937), neurofibrillary tangles emerged as the leading candidate for the critical pathological lesion underlying AD. However, the identification of a series of causative familial AD (FAD) gene mutations in the early to mid-1990s and the subsequent recognition that they all altered the production of Aβ from APP (Goate et al 1991; Levy-Lahad et al 1995; Murrell et al 1991; Rogaev et al 1995; Sherrington et al 1995) made it clear that abnormal accumulation of Aβ in the brain is one of the earliest events in the AD pathological cascade (Hardy &

Higgins 1992; Selkoe 1991; 2003; 2004). With the increased ability to monitor Aβ levels *in vivo*, it is now widely recognized that the shift from normal, low levels of Aβ in the brain to elevated levels of Aβ production and aggregation into amyloid plaques can begin decades prior to the onset of cognitive impairment (Jack Jr et al 2010; Sperling et al 2011).

Given the proposed functional role for LR11 in helping to maintain low Aβ levels in the brain (Andersen et al 2005; Offe et al 2006), it stands to reason that for the loss of LR11 to impact Aβ accumulation in a meaningful way so as to effect the course of AD onset and/or progression, the loss of LR11 must likewise occur very early in the disease, during the period of dynamic increase in Aβ levels in the brain. As such, low LR11 levels should be detectable in the earliest stages of the disease. In the previous two chapters, we have shown widespread reduction in LR11 expression in at least a subset of cases in the MCI and AD diagnostic groups in both of our study cohorts as well as in the NCI cases in the ROS 2.0 cohort. In fact, the only diagnostic group in either cohort with no cases with low LR11 expression is the ROS 1.0 NCI control group. Notably, this is also the only diagnostic group without any cases harboring amyloid deposition.

In order to determine if LR11 expression is related to other known early changes in the development of AD, and to more directly examine the specific relationship between LR11 expression and amyloid burden, we performed an extensive series of statistical analyses to identify correlates of LR11 across all stages of the disease. In addition to allowing us to determine if changes in LR11 expression

are related to other early events in the progression of AD or susceptibility factors, identifying correlates of LR11 could also suggest potential mechanistic links between LR11 function and other pathological changes in AD, adding additional value to this work.

We first examined a series of demographic variables to determine if low LR11 expression was associated with any particular subpopulations of individuals within our cohorts. We also looked at the association between LR11 expression and apoE genotype, the primary genetic risk factor for late onset, sporadic AD. We then examined a series of cognitive measures to determine if reduced LR11 expression was associated with cognitive symptomatology. Finally, as noted above, we looked at the correlation between LR11 expression and a series of global pathology measures as well as the frequency of specific AD-associated lesions (including both diffuse and neuritic plaques) in order to better clarify the relationship between LR11 and AD pathological events. Because of the relatively small size of each of our individual diagnostic groups, correlations with LR11 expression were examined across all of the cases in a given cohort regardless of final diagnosis rather than within each individual diagnostic group.

5.2 Results

Due to the density of information presented in this chapter, this results section is organized into four subsections based on the type of variables being

discussed (demographic, genetic, cognitive or pathological). Within each subsection, the results from the ROS 1.0 cohort are presented first, followed by the results from the ROS 2.0 cohort. A comparison of each variable examined between diagnostic groups is provided before a synopsis the results of the correlational analyses with LR11 expression, for each cohort and set of variables.

Demographic Variables

The relationship between frontal cortex LR11 expression and a series of demographic factors was first examined to determine if low LR11 expression is associated with any specific subpopulations of individuals as well as to identify any potential confounding factors. The demographic variables examined were age at death (in years), gender, years of education and post mortem interval (PMI, measured in hours).

As noted in Chapter 2, there were no significant differences in the number of males ($p = 0.72$), years of education ($p = 0.20$) or PMI ($p = 0.51$) between the NCI, MCI and AD diagnostic groups in the ROS 1.0 cohort. As a result of excluding control cases with any amyloid pathology, the NCI group was significantly younger at the time of death than either the MCI or AD groups ($p = 0.0034$, Table 5.1). Mixed models analysis revealed no significant correlation between frontal cortex LR11 expression and age at death or PMI. Likewise, there was no difference in LR11 expression between males and females. The number of years of education was weakly correlated with LR11 expression in this cohort ($p = 0.046$, Table 5.2).

Table 5.1 – ROS 1.0 Demographic Variable Comparison Across Groups*

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group	Pair-wise Comparisons
Age at death, years	75.4 ± 5.2 (67 - 82)	84.2 ± 6.0 (75 - 97)	86.8 ± 4.8 (80 - 94)	82.7 ± 6.9 (67 - 97)	p = 0.0011[a]	NCI < MCI, AD
Number (%) of males	6 (67%)	7 (47%)	4 (40%)	17 (50%)	p = 0.72[b]	
Years of education	19.2 ± 4.2 (12 - 26)	18.1 ± 4.7 (8 - 30)	16.3 ± 3.9 (6 - 20)	17.9 ± 4.4 (6 - 30)	p = 0.30[a]	
PMI, hours	6.5 ± 3.9 (3.5 - 16)	6.6 ± 4.2 (3 - 16)	6.1 ± 2.7 (3 - 10.7)	6.5 ± 3.6 (3.0 - 16)	p = 0.97[a]	

[a]Kruskal-Wallis test

[b]Fisher's Exact test

*Unless otherwise noted, data are presented as Mean ± SD (range). Pair-wise comparisons are provided for all variables with significant group differences.

Table 5.2 – Association Between ROS 1.0 Demographic Variables and LR11 Expression*

	Frontal Cortex[a]
Age at death, years	$F_{(1,32)}$ = 3.30 (p = 0.079)
Number of males	$F_{(1,32)}$ = 0.00 (p = 0.95)
Years of education	**$F_{(1,32)}$ = 4.29 (p = 0.046)**
Post-mortem interval, hours	$F_{(1,32)}$ = 2.73 (p = 0.11)

[a]By mixed models analysis with random intercept, fixed covariate, Kenward-Roger denominator degrees of freedom, unstructured covariance structure, and square-root transformed LR11 values.

*Association data are presented as F-statistic (p – value). Significant associations with LR11 expression are in bold.

As in the ROS 1.0 cohort, there were no significant differences in the number of males ($p = 0.60$), years of education ($p = 0.99$) or PMI ($p = 0.49$) between the NCI, MCI and AD diagnostic groups in the ROS 2.0 cohort. The mean age at death of the NCI group was slightly younger than the mean age at death of the AD group ($p = 0.031$, Table 5.3). LR11 expression in the precuneus was weakly correlated with age at death ($p = 0.038$, Table 5.4). However, there was no correlation between LR11 expression in either the frontal cortex ($p = 0.056$) or the visual cortex ($p = 0.084$) in these same cases. Likewise, there was no difference in LR11 expression between males and females (p-values ranging from 0.32 – 0.72) and no correlation between PMI and LR11 expression (p-values raning from 0.38 – 0.83) in any of the brain regions examined. Finally, in contrast to the ROS 1.0 cohort, there was no correlation between the number of years of education and LR11 expression in any of the brain regions examined in the ROS 2.0 cohort (p-values ranging from 0.54 – 0.79).

Genetic Variables – apoE Genotype

ApoE genotype is the primary genetic susceptibility factor for late onset AD (Buerger et al 2005; Herukka et al 2007), with carriers of the ε4 allele having a significantly increased likelihood of developing AD in their lifetimes compared to non-ε4 carriers (National Institute on Aging/Alzheimer's Association Working Group & Relkin 1996). While the association between apoE and AD is well known, the direct influence of apoE on the pathogenic mechanisms of AD is still not fully

Table 5.3 – ROS 2.0 Demographic Variable Comparison Across Groups*

	NCI (N=14)	**MCI (N=15)**	**AD (N=14)**	**Total (N=43)**	**Comparison by group**	**Pair-wise Comparisons**
Age at death, years	84.6 ± 4.5 (78 – 93)	86.2 ± 4.4 (79 – 94)	89.0 ± 4.8 (76 – 95)	86.6 ± 4.8 (76 – 95)	p = 0.031[a]	NCI < AD
Number (%) of males	5 (36%)	7 (47%)	4 (29%)	16 (37%)	p = 0.60[b]	
Years of education	17.6 ± 4.0 (10 – 25)	17.8 ± 3.6 (10 – 25)	18.2 ± 3.4 (14 – 26)	17.9 ± 3.6 (10 – 26)	p = 0.99[a]	
PMI, hours	5.4 ± 2.4 (1.0 – 9.8)	6.2 ± 2.6 (2.0 – 11.5)	4.9 ± 2.0 (1.5 – 8.2)	5.5 ± 2.4 (1.0 – 11.5)	p = 0.49[a]	

[a]Kruskal-Wallis test

[b]Chi-square test

*Unless otherwise noted, data are presented as Mean ± SD (range). Pair-wise comparisons are given for all variables with significant group differences.

Table 5.4 – Association Between ROS 2.0 Demographic Variables and LR11 Expression*

	Frontal Cortex	**Precuneus**	**Visual Cortex**
Age at death, years[a]	r = 0.72 (p = 0.056)	**r = 0.32 (p = 0.038)**	r = 0.084 (p = 0.59)
Number of males[b]	p = 0.72	p = 0.32	p = 0.45
Years of education[a]	r = -0.077 (p = 0.62)	r = -0.096 (p = 0.54)	r = 0.042 (p = 0.79)
Post-mortem interval, hours[a]	r = 0.14 (p = 0.38)	r = -0.034 (p = 0.83)	r = 0.038 (p = 0.81)

[a]By Spearman correlation

[b]By t-test

*Association data are presented as Spearman r (p – value) or just the p-value as appropriate. Significant associations with LR11 are in bold.

understood. The apoE protein is known to interact with the majority of low density lipoprotein receptor (LDLR) family members (Fagan et al 1996; Fagan et al 2002; Hoe & Rebeck 2005; Ljungberg et al 2003), including LR11 (Taira et al 2001), with the ε4 isoform having increased receptor binding affinity compared to the ε2 and ε3 isoforms (Contois et al 1996; Rall & Mahley 1992). Given these intriguing observations, we were very interested to look at the association between apoE genotype and LR11 expression in both of our cohorts.

We first looked at the distribution of apoE ε4 carriers across diagnostic groups. In the ROS 1.0 cohort, there were no ε4 carriers in the NCI group. There were five ε4 carriers in both the MCI and AD groups for a total of ten ε4 carriers in the population (Table 5.5A). There was no significant difference in the distribution of ε4 carriers across groups (Fisher's exact test, $p = 0.086$). There was no significant association between the presence of an apoE ε4 allele and frontal cortex LR11 expression in the ROS 1.0 cohort ($p = 0.45$, Table 5.6A).

In the ROS 2.0 cohort, there was one ε4 carrier in the NCI group and six ε4 carriers in both the MCI and AD diagnostic groups, for 13 total ε4 carriers in the population (Table 5.5B). There was no significant difference in the distribution of ε4 carriers across groups (chi square test, $p = 0.072$). There was no significant difference in LR11 expression in the frontal cortex ($p = 0.87$), precuneus (0.33) or visual cortex ($p = 0.81$) between ε4 carriers and non-carriers in the ROS 2.0 cohort (Table 5.6B).

Table 5.5 – ROS 1.0 and ROS 2.0 ApoE Genotype Distribution Across Groups

A. ROS 1.0 Cohort

	NCI (N=9)	**MCI (N=15)**	**AD (N=10)**	**Total (N=34)**	**Comparison by group[a]**
Subjects with *APOE* ε4 allele (%)	0 (0%)	5 (33%)	5 (50%)	10 (29%)	p = 0.086

[a]Fisher's Exact test

B. ROS 2.0 Cohort

	NCI (N=14)	**MCI (N=15)**	**AD (N=14)**	**Total (N=43)**	**Comparison by group[a]**
Subjects with *APOE* ε4 allele (%)	1 (7%)	6 (40%)	6 (43%)	13 (30%)	p = 0.072

[a]Chi-square test

Table 5.6 – Association Between Presence of *APOE* ε4 allele and LR11 Expression*

A. ROS 1.0 Cohort

	Frontal Cortex[a]
Presence of *APOE* ε4 allele	$F_{(1,32)} = 0.59$ ($p = 0.45$)

[a]By mixed models analysis with random intercept, fixed covariate, Kenward-Roger denominator degrees of freedom, unstructured covariance structure, and square-root transformed LR11 values.

B. ROS 2.0 Cohort

	Frontal Cortex[a]	**Precuneus[a]**	**Visual Cortex[a]**
Presence of *APOE* ε4 allele	$p = 0.87$	$p = 0.33$	$p = 0.81$

[a]By t-test

*Association data for ROS 1.0 are presented as F-statistic (p – value). Association data for ROS 2.0 is presented as just the p-value.

Cognitive Variables

For the ROS 1.0 cases, two measures of global cognitive ability were examined: the global cognitive score (GCS) and the Mini-Mental State Examination (MMSE). The GCS is a composite z-score compiled from 19 neuropsychological tests of cognition (Wilson et al 2002). A GCS of +1 (or -1) indicates cognitive function that is 1 standard deviation above (or below) the average of the reference population (Ikonomovic et al 2005). The MMSE is a short clinical test that was developed in 1975 and is still broadly used today to quickly assess the cognitive aspects of mental function (Folstein et al 1975). As expected, there were significant group differences in both GCS and MMSE scores in the ROS 1.0 population ($p < 0.0001$ for both comparisons). Post-test pairwise comparisons revealed that the NCI and MCI cases had higher global cognitive scores ($p < 0.001$) and performed better on the MMSE than the AD cases ($p < 0.01$). The NCI cases also had slightly higher global cognitive scores than the MCI cases ($p < 0.05$), although there was no significant difference in mean MMSE score between the two groups (Table 5.7). A mixed models repeated measures analysis found that frontal cortex LR11 expression was significantly correlated with cognitive ability as measured by GCS ($p = 0.0020$, Table 5.8), with the cases with the highest LR11 expression performing best on cognitive tests while those with the lowest levels of LR11 expression were the most impaired, regardless of clinical diagnosis (Figure 5.1). In contrast, even though there was a strong correlation between GCS and MMSE in the ROS 1.0 cases ($p < 0.0001$), there was no significant correlation between MMSE and frontal cortex LR11 expression ($p = 0.055$, Table 5.8). As noted above, there was no significant difference in mean MMSE

Table 5.7 – ROS 1.0 Cognitive Variable Comparison Across Groups*

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group[a]	Pair-wise Comparisons
Global Cognitive Score (GCS)	0.1 ± 0.2 (-0.4 - 0.3)	-0.5 ± 0.3 (-1.0 – 0.1)	-1.8 ± 0.6 (-3.0 - -1.1)	-0.7 ± 0.8 (-3.0 – 0.3)	$p < 0.0001$	NCI > MCI > AD
MMSE	27.9 ± 1.8 (25 - 30)	26.4 ± 1.9 (22 – 29)	20.0 ± 6.0 (7 - 25)	24.9 ± 4.8 (7 - 30)	$p < 0.0001$	NCI, MCI > AD

[a]Kruskal-Wallis test

*Data are presented as Mean ± SD (range).

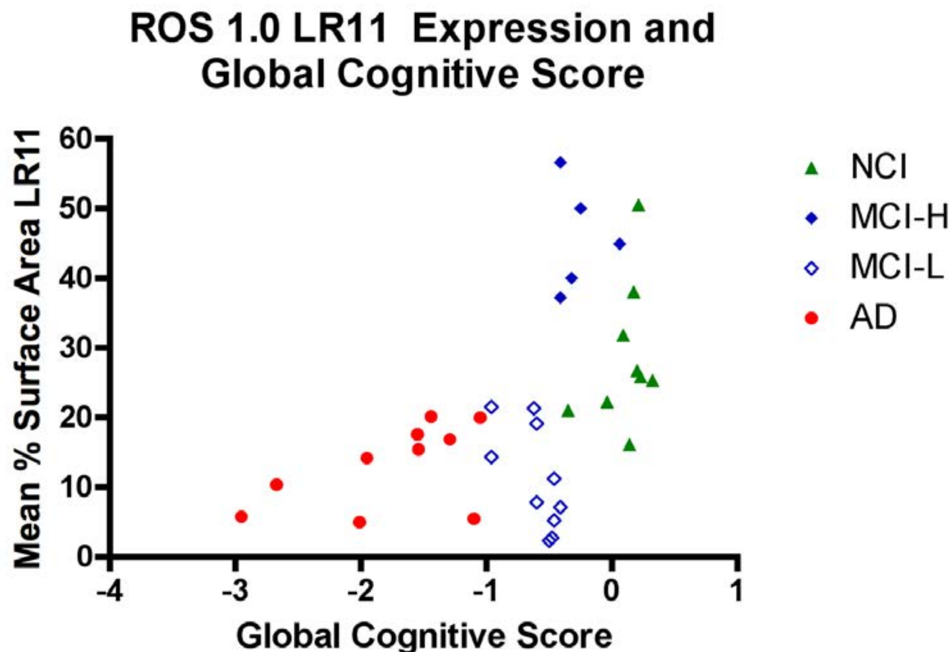
Table 5.8 – Association Between ROS 1.0 Cognitive Variables and LR11 Expression*

	Frontal Cortex[a]
Global Cognitive Score (GCS)	**$F_{(1,29)} = 11.48$ ($p = 0.0020$)**
MMSE	$F_{(1,31)} = 3.97$ ($p = 0.055$)

[a]By mixed models analysis with random intercept, fixed covariate, Kenward-Roger denominator degrees of freedom, unstructured covariance structure, and square-root transformed LR11 values.

*Association data are presented as F-statistic (p – value). Positive associations with LR11 are in bold.

Figure 5.1 – Frontal cortex LR11 expression in ROS 1.0 is related to cognitive performance as measured by GCS.



LR11 expression was found to be strongly correlated with global cognitive score across all cases by repeated-measures analysis ($F_{(1,29)}$ = 11.48, p = 0.0020). Diamonds designate AD cases; filled squares designate MCI cases with low LR11 expression; open squares designate MCI with high LR11 expression; triangles designate NCI cases.

score between the NCI and MCI groups. Moreover, the range of MMSE scores across all cases was quite narrow, with 24 of the 34 cases having MMSE scores of 25 or greater. Therefore, it is likely that the degree of difference in cognitive impairment, especially at the higher end of cognitive performance, may be too minor to be detected on the less sensitive MMSE, leading to a lack of correlation with LR11 expression.

As in the ROS 1.0 cohort, there were also significant differences in GCS and MMSE scores between groups in the ROS 2.0 cohort ($p < 0.0001$ for both comparisons). Post-test pair-wise comparisons revealed that the NCI and MCI cases had higher global cognitive scores ($p < 0.01$) and performed better on the MMSE than the AD cases ($p < 0.001$). There was no difference between the NCI and MCI groups in either GCS or MMSE (Table 5.9A). Group differences in z-scores for five separate cognitive domains were also examined in this cohort. Significant group differences were found in episodic memory z-score ($p < 0.0001$, composite of seven test scores), perceptual speed z-score ($p = 0.0004$, composite of two test scores) and visuospatial ability z-score ($p = 0.0080$, composite of two test scores). Weakly significant group differences were found in semantic memory z-scores ($p = 0.024$, composite of four test scores) and working memory z-scores ($p = 0.017$, composite of four test scores) (Table 5.9B). Post-test pairwise comparisons found significant differences between the NCI and AD groups in all five z-scores (p values ranging from $p < 0.001$ for perceptual speed to $p < 0.05$ for semantic and working memory). Significant differences were also found between the MCI and AD groups in episodic

Table 5.9 – ROS 2.0 Cognitive Variable Comparison Across Groups*

A. Global Cognition Scores

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[a]	Pair-wise Comparisons
Global Cognitive Score (GCS)	0.5 ± 0.2 (0.3 – 0.8)	0.2 ± 0.3 (-0.5 – 0.9)	-0.6 ± 0.5 (-1.4 – 0.6)	0.03 ± 0.6 (-1.4 – 0.9)	p < 0.0001	NCI, MCI > AD
MMSE	28.1 ± 1.5 (26 - 30)	27.1 ± 2.6 (22 - 30)	18.8 ± 5.8 (10 - 28)	24.7 ± 5.6 (10 - 30)	p < 0.0001	NCI, MCI > AD

[a]Kruskal-Wallis test

B. Individual Cognitive Domain Z-Scores

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[a]	Pair-wise Comparisons
Episodic Memory z-score	0.8 ± 0.3 (0.3 – 1.4)	0.4 ± 0.5 (-0.3 – 1.7)	-0.9 ± 0.7 (-2.0 – 0.7)	0.1 ± 0.9 (-2.0 – 1.7)	p < 0.0001	NCI, MCI > AD
Semantic Memory z-score	0.3 ± 0.6 (-1.0 – 1.1)	0.1 ± 0.6 (-1.3 – 1.0)	-0.3 ± 0.6 (-1.6 – 0.6)	0.05 ± 0.6 (-1.6 – 1.1)	p = 0.024	NCI > AD
Working Memory z-score	0.3 ± 0.4 (-0.3 – 0.9)	0.2 ± 0.5 (-0.7 – 0.9)	-0.3 ± 0.6 (-1.4 – 1.1)	0.08 ± 0.5 (-1.4 – 1.1)	p = 0.017	NCI > AD
Perceptual Speed z-score	0.3 ± 0.5 (-0.5 – 1.1)	-0.05 ± 0.6 (-1.2 – 0.8)	-0.9 ± 0.6 (-1.7 – 0.4)	-0.2 ± 0.8 (-1.7 – 1.1)	p = 0.0004	NCI, MCI > AD
Visuo-spatial Ability z-score	0.2 ± 0.4 (-0.6 – 0.8)	-0.2 ± 0.7 (-1.2 – 1.0)	-0.6 ± 0.7 (-2.0 – 0.8)	-0.2 ± 0.7 (-2.0 – 1.0)	p = 0.0080	NCI > AD

[a]Kruskal-Wallis test

*Data are presented as Mean ± SD (range).

memory ($p < 0.01$) and perceptual speed ($p < 0.05$). There were no differences between the NCI and MCI groups for any cognitive domain z-scores.

In light of the findings in the ROS 1.0 cohort, we expected to find a strong positive correlation between LR11 expression and global cognitive ability. Moreover, because individuals with episodic memory complaints often progress to AD at a greater rate (Bäckman 2008; Sperling et al 2010), we hypothesized that in this larger cohort we would see the strongest associations between LR11 expression and episodic memory impairment. However, no association was found between GCS and LR11 expression in the frontal cortex ($p = 0.60$), precuneus ($p = 0.98$) or primary visual cortex ($p = 0.75$). Likewise, there was no correlation between MMSE score and LR11 expression in the same brain regions (See Table 5.10A for correlation coefficients and p-values). We also looked at the correlation between LR11 expression in each of the three brain regions and the z-scores for each cognitive domain and found no significant associations (Table 5.10B), including with episodic memory impairment. The correlation between LR11 expression in each brain region and scores on each of 21 individual cognitive tests were also examined. Only one test (Complex Ideation Material Test) showed a weakly significant correlation with frontal cortex LR11 expression ($p = 0.023$, Table 5.11). There were no significant correlations with LR11 expression in the precuneus or the visual cortex and any individual cognitive scores (data not shown).

Finally, because individuals with the amnestic subtype of MCI are known to progress to AD at a greater rate than individuals with non-amnestic MCI

Table 5.10 – Association Between ROS 2.0 Cognitive Variables and LR11 Expression*

A. Global Cognition Scores

	Frontal Cortex[a]	**Precuneus**[a]	**Visual Cortex**[a]
Global Cognitive Score (GCS)	r = 0.083 (p = 0.60)	r = 0.0030 (p = 0.98)	r = 0.050 (p = 0.75)
MMSE	r = -0.0025 (p = 0.99)	r = 0.10 (p = 0.52)	r = 0.10 (p = 0.50)

[a]By Spearman correlation

B. Individual Cognitive Domain Z-Scores

	Frontal Cortex[a]	**Precuneus**[a]	**Visual Cortex**[a]
Episodic Memory z-score	r = 0.063 (p = 0.69)	r = 0.047 (p = 0.77)	r = 0.033 (p = 0.83)
Semantic Memory z-score	r = -0.0009 (p = 0.99)	r = -0.14 (p = 0.39)	r = -0.020 (p = 0.90)
Working Memory z-score	r = 0.23 (p = 0.13)	r = 0.18 (p = 0.25)	r = 0.23 (p = 0.14)
Perceptual Speed z-score	r = 0.011 (p = 0.94)	r = -0.095 (p = 0.55)	r = -0.070 (p = 0.66)
Visuospatial Ability z-score	r = 0.059 (p = 0.71)	r = 0.0040 (p = 0.80)	r = -0.0005 (p = 0.99)

[a]By Spearman correlation

*Association data are presented as Spearman r (p – value).

Table 5.11 – Positive Associations Between ROS 2.0 Cognitive Test Scores and LR11 Expression*

	Frontal Cortex[a]	**Precuneus[a]**	**Visual Cortex[a]**
Complex Ideation Material Test Score	**r = 0.35 (p = 0.023)**	r = 0.074 (p = 0.63)	r = 0.18 (p = 0.25)

[a]By Spearman correlation

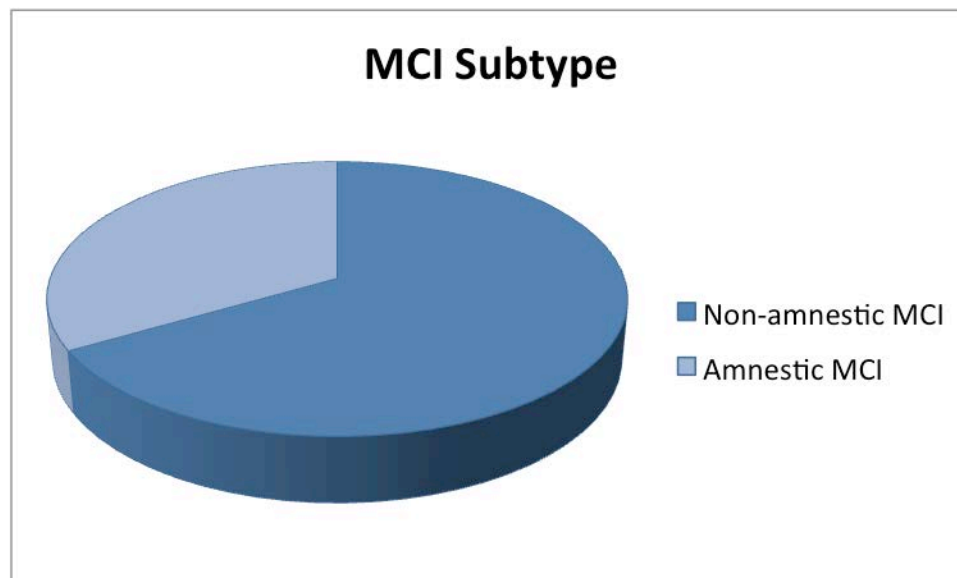
*Association data are presented as Spearman r (p – value). Positive associations with LR11 expression are shown in bold.

(Markesbery et al 2006; Petersen et al 2006; Wolk et al 2009), we questioned if low LR11 expression was more prevalent in individuals with amnestic MCI (aMCI) compared to those with non-aMCI. Within the ROS 2.0 MCI diagnostic group, we had five individuals that were classified as having aMCI and 10 individuals that had non-aMCI (Figure 5.2). Notably, there were only two cases in the MCI diagnostic group with low LR11 expression. One of these cases was aMCI and one was non-aMCI. As a result, there was no difference in mean LR11 expression in the frontal cortex, precuneus or primary visual cortex between aMCI and non-aMCI individuals (Table 5.12).

Pathological Variables

The two hallmark lesions of AD are NFTs, which result from the hyperphosphorylation of tau and amyloid plaques, which are composed of aggregated Aβ peptides. Amyloid plaques can take two forms: neuritic plaques, which are associated with dystrophic neurites and diffuse plaques, which are not. The frequency of each of these lesions was assessed in the frontal cortex, the superior temporal cortex and the inferior parietal cortex for each case in the ROS 1.0 cohort. Semi-quantitative scores for the frequency of each lesion were assigned on a five point scale, with a score of 0 denoting no lesions in a given brain region and a score of 5 denoting frequent lesions (20+) in that brain region. Because the ROS 1.0 cohort specifically excluded any NCI cases with amyloid pathology and any AD cases lacking amyloid pathology, there were significant group differences in both neuritic and diffuse plaque frequencies for all three brain regions examined ($p \leq 0.0007$ for

Figure 5.2 – Distribution of MCI subtypes in the ROS 2.0 cohort.



ROS 2.0 MCI cases were dichotomized into amnestic and non-amnestic subgroups based on specific memory impairment. Five cases were classified as amnestic MCI (aMCI) and ten cases were classified as non-amnestic MCI (non-aMCI).

Table 5.12 – Association Between ROS 2.0 MCI Subtype and LR11 Expression

	Frontal Cortex[a]	**Precuneus[a]**	**Visual Cortex[a]**
MCI Subtype (amnestic v. non-amnestic)	$p = 0.57$	$p = 0.96$	$p = 0.59$

[a]By t-test

all comparisons, Tables 5.13A - C). There were also significant differences in NFT frequency between diagnostic groups for all three brain regions, but these differences were weaker than for either plaque measure (p values ranging from 0.0010 to 0.034). Notably, post-test pairwise comparisons revealed no significant difference in neuritic plaque, diffuse plaque or NFT frequency between the MCI and AD groups in any of the brain regions examined, suggesting that while the NCI cases in the ROS 1.0 cohort were pathologically clear, the cases in the MCI group are predominantly pathologically AD-like.

Three measures of global AD pathology were also examined: Braak score, which is determined based on the extent and topographical distribution of NFTs (Braak & Braak 1991); NIA Reagan diagnosis, which incorporates both amyloid plaque burden and Braak score as a means of determining the likelihood that the degree of cognitive impairment observed is due to the underlying AD pathology in the brain (1997); and CERAD diagnosis, which is determined based on the extent of amyloid plaques in the brain relative to the age of the individual and serves as an assessment as to whether the patient had AD at the time of death (Mirra et al 1991; Morris et al 1989). Again, as expected due to the established exclusion criteria for the selection of the ROS 1.0 cases, there were significant group differences for all three of these measures of global pathology ($p < 0.0001$ for all three sets of variables, Table 5.14). Post-test pairwise comparisons between groups revealed that for all three measures, the NCI group was significantly different than both the AD group ($p < 0.001$, all three variables) and the MCI group ($p < 0.01$, all three

Table 5.13 - ROS 1.0 Frequency of AD Pathological Lesions Comparison Across Groups*

A. Frontal Cortex Lesions

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group[b]	Pairwise Comparisons
Neuritic Plaque Frequency[a]	0	2.4 ± 1.8 (0 – 5)	3.7 ± 1.2 (2 – 5)	2.1 ± 1.9 (0 – 5)	p = 0.0002	NCI < MCI, AD
Diffuse Plaque Frequency[a]	0	3.0 ± 2.2 (0 – 5)	3.9 ± 1.4 (1 – 5)	2.5 ± 2.2 (0 – 5)	p = 0.0003	NCI < MCI, AD
NFT Frequency[a]	0.2 ± 0.4 (0 – 1)	0.6 ± 0.9 (0 – 3)	1.1 ± 0.6 (0 – 2)	0.6 ± 0.8 (0 – 3)	p = 0.013	NCI < AD

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

B. Superior Temporal Cortex Lesions

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group[b]	Pairwise Comparisons
Neuritic Plaque Frequency[a]	0	2.5 ± 1.6 (0 – 5)	3.8 ± 1.1 (2 – 5)	2.2 ± 1.9 (0 – 5)	p < 0.0001	NCI < MCI, AD
Diffuse Plaque Frequency[a]	0	3.0 ± 2.1 (0 – 5)	4.2 ± 1.0 (3 – 5)	2.6 ± 2.2 (0 – 5)	p < 0.0001	NCI < MCI, AD
NFT Frequency[a]	0.1 ± 0.3 (0 – 1)	1.4 ± 1.4 (0 – 5)	2.6 ± 1.8 (0 – 5)	1.4 ± 1.6 (0 – 5)	p = 0.0010	NCI < MCI, AD

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

C. Inferior Parietal Cortex Lesions

	NCI (N=9)	**MCI (N=15)**	**AD (N=10)**	**Total (N=34)**	**Comparison by group[b]**	**Pairwise Comparisons**
Neuritic Plaque Frequency[a]	0	2.4 ± 2.0 (0 – 5)	3.7 ± 1.3 (1 – 5)	2.1 ± 2.0 (0 – 5)	p = 0.0003	NCI < MCI, AD
Diffuse Plaque Frequency[a]	0	2.8 ± 2.2 (0 – 5)	3.7 ± 1.6 (0 – 5)	3.7 ± 1.6 (0 – 5)	p = 0.0007	NCI < MCI, AD
NFT Frequency[a]	0.1 ± 0.3 (0 – 1)	0.8 ± 1.3 (0 – 4)	1.3 ± 1.2 (0 – 3)	0.8 ± 1.1 (0 – 4)	p = 0.034	NCI < AD

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

*Data are presented as Mean ± SD (range).

Table 5.14 – ROS 1.0 Global Pathological Variables Comparison Across Groups*

	NCI (N=9)	MCI (N=15)	AD (N=10)	Total (N=34)	Comparison by group[c]	Pairwise Comparisons
Braak Score	1.6 ± 1.1 (0 – 4)	3.8 ± 1.0 (1 – 5)	4.6 ± 0.5 (4 – 5)	3.5 ± 1.5 (0 - 5)	p < 0.0001	NCI < MCI, AD
NIA Reagan Diagnosis[a]	3.1 ± 0.3 (3 – 4)	2.2 ± 0.6 (1 - 3)	1.6 ± 0.5 (1 - 2)	2.3 ± 0.8 (1 - 4)	p < 0.0001	NCI > MCI, AD
CERAD Diagnosis[b]	4.0 ± 0.0 (4)	2.1 ± 1.0 (1 - 4)	1.6 ± 0.5 (1 - 2)	2.5 ± 1.2 (1 - 4)	p < 0.0001	NCI > MCI,AD

[a]NIA Reagan Diagnosis was reported on the following scale: 1=High likelihood, 2=Intermediate likelihood, 3=Low likelihood, 4=No AD

[b]CERAD Diagnosis was reported on the following scale: 1=Definite AD, 2=Probable AD, 3=Possible AD, 4=No AD

[c]Kruskal-Wallis test

*Data are presented as Mean ± SD (range).

variables). As with the specific lesion frequencies, the MCI and AD groups did not differ in any measure of global AD pathology.

Given the proposed mechanistic role for LR11 in regulating APP processing, we hypothesized that frontal cortex LR11 expression would be closely associated with the frequencies of both neuritic plaques and diffuse plaques, also in the frontal cortex. We also hypothesized that because CERAD diagnosis is predominantly driven by the extent of amyloid pathology in the brain, it would also be significantly associated with LR11 expression. However, there was no correlation between LR11 expression in the frontal cortex and the frequency of neuritic plaques ($p = 0.84$), diffuse plaques ($p = 0.73$) or NFTs ($p = 0.81$), also in the frontal cortex (Table 5.15A). Likewise, no correlation was seen between LR11 expression and any measure of global AD pathology (p values ranging from 0.092 – 0.18, Table 5.15B), including CERAD diagnosis.

Unlike in the ROS 1.0 cohort, there were no exclusion criteria based on pathology for the selection of the ROS 2.0 cohort. As a result, there were extensive neuritic and diffuse plaques present in the NCI cases in the ROS 2.0 cohort in all five brain regions for which data was available (frontal cortex, hippocampus CA1, entorhinal cortex, superior temporal cortex and inferior parietal cortex), a striking difference from the complete absence of such pathology in the ROS 1.0 NCI cases. As a result, there were no significant group differences in neuritic plaque, diffuse plaque or NFT frequency in the hippocampus CA1, entorhinal cortex, superior temporal cortex or inferior parietal cortex (See Tables 5.16 B – E for p-values) in the

Table 5.15 – Association Between ROS 1.0 Pathological Variables and LR11 Expression*

A. Frequency of AD Pathological Lesions in Frontal Cortex

	Frontal Cortex[a]
Neuritic Plaque Frequency	$F_{(1,32)} = 0.04$ ($p = 0.84$)
Diffuse Plaque Frequency	$F_{(1,32)} = 0.12$ ($p = 0.73$)
NFT Frequency	$F_{(1,31)} = 0.06$ ($p = 0.81$)

[a]By mixed models analysis with random intercept, fixed covariate, Kenward-Roger denominator degrees of freedom, unstructured covariance structure, and square-root transformed LR11 values.

B. Global Pathology Scores

	Frontal Cortex[a]
Braak Score	$F_{(1,32)} = 2.03$ ($p = 0.16$)
NIA Reagan Diagnosis	$F_{(1,32)} = 3.02$ ($p = 0.092$)
CERAD Diagnosis	$F_{(1,31)} = 1.92$ ($p = 0.18$)

[a]By mixed models analysis with random intercept, fixed covariate, Kenward-Roger denominator degrees of freedom, unstructured covariance structure, and square-root transformed LR11 values.

*Association data are presented as F-statistic (p – value).

Table 5.16 - ROS 2.0 Frequency of AD Pathological Lesions Comparison Across Groups*

A. Frontal Cortex Lesions

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[b]	Pairwise Comparisons
Neuritic Plaque Frequency[a]	2.1 ± 1.4 (0 – 4)	2.1 ± 2.0 (0 – 5)	3.4 ± 1.4 (1 – 5)	2.6 ± 1.7 (0 – 5)	p = 0.098	
Diffuse Plaque Frequency[a]	2.8 ± 2.2 (0 – 5)	2.4 ± 2.2 (0 – 5)	4.6 ± 0.9 (2 – 5)	3.2 ± 2.1 (0 – 5)	p = 0.012	MCI < AD
NFT Frequency[a]	0 (0)	0.3 ± 0.6 (0 – 2)	0.7 ± 0.9 (0 – 3)	0.3 ± 0.7 (0 – 3)	p = 0.0084	NCI < AD

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

B. Hippocampus CA1 Lesions

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[b]
Neuritic Plaque Frequency[a]	1.4 ± 1.4 (0 – 4)	2.1 ± 1.3 (0 – 4)	1.4 ± 2.0 (0 – 5)	1.6 ± 1.6 (0 – 5)	p = 0.31
Diffuse Plaque Frequency[a]	0.7 ± 1.4 (0 – 5)	1.1 ± 1.6 (0 – 5)	0.7 ± 1.3 (0 – 4)	0.8 ± 1.4 (0 – 5)	p = 0.60
NFT Frequency[a]	3.4 ± 1.8 (0 - 5)	3.6 ± 1.8 (0 – 5)	3.4 ± 1.8 (0 – 5)	3.5 ± 1.8 (0 – 5)	p = 0.89

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

C. Entorhinal Cortex Lesions

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[b]
Neuritic Plaque Frequency[a]	2.4 ± 1.9 (0 – 5)	2.5 ± 1.6 (0 – 5)	2.4 ± 1.8 (0 – 5)	2.4 ± 1.7 (0 – 5)	p = 0.96
Diffuse Plaque Frequency[a]	2.8 ± 1.9 (0 – 5)	3.0 ± 1.6 (0 – 5)	2.1 ± 2.0 (0 – 5)	2.6 ± 1.8 (0 – 5)	p = 0.46
NFT Frequency[a]	3.6 ± 1.6 (1 - 5)	4.1 ± 1.1 (2 – 5)	4.2 ± 1.2 (2 – 5)	4.0 ± 1.3 (1 – 5)	p = 0.55

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

D. Superior Temporal Cortex Lesions

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[b]
Neuritic Plaque Frequency[a]	2.1 ± 1.8 (0 – 5)	3.2 ± 1.5 (0 – 5)	2.7 ± 1.7 (0 – 5)	2.7 ± 1.7 (0 – 5)	p = 0.22
Diffuse Plaque Frequency[a]	3.5 ± 2.1 (0 – 5)	3.6 ± 1.7 (0 – 5)	3.3 ± 2.2 (0 – 5)	3.5 ± 2.0 (0 – 5)	p = 0.96
NFT Frequency[a]	0.6 ± 1.2 (0 - 4)	1.7 ± 1.8 (0 – 5)	1.2 ± 1.4 (0 – 4)	1.2 ± 1.5 (0 – 5)	p = 0.11

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

E. Inferior Parietal Cortex Lesions

	NCI (N=14)	MCI (N=15)	AD (N=14)	Total (N=43)	Comparison by group[b]
Neuritic Plaque Frequency[a]	2.6 ± 1.6 (0 – 4)	3.2 ± 1.5 (0 – 5)	2.9 ± 1.5 (0 – 5)	2.9 ± 1.5 (0 – 5)	p = 0.63
Diffuse Plaque Frequency[a]	2.6 ± 2.1 (0 – 5)	3.0 ± 1.8 (0 – 5)	3.3 ± 1.9 (0 – 5)	3.0 ± 1.9 (0 – 5)	p = 0.62
NFT Frequency[a]	0.1 ± 0.4 (0 - 1)	0.7 ± 1.0 (0 – 3)	0.5 ± 0.8 (0 – 2)	0.4 ± 0.8 (0 – 3)	p = 0.22

[a]Lesion Frequency was reported on the following scale: 0 = none, 1=sparse (1-2), 2=sparse to moderate (3-5), 3=moderate (6-12), 4=moderate to frequent (13-19), 5-frequent (20+)

[b]Kruskal-Wallis test

*All data are presented as Mean ± SD (range). Pair-wise comparisons are provided for all variables with significant group differences.

ROS 2.0 cohort. Moreover, there was no significant difference between diagnostic groups in neuritic plaque frequency in the frontal cortex either ($p = 0.098$, Table 5.16A). Only the difference in NFT frequency in the frontal cortex between the three diagnostic groups reached statistical significance ($p = 0.0084$), with a significantly higher frequency of NFTs in the AD cases compared to NCI ($p < 0.01$, post test pairwise comparison). However, it is worth noting that this difference is likely attributable to the complete absence of NFTs in all of the NCI cases in this cohort and that even in the AD cases, the frequency of NFTs was relatively low, with a mean frequency score of less than one on a 5-point scale. Finally, there was a weakly significant difference in diffuse plaque frequency in the frontal cortex as well ($p = 0.012$), with the MCI cases having slightly higher frequency of diffuse plaques than the AD cases. There was no significant difference in diffuse plaque frequency between NCI and MCI or NCI and AD.

As in the ROS 1.0 cases, we also examined a series of measures of global AD pathology in the ROS 2.0 cases as well. While the mean Braak score, NIA Reagan diagnosis and CERAD diagnosis for the MCI and AD groups were highly similar between the two cohorts, there were notable differences in the NCI groups, with the ROS 2.0 NCI group more closely resembling the MCI and AD groups in all three measures. In fact, while there were highly significant group differences for all three global AD pathology measures in ROS 1.0, there were only weakly significant group differences for these same measures in ROS 2.0 (p values ranging from 0.012 – 0.020, Table 5.17). Pairwise comparisons revealed while the NCI group did differ from the AD group for all three measures ($p < 0.05$, all measures), the MCI group

Table 5.17 - ROS 2.0 Global Pathological Variables Comparison Across Groups*

	NCI (N=14)	**MCI (N=15)**	**AD (N=14)**	**Total (N=43)**	**Comparison by group[c]**	**Pairwise Comparisons**
Brain Weight (g)	1161 ± 99.0 (1000 – 1320)	1178 ± 167.6 (890 – 1480)	1167 ± 118.4 (1006 – 1460)	1169 ± 129.5 (890 – 1480)	$p = 0.99$	
Braak Score	2.8 ± 1.3 (1 – 5)	3.4 ± 1.2 (1 – 5)	4.1 ± 1.1 (1 – 5)	3.4 ± 1.3 (1 – 5)	$p = 0.020$	NCI < AD
NIA Reagan Diagnosis[a]	2.6 ± 0.5 (2 – 3)	2.3 ± 0.8 (1 – 3)	1.8 ± 0.6 (1 – 3)	2.2 ± 0.7 (1 – 3)	$p = 0.012$	NCI > AD
CERAD Diagnosis[b]	2.6 ± 0.9 (1 – 4)	2.5 ± 1.2 (1 – 4)	1.6 ± 0.5 (1 – 2)	2.2 ± 1.0 (1 – 4)	$p = 0.018$	NCI > AD

[a]NIA Reagan Diagnosis was reported on the following scale: 1=High likelihood, 2=Intermediate likelihood, 3=Low likelihood, 4=No AD

[b]CERAD Diagnosis was reported on the following scale: 1=Definite AD, 2=Probable AD, 3=Possible AD, 4=No AD

[c]Kruskal-Wallis test

*Data are presented as Mean ± SD (range). Pair-wise comparisons are provided for all variables with significant group differences.

was not significantly different from either the NCI or the AD groups for any global pathology measure. Finally, there was no significant group difference in brain weight between the three diagnostic groups (p = 0.99) which is used here as a surrogate measure for neuronal atrophy. Together, these data suggest that many of our NCI and MCI cases harbor significant levels of AD-related pathology and may actually represent prodromal AD cases.

Given the AD-like levels of plaques and tangles and the similar variability in LR11 expression across all three diagnostic groups in the ROS 2.0 cohort, we were particularly interested in examining whether LR1 expression in the frontal cortex was related to the frequency of specific lesions in the same brain region. However, no correlation was found between LR11 expression in the frontal cortex and the frequency of neuritic plaques (p = 0.98), diffuse plaques (p = 0.33) or NFTs (p = 0.10) in the same brain region (Table 5.18). We also looked at the correlations between LR11 expression in the precuneus or in the primary visual cortex and the frequency of each lesion in the frontal cortex. There was a weakly significant negative correlation between NFT frequency in the frontal cortex and LR11 expression in the primary visual cortex (Spearman r = -0.32, p = 0.036). However, given the weakness of this association, the topographical differences in the brain locations of these specific measures and the large number of correlational analyses being run, it is likely that this association is spurious. No other significant correlations were observed between LR11 expression in either the precuneus or primary visual cortex and lesions in the frontal cortex. Because of the topographical proximity, we also chose to specifically look at the association between LR11

Table 5.18 – Association Between ROS 2.0 AD Pathological Lesions in Frontal Cortex and LR11 Expression*

	Frontal Cortex[a]	**Precuneus[a]**	**Visual Cortex[a]**
Neuritic Plaque Frequency	r = -0.0033 (p = 0.98)	r = -0.097 (p = 0.53)	r = -0.074 (p = 0.64)
Diffuse Plaque Frequency	r = -0.15 (p = 0.33)	r = -0.015 (p = 0.93)	r = -0.12 (p = 0.44)
NFT Frequency	r = -0.25 (p = 0.10)	r = -0.27 (p = 0.08)	**r = -0.32 (p = 0.036)**

[a]By Spearman correlation

*Association data are presented as Spearman r (p – value). Significant associations with frontal cortex LR11 expression are shown in bold.

expression in the precuneus and the frequency of AD lesions in the superior temporal and inferior parietal cortices. No association with LR11 expression was found between any of these measures (Table 5.19). Finally, no significant relationship was seen between LR11 expression in any of the three brain regions and brain weight, Braak score, NIA Reagan diagnosis or CERAD diagnosis (Table 5.20).

5.3 Discussion

In recent years, work in the field of Alzheimer's disease research has increasingly shifted towards identifying the earliest detectable pathological and cognitive changes in the brain that can serve as diagnostic indicators for the presence of disease before it is too late for effective treatment (Jack Jr et al 2010; Sperling et al 2011). To better understand how low LR11 expression relates to these other early events, we performed a series of correlational analyses designed to identify cognitive, pathological and/or genetic correlates of LR11 across all stages of the disease. While we had hypothesized that low LR11 expression may be associated with apoE genotype, episodic memory impairment, MCI subtype and/or amyloid burden, we found that within the limits of this study, no correlates of LR11 expression consistently emerged.

Table 5.19 – Association Between ROS 2.0 AD Pathological Lesions in Superior Temporal Cortex or Inferior Parietal Cortex and LR11 Expression in Precuneus*

A. Superior Temporal Cortex Lesions

	Precuneus[a]
Neuritic Plaque Frequency	r = 0.098 (p = 0.53)
Diffuse Plaque Frequency	r = -0.012 (p = 0.94)
NFT Frequency	r = 0.12 (p = 0.45)

[a]By Spearman correlation

B. Inferior Parietal Cortex Lesions

	Precuneus[a]
Neuritic Plaque Frequency	r = 0.086 (p = 0.59)
Diffuse Plaque Frequency	r = 0.062 (p = 0.69)
NFT Frequency	r = -0.069 (p = 0.66)

[a]By Spearman correlation

*Association data are presented as Spearman r (p – value).

Table 5.20 – Association Between ROS 2.0 Global Pathological Variables and LR11 Expression*

	Frontal Cortex[a]	**Precuneus[a]**	**Visual Cortex[a]**
Brain Weight	r = 0.053 (p = 0.74)	r = 0.027 (p = 0.86)	r = 0.19 (p = 0.22)
Braak Score	r = 0.055 (p = 0.73)	r = 0.049 (p = 0.75)	r = -0.039 (p = 0.81)
NIA Reagan Diagnosis	r = -0.0074 (p = 0.96)	r = -0.032 (p = 0.84)	r = 0.067 (p = 0.67)
CERAD Diagnosis	r = -0.041 (p = 0.79)	r = -0.068 (p = 0.66)	r = -0.032 (p = 0.84)

[a]By Spearman correlation

*Association data are presented as Spearman r (p – value).

Of the demographic, genetic, cognitive and pathological measures examined, only the association between GCS and frontal cortex LR11 expression in the ROS 1.0 cohort reached statistical significance. Specifically, we found that across all cases, the individuals with the highest levels of LR11 expression had the lowest degree of cognitive impairment and vice versa, suggesting that LR11 expression may serve as a marker of disease severity. Much effort has been devoted to the identification of pathological correlates for symptom severity in AD (Guillozet et al 2003; Markesbery et al 2006; Petersen et al 2006). However, the lack of correlation between cognitive ability and amyloid pathology and the weak correlation with NFTs has been disappointing (Delaère et al 1989; Fukumoto et al 2003; McKee et al 1991; Näslund et al 2000). The best known correlate of cognitive dysfunction in AD is synaptic density (Scheff et al 1990; Scheff et al 2006; Terry et al 1991) and it is interesting to note that the loss of other LDLR family members may contribute to synaptic loss and the resultant cognitive impairment (Motoi et al 1999; Weeber et al 2002). Our findings here in the ROS 1.0 cohort seem to suggest that reduced LR11 expression may predispose individuals to cognitive impairment and the development of AD. Based on these results, we hypothesized that in the ROS 2.0 cohort (for which additional cognitive data was available), we would not only replicate this finding, but that we would further find that LR11 expression correlated particularly well with episodic memory impairment, which is known to be affected in the earliest stages of AD. Moreover, because individuals with amnestic MCI are known to have a greater rate of conversion to AD than individuals with non-amnestic MCI, we also predicted that aMCI cases would have lower LR11 expression

than non-aMCI cases. However, within the ROS 2.0 cohort, there were no positive correlations between LR11 expression in any of the three brain regions examined and any of the measures of cognitive ability, including GCS. While this does not negate the positive association between LR11 expression and global cognitive ability that we observed in the ROS 1.0 cohort, it does suggest that significant additional work will be needed to elucidate the true relationship between LR11 and cognitive impairment, especially in MCI.

While it would be easy to conclude from the results presented in this chapter that LR11 expression is not related to any specific aspect of AD development, including amyloid accumulation, it is important to remember that correlational studies done on post-mortem brain are highly limited and cannot detect active change in the brain. Just as detecting a correlation between two variables does not automatically mean that the two are causally related, it is equally true that a lack of correlation does not necessarily mean that there is a lack of causation. Mechanistic *in vitro* studies have shown that LR11 plays an important role in promoting non-amyloidogenic processing of APP, thereby helping to maintain low levels of Aβ production (Andersen et al 2005; Herskowitz et al 2011; Offe et al 2006). Moreover, reducing LR11 expression in cell culture and/or in animal models has been shown to result in increased Aβ levels (Dodson et al 2008). Given this important regulatory role, it stands to reason that a lack of LR11 protein expression in the human brain would be accompanied by enhanced or accelerated amyloidosis, leaving the individual at an increased risk for developing dementia. While we have focused here on the relationship between LR11 expression and amyloid plaques, a growing body

of evidence suggests that certain smaller, soluble Aβ oligomers may actually be more toxic than amyloid plaques (Catalano et al 2006; Shankar et al 2007; Walsh et al 2005; Walsh & Selkoe 2007). Additional studies examining the relationships between LR11 expression and soluble Aβ levels could prove particularly enlightening. Likewise, the development of methods for imaging LR11 expression *in vivo* and subsequent association studies between live imaging of LR11 expression and amyloid biomarkers will be needed to directly test these hypotheses and to better clarify the temporal relationship between a change in LR11 expression and the onset of amyloid accumulation.

The work presented in this dissertation was primarily designed to characterize LR11 expression in subjects with MCI. The MCI diagnosis was initially introduced as a clinical concept to separate cognitively impaired individuals from those with frank dementia (Zaudig 1992). Many, but not all, individuals diagnosed with MCI progress to greater stages of cognitive impairment, leading to an eventual diagnosis of AD. As a result, in research settings this diagnostic group has often been used to represent a state of prodromal AD. Brains from individuals with MCI have often been used in studies designed to identify "early" changes in AD. However, it is now widely believed that cognitive impairment is a lagging indicator for the presence of disease, with the triggering events that lead to the development of AD potentially beginning decades before the first signs of cognitive difficulty (Jack Jr et al 2010). Extensive cortical and hippocampal amyloid pathology and NFTs in the medial temporal lobe are common in post mortem MCI brains, making MCI and AD virtually indistinguishable upon autopsy (Markesbery et al 2006; Petersen et al

2006). In fact, by the time cognitive changes are apparent, many of the neuropathological processes may have begun to plateau, including the production and deposition of Aβ (Engler et al 2006). As a result, the active period during which any pathological events directly affecting Aβ accumulation are likely to occur is almost entirely contained within the disease stage represented by the NCI diagnostic groups. Therefore, it is possible that any correlation between LR11 expression and pathological variables would only be detectable within the NCI diagnostic group.

In the ROS 1.0 cohort, case selection was based at least in part on pathological criteria. This ensured that the NCI cases were free of amyloid pathology, making it a true disease-free control group. In the ROS 2.0 cohort, brains from cognitively normal individuals were included in the NCI group irrespective of underlying AD pathology. As a result, both the NCI and MCI groups had significant AD pathology, suggesting that nearly all of the cases examined in the ROS 2.0 cohort had already developed some disease related neurodegenerative pathology prior to death. In fact, thirteen of the fourteen NCI cases in the ROS 2.0 cohort were found to have some degree of amyloid pathology, far more than the 20 – 40% of the cognitively intact elderly population that is believed to harbor "silent" amyloid accumulation (Arriagada et al 1992; Morris et al 1996). Therefore, it appears that neither the ROS 1.0 or ROS 2.0 NCI groups are truly representative of the aged, cognitively intact population at large, making them both less than ideal for studying associations between LR11 and pathology in presymptomatic AD cases. Moreover, even if our NCI groups were pathologically representative, both groups are

relatively small, making it impossible to draw any meaningful conclusions about the relationship between LR11 expression and pathological variables within these diagnostic groups. Given these limitations, it is apparent that significant additional work will be needed to more clearly elucidate the relationship between LR11 expression and clinical or pathological variables, both across all stages of the disease and within the pre-clinical stages of the disease specifically.

CHAPTER 6. DISCUSSION

6.1 Summary

LR11 was first recognized as a down-regulated transcript in lymphoblasts cultured from Alzheimer's disease (AD) patients in 2004 (Scherzer et al 2004). Subsequent work confirmed qualitatively that LR11 protein expression is also reduced in AD brain and that LR11 can regulate Aβ levels by directing APP trafficking away from intracellular compartments containing γ-secretase *in vitro* (Andersen et al 2005; Andersen et al 2006; Herskowitz et al 2011; Offe et al 2006; Schmidt et al 2007; Spoelgen et al 2006). These findings led us to hypothesize that the loss of LR11 is a primary, contributing event in the development of AD. This hypothesis gained considerable strength with the findings of Dodson et al in 2008 that a well-established AD mouse model with an additional LR11 deficiency demonstrates accelerated amyloidosis compared to the same AD mouse model with intact LR11 expression (Dodson et al 2008). Given this seemingly critical role for LR11 in modulating Aβ production both *in vitro* and *in vivo*, as well as recent reports that SNPs in the *SORL1* gene are associated with an increased risk of developing AD (Bettens et al 2008; Kölsch et al 2009; Lee et al 2007a; Meng et al 2007; Rogaeva et al 2007), we hypothesized here that low LR11 expression should be apparent even in the earliest stages of AD, including in at least a subset of mild cognitive impairment (MCI) cases. Moreover, we further hypothesized that LR11 expression

levels would be closely related to other early events in the progression of AD, including amyloid plaque frequency and episodic memory impairment.

To test this hypothesis, we developed a novel quantitative immunohistochemical approach to measure LR11 expression in a number of brain regions from cases with a clinical diagnosis of no cognitive impairment (NCI), MCI or AD. LR11 expression was measured in two distinct cohorts that were obtained through our longtime collaboration with the Religious Orders Study. The first cohort, ROS 1.0, consisted of 34 cases whose final clinical diagnosis at the time of death was pathologically confirmed on autopsy. The second cohort, ROS 2.0, consisted of 43 cases. Clinical diagnoses in this cohort were not autopsy-confirmed, making this population more representative of that commonly seen in a neurological clinic. As a result, almost all of the individuals in the ROS 2.0 study harbored at least some degree of AD-related pathological lesions, even in the absence of clinically detectable cognitive impairment.

In chapter 3, we tested the hypothesis that the level of LR11 protein expression in the frontal cortex of MCI brain is similar to that seen in AD brain and markedly less than that seen in control brain, in at least a subset of cases. In the ROS 1.0 cohort, we found low LR11 expression in all ten AD cases examined and robust LR11 expression in nearly all of the NCI cases. LR11 expression in the MCI group was highly variable in ROS 1.0, with five cases having robust, control-like LR11 expression and ten cases having low, AD-like LR11 expression. In the ROS 2.0 cohort, we found low LR11 expression in approximately 30% of the AD cases examined, far less than originally expected. Moreover, we also found low LR11

expression in a similar proportion of cases in both the MCI and NCI groups. Together, these results suggest that LR11 expression is low in at least a subset of cases diagnosed with MCI, similar to what was observed in AD.

In chapter 4, we tested the hypothesis that low LR11 expression would be detectable earlier in the progression of AD in areas of the brain that are known to develop amyloid plaques very early and that LR11 expression would be persistently robust until very late in the disease in brain areas that are generally spared in AD. To test this hypothesis, LR11 expression was measured in the ROS 2.0 cohort in two additional brain areas: the precuneus, a known predilection site for amyloid accumulation and the primary visual cortex, an area of the brain that is generally spared in AD. In both brain regions examined, we found reduced LR11 expression in a similar proportion of cases as in the frontal cortex in all three diagnostic groups. Moreover, of the 14 cases that were found to have low LR11 expression in at least one brain region, ten of them had low LR11 expression in two or more brain regions, suggesting that LR11 expression is either consistently high or consistently low throughout the brain.

In chapter 5, we presented the results of an extensive series of statistical analyses that were designed to identify correlates of LR11 expression in order to determine if LR11 expression is related to other known early changes in the development of AD. We examined a wide range of demographic, genetic, cognitive and pathological variables. While we found a strong relationship between LR11 expression in the frontal cortex and global cognitive score in the ROS 1.0 cohort, no

correlates of LR11 expression consistently emerged in both cohorts within the limits of this study.

6.2 Revisiting LR11 in Alzheimer's Disease

While this study was primarily designed to characterize LR11 expression in MCI, another important aspect of this study was the confirmation of previous findings regarding the expression of LR11 in AD brain. To date, only four publications have looked at LR11 protein expression in human AD brain, three of which have come out of our lab group. Two of these reports, Scherzer et al (2004) and Offe et al (2006) used qualitative immunohistochemistry and western blotting to establish that LR11 expression is reduced in AD brain compared to control brain (Offe et al 2006; Scherzer et al 2004). In a third study from our group, Dodson et al (2006) used a semi-quantitative assessment of LR11 immunohistochemistry to determine that LR11 expression in sporadic AD brain is lower than in FAD brain or control (Dodson et al 2006). Finally, Andersen et al (2005) used western blotting to confirm the reports from our lab group that LR11 expression is reduced in AD brain (Andersen et al 2005). The work presented in this dissertation, then, is the only quantitative measurement to date of LR11 protein expression in neurons in human AD brain compared to control as well as MCI brain.

While many of our most interesting observations involve LR11 in the pre-AD stages of disease progression, two important findings regarding LR11 expression in

AD brain are worth noting. First, we have reported here for the first time that low LR11 expression is not found in all AD cases and therefore is not required for the development of full-blown AD. Second, we have shown that low LR11 expression in the brain is not restricted to AD-vulnerable brain regions only, as had been previously believed.

Low LR11 Expression is not required for the development of full-blown AD

Using our quantitative immunohistochemistry technique, we found low LR11 expression in the frontal cortex in four of the 14 AD cases in the ROS 2.0 cohort (Figure 3.6). Moreover, we found a similar number of AD cases with low LR11 expression in the precuneus (6 cases, Figure 4.4) and the primary visual cortex (5 cases, Figure 4.6). Based on the findings from this cohort, we hypothesize that low LR11 expression is likely to be part of the pathological array in approximately 35% of all AD cases.

This finding stands in contrast to the majority of previous studies that have looked at LR11 protein expression in human brain, all of which reported "remarkably consistent" findings of low LR11 expression in AD brain (Andersen et al 2005; Ma et al 2009; Offe et al 2006; Scherzer et al 2004). However, while this finding was unexpected, it is not unprecedented. In the report from Dodson et al in 2006, the authors reported that mean LR11 expression in FAD brain was significantly higher than in sporadic AD brain and was not different from control, demonstrating that low LR11 expression is not a universal element of AD pathology (Dodson et al 2006). Moreover, while the mean LR11 expression in the sporadic AD

cases corresponded to a semi-quantitative ranking of “light” staining, five of the 16 sporadic AD cases were found to have “moderate” levels of LR11 expression. This is the same level of LR11 expression that was seen on average in the FAD and control brains, suggesting that even in sporadic AD, notable LR11 expression was present in at least some of the cases examined. Finally, also in that same study, LR11 expression was high throughout the lifetime of an AD mouse model where amyloidosis was driven by mutations in the APP and PS1 FAD genes, again demonstrating that extensive amyloid deposition can occur even in the presence of LR11 protein expression.

The limited nature of LR11 loss in sporadic AD is similar to that seen for the susceptibility gene *APOE.* While carriers of the apoE ε4 allele are known to have an increased risk for developing AD during their lifetimes (National Institute on Aging/Alzheimer's Association Working Group & Relkin 1996), the inheritance of a copy of the ε4 allele is not required for full-blown AD to develop. In fact, more than half of the individuals that are diagnosed with AD are not apoE ε4 carriers (Herz & Beffert 2000). There are many interconnected but distinct pathological pathways that can result in what is commonly identified as AD. Low LR11 levels may be an important contributing factor in only a subset of people that will ultimately develop AD. While the size and scope of this study restrict our ability to speculate on increased susceptibility for developing AD in individuals with low LR11 expression, this is an area the merits further examination.

While it is worth noting that all of the AD cases in the ROS 1.0 cohort had low LR11 expression, we believe that low LR11 expression is likely to be over-

represented in this smaller cohort due to natural variability and sampling differences. While significant additional work will be required to determine the true prevalence of low LR11 expression in AD, we hypothesize that low LR11 expression is likely to be part of the pathological profile of approximately ~35% of AD cases, closer to the proportion of affected cases seen in the ROS 2.0 cohort. Regardless of the actual prevalence of low LR11 expression in AD, the primary conclusion from these results is that low LR11 expression is not required for the development of full-blown AD and that AD can still develop even in the presence of robust LR11 expression.

Low LR11 expression is not restricted to AD-vulnerable brain regions

In the experiments described in Chapter 3, we quantified LR11 expression in the frontal cortex, one of the most studied regions in the brain in AD. In the experiments described in Chapter 4, we also quantified LR11 expression in two additional brain regions in the ROS 2.0 cases in order to better understand the pattern of LR11 loss in the brain both topographically within one diagnostic group and temporally over the course of disease progression from NCI to MCI to AD. LR11 expression was examined in the precuneus, a region of the brain known to accumulate amyloid plaques very early in the disease and in the primary visual cortex, an area of the brain that is generally spared in AD. In the precuneus, we found low LR11 expression in a comparable proportion of NCI and MCI cases to that which had low LR11 expression in the frontal cortex, as predicted (Figure 4.4).

Given that the primary visual cortex does not develop pathological lesions in AD until very late in the disease (if at all) (Arnold et al 1991; Duyckaerts & Hauw 1997; Metsaars et al 2003) and that a previous study had found that LR11 expression was only reduced in AD-vulnerable brain regions (Offe et al 2006), we had predicted that LR11 expression would be persistently robust in this brain region in almost all of the NCI and MCI cases examined. Moreover, we predicted that the majority of the AD cases examined would also have high LR11 expression. However, we found that LR11 expression in the primary visual cortex was very similar to that seen in the other two brain regions, with a subset of cases in each diagnostic group having low LR11 expression (Figure 4.6).

When we looked at the topographical distribution of LR11 loss within each case, we found that when one brain region had altered LR11 expression, other areas of the brain were likely to have low LR11 expression as well. Of the fourteen cases that were found to have low LR11 expression in at least one brain region, eight of them actually had low LR11 expression in all of the brain regions examined with another two cases having low LR11 expression in all but one brain region examined (Figure 4.7). Based on these findings, we conclude that LR11 expression is generally either universally high or universally low in the brain.

While these results were unexpected, given the previous report by Offe et al (2006), they are logical. LR11 was originally identified as a downregulated transcript in cultured lymphoblasts from AD patients, suggesting that LR11 expression may be reduced not just in the central nervous system of AD patients but

also in the cells of the immune system (Scherzer et al 2004). A growing body of evidence has shown that SNPs in the *SORL1* gene may be associated with an increased risk of developing AD (Bettens et al 2008; Kölsch et al 2009; Lee et al 2007a; Meng et al 2007; Rogaeva et al 2007) and splice-variants with different expression profiles in AD have been identified (Grear et al 2009), raising the possibility that global LR11 expression may be a function of *SORL1* genotype, rather than locally determined.

6.3 LR11 in Pre-Alzheimer's Disease Stages

Early Events in the Development of AD: The Biomarker Model

It has been over 100 years since Dr. Alzheimer first described the neurodegenerative disease that bears his name and over 25 years since the first cholinergic replacement therapies were introduced (Alzheimer 1906; 1907; Summers et al 1986). While cholinergic replacement based treatments are still considered to be the primary means of treating Alzheimer's disease (AD) today, this therapeutic approach merely alleviates the symptoms of AD, rather than treating the root pathological cause (Lleó et al 2006). Moreover, due at least in part to our limited understanding of the early clinical manifestations of the disease, treatment for AD often doesn't begin until a patient has been diagnosed with dementia. Unfortunately, by the time dementia, or even MCI is clinically detectable, the

pathological processes of AD have been long underway, beginning with the creeping appearance of senile plaques throughout the brain and ultimately resulting in the widespread synapse loss and cortical atrophy that directly contributes to the clinical manifestation of the disease (Jack Jr et al 2010; Sperling et al 2011), as will be described in more detail below. While it is true that the cholinergic replacement therapies that are used today are the most effective once cognitive impairment is evident, the fact remains that these symptomatic treatments merely mimic the function of cells that have been permanently lost. By waiting for a state of dementia to develop before "diagnosing" a patient with AD, and waiting for a diagnosis to begin treatment on top of that, we are simply waiting too long to have any expectation of modifying the disease course in a way that preserves or restores cognitive function. Truly effective therapies must alter the pathogenic processes that occur prior to this neurodegenerative stage of the disease. In short, there are two fundamental challenges to curing or preventing AD facing scientists today: we need to be able to predict who will develop the disease in the future and we need to develop disease-modifying treatments that are likely to have the greatest efficacy during this "pre-symptomatic" disease stage. To order to address both of these needs, the focus of recent research on this disease has increasingly turned towards identifying early changes or characteristics related to incipient AD that can be used to identify individuals at the greatest risk for developing AD, as potential therapeutic targets, or both.

While incipient familial AD (FAD) can often be anticipated due to family history and/or the presence of known mutations in APP or the presenilins, incipient

sporadic AD can be very difficult to diagnose in the preclinical stages. However, because of the highly similar disease processes underlying both disorders, longitudinal studies of individuals at high risk of developing AD have provided important insights into the earliest pathological and clinical events in the progression of AD. As a result, a more comprehensive understanding of the early signatures of incipient sporadic AD is beginning to emerge.

An extensive body of evidence has now established that the first measurable change in the brain associated with the development of AD is the abnormal accumulation of Aβ into first diffuse plaques (DPs) and then neuritic plaques (NPs) (Hardy & Higgins 1992; Selkoe 1991; 2003; 2004). Longitudinal studies following individuals at an increased risk for developing AD have also shown that amyloid biomarkers, including CSF $A\beta_{42}$ levels and PiB binding of insoluble amyloid undergo dynamic change very early in AD and largely reach a plateau by the time clinical symptoms appear (Bacskai et al 2007; Clark et al 2003; Edison et al 2007; Grimmer et al 2009; Ikonomovic et al 2008; Jagust et al 2009; Klunk et al 2004; Rosen et al 2010; Rowe et al 2007; Schoonenboom et al 2008; Shaw et al 2009; Strozyk et al 2003). Moreover, it has been estimated that 20 - 40% of cognitively intact older individuals harbor significant amyloid accumulation, despite being symptom-free (Aizenstein et al 2008; Bouwman et al 2009; Knopman et al 2003; Mintun et al 2006; Peskind et al 2006; Price et al 2009; Price & Morris 1999; Savva et al 2009; Shaw et al 2009), with accepted wisdom in the field holding that these individuals will develop AD should they live long enough. Following amyloid accumulation, other known pathological changes begin to occur after a variable lag period that can

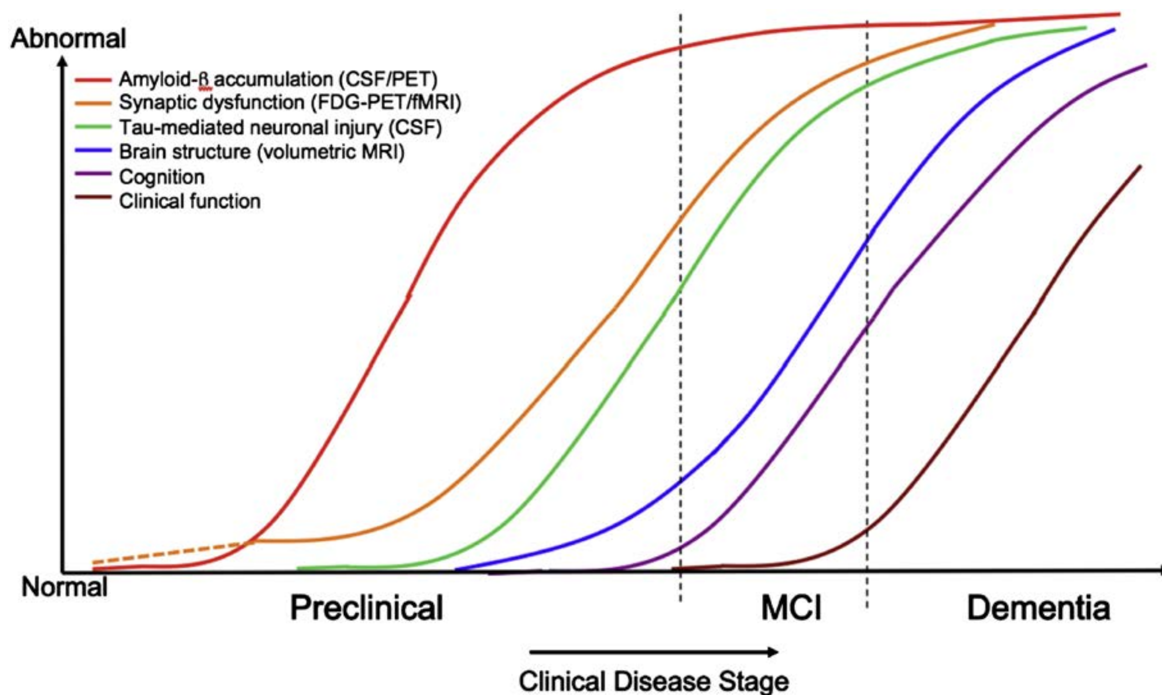
last as long as a decade or more (Stern 2006). A notable increase in the concentrations of tau and particularly phosphorylated tau in the CSF can often be detected in presymptomatic AD patients, indicating the presence of neurofibrillary tangles in the brain prior to the onset of clinical symptoms (Buerger et al 2006). Imaging studies have also demonstrated increased synaptic dysfunction (Minoshima et al 1997) and cortical atrophy (Jack Jr et al 1992) occurring shortly before the onset of overt memory impairment and often paralleling cognitive decline (Engler et al 2006; Fox et al 1999; Jack Jr. et al 2009; Vemuri et al 2009a; b).

Subtle changes in cognitive ability can also signal the presence of underlying disease in the brain long before the patient experiences impairment severe enough to warrant a diagnosis of AD dementia or even mild cognitive impairment (MCI) (Howieson et al 2008). These states exist along a spectrum of degrees of cognitive impairment, ranging from absolutely no cognitive impairment to a state best described as “not normal, not MCI” (Albert et al 2011). Almost always, the first cognitive complaints that an individual has are related to difficulties with episodic memory. These specific complaints often progress to greater levels of cognitive impairment, resulting first in a diagnosis of amnestic MCI (aMCI) before widening out to a general state of global dementia of the Alzheimer’s type. Given that patients with episodic memory deficiencies and/or aMCI are known to progress to AD at a greater rate (Gauthier et al 2006; Petersen & Negash 2008; Petersen et al 1999), these behavioral endophenotypes are considered to be early events in the progression of AD and are increasingly being used as diagnostic tools for impending AD prior to the onset of dementia.

Finally, while not directly a part of the AD pathogenic cascade, it is also important to note that certain susceptibility factors, such as being a carrier of an apoE ε4 allele, are often present throughout a person's lifetime, ultimately leaving that person at an increased risk for developing sporadic AD late in life. Recognizing these factors in presymptomatic individuals is an important part of the emerging "pre-AD" profile that may someday be used to identify those at the greatest risk for developing AD in the future.

While many of the pathological and cognitive hallmarks described above have been associated with the development of AD for decades, it has only been recently that we have been able to observe *in vivo* how these events relate to each other temporally over the development of the disease, especially in the earliest, pre-clinical stages. Changes in biomarkers of amyloid deposition (reduced CSF $A\beta_{42}$ levels, increased PiB binding to insoluble Aβ), synaptic dysfunction (decreased regional cerebral glucose metabolism as detected by FDG-PET, fMRI), tau-mediated neuronal injury (increased levels of tau and phosopho-tau in CSF) and cortical atrophy (volumetric MRI) are now widely accepted as precursors to the eventual development of AD. By studying and monitoring these specific biomarkers in patients who are at a high risk for developing AD due to family history, apoE genotype or the presence of trisomy 21, an ordered model of disease progression has begun to emerge that is rapidly gaining acceptance across the field (Figure 6.1) (Jack Jr et al 2010; Sperling et al 2011). This biomarker-based model proposes that the primary pathological events in early AD occur in two stages. In the first stage, amyloid levels in the brain become increasingly elevated, eventually reaching a

Figure 6.1 – Biomarker Model of AD Progression



Hypothetical model of dynamic biomarkers of AD as proposed by Jack et al (Jack Jr et al 2010) and expanded by Sperling et al (Sperling et al 2011). The temporal trajectory of change from normal to maximally abnormal of known biomarkers of AD is shown as a function of disease stage. Changes in cognitive ability and clinical function with advancing disease are also illustrated, demonstrating that most of the pathological changes associated with AD are present to some degree in the preclinical, asymptomatic stage of the disease.

Reprinted from Sperling, R.A. et al (2011) *Alzheimer's and dementia: the journal of the Alzheimer's Association* with permission from Elsevier (Sperling et al 2011).

plateau of maximal abnormality. This "amyloid stage" occurs almost entirely before the onset of any clinical symptoms, with the first signs of abnormality becoming apparent decades before any cognitive impairment is detectable. The second pathological stage, which is also known as the "neurodegeneration stage", follows the amyloid stage at a variable lag period that can be affected by a number of factors including apoE genotype, lifestyle and environmental influences and cognitive reserve. In this stage, biomarkers of synaptic dysfunction, tau-mediated neuronal injury and cortical atrophy become abnormal in an ordered manner, often beginning with a period of slow change and becoming more rapid with the eventual onset of cognitive impairment. Of the biomarkers that are known to predict future AD, changes in neurodegeneration biomarkers, and especially those of gross cortical atrophy most closely mirror changes in cognitive ability (Terry et al 1991), suggesting that it is the magnitude of cell loss and not the total lesion burden in the brain that is the substrate for the clinical symptoms associated with AD. While changes in neurodegeneration biomarkers are often apparent in preclinical individuals, these biomarkers are undergoing their most dynamic period of change during the MCI and early AD stages of the disease (Fox et al 1999; Jack Jr et al 1992; Minoshima et al 1997). While some of the specific details in the biomarker model of AD are still being debated, the primary tenet of this model has gained widespread acceptance since Jack Jr et al first proposed it in 2010 (Jack Jr et al 2010). This tenet proposes that the majority of potentially reversible, causative pathological change in the development of AD occurs years to decades in advance of the onset of cognitive impairment. The corollary to this, then, is that by the time cognitive impairment is

clinically detectable, irreversible neuronal loss is already underway. By this stage of the disease, successful therapeutic interventions may delay the progression of the disease, but they are unlikely to significantly alter the disease's relentless march towards full-blown dementia and death.

One of the notable benefits of this biomarker model is that it provides a useful framework for assigning cases to clinical and/or preclinical disease stages based on the degree of cognitive impairment an individual displays and their biomarker signature. Because we don't have comprehensive biomarker data on the cases in the experimental cohorts used in this dissertation, we cannot assign individual cases to the specific disease stages laid out above. However, by looking at the degree of cognitive impairment present at the time of death (NCI, MCI or AD dementia) together with the presence or absence of pathological lesions on autopsy, we can roughly estimate where our diagnostic groups fall along the spectrum of disease described by this model. By doing this, it becomes evident that our diagnostic groups each correspond to one of four stages of AD progression: clinical AD, clinical MCI, preclinical AD or no disease. Both of the MCI groups and both of the AD groups in the cohorts used in the studies presented here had similarly high levels of both neuritic and diffuse plaques, as would be expected in groups corresponding to each of these clinical disease stages (Tables 5.13 and 5.16). The cases comprising the NCI group from the ROS 2.0 cohort also harbored significant levels of amyloid accumulation, despite the lack of cognitive impairment exhibited by those individuals (Table 5.16). Therefore, we conclude that the ROS 2.0 NCI group corresponds to the long preclinical stage of AD. Finally, the cases comprising

the NCI group from the ROS 1.0 cohort displayed neither cognitive impairment nor the any sign of AD-related pathology (Table 5.13). Therefore, based on the biomarker model described above, we conclude that this group corresponds to the "normal" or "no disease" stage that precedes the pathological onset of disease. By characterizing LR11 expression across each of these disease stages, as we have done in this dissertation, we can begin to determine if the loss of LR11 is an early event in the development of AD, as initially hypothesized at the outset of this study and if so, where the loss of LR11 fits into the biomarker model of AD.

Placing Low LR11 Expression into the Biomarker Model of AD

Based on the findings presented in this dissertation, I believe that in cases with end-stage AD with accompanying low LR11 expression, the loss of LR11 protein expression occurs very early in the development of the disease, prior to the onset of cognitive impairment. As noted above, we found low LR11 expression in at least 30% of the AD cases that were examined. Moreover, a similar proportion of MCI cases also had low levels of LR11 protein expression. Therefore, it appears that as cases progress from MCI to AD, there is little to no additional loss of LR11 suggesting that LR11 expression has likely become abnormal in the maximum number of cases that will be affected by this stage. Moreover, we also found low LR11 expression in a similar proportion of cases in the NCI group from the ROS 2.0 cohort, a group that I believe corresponds to the preclinical stage of AD as described in the previous section. Based on this result, it appears that LR11 expression has

already become maximally abnormal in this preclinical stage of the disease as well, with no additional loss of LR11 apparent as cases progress to MCI. This is highly similar to the profile of change exhibited by other pathological elements in AD, many of which become maximally abnormal prior to the onset of cognitive impairment. Finally, in the normal cases with no signs of incipient disease that comprise the NCI group from the ROS 1.0 cohort, no cases were found with low LR11 expression. Therefore, I conclude that a change from normal, robust levels of LR11 expression to low LR11 expression, if it occurs at all, is most likely to occur at some point during the progression from normal to preclinical AD, with no further loss of LR11 at the later stages of the disease.

It has been reported that 20-40% of cognitively intact elderly individuals have some degree of "silent" AD pathological changes in their brains (Arriagada et al 1992; Morris et al 1996). Given our estimation that approximately 35% of AD cases will also have low LR11 expression, I predict that between 7% and 14% of the cognitively intact elderly population will have low LR11 expression that I believe may contribute to the onset and/or development of AD over the course of their lifetimes, should they live long enough. While this figure is obviously speculative and requires future experimental confirmation, it should prove to be a helpful starting guideline in interpreting future results regarding LR11 expression in presymptomatic AD.

As noted in previous chapters, the "all cases have pathology or no cases have pathology" nature of the two NCI groups characterized for this dissertation does not

allow us to directly examine the relationship between LR11 expression and the presence or absence of pathological lesions in the asymptomatic NCI cases within one experimental cohort. Moreover, the relatively small size of the NCI group in the ROS 2.0 cohort makes it very difficult to conduct statistically meaningful analyses of the relationships between LR11 expression and other pathological changes within this one group. Nonetheless, given what is known about the functional role of LR11 in regulating the intracellular trafficking of APP and the production of Aβ, we can rationally speculate that a loss of LR11 in the preclinical stage of AD is likely to influence the rapid accumulation of Aβ that occurs in the earliest phases of this stage of the disease. Depending on the specific timing of LR11 loss, the effect of this change in LR11 expression on amyloid accumulation could manifest itself as an earlier age of onset for pathological change, a more rapid accumulation of Aβ leading to a shorter lag phase before the initiation of downstream pathological events or some combination thereof.

Because of the limited nature of the experiments performed and the characteristics of the cases being studied, the interpretations laid out in this section are highly speculative and will require significant additional confirmation in the future. In particular, post-mortem studies similar to those done as part of this thesis work characterizing LR11 expression in a truly representative NCI population would be particularly informative. Ideally, this experimental group would include young, pathology free individuals as well as aged individuals both with and without AD lesions in addition to the MCI and AD groups examined here. If our hypotheses about the time course of LR11 loss are correct, we would anticipate that 100% of

the young, no pathology NCI cases would have normal, high LR11 expression and that approximately 35% of the aged NCI cases harboring AD pathology would have low LR11 expression, as seen in the ROS 2.0 NCI cohort. In the aged NCI cases with no pathology, we would predict robust LR11 expression in close to 100% of the cases, similar to that seen in the ROS 1.0 NCI cohort. This larger, multi-faceted NCI population could also prove to be an ideal group in which the interplay between LR11 expression and amyloid accumulation in preclinical AD could be examined.

Much of what is known about the relationship between amyloid accumulation and other early events in the development of AD was determined through *in vivo* monitoring of biomarkers in longitudinal studies of individuals at an increased risk for developing AD. With the development of similar means of measuring LR11 expression in living patients, researchers would be able to observe how and when LR11 expression changes relative to other known biomarkers of pathological events and the onset of cognitive impairment. LR11 is a single transmembrane protein that is known to undergo proteolytic cleavage (Böhm et al 2006; Hampe et al 2000), resulting in the release of the soluble ectodomain (or sLR11) from cells. Recent work by Ma et al (2009) has shown that sLR11 can be detected by western blotting CSF samples from control patients (Ma et al 2009). Using this approach, they showed that CSF sLR11 levels are significantly reduced in patients with mild to moderate AD and that CSF sLR11 levels were correlated with CSF sAPPβ, with the cases having the lowest LR11 levels also having the lowest levels of sAPPβ. Of particular note from this study are five control cases that had low, AD-like levels of both proteins in the CSF, further supporting our hypothesis

that loss of LR11 expression occurs during the preclinical stages of the disease. In addition to the western blot approach used in the study that was just described, soluble LR11 can also be measured in both CSF and in serum using ELISA (Matsuo et al 2009). Longitudinal monitoring of sLR11 levels in CSF relative to other known pathological biomarkers and cognitive testing over the course of the disease will provide important insights into the temporal relationship of these events that may prove useful for identifying patients in the preclinical stages of the disease and that are at the greatest risk for developing full-blown AD.

While measuring sLR11 in CSF is relatively straightforward, the downside to this approach is the lack of topographic specificity in determining where in the brain LR11 expression is being lost and in which cell types. While LR11 expression is markedly lost in neurons in AD, glial cells generally maintain robust LR11 expression even in the latest stages of the disease (Offe et al 2006). As a result, the differences in LR11 expression between individuals is often blunted in measurements that do not differentiate between cell types, like western blotting of tissue homogenates or CSF. Live imaging of full length LR11 in the brains of living patients would allow for a more specific characterization of neuronal LR11 expression at various stages of the disease. Moreover, while we found that LR11 loss was generally widespread in the brain, it remains a possibility that small, transient differences in regional LR11 expression do exist in the earliest preclinical stages of AD. The development of small, radiolabeled ligands that are specific for LR11 would enable researchers to characterize topographic as well as temporal changes in LR11 expression relative to other AD pathological changes and cognitive decline.

Improving our understanding of the relationship between LR11 expression and disease progression broadly and between LR11 and other pathological events in particular has important implications for both the diagnosis and treatment of AD. It has already been shown that Aβ levels can be controlled *in vitro* through changes in LR11 expression. For individuals that have begun to show signs of incipient AD but who have stable LR11 expression, therapies that take advantage of the presence of LR11 could prove particularly effective at slowing the accumulation of Aβ in the brain. Moreover, while it is unlikely that low LR11 expression in and of itself will be predictive of a future diagnosis of AD, a change in LR11 expression could prove to be an important part of the emerging "pre-AD" profile of individuals at the greatest risk for developing AD in the near future. Given the timeline for LR11 loss proposed here and the potential functional impact of that loss on disease progression, it is likely that low LR11 expression will emerge as an important susceptibility factor indicating increased risk for future disease, similar to depression, cardiovascular risk factors and apoE genotype.

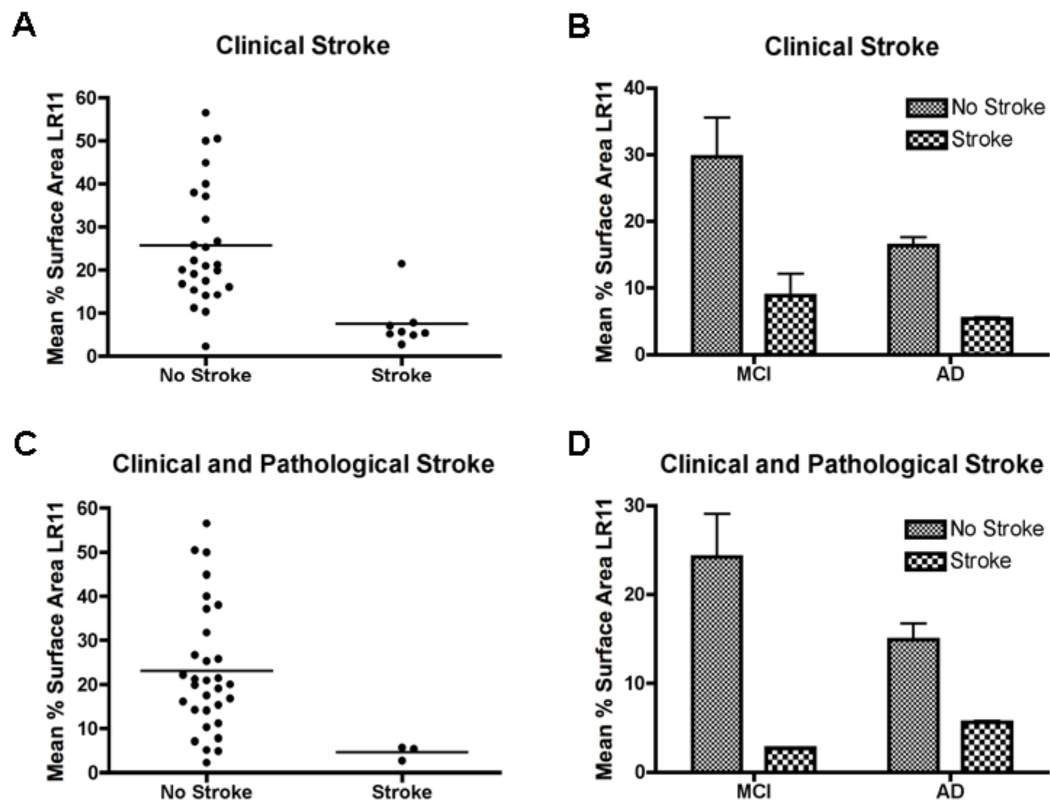
6.4 LR11 and Stroke

It has long been recognized that classic cardiovascular risk factors such as hypertension, high cholesterol levels and obesity are associated not just with an increased risk of cardiovascular disease, but also with an increased risk of

developing dementia (de la Torre 2004; Panza et al 2006; Viswanathan et al 2009; Waldstein & Wendell 2010). Moreover, specific cardiovascular diseases themselves, including myocardial infarction, atherosclerosis and stroke are often found alongside dementia disorders (including AD and vascular dementia) in the same patients (Panza et al 2006). Traditionally, the presence of cerebrovascular pathology was believed to be associated with vascular dementia only and was considered to be exclusion criteria for a final diagnosis of AD. However, a growing body of evidence now suggests that cardiovascular risk factors and/or diseases may contribute directly to AD pathogenesis (Panza et al 2006). As a result, recent research has focused on elucidating the potential mechanisms by which cardiovascular pathology can contribute to an increased risk of developing AD.

During the analysis of the data from the ROS 1.0 cohort, we observed that cases with a reported clinical history of stroke had lower neuronal LR11 expression (7.6 $\pm$ 2.1 percent surface area LR11, n = 8) compared to those with no reported history of stroke (25.7 $\pm$ 2.7 percent surface area LR11, n = 26; p = 0.0011, t-test) (Figure 6.2A). Moreover, a two-way ANOVA confirmed a significant effect of clinical stroke history on LR11 expression (p = 0.0093) while the effect of diagnostic group on LR11 expression was not significant (p = 0.14) (Figure 6.2B). This observation was especially striking in the three cases (two AD, one MCI) that had both a clinical history of stroke and gross cerebral infarcts on autopsy (Figure 6.2C and D). In light of this observation, in order to avoid any potential confounding factors in the ROS 2.0 study, we chose to exclude the four cases (one NCI, two MCI and one AD) in that cohort that had both a history of clinical stroke and gross cerebral infarcts on

Figure 6.2 – LR11 expression is low in ROS 1.0 cases with a history of clinical stroke



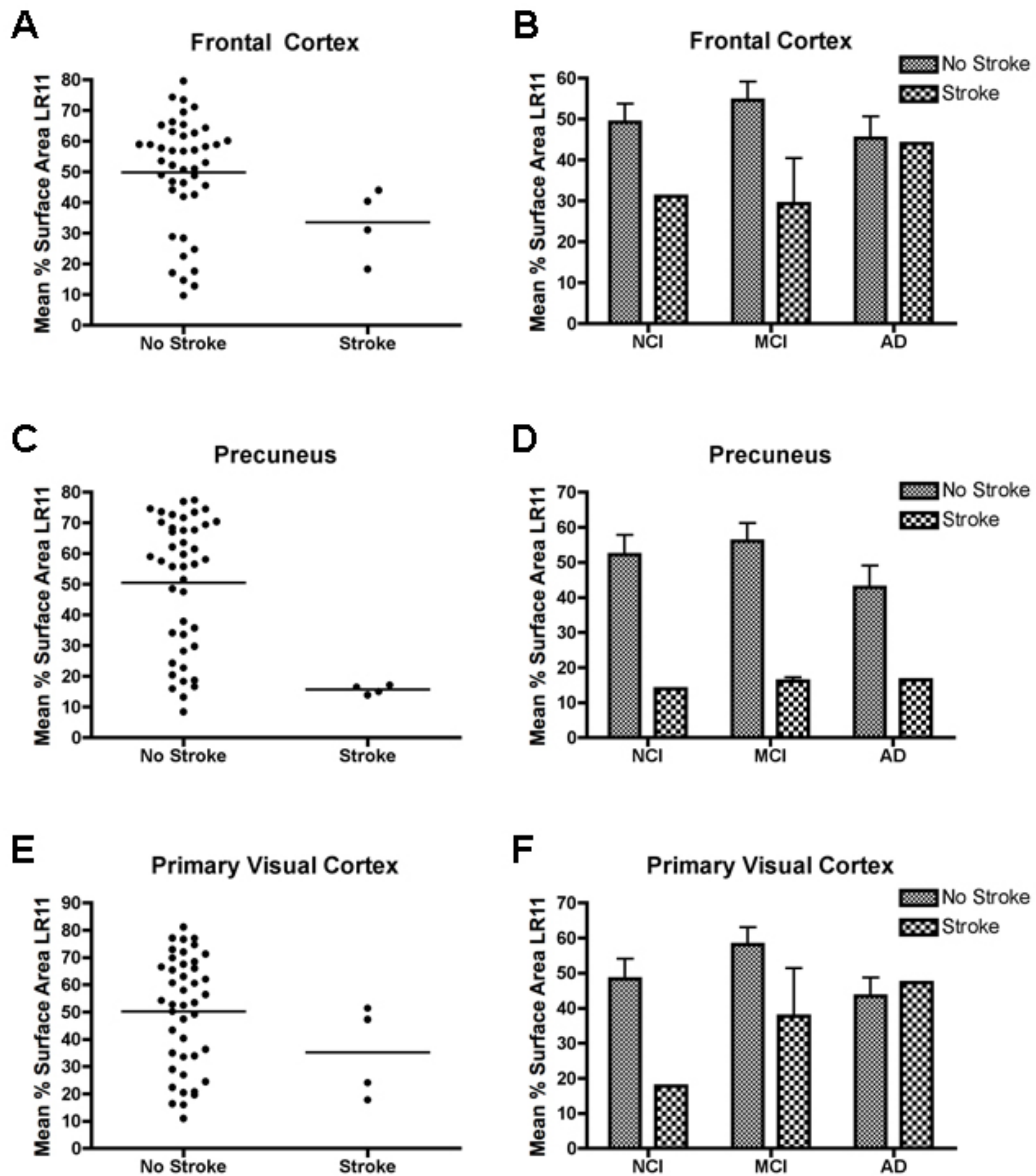
(A) LR11 expression is reduced in cases with a history of clinical stroke (7.6 ± 2.1 percent surface area LR11) compared to cases with no such history (25.7 ± 2.7 percent surface area LR11; p = 0.0011, t-test). Each point on the graph represents individual cases and the short horizontal bars indicate the mean LR11 expression for the group. Data is given as mean ± SEM. (B) Two way ANOVA confirmed a significant effect of clinical stroke on LR11 expression (p = 0.0093) while the effect of diagnostic group on LR11 expression was not significant (p = 0.14). Error bars represent SEM. (C) This trend was especially prominent in the three cases (1 MCI, 2 AD) that had gross cerebral infarcts on autopsy in addition to a history of clinical stroke (4.6 ± 1.0 percent surface area LR11) compared to cases without both cerebral infarcts and clinical stroke (23.1 ± 2.6 percent surface area LR11). This effect was only weakly significant (p = 0.04, t-test) due to the small number of cases with both clinical and pathological stroke. (D) While neither effect reached

significance due to the low power of the comparison, the effect of stroke history on LR11 expression ($p = 0.12$) was again stronger than the effect of diagnostic group ($p = 0.74$), as assessed by two-way ANOVA.

autopsy from the final data analysis that has been presented thus far. However, subsequent analysis of these four cases relative to the rest of the ROS 2.0 cohort again revealed low neuronal LR11 protein expression in cases with evidence of both clinical and pathological stroke, similar to what was originally observed in the ROS 1.0 stroke cases (Figure 6.3A, C and E). In all three brain regions examined, the effect of clinical plus pathological stroke on LR11 expression was stronger than the effect of diagnostic group although again, only the effect of stroke on LR11 expression in the precuneus reached significance ((B) frontal cortex: effect of stroke $p = 0.14$, effect of diagnostic group $p = 0.94$; (D) precuneus: effect of stroke $p = 0.0043$, effect of diagnostic group $p = 0.89$; (F) primary visual cortex: effect of stroke $p = 0.16$, effect of diagnostic group $p = 0.51$). Information on additional cardiovascular risk factors was not available.

While the cardiovascular risk factors described above have long been associated with an increased risk for developing AD, the high comorbidity of both stroke and AD has generally been attributed to the commonality of the two disorders in the elderly population (Honig et al 2003). However, it is becoming increasingly well accepted that stroke can cause an increased risk of future AD, with approximately 30% of stroke patients developing dementia within three years of the stroke event (Hénon et al 2001). The presence of additional cardiovascular pathologies has been shown to increase this risk even further (Honig et al 2003). The presence of cerebral infarcts has also been shown to lead to increased cognitive impairment and higher prevalence of dementia, even in the absence of symptomatic

Figure 6.3 – LR11 expression is generally low in ROS 2.0 cases with both clinical and pathological stroke



LR11 expression in the frontal cortex (A-B), precuneus (C-D), primary visual cortex (E-F) is shown for cases with both a clinical history of stroke and gross cerebral infarcts on autopsy compared to cases without both clinical and pathological stroke. In all brain regions, mean LR11 expression was lower for the cases with both clinical and pathological stroke (n = 4; 1 NCI, 2 MCI and 1 AD) compared to the rest of the

cohort (n = 43). However, due to the small number of cases with stroke, only the difference in LR11 expression in the precuneus reached significance ((A) frontal cortex $p = 0.06$, (C) precuneus $p < 0.0001$, (E) primary visual cortex $p = 0.19$). In all three brain regions, the effect of clinical plus pathological stroke on LR11 expression was stronger than the effect of diagnostic group although again, only the effect of stroke on LR11 expression in the precuneus reached significance ((B) frontal cortex: effect of stroke $p = 0.14$, effect of diagnostic group $p = 0.94$; (D) precuneus: effect of stroke $p = 0.0043$, effect of diagnostic group $p = 0.89$; (F) primary visual cortex: effect of stroke $p = 0.16$, effect of diagnostic group $p = 0.51$). In panels A, C, and E, individual cases are represented by each point on the graph and the mean LR11 expression for each group is indicated by the short horizontal bars. In panels B, D, and F, the error bars for all groups with more than case represent the SEM.

stroke (Snowdon et al 1997; Vermeer et al 2003). Finally, a study by Snowdon et al in 1997 made the intriguing observation that in patients who had had a stroke, a lower burden of AD related-lesions was required for the development of dementia (Snowdon et al 1997).

While these results clearly suggest a strong association between stroke and AD, the specifics pertaining to how or why a stroke event could lead to increased risk of AD are still under debate. A number of potential scenarios have been proposed, which were reviewed at length by Honig et al (Honig et al 2003) and which will be briefly discussed here. First, it has been hypothesized that ischemic events could act as a trigger event for the AD pathogenic cascade, resulting first in the formation of amyloid plaques and subsequently synaptic dysfunction, the appearance of NFTs and cerebral atrophy. Work by Hall et al in the mid 1990s showing that the expression of APP and Aβ are altered following a stroke adds considerable support to this hypothesis (Hall et al 1995). A related hypothesis has also been proposed that suggests that rather than acting as a trigger event, ischemic events may instead exacerbate symptoms of incipient AD. It is also possible that pre-clinical AD-related pathological changes could predispose patients to stroke, resulting in the appearance of stroke event that precedes the development of dementia. Finally, given that the cardiovascular risk factors that predispose an individual to increased risk of AD are nearly identical to the risk factors that predispose individuals to stroke, it remains possible that while the association between the two diseases is high, there is no mechanistic connection between them.

Additional work in the field will no doubt help to clarify the relationship between stroke and AD risk in the future.

In light of these proposed explanations for the strong association between stroke and AD risk, a number of interesting questions regarding the relationship of LR11 expression to both stoke and AD arise. First and foremost, is LR11 expression in the brain reduced in response to stroke? Conversely, could low LR11 expression predispose individuals to stroke? Likewise, does low LR11 expression exacerbate the response to ischemic insult? Finally, much like has been proposed for other cardiovascular risk factors, could low LR11 expression simply predispose individuals to both stroke and AD independently? While our observations here are highly preliminary, additional work examining the relationship between stroke, AD and LR11 is certainly warranted. In particular, studies looking at LR11 expression in mice that have been subjected to ischemic insult and studies looking at the stroke response in mice with deficient LR11 expression should be particularly enlightening.

Our observations regarding low LR11 expression in cases with a history of stroke are also notable in light of the important role that the low-density lipoprotein receptor-related protein 1 (LRP1) plays in mediating the cell death response to an ischemic event. LRP1 is the largest member of the LDL receptor family and shares a highly similar domain structure with LR11. Moreover, LR11 and LRP1 share a number of extracellular ligands, including apoE, RAP, uPA, tPA and other components of the plasminogen activating system (Gliemann et al 2004). Following

an ischemic event, the activity of tPA (a highly specific serine protease) is increased, resulting in increased binding of tPA to LRP1 and a subsequent increase in LRP1 expression and processing. LRP1 is ultimately cleaved by γ-secretase, releasing the LRP intracellular domain which then translocates into the nucleus where it initiates downstream signaling cascades resulting in increased NFκB signaling, iNOS expression, caspase-3 cleavage and apoptotic cell death. Inhibiting either the binding of tPA and/or the cleavage of LRP1 by γ-secretase results in a decrease in both caspase-3 cleavage and apoptotic cell death in ischemic tissues (Polavarapu et al 2008; Zhang et al 2007). While LR11 or a lack thereof has not previously been implicated in the cellular response to stroke, the ability of LR11 to competitively bind tPA and sequester it away from LRP1 suggests an interesting potential mechanistic link between low LR11 expression and stroke that also warrants future investigation (Gliemann et al 2004).

Finally, given the novelty of this observation, it is possible that an over inclusion of cases with a history of stroke and/or cerebral infarcts in previous experimental cohorts could account for the discrepancy between our results reporting loss of LR11 in a subset of AD cases in the ROS 2.0 cohort and the near universal loss of LR11 expression in AD cases reported in earlier studies (Andersen et al 2005; Offe et al 2006; Scherzer et al 2004).

6.5 Final Words

The first 100 years of Alzheimer's disease research have been primarily focused on identifying the pieces of what has turned out to be a very complicated puzzle. Starting with Dr. Alzheimer's early reports of widespread neuronal loss and his prescient recognition of the importance of neuritic plaques and neurofibrillary tangles, neuroscientists have worked throughout the last century to identify the critical contributory pathologic events, susceptibility genes and behavioral hallmarks that comprise the bulk of our knowledge on the disease today.

In this new century, the challenges facing scientists and clinicians have broadened, with an increased focus not just on identifying these crucial puzzle pieces, but rather on how these pieces come together to cause Alzheimer's disease. The first big step forward in understanding how these pathological events relate to each other came with the identification of the APP and presenilin familial AD genes. This discovery, together with other more recent findings led to the proposal of the amyloid cascade hypothesis; that is, that the initiating trigger for AD is the abnormal accumulation of Aβ in the brain and that the other known pathological events associated with AD, including the formation of NFTs, synaptic dysfunction and cortical atrophy, all occur downstream of this event. Now, with recent advances in technology, we can more directly observe the topographic and temporal relationships between seemingly disparate events. The full puzzle is now beginning to emerge. We're getting closer every day to not only being able to identify those patients who are essentially destined to develop sporadic AD, but also to being able to modify their disease course in a way that preserves cognitive function over the long term.

The story of LR11 in AD is a comparatively new one. Through the work presented in this dissertation, we now have a better understanding of the extent of LR11 loss in AD and when in the course of the disease LR11 expression may become abnormal. While there is still much work to be done before we can truly understand the role this protein plays in the healthy brain and the consequences of having low LR11 expression for the development of AD, hopefully the work presented here can begin to help us to see how low LR11 expression fits in to the complicated puzzle that is Alzheimer's disease.

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