INTERACTIONS BETWEEN EXCITOTOXICITY AND LYSOSOMAL INHIBITION: IMPLICATIONS FOR ALZHEIMER’S DISEASE PATHOGENESIS

by

Michael Paul Murphy

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Psychology
University of Toronto

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0-612-35262-5
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Michael Paul Murphy
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ABSTRACT

The peptide β-amyloid (Aβ) is believed to be the crucial element in Alzheimer’s disease (AD) pathogenesis, being terminally responsible for neuronal death and ensuing dementia. Lysosomal inhibition alters the processing of the β-amyloid precursor protein (βAPP), from which Aβ is derived, such that potentially amyloidogenic fragments (fβAPP) accumulate intracellularly, largely within lysosomes. Although it has been hypothesized that the lysosomal dysfunction accompanying normal aging (and modeled with lysosomal inhibitors) may be connected to AD, the consequences of this manipulation on the CNS are not known, nor is the potential importance of intracellular Aβ (iAβ) understood. Hence, monkeys, rats and βAPP transgenic mice received direct brain infusions (i.c.v.) of the lysosomal inhibitors leupeptin or chloroquine (CHL) for up to 2 months. Both chemicals caused an increase in iAβ, but did not cause significant neuronal death. Both also caused a strong astrocytic response, and CHL caused blood-brain barrier (BBB) dysfunction. These changes do not resemble major aspects of AD pathology. It has been shown that AD is associated with neurotrauma, such as head injury, and that excitotoxic processes cause similar degenerative changes. Also, Aβ renders cultured neurons vulnerable to excitotoxic damage. Therefore, if dysfunctional
lysosomes and iAβ represent a risk factor for AD, excitotoxins such as kainic acid (KA) may combine synergistically with lysosomal inhibitors. Hence, CHL (over a range of systemic doses and i.c.v.; 1 week, mice and rats) was combined with KA followed by a 1 week survival period; multiple behavioral and histological analyses were conducted at several time points. Accumulation of iAβ was evident at high doses, as was astrocytic activation. No deficits in sensorimotor ability (balance, muscle strength, vision, exploratory behavior) were detected. However, animals given KA and doses of CHL sufficient to cause iAβ accumulation were markedly impaired on the Morris watermaze test of spatial learning and exhibited increased limbic system damage, particularly in the amygdala. These results indicate that although iAβ may not be immediately harmful, it may nevertheless be coupled to a pathogenic process relevant to the development of AD and should be further examined in the context of the development of appropriate treatment strategies.
ACKNOWLEDGMENTS

Next to the torture of constructing a coherent introduction out of the roughly 6000+ AD related articles in the past 5 or 6 years, I could make a case for this being one of the toughest sections to write. It’s difficult to find the right words to do justice to the contributions that everyone has made to this work (ordeal?), and at times it seems like I’m selling them short. On the other hand, I also feel like I’m giving an acceptance speech at the Academy Awards, and that I’m going to be dragged off the stage at any minute. So......be patient, it’s a long one.

My loving wife Sharon has to appear at the top of the list. For her, the words don’t exist to describe how good she’s been throughout this whole experience. I’ve been told that if your marriage can survive a thesis, there just ‘ain’t much more to worry about. You’ve stuck with me through the whole arduous journey - the long hours, the sacrifices, and my (sometimes very) short temper. Kitten, I love you all the more for your patience, and look to the future with the comfort that you’ll always be there for me (and that we can spend more time together, after I do all those dishes).

Although they may not know it, over the years my friends have provided indispensable support and encouragement. Thanks to Bruce, Doug, Faiz, Jared, Jason and Steve for getting me through those critical formative years and helping me to develop my creative edge, as well as fostering my skills of global domination. I owe a special debt to Steve for allowing me ample practice at those special abilities that all Irishmen need, as well as much of my Pythonesque sense of humor and affinity for lampposts. Well done, Brother Fat Man!

Thanks are also in order for all of the regular Euchre and weekend crowd, for providing much needed distraction and all round good times. Thanks to Brian and Crystal Leila for all those dusk-‘til-dawn card sessions (in spite of Crystal’s frightening dominance), and Marc and Leona Nicholas for some of the most memorable pub crawl moments (the best of which, unfortunately, cannot be mentioned in print). You guys are great, and I would’ve went totally mental without you.

I would never have made it this far if it weren’t for the mutual love and support of my parents, Michael and Sylvia Murphy, and their patience with their son (the perpetual
student) in letting me find my own path. My other set of parents, Jennie and Jim Sword, have been terrific in lending that little bit of extra encouragement when needed. Over the years, you’ve all shown genuine interest and enthusiasm for my sometimes cryptic studies, and let me know that I wasn’t working in a vacuum.

My sister, Joanne McCluskey, has always been there for me when I needed her. From the .... aggressive interactions of our youth (and all those times that I forced her to endure Dr. Who), to all those long hours at the zoo, introducing me to bingo, and being one of my closest friends and confidants - love ‘ya, Acha! In the same breath, I couldn’t have asked for a better brother in law in Paul. For my eternal comrade in numerous video game extravaganzas (thanks to Jo for permitting all those hours at the TV, with only occasional psychotic explosions - wocka-wocka-wocka), the many memorable laughs (Mr. Snuffalopagos, ah, comes to mind - better get a life preserver!), and all the late night conversations, I have only these words of wisdom: Always gain ground, show no mercy, and remember - you .... are my number one .... *guy*!

I probably would never have made it as a grad student if it weren’t for my close friend and nearly constant companion, Liz (now Dr.) Head. It still amazes me that after all these years we can still get together and talk (more like free association, as you say) for 12+ hours. Notice how the campus pub collapsed after we stopped going - coincidence? I think not. Magic cards, cribbage, bingo, Washington, Miami, hurricanes (real and the bar), pumpkin carving, Washington (again, but better), and a legendary pub crawl through the streets of Toronto......shall I go on? Hey! Take care, MFB.

While still on the lab front, thanks to all those who’ve been my comrades and companions over the years. To those that are long gone, Dr. Mona Sazgar, Mark Hlousek, Andrew Kohn, Janina Ferbinteanu and Chris Reid......thanks for the memories? Thanks to Mike Michael for all the enlightened philosophical banter, and Dr. Moshe Khurgel for showing me the ropes and for some unforgettable Neuroscience Meeting moments (particularly in wind swept Miami). For John Rick, thanks for the many years of good conversation in lounge number 2, the months of gaming (no doubt delaying both our dissertations), and, uh, teaching me the compulsive side of science. And where would I be without my fellow gossip monger and sometimes roommate, Candace Douglas? Insane, perhaps? Good luck - I already miss you.
This work would not have been possible without the friendship and lengthy technical assistance of John Mielke (to the Milgram lab!) and Suzi Barsoum (friend-who-is-a-girl), whose loyalty to the project was never questioned. Thanks for patiently listening to all my manic ranting and giving me years of more fun than one person should have in a lab o’ rats (roll Conan soundtrack, please). Many others have contributed to various components of these studies: Alice Koo, Monika Kaila, Jonathan Maritz, Karen Bengualid, Asheel Sharma, Rahim Hirji, Rishi Narine, Christina Beckett, Amin Ladak, Deva Thiruchelvam, and Hance Clarke. Thanks also to latecomers Lara Chebaro and Luke Shannon for filling in the details, the interesting textures, and the indispensable humor. Thanks to the Wild Strawberries, Björk, Depeche Mode, New Order and Erasure for providing the atmosphere.

Much thanks to Dr. Bill Milgram, who’s been a good friend and colleague, and has yielded many items of good advice over an equal number of good beers. Bill, as my first exposure to the field of neuroscience, I owe you a great deal. Mind you, this was as I dozed contentedly in Brain Mech I after working my night job. Time, I must say, that’s been made up many fold since.

Gwen, you’ve been more than I ever could’ve expected of an advisor - you’ve been a good friend. I can’t think of many supervisors that would allow a student so much freedom, while simultaneously providing those crucial little pushes through an often bewildering scientific quagmire (oooo.....metaphor!). We’ve had some unforgettable laughs - and some truly impressive parties (remember Chris and the 3 hour thing?). Here’s to many fruitful future collaborations, a book, and a one hour television special.

Finally, thanks to all the members of my committee for their helpful advice and guidance. Thanks also to Drs. Yasuo Ihara (Univ. of Tokyo), Dora Games and Dale Schenk (Athena Neurosciences) for providing antibodies, Dr. Mark Dubach (Univ. of Washington) for the primate surgical work, and Dr. Sayeeda Zain (Univ. of Rochester) for the transgenic mice. I’d also like to extend my gratitude to Deprenyl Animal Health and the Ontario Graduate Scholarship program that allowed me to start and complete my graduate studies (respectively) at the University of Toronto, and the Natural Sciences and Engineering Research Council of Canada which provided the direct support for most of the following work.
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LIST OF ABBREVIATIONS

αACT  Alpha₁-Antichymotrypsin
Aβ    Alzheimer’s Beta-Amyloid Peptide (general)
Aβ₄₂  Alzheimer’s Beta-Amyloid Peptide (number denotes subtype)
ACh   Acetylcholine
AD    Alzheimer’s Disease
AHip  Amygdala/Hippocampal Transition Area
ALS/PDC Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex
APir  Amygdala/Piriform Transition Area
ApoE  Apolipoprotein E (general)
ApoE4  Apolipoprotein E [number(s) denotes subtype and/or genotype]
βAPP  Beta-Amyloid Precursor Protein (general)
βAPP₆₉₅ Beta-Amyloid Precursor Protein (number denotes subtype)
BBB   Blood-brain Barrier
CHL   Chloroquine
CL    Ceroid Lipofuscin
CNS   Central Nervous System
CP    Compact or Burnt-Out Plaque
CSPG  Chondroitan Sulfate Proteoglycan
DP    Diffuse Plaque
EAA   Excitatory Amino Acid
EC    Entorhinal Cortex
ECM   Extracellular Matrix
EEG   Electroencephalogram
FAD   Familial Alzheimer’s Disease
fβAPP Fragments of βAPP containing Aβ
FD    First Discharge
FS    First Seizure
<table>
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<tr>
<td>SP</td>
<td>Senile Plaque</td>
</tr>
<tr>
<td>SSF</td>
<td>Single Straight Filaments</td>
</tr>
<tr>
<td>τ</td>
<td>Tau Protein</td>
</tr>
<tr>
<td>TG</td>
<td>Transgenic Mouse</td>
</tr>
<tr>
<td>veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VMCo</td>
<td>Ventromedial Cortical Nucleus</td>
</tr>
<tr>
<td>WM</td>
<td>Watermaze Test</td>
</tr>
<tr>
<td>WS</td>
<td>Wire Suspension Test</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type Mouse</td>
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SECTION A:

GENERAL INTRODUCTION AND HYPOTHESIS
Alzheimer’s disease (AD), the most common cause of dementia in the elderly, is characterized neuropathologically by two major features: neurofibrillary tangles (NFT) and senile plaques (SP). Both elements are weakly associated with symptoms of cognitive deterioration, since the direct cause of the cognitive dysfunction is the loss of neurons and synapses. This neurodegenerative process is probably related to one, or both, of these features. Only SP can be considered to be specific for AD, since NFT-like pathology is common in many disorders. Existing genetic evidence provides strong support for the importance of the β-amyloid peptide (Aβ), the principal component of SP, in the disease process. Overexpression of the β-amyloid precursor protein (βAPP) in Down’s syndrome leads to the development of AD-like pathology at a young age. Further, several mutations in the vicinity of the Aβ portion of βAPP are known to cause the disease. Other genetic linkages to chromosomes 19 (apolipoprotein E), 14 (presenilin 1) and 1 (presenilin 2) have also been shown to either influence or cause (in the case of the presenilins) AD, and may do so via interactions with Aβ. Hence, most research has focused on the processing and deposition of Aβ as the cause of AD.

Another possibility relates to the presence of intracellular Aβ (iAβ). Logically, iAβ must exist at some point, since no evidence has been uncovered to indicate that the peptide is generated extracellularly. Given the acknowledged importance of Aβ and that SP are such a prevalent lesion in the disorder, most research efforts have concentrated on the investigation of the extracellular Aβ deposits. However, the possibility has never been directly addressed that the peptide exerts its toxic effects from an intracellular location, resulting in neuronal dysfunction and death. In the case of AD, this could result in the liberation of the insoluble Aβ peptide, which may then seed the subsequent growth of SP. These extracellular deposits could then drive the pathology by promoting additional insults through such mechanisms as chronic inflammation.

This thesis had two main goals dealing with the examination of iAβ. First, is iAβ directly damaging to neurons? If so, can this damage lead to the formation of SP in suitable subject populations? This is the direct or causal hypothesis of iAβ toxicity. Examination of the alternative hypothesis was the second goal of this thesis. That is,
even if iAβ is not directly toxic, it may impart increased susceptibility to injury or neurodegeneration caused by another factor. I call this the indirect or permissive hypothesis of iAβ toxicity. Both of these hypotheses will be addressed using a combination of histology, immunohistochemistry and behavioral analyses.

The endosomal/lysosomal system can be perturbed through the administration of lysosomal inhibitors, such as chloroquine and leupeptin, and this manipulation leads to the intralysosomal accumulation of a lipofuscin- or age pigment-like substance in vivo. Hence, this treatment has been hypothesized to create artificial cellular aging. Such a manipulation also leads to the intracellular accumulation of Aβ and potentially amyloidogenic fragments of βAPP (fβAPP) in vitro, but has not been assessed for this purpose in vivo. Since lysosomal inefficiency leading to lipofuscin accumulation is characteristic of the aging process, and since AD is closely associated with increasing age, it is therefore possible that the two processes are linked in a causative manner. Therefore, the direct brain administration of both of these compounds (chloroquine and leupeptin) was explored as a means to induce the formation of iAβ in vivo and to examine the consequences of this induction in experiments 1 and 2. This manipulation did not lead to any manifestations of AD-like pathology (making the direct hypothesis of iAβ toxicity unlikely), but did cause increased iAβ immunoreactivity.

The indirect hypothesis of iAβ toxicity was examined in the remainder of the thesis. One likely candidate for a precipitating factor is an excitotoxic mechanism, since Aβ is known to amplify this process in vitro. Hence, additional experiments were conducted to determine if intracellular Aβ (induced via the administration of lysosomal inhibitors) could increase the risk for excitotoxic damage following administration of an excitotoxic compound, kainic acid. Induction of iAβ was accomplished in two ways: by direct brain infusion of chloroquine (experiment 3) or via a systemic route (experiments 4-7). Injection of kainic acid following this treatment lead to increased neuronal loss and a deficit in spatial learning and memory.

The findings of this thesis thus indicate that iAβ, although it may not be directly or immediately toxic, may nevertheless be an important element in the degenerative process and should thus be further explored as a possible target for future therapeutics.
A2 Neuropathology of Alzheimer’s Disease

A2.1 Background

Alzheimer’s disease (AD) is a crippling neurodegenerative disorder that is closely associated with aging in humans. The incidence rate of the disorder has been estimated to be as high as 123 new cases per 100,000 individuals/year, or approximately 10% of those persons over the age of 65 and 40% of those over the age of 85 (Evans et al, 1989). Death usually occurs within 5-10 years of the age of onset (McKhann et al, 1984). This age-adjusted reduction in life expectancy (2.6% survival versus 16.6% expected; Mölsä et al, 1995) renders AD the 4th leading cause of death in the developed world, following cancer, heart disease and stroke (Evans et al, 1989). Given the steady increase in the proportion of elderly individuals in the population, AD is likely to become an ever greater societal burden in the coming century.

Alzheimer’s disease is the most common cause of dementia in the elderly, accounting for slightly more than 50% of all cases (Katzman, 1986). Most of the remaining cases (about 33% of the total) are made up of individuals with vascular or multi-infarct dementia (MID; Alafuzoff et al, 1987b), although some overlap probably exists. Another 10% of demented patients can be classified as frontotemporal dementia, which includes nonspecific frontotemporal degeneration, frontal degeneration with anterior spinal neuron loss, and Pick’s disease (Brun et al, 1994; Mendez et al, 1996). The small portion of residual dementias can be attributed to other causes, both primary (e.g., Korsakoff’s syndrome, Huntington’s disease, etc.) and secondary (e.g., tumors, metabolic encephalopathies, etc.)(see Fig. A2-1).

For a disorder with such broad economic implications, it is astonishing that so little is known about AD etiology. No association has been found between the incidence of AD and type of dwelling, occupation, gender or marital status (Beard et al, 1992; Breteler et al, 1992). Although it has been postulated that diet (particularly a Western style diet rich in saturated fats) is an important factor (Newman, 1992), actual evidence for this is slim. In fact, much of the variation in incidence rates between different cultures is probably attributable to differences in diagnosis. In Western countries, approximately 2/3 of demented elderly patients are diagnosed with AD, while in Asia the figure is very
A2-1. Distribution of types of dementia in the elderly. Although AD is by far the most common, most other cases of dementia can be grouped into the broad categories of vascular/multi-infarct or frontotemporal dementia.

close to 1/3 (Breteler et al, 1992). In contrast, a Shanghai study in which the participating diagnosticians were either trained in the United States, or trained by U.S. practitioners, classified 2/3 of demented patients as AD cases (Zhang et al, 1990).

Much has been made of an apparent association between AD and low education or illiteracy (Mortimer and Graves, 1993). However, this association is typically found in women (Beard et al, 1992; Zhang et al, 1990), and may be related to lower levels of education in elderly women in general, due to the societal trends dominant earlier this century (Beard et al, 1992). Further, although it is possible that educated individuals have greater cognitive "reserves" to protect them from AD, or that they are less vulnerable due to higher levels of intellectual stimulation, perhaps education in this sense is serving as a surrogate variable for differences in life-style and general health associated with socioeconomic status (Breteler et al, 1992; Mortimer and Graves, 1993).
The most robust factor linked to AD (apart from age and family history of the disease) is a prior history of head trauma involving loss of consciousness. Estimated increase in relative risk is about 80%, which could account for 2-20% of all cases of AD (Mortimer et al, 1985, 1991). Unlike other environmental factors implicated in AD etiology (such as aluminum exposure; McLachlan et al, 1996; c.f. section A2.2, below), head trauma has received significant experimental support. For instance, individuals suffering a single head injury leading to a loss of consciousness exhibit numerous neuropathological changes resembling early phases of AD when examined from 4 hours to 2.5 years later (Roberts et al, 1991, 1994; Newman et al, 1995). Similarly, patients suffering from dementia pugilistica, believed to be caused by repeated blows to the head, possess lesions characteristic of AD (senile plaques, neurofibrillary tangles, neuropil threads and congophilic angiopathy; Roberts et al, 1990; Tokuda et al, 1991).

The earliest phase of AD resembles a myriad of other neurological and psychological conditions. Often, it is the patient who first notices the abnormalities, which are best characterized as “diffuse”. Common complaints include tiredness, poor concentration, anxiety, depression and paranoia; as the disease progresses, additional symptoms indicative of temporoparietal degeneration become apparent (Gustafson et al, 1995). Detailed consensus criteria for AD diagnosis have been published (McKhann et al, 1984; Khachaturian, 1985), as have methods for distinguishing MID (Hachinski et al, 1975) and frontotemporal dementia (Brun et al, 1994; Mendez et al, 1996) from AD.

In later stages of the disorder, AD is characterized by a complex pattern of cognitive deterioration. Currently, a preliminary diagnosis of AD is made based on an extensive (and costly) battery of neuropsychological and neurological tests designed to exclude other causes of dementia. Patients are first given a series of standardized tests designed to assess global cognitive function, such as standard IQ tests and the Mini-Mental State Exam, or MMSE (Folstein et al, 1975). The earliest signs of decline are usually seen on subtests of these composite indices that measure episodic or explicit memory (such as word/story recall or reproduction of a complex geometric figure) and attention (such as the digit-symbol test or the trail-making test)(Grady et al, 1988; Almkvist et al, 1995; Growdon, 1995). Additional tests are often administered to determine the patient’s ability to function independently and interact normally in society,
and these indices also show a steady deterioration over the course of the disease (Feldman et al., 1995). In more advanced cases, a constellation of additional symptoms begin to appear: dysphasia (loss of language ability), dyspraxia (loss of manual dexterity), dysgnosia (loss of knowledge), and spatial disorientation (Gustafson et al., 1995). In about 30% of these cases, noticeable sensorimotor impairment is also present; in some individuals (6-7%), the extrapyramidal symptoms may be severe enough to warrant a concurrent diagnosis of Parkinson’s disease (Growdon, 1995). In the terminal stage, serious psychological (delusions and psychotic episodes) and neurological (seizures and myoclonic twitches; Hesdorffer et al., 1996) disturbances may be present, and the patient is completely unable to care for themselves (Gustafson et al., 1995).

Despite considerable improvement in diagnostic accuracy in recent years, correct clinical diagnosis of AD currently hovers at approximately 80% (DeKosky et al., 1992; Growdon, 1995). At autopsy or biopsy, individual cases are evaluated based on well-documented anatomical criteria. For AD, this involves a determination of the density and distribution of senile plaques and neurofibrillary tangles. In combination with available clinical data, a diagnosis can be delivered once all other likely causes of dementia are excluded (Khachaturian, 1985; Alafuzoff et al., 1987b; DeKosky et al., 1992). Clearly, a better means of assessment is required if future therapeutic measures are to be administered effectively. Although neuroimaging technology is improving rapidly, it is of little use in diagnosing AD, except for the exclusion of other neurological causes of dementia (Growdon, 1995). One other possibility may be early detection by determining pupilar dilation hypersensitivity to dilute cholinergic agonists (such as tropicamide, a synthetic analog of atropine), which are routinely used in ophthalamogic exams. The enhanced mydriatic response shown by AD patients may be used to discriminate between AD and otherwise normal aged individuals, as well as subjects with dementia stemming from other causes (Scinto et al., 1994).

A2.2 General Pathology

A2.2.1 Neuronal Loss

At the gross anatomical level, AD patients have been shown to exhibit gyral atrophy, dilation of the cerebral ventricles, and a decrease in overall brain weight - all of
which are probably related to neuronal death, which may be more than 50% in some cortical and subcortical areas (Kemper, 1984; Hyman et al, 1995; Gómez-Isla et al, 1997). Similarly, there is also a significant loss of synapses (Lassmann, 1996) and a reduction in dendritic arborizations and spine density (Mehruein et al, 1975). Accentuated destruction of myelinated fibres throughout the brain is evident, particularly the longer corticocortical association fibres (Kemper, 1984) and axons in the olfactory tract (Davies et al, 1993). The damage can be either Wallerian (where the axis cylinder is fragmented and degenerating) or a primary demyelinating event (Terry and Wisniewski, 1972). This leads to a functional disconnection of numerous neocortical areas. For example, AD patients show a marked reduction in electroencephalographic (EEG) coherence between activity in the parietal and frontal cortices, which are normally strongly connected via the superior longitudinal fasciculus (Leuchter et al, 1992). In a similar manner, loss of specific populations of neurons can also leave some areas functionally disconnected. Of greatest relevance to AD are the loss of neurons of the hippocampus (especially CA1), subiculum and entorhinal cortex (layer II stellate cells, among others), creating a situation in which the hippocampal formation is isolated from both input and output, and may be functionally useless in AD patients (Hyman et al, 1984; Van Hoesen et al, 1991; Braak and Braak, 1985, 1991, 1993; Nitsch, 1993).

End-stage AD appears to affect most of the brain. However, although most neurotransmitter systems are dysfunctional to some extent (Selkoe and Kosik, 1984), not all may be affected equally, particularly in the case of acetylcholine (ACH; Davies and Maloney, 1976; Whitehouse et al, 1982; Coyle et al, 1983; DeKosky et al, 1992). Similarly, Parkinson’s disease patients presenting signs of dementia (and other signs of AD-like pathology) may show some loss of basal forebrain neurons (Whitehouse et al, 1983). Although cholinergic neurons of the basal forebrain (especially the nucleus basalis of Meynert) were originally thought to be lost in AD, a significant amount of neuronal “loss” may actually be cell atrophy and shrinkage (Salehi et al, 1994).

A2.2.2 Vascular Pathology

Most AD patients (80-90%) exhibit congophilic angiopathy, which is a characteristic red-green birefringence of the cerebrovasculature observed when tissue
samples are viewed under polarized light following staining with the dye Congo red. This is caused by a deposition of amyloid fibrils within the walls of the blood vessels (Glenner et al, 1981; Ko et al, 1991). Perivascular microglia, pericytes, and myocytes have all been implicated in this process (Wisniewski et al, 1992; Wisniewski and Wegiel, 1994). It has frequently been suggested that AD is related to a disturbance of the vascular system, eventually leading to a break down of the blood-brain barrier (BBB; Glenner et al, 1981). Indeed, the β-amyloid peptide (Aβ), the primary constituent of senile plaques and angiopathic deposits within the CNS microvasculature, has been shown to directly damage the vascular endothelium and cause severe degenerative changes in endothelial cells (Thomas et al, 1996). It is therefore not surprising that AD patients show a striking reduction in the density of CNS microvessels, with those remaining appearing twisted, tortuous (occasionally possessing loops or kinks) and fragmented with a significant loss of branching (Fischer et al, 1990; Buée et al, 1994). Also, major components of the vascular basement membrane (laminin, collagen type IV and heparan sulfate proteoglycan; Perlmutter et al, 1991) and serum proteins such as immunoglobins (Ishii et al, 1975; Ishii and Haga, 1976), albumin (Wisniewski and Kozlowski, 1982), complement factors (Alafuzoff et al, 1987a), and amyloid P component/α1-glycoprotein (Kalaria and Grahovac, 1990) are found within senile plaques, further implicating the involvement of the CNS circulation and a dysfunctional BBB. Although some of these proteins may be manufactured locally within the brain, amyloid P component is made only in the liver (Kalaria and Grahovac, 1990). Nevertheless, any compromise of the BBB is likely to be transient rather than continuous, since direct measurements of BBB integrity in vivo have failed to consistently demonstrate increased permeability (Mooradian, 1988; Dysken et al, 1990).

At the ultrastructural level, normal aged human brain possesses characteristics indicative of a fully functional BBB (Stewart et al, 1987; Mooradian, 1988). In AD, scanning electron micrographs reveal that many cerebral blood vessels have a lumpy, nodulated appearance, with about 50% of patients also showing pitting or lacunae in the vessel walls (Scheibel et al, 1989). The pathology is much more subtle in transmission electron microscopy, where the major alterations are increases in the number and/or
size of pericytes, reduced mitochondrial density, and an increase in the size of junctional clefts between endothelial cells (Stewart et al., 1992). The altered pericyte profile may be indicative of a chronically faulty BBB, since pericytes are known to enter a phagocytic state and become hypertrophic in response to BBB breakdown (Van Deurs, 1976). Although the bulk of the evidence points towards a vascular abnormality of some form, the results are not always compelling (Mooradian, 1988). One possibility may be that AD patients with the most severe BBB/vascular dysfunction are those that have additional mild vascular disorders that might often go undetected (Blennow et al., 1990).

**A2.2.3 Other Minor Pathologies**

Two additional abnormalities found in AD are granulovacuolar degeneration (GVD) and the Hirano body (Kemper, 1984). Granulovacuolar degeneration is typically observed intracytoplasmically in the hippocampus (usually CA1, more common in posterior areas; Ball, 1977). Granulovacuolar degeneration manifests as transparent vesicles, from 1-5 μm in diameter, with a dense central granule (about 0.5-1.5 μm in size) which may resemble lipofuscin. A clear relationship does not exist between GVD and other, more specific AD pathologies (Kemper, 1984). The Hirano body, an eosinophilic spindle shaped structure approximately 15 μm long and 30 μm in diameter, can also be found within the hippocampus (again, CA1), although it may occur extracellularly as well as intracellularly (Hirano et al., 1968). As with GVD, its relationship to other aspects of AD is largely unknown.

**A2.2.4 Diagnostic Pathologies**

All of the above pathologies are also found, to a greater or lesser degree, in other neurological disorders besides AD (Hirano et al., 1968; Kemper, 1984). The characteristic anatomical hallmarks that define AD are the extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFT). Although it is essentially certain that the downstream pathology of neuronal (and synapse) loss is largely responsible for the actual symptoms of cognitive deterioration, it is the combined presence of SP and NFT
at high levels that confirm the presence of Alzheimer's disease (Okazaki, 1989; Khachaturian, 1985).

A2.2.4.1 Neurofibrillary Tangles

Neurofibrillary tangles occur most frequently in the entorhinal cortex (EC), subiculum, and hippocampal formation (Brady and Mufson, 1991; Braak and Braak, 1985, 1991, 1993). The pathology first appears in the transentorhinal area, then progresses to the rest of EC, followed by the hippocampus. In advanced cases of AD, neocortical association areas are severely affected (Braak and Braak, 1991), as are some hypothalamic nuclei (Swaab et al, 1992) and the nucleus basalis of Meynert (Iqbal and Grundke-Iqbal, 1991). Locally, the distribution of NFT displays several characteristic patterns. For example, area CA1 is more vulnerable than other hippocampal cell fields (Braak and Braak, 1991, 1993), and EC layer II stellate neurons are more prominently affected than other EC neurons (Brady and Mufson, 1991; Braak and Braak, 1985, 1991, 1993; Van Hoesen et al, 1991). Although normally an intracellular lesion found in the somatodendritic compartment of neurons (Kosik et al, 1989), NFT are also found extracellularly as isolated "ghost tangles", which are believed to be the insoluble remnants of deceased cells (Beyreuther and Masters, 1996). Another common pathological feature of AD, neuropil threads, are nearly identical to NFT (Braak et al, 1986), although they are localized dendritically (Braak and Braak, 1988).

Neurofibrillary tangles contain two primary elements: single straight filaments (SSF) and paired helical filaments (PHF), with the latter being the major component (Kidd, 1963; Shibayama and Kitoh, 1978; Goedert et al, 1995). Perikaryal NFT are composed mostly of PHF, whereas those in the neuronal processes contain an approximately equal mixture of the two types (Yagishita et al, 1981). Single straight filaments have a caliber of approximately 10-15 nm (Shibayama and Kitoh, 1978; Goedert et al, 1995, 1996), whereas PHF have a maximum diameter of about 20 nm, a minimum diameter of between 2-9 nm at the constrictions, and a twist period of about 160 nm (Ruben et al, 1992; Goedert, 1995). Although the original term based on the appearance of PHF on electron micrographs (Kidd, 1963) implies the existence of a pair of intertwined filaments, recent evidence indicates that the fine structure of PHF is
probably a slightly irregular, single-stranded twisted helical ribbon (Pollanen et al, 1994; Ruben et al, 1995).

The PHF are composed almost entirely of the microtubule-associated protein tau (τ) (Grundke-Iqbal et al, 1986a), which is highly polymerized (Ruben et al, 1991), abnormally phosphorylated (Iqbal et al, 1986), and tagged with ubiquitin (Mori et al, 1987). The abnormal state of phosphorylation is likely responsible for abnormal microtubule assembly in AD (Iqbal et al, 1986). Tau is now known to be "hyperphosphorylated", since treatment of τ isolated from brains of AD patients with alkaline phosphatase reduces the apparent molecular weight of AD-τ to that of normal τ (Lee et al, 1991), restores its ability to promote microtubule assembly (Iqbal et al, 1994), and enables or disables labeling with phosphorylation sensitive antibodies (Grundke-Iqbal et al, 1986b; Drewes et al, 1992). Over-phosphorylation of τ increases the proportion of β-sheet composition, which may be related to the reduction in the affinity of τ for microtubules and its propensity towards aggregation and insolubility (Lang et al, 1992). Ubiquitin, which tags cytosolic proteins for degradation (Reichsteiner, 1987), is thought to be a late addition in the development of PHF pathology, since hyperphosphorylation of τ and the formation of early PHF filaments precedes ubiquitination (Bancher et al, 1989a, 1991).

The discovery that the AD brain contained high levels of aluminum in areas associated with large amounts of neurofibrillary pathology, and that administration of aluminum salts in cats produced neurofibrillary degeneration and impaired learning (McLachlan et al, 1973), initially generated intense excitement as a possible environmental pathogen for at least this aspect of AD. Later studies confirmed that NFT bearing neurons in AD did, in fact, contain significant amounts of aluminum (Perl and Brody, 1980). Further, the South Pacific island of Guam, which has inordinately high levels of aluminum in both its soil and drinking water, possesses an endemic disorder (Guam amyotrophic lateral sclerosis and Parkinsonism-dementia complex - Guam ALS/PDC; Hirano et al, 1968) that is characterized by large numbers of NFT that are similarly accompanied by intraneuronal accumulations of aluminum (Perl et al, 1982). However, the experimental neurofibrillary pathology produced by aluminum intoxication
differs markedly from that of AD, in that the filamentous inclusions are 10 nm straight filaments composed of medium and high molecular weight neurofilament proteins rather than τ (Savory et al, 1995), although they are hyperphosphorylated. As for Guam ALS/PDC, the disorder is now believed to be caused by an excitotoxic compound (L-BMAA) from a native species of cycad present in both the diet and medicinal preparations of the native Chamorro (Spencer et al, 1987). In any case, even though the NFT found in Guam ALS/PDC have been shown to be indistinguishable chemically, histologically, and ultrastructurally from those of AD, the presence of SP in these patients is not a feature of the disorder (Hirano et al, 1968; Mawal-Dewan et al, 1996). Hence, the presence of aluminum in NFT is probably not directly related to AD pathology. Aluminum may become associated with tangle-bearing neurons at a later stage, at which point it may contribute to subsequent degenerative changes. This may explain why it is still found to be epidemiologically linked to AD (McLachlan et al, 1996).

The six primate τ isoforms, ranging in length from 352-441 amino acids, are produced from a single gene by alternative mRNA splicing (Goedert et al, 1995). Mice, in contrast, appear to possess only two isoforms (Lee et al, 1988). Three of the human isoforms possess 3 microtubule binding domains (both murine forms also have 3 binding domains) localized to the C-terminal region, while the remaining three possess 4; all 6 are found in NFT (Goedert et al, 1995). Although it has long been known that PHF antibodies are somewhat crossreactive with SSF, only recently has it been determined that both are composed of abnormal τ: PHF are generated from isoforms containing 3 microtubule binding domains, and SSF are generated from those containing 4 (Goedert et al, 1996). The glycosaminoglycan (GAG) component of heparan sulfate proteoglycan (HSPG), which is normally an extracellular matrix protein, may contribute to the formation of both types of filaments at an early stage (Goedert et al, 1996).

The actual enzymes involved in the over-phosphorylation of τ are not known, and most protein kinases are actually reduced in AD (Saitoh et al, 1991). However, the over phosphorylation appears to occur mainly at serine-proline sites, raising the possibility that a proline-directed serine/threonine kinase is involved (Drewes et al, 1992). For example, mitogen-activated protein kinase p42 (Drewes et al, 1992) and glycogen-
synthase kinase-3 (Mandelkow et al., 1992), both of which are found associated with microtubules, are capable of converting normal \( \tau \) into an AD-like state. Interestingly, both of these enzymes are activated by tyrosine phosphorylation and are involved in signal transduction processes (Mandelkow et al., 1992).

A2.2.4.2 Senile or Neuritic Plaques

Unlike NFT, the distribution of SP is not well defined, with early patterns of deposition exhibiting considerable individual variability, in both global and laminar distribution (Braak and Braak, 1991, 1993). The earliest regions affected are basal areas of the frontal and temporal lobes, especially the amygdala, followed by nearly all neocortical association areas, hippocampal complex, and then finally the primary sensory cortices (Braak and Braak, 1991). Subcortical regions (such as the thalamus and hypothalamus) are affected only in more advanced cases, and diffuse plaques may also appear in the striatum and cerebellar cortex (Braak and Braak, 1991).

The generic term amyloid refers to a group of diverse (but disease specific) deposits of extracellular protein found in several disorders (such as the prion disorders Creutzfeldt-Jakob disease and Kuru), of which AD is only one. All amyloid deposits share certain features, the most common of which is a large proportion of \( \beta \)-sheet structure, as seen by \( \beta \)-sheet sensitive dyes such as Congo red and thioflavin S (Kisilevsky, 1987). Senile plaques contain, as their major constituent, a 39-43 amino acid peptide called the Alzheimer \( \beta \)-amyloid peptide, or A\( \beta \) (Glenner and Wong, 1984; Masters et al., 1985). All types of AD related amyloid deposits contain some form of this peptide, including those of congophilic angiopathy and the plaques found in Down’s syndrome, which is intimately related to AD (Masters et al., 1985).

The earliest type of plaque consists of a small diffuse deposit of A\( \beta \) (DP I; Yamaguchi et al., 1988a,b), which may then evolve into a larger diffuse deposit (DP II; Yamaguchi et al., 1988a,b, 1989, 1991; Rozemuller et al., 1989) and/or more advanced types. The more advanced forms of SP include primitive plaques (PP; a diffuse deposit of A\( \beta \) with some reactive glia and degenerating neurites), classic or neuritic plaques (NP; a core of condensed A\( \beta \) surrounded by a halo of degenerating neurites and
reactive glia), and burnt out or compact plaques (CP; dense core of Aβ surrounded by an area largely devoid of cellular elements) (Terry and Wisniewski, 1972; Wisniewski and Terry, 1973). The proposed scheme of plaque evolution is based primarily on the relative proportions of the different plaque subtypes (Rozemuller et al, 1989). Although actual estimates vary, the typical values are approximately 80% DP (both types), 15% PP, 4% NP and 1% CP (Tagliavini et al, 1988; Giaccone et al, 1989; Rozemuller et al, 1989; Yamaguchi et al, 1989). As SP progress along this path at least 40 distinct elements can be detected at various stages, of which only those believed to be of pathological significance will be discussed here (see Table A2-1).

Small diffuse plaques contain only the Aβ peptide in a non-fibrillar or pre-amyloid form lacking the characteristic argyrophilic and congophilic staining characteristics of the more advanced plaque subtypes, likely indicating minimal amounts of β-sheet structure (Yamaguchi et al, 1988b; Rozemuller et al, 1989). As the Aβ deposit becomes larger, some prominent astroglial processes and rare degenerating neurites may be detected (Yamaguchi et al, 1991), along with the appearance of a series of other biochemical markers.

The plaque associated markers found within DP (usually type II only) obviously suggest that at least some of them may be involved in early phases of amyloidogenesis (c.f. section A3.2, below and table A2-1). The low-density lipoprotein transport molecule, apolipoprotein E (ApoE) appears in larger diffuse deposits of Aβ (Namba et al, 1991), and may be part of a pathway involved in amyloid clearance. The extracellular matrix protein heparan sulfate proteoglycan (HSPG; Snow et al, 1988, 1990) may be involved in fibril stabilization and contribute to the insolubility of Aβ (Snow and Malouf, 1993). The recent finding that HSPG may also be involved in the formation of both types of filaments found in NFT strengthens the possible relationship between this macromolecule and AD pathology (Goedert et al, 1996).

The acute phase response protein α-1-antichymotrypsin (αACT; Abraham et al, 1988; Rozemuller et al, 1991; Shoji et al, 1991), inflammatory cytokines IL-1 (Griffin et
Table A2-1. Senile Plaque Subtypes and Components

<table>
<thead>
<tr>
<th>Type</th>
<th>Frq.</th>
<th>Aβ</th>
<th>Components/Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse (DP)</td>
<td>80%</td>
<td>Diffuse</td>
<td>HSPG, ApoE, αACT*</td>
</tr>
<tr>
<td>Primitive (PP)</td>
<td>15%</td>
<td>Diffuse</td>
<td>reactive glia, dystrophic neurites, inflammatory markers</td>
</tr>
<tr>
<td>Neuritic (NP)</td>
<td>4%</td>
<td>Core</td>
<td>highly amplified PP characteristics, lysosomal enzymes</td>
</tr>
<tr>
<td>Compact (CP)</td>
<td>1%</td>
<td>Core</td>
<td>lesion devoid of most cellular elements; also lacks enzymes and inflammatory markers (&quot;burnt out&quot;)</td>
</tr>
</tbody>
</table>

*These 3 elements are also found in all subsequent SP subtypes

al, 1995) and IL-6 (Huell et al, 1995), activated complement (Eikelenboom et al, 1991; McGeer et al, 1989; Lue et al, 1996) and serum amyloid P component (Rozemuller et al, 1989; Kalaria and Grahovac, 1990) all appear in later stages of DP II and PP. A patchy distribution of ICAM-1, a leukocyte adhesion ligand expressed constitutively on the surface of endothelial cells and upregulated in inflammatory states, also occurs throughout the NP subtype (Akiyama et al, 1993; Verbeek et al, 1996). McGeer and McGeer (1995) have proposed that these findings suggest that a state of chronic inflammation may be connected to the evolution of the neuropathology. Potentially, this hypothesis could explain the consistent epidemiological connection between increased risk of AD and history of head trauma (Mortimer et al, 1991), the observation that patients undergoing anti-inflammatory therapy for chronic disorders such as rheumatoid arthritis have a lowered risk of AD (McGeer et al, 1990; McGeer and McGeer, 1995; Rich et al, 1995; Andersen et al, 1995; McGeer et al, 1996), and that the anti-inflammatory drug indomethacin may slow AD progression (Rogers et al, 1993). Hence, the appearance of reactive glia (both astrocytes and microglia) are believed to herald a turning point in the disease process.

Reactive neuroglia first become noticeable in some (but not all) PP, are always present in NP, and almost never in CP. Both reactive astrocytes and microglia are usually absent from diffuse plaques (Itagaki et al, 1989; Grundke-Iqbal et al, 1990), although this may not be true of very large Aβ deposits (DP II; Yamaguchi et al, 1991) or
in very advanced AD cases (Ohgami et al, 1991). The relationship of the glial elements
to the SP differs markedly. In general, the number of astrocytes may increase in AD
brain nonspecifically (Schechter et al, 1980). Reactive astrocytes, however, are typically
found around the periphery of the plaque, from which they extend numerous, finger-like
processes through the halo towards the plaque core (Dickson et al, 1988; Itagaki et al,
1989). Activated microglia may be more frequently associated with plaques than
astrocytes (Dickson et al, 1988), and are the only cellular component associated with
small dense deposits of Aβ found in the white matter (Uchihara et al, 1995). Although
activated microglia may occasionally be found associated with ghost tangles (Perlmutter
et al, 1992), the significance of this relationship is poorly defined (although they are likely
engaged in degradation of the NFT). Also, whereas the astrocytes are located around
the outside of the plaque (possibly forming a barrier against further spread of the lesion),
microglia are tightly bound to the plaque core where they may be either phagocytosing
(Itagaki et al, 1989) or manufacturing (Wegiel and Wisniewski, 1990) the Aβ fibrils.

The β-amyloid peptide has several notable effects on neuroglia. Perhaps not
surprisingly (since they have been observed in plaques), Aβ can directly trigger both
astrocytes and microglia to produce a variety of substances known to be involved in the
CNS inflammatory response. β-Amyloid will cause the secretion of bFGF and IL-1 from
both types of cell (Araujo and Cotman, 1992) and TNFα in microglia (Meda et al, 1995;
Yan et al, 1996). Interleukins-1 and 6 (both found in SP) are potent inducers of αACT
production in astrocytes (McGeer and McGeer, 1995; Huell et al, 1995), which appear to
be the major (if not only) producers of this SP related compound in AD brain
(Pastemack et al, 1989). It is also well known that TNFα causes a large upregulation of
ICAM-1 in endothelial cells (Kwiatkowski, 1992), which is also associated with SP.

The β-amyloid peptide is known to be a potent chemotactic stimulus for microglia
(Davis et al, 1992b). Further, once microglia bind to Aβ fibrils, they become highly
resistant to further chemotactic stimuli, possibly explaining the strong adherence of
microglia to the core of SP (Khoury et al, 1996; Yan et al, 1996). This
chemotactic/haptotactic response appears to be mediated through Aβ recognition by the
class A scavenger receptor for low density lipoproteins expressed on the surface of
microglia (Christie et al, 1996; Khoury et al, 1996) and/or the receptor for advanced glycation end-products (RAGE; Yan et al, 1996). Once bound to either of these receptors, Aβ will cause microglia to engage in respiratory burst activity (Banati and Kreutzberg, 1993), causing the release of large quantities of reactive oxygen radicals (Khoury et al, 1996; Yan et al, 1996) and cytotoxic nitrogen intermediates, such as NO₂⁻ (Meda et al, 1995).

All of these data are to some extent intertwined with an important unresolved issue in the pathogenesis of AD: what is the source of the Aβ peptide? The lack of a consistent localization of reactive glia within DP would seem to argue against a glial origin for Aβ. However, this would not necessarily rule out the involvement of glial cells at later stages of amyloidogenesis. For example, the strong association between microglia and the plaque core, and the presence (at the ultrastructural level) of Aβ fibrils within cytoplasmic channels and vesicles fusing with the Golgi apparatus, can be taken as indicative of microglia manufacturing the insoluble deposits (Wegiel and Wisniewski, 1990). Both microglia and astrocytes are known to produce all major transcripts of the β-amyloid precursor protein (βAPP, from which Aβ is derived; Haass et al, 1991), although they appear to actually secrete very little. In addition, neurodegeneration, such as following excitotoxic lesions with kainic acid (KA; Siman et al, 1989) and quinolinic acid (Töpper et al, 1995), as well as being in the vicinity of a NP (Yamaguchi et al, 1992), results in a large and persistent increase in βAPP production in astrocytes. Non-excitotoxic lesions with colchicine leads to the same result, whereas seizure induction with bicuculline (not causing neuronal death) does not (Siman et al, 1989). Similarly, microglia will also scale up βAPP production in response to neurodegeneration (Banati et al, 1994). These data could be taken to indicate that neuronal injury could begin a cascade involving glial cells that could accelerate the development of the pathology. The relationship of Aβ and a glial-mediated inflammatory response, as discussed above, certainly implicates glial cells as major harbingers of neuronal destruction.

Sparks et al (1993) have demonstrated a convincing correlation between the number of SP and the presence or absence of coronary artery disease in a large number of cognitively normal patients, indicating that the vascular system may be
involved to some degree in amyloidogenesis. Similarly, the presence of ApoE within SP (Namba et al, 1991), and its genetic association with both AD (Corder et al, 1993) and vascular disorders (Zhang et al, 1992; Breslow, 1996) supports this conclusion. However, although numerous examples of perivascular SP can be found, most DP can be shown to not be associated with blood vessels, and those that are do not exhibit Aβ deposition in the vessel walls (Yamaguchi et al, 1989). Also, circulating Aβ cannot enter the brain in significant quantities (Banks et al, 1991). Hence, the dissociation of the cerebrovasculature from the earliest phase of SP formation also seemingly eliminates this system as the origin of Aβ.

Since both glial cells and the vascular system are an unlikely source for the formation of diffuse deposits of Aβ, the pathogenic cascade must therefore logically begin with neurons. Unfortunately, the evidence for this is merely suggestive. In cases of progressive aphasia and frontal lobe dementia, Aβ deposition occurs in minimal quantities (in comparison to the rest of the brain) in the areas of principal pathology (Mann et al, 1992). Therefore, Aβ deposition appears to only occur in areas where functional neurons are still present. Also, since severe gliosis occurs in the areas afflicted with neurodegeneration in these disorders, this provides additional evidence against a glial origin for the peptide (Mann et al, 1992). In further support of this hypothesis, neurons that appear normal within DP exhibit Aβ immunoreactivity localized to presynaptic terminals within the Aβ deposit (Probst et al, 1991). Similarly, Aβ deposition in the aged canine appears to occur first in and around neurons (Cummings et al, 1996a), and transgenic mice overexpressing a mutant form of neuronal βAPP develop SP (closely associated with neurons) indistinguishable from those of humans (Hsiao et al, 1996; Masliah et al, 1996). On the other hand, there is no clear relationship between neurons expressing high levels of βAPP and concentration of SP (Chou et al, 1990; Johnson-Wood et al, 1997), which may indicate either that many of the neurons in the area are already dead, or that regional factors responsible for processing and/or clearance of Aβ are more important for SP development.

The finding that a form of the lysosomal enzyme β-hexosaminidase not found in glia is present in about 30-40% of diffuse plaques (and all higher level plaques) can also
be taken to show that neurons are of potentially greater importance than glia in the process of SP formation (Cataldo et al, 1996). Other lysosomal enzymes are also found in plaques, both in AD and Down’s syndrome (DS), although usually only in the PP and NP (some CP may stain weakly; Cataldo et al, 1996). The cathepsins B and D (Cataldo and Nixon, 1990; Cataldo et al, 1990) and acid phosphatase (Friede, 1965) are consistently found within plaques, usually within the core region. The hydrolases are only weakly associated with the DP, and can be easily washed out (Cataldo et al, 1990). They are, however, enzymatically active (Cataldo and Nixon, 1990). Whatever the source of the hydrolase deposits, they have been put forward as key evidence for the involvement of the endosomal/lysosomal system in AD (c.f. A4.1, below).

Dystrophic neurites, which are degenerating neuronal processes, are a later element to appear associated with senile plaques. Dystrophic neurites can be labeled by antibodies directed against PHF or abnormally phosphorylated \(\tau\) epitopes (Dickson et al, 1988; Cole et al, 1991; Wang and Munoz, 1995) and \(\beta\)APP (Arai et al, 1990; Cole et al, 1991; Wang and Munoz, 1995). Levels of neuronal \(\beta\)APP are significantly elevated in AD brain, particularly in association with SP (Cole et al, 1989b), although it is unknown whether this is greater than the elevation often seen in plaque-associated glial cells (Yamaguchi et al, 1992).

A2.3 Specificity and Significance of AD Neuropathology

Obscured amongst all of the complexities of AD neuropathology lies a mystery: what aspect of the disorder is responsible for the clinical manifestations of the disease? A clear answer has never really emerged, despite extensive study. These studies have mainly focused on the relationship of each factor with cognitive deterioration, and their association with AD as a specific component of the disorder. Not surprisingly, the best correlate with clinical pathology is the massive loss of neurons (Mann et al, 1988; Gómez-Isla et al, 1997) and synapses (DeKosky et al, 1990; Terry et al, 1991) that occurs throughout the AD brain. That AD is primarily a disorder of neural degeneration is not in doubt; the problem lies in the determination of the cause of this loss, and in the relative importance of the different putative mechanisms.
One obvious approach to the problem of assessing the specificity and significance of AD pathology has been the examination of SP and NFT and their relation to dementia. Although the research in this area has been more successful than others, not much has been resolved despite decades of intensive study. This problem is compounded by the issue of specificity. It is well known, for example, that both NFT and SP increase in frequency with normal aging (Berg, 1985; Ulrich, 1985; Arriagada et al, 1992; Jellinger, 1995; Langui et al, 1995), and these persons are not necessarily demented (Tomlinson et al, 1970). Alternatively, the development of these changes may herald the onset of early AD (Ulrich, 1985; Langui et al, 1995). It has been suggested by a variety of investigators that AD in fact represents exaggerated or “abnormal” aging of the nervous system, although there are several problems with this hypothesis (see Berg, 1985, for discussion).

**A2.3.1 Significance of Vascular Pathology**

The earliest views on AD pathology centered on the involvement of the vascular system as the potential driving force behind the degenerative changes. These hypotheses were based largely on the similarities between AD and systemic amyloidoses, which were known to possess a significant vascular component (Glenner et al, 1981; Kisilevsky, 1987; Kisilevsky et al, 1992). Severe congophilic angiopathy may compromise the BBB, in some cases actually leading to a stroke (Mandybur and Bates, 1978). This is perfectly reasonable, given the ability of the Aβ peptide to damage blood vessels (Thomas et al, 1996), and at least one mutant form of βAPP appears to cause this directly (see A3.1.1, below). Further, DS patients have been shown to exhibit AD-like vascular abnormalities (Bueé et al, 1994).

However, it has become clear that the cerebrovascular system is an unlikely source for the Aβ found in plaques (see A2.2.2, above). Also, many of the serum proteins found in the brain in AD are also found in several other disorders (Kalaria et al, 1991), particularly in MID (Alafuzoff et al, 1985). Multi-infarct/vascular dementia, which is a the second largest category of dementias in the elderly, is characterized (among other things) by a highly dysfunctional blood-brain barrier (Alafuzoff et al, 1983; Leonardi et al,
1985; Wallin et al, 1990), whereas this cannot be directly demonstrated in AD (see A2.2, above). Given the difficulty in obtaining an accurate pre-mortem diagnosis of AD, and since the two disorders are known to overlap considerably in some cases (Alafuzoff et al, 1987b), it is understandable why the involvement of the vascular system in AD was perhaps overestimated in the past. This is not to say that the cognitive component of AD is without vascular influence. A recent study found a significant association between SP and dementia when AD was concurrent with vascular disease, but no relationship when vascular abnormalities were taken into account (Nagy et al, 1997).

A2.3.2 Significance of Cholinergic Deficits

Focus in the early 80s shifted towards the role of the cholinergic system as a possible mediator of dementia (Whitehouse et al, 1982; Coyle et al, 1983; see A2.2, above). It has also been shown that the decline in choline acetyltransferase activity is correlated with the degree of dementia (Wilcock et al, 1982; DeKosky et al, 1992). In addition to the apparent loss of basal forebrain cholinergic neurons and markers, analogies can be drawn with the cognitive impairments following administration of cholinergic antagonists, such as scopolamine (Bartus et al, 1982). In rats, destruction of the same basal forebrain neurons that are affected in AD results in a severe impairment in spatial learning and memory, and this can be partially reversed by implants of cells that constitutively secrete ACh (Winkler et al, 1995). Also, since ACh can regulate normal secretion of βAPP, loss of cholinergic innervation could shift the balance towards an alternative pathway generating Aβ (Buxbaum et al, 1992; c.f. A3, below), and Aβ can directly cause spatial learning and memory deficits in mice that can be attenuated by cholinergic agonists (Maurice et al, 1996).

Given these findings, it is puzzling that cholinergic replacement therapy has been largely disappointing in AD. The only currently available pharmaceutical for the treatment of AD, tacrine or THA (Cognex, Parke-Davis), benefits only a small subpopulation of AD patients (Francis et al, 1995). Furthermore, the effect is usually quite modest, and paradoxically appears to benefit severe AD cases significantly more than mild ones (Farlow et al, 1995). This limited effect contrasts markedly with the relative success of
dopaminergic replacement (primarily through L-DOPA administration) in Parkinson’s disease (Rinne, 1989). Likely, the discrepancy between the two disorders can be explained on the basis of additional pathology. For instance, it is possible that the role of acetylcholine in the loss of cognitive function in AD is related to a loss of its modulatory function. That is, ACh in the hippocampus acts mainly presynaptically to increase the efficiency of glutamate release (Gray et al, 1996). Glutamate levels (Ellison et al, 1986) and receptors (Chalmers et al, 1990) are abnormal in AD, and degeneration of glutamatergic neurons is associated with dementia (Francis et al, 1993). Since the target cells of the cholinergic system are disrupted, the replacement of ACh by any means would therefore be of limited utility in the treatment of AD symptoms. This line of reasoning, then, results in the relegation of ACh to a secondary role in the clinical features of the disorder.

A2.3.3 Significance of NFT Pathology

Numerous studies have shown a correlation between the number and/or density of NFT and severity of cognitive deterioration (Wilcock and Esiri, 1982; Barcikowska et al, 1989; McKee et al, 1991; He et al, 1993; Lue et al, 1996; Nagy et al, 1997). Similarly, numerous cases of nondemented patients have been documented with large numbers of senile plaques with minimal NFT, suggesting that the NFT are necessary for dementia to occur (Barcikowska et al, 1989; McKee et al, 1991). However, the issue arises as to whether or not the NFT are part of the disease process or merely an indicator of neuronal dysfunction brought about by other aspects of the disease process. Many otherwise normal individuals show NFT-like changes in entorhinal cortex before any other pathology (e.g., Braak and Braak, 1991), but this could represent any number of disorders other than early AD. Neurofibrillary tangles which are structurally and biochemically very similar to those of AD are found in progressive supranuclear palsy (Yagashita et al, 1981; Schmidt et al, 1996a), Guam ALS/PDC (Mawal-Dewan et al, 1996), dementia pugilistica (Roberts et al, 1990), Parkinson’s disease (Bancher et al, 1993), subacute sclerosing panencephalitis (Wisniewski et al, 1979), Hallervordan-Spatz disease (Wisniewski et al, 1979), and Niemann-Pick disease (Feany and Dickson, 1996). Further, neuropil threads, which are also composed mainly of abnormal tau, are
also seen in progressive supranuclear palsy (Probst et al, 1988; Davis et al, 1992a) and corticobasal degeneration (Feany and Dickson, 1996). Patients suffering from Pick’s disease also show neuropil threads, and Pick bodies (which are characteristic of the disorder) label with NFT antibodies (Yasuhara et al, 1995). Therefore, although a definitive characteristic of AD, the presence of NFT can hardly be said to be specific to this disease.

Since microtubules are an essential component of the cellular cytoskeleton, impaired axoplasmic flow becomes a plausible explanation for neuronal death in AD (Bancher et al, 1989a; Iqbal and Grundke-Iqbal, 1991). However, there is a clear dissociation between NFT pathology and cell loss in several nuclei of the hypothalamus (Swaab et al, 1992) and hippocampal CA2 neurons, which are relatively unaffected in AD, show very high levels of Alz-50 immunoreactivity (IR) and NFT (Brady and Mufson, 1991). Also, although the amount of NFT pathology correlates strongly with the duration of AD and is moderately correlated with the degree of cell loss, the presence of the (presumably) insoluble ghost tangles in the extracellular space cannot (by several orders of magnitude) account for the missing neurons (Hyman et al, 1995; Gómez-Isla et al, 1997). Recently, some evidence has indicated that extracellular deposits of \( \tau \) may continue to undergo degradation (Mena et al, 1995), which may at least account for some of the discrepancy with the observed loss of neurons.

**A2.3.4 Significance of SP Pathology**

Senile plaques have also been found to correlate with degree of dementia, although this correlation is generally acknowledged to be weaker than that of NFT (Roth et al, 1966; Wilcock and Esiri, 1982; McKee et al, 1991; He et al, 1993). In terms of SP subtype, it appears that the more advanced forms (NP+) are required (Mochizuki et al, 1996; Morris et al, 1996a), and weaker correlations are obtained when the far more numerous diffuse plaques are included. This is not surprising since the more advanced types of SP are closely associated with neurodegeneration, whereas DP, for example, are usually not. Senile plaques (with Ab as the principal component) are also more specific for AD than NFT: only Down’s syndrome patients are known to develop true SP
pathology. Since this occurs along with NFT, it indicates that DS patients are actually developing a variant form of AD rather than a distinct disorder (see A3.1, below).

The issue of whether or not the Aβ deposits are neurotoxic has not been resolved. Low concentrations of soluble Aβ actually appear to be neurotrophic (Whitson et al., 1989; Yankner et al., 1990). However, increasing the concentration of soluble Aβ (Yankner et al., 1990) or administration of insoluble Aβ in the form of plaque cores (Frautschy et al., 1991) is extremely neurotoxic. Interestingly, in both the rat (Kowall et al., 1991) and non-human primate (Kowall, 1994), neurotoxic concentrations of Aβ also induce some signs of PHF immunoreactivity around the lesion site, although it is not at all clear how presumably insoluble extracellular aggregates influence the development of intracellular pathology. It should also be mentioned that the actual relevance of direct Aβ toxicity achieved through administration of the peptide for actual AD pathology is highly uncertain. For instance, the experimentally demonstrated degenerative changes in vivo are exceedingly rapid (under a month), whereas they likely take decades in humans. Also, it has never been established that the amounts of extracellular Aβ required to induce degenerative changes truly reflect the situation at any stage of Alzheimer’s disease. It is also puzzling that huge deposits of Aβ occur “naturally” in humans without any evidence of neurodegeneration, such as in the striatum or cerebellum, as well as in areas known to otherwise develop characteristic AD pathology (McGeer et al., 1994). Even SP found in AD cases often have neurons in close proximity to the deposit (even occasionally within the deposit) that do not in any way appear to be abnormal. Finally, transgenic mice that develop huge loads of extracellular Aβ (more than even the most severe cases of AD) show little or no evidence of cell death in the affected regions (Irizarry et al., 1997).

A2.3.5 Significance of Reactive Gliosis

There is significant evidence for oxidative damage in AD (Smith et al., 1996; Good et al., 1996). Although Aβ is capable of generating free radicals under some circumstances in vitro (Harris et al., 1995; Thomas et al., 1996), reactive microglia are probably responsible for the oxidative stress observed in vivo (McGeer and McGeer,
In support of this hypothesis, (1) the nonsteroidal anti-inflammatory drug indomethacin may be able to slow the rate of cognitive deterioration in AD (Rogers et al., 1993) and (2) inflammatory reactivity correlates with loss of synapses (as measured by synaptophysin immunoreactivity; Lue et al., 1996). Hence, the involvement of reactive microglia is a secondary consequence of SP formation, with Aβ as a necessary factor in initiating this cascade (McGeer and McGeer, 1995; Khoury et al., 1996; Yan et al., 1996).

**A2.3.6 Significance of Intracellular Aβ?**

Although the presence of extracellular Aβ deposits is a diagnostic feature of AD, this does not necessarily mean that the SP are the cause of neurodegeneration (see A2.3.4, above). That is, neurons containing large amounts of Aβ could degenerate and release insoluble Aβ into the extracellular space, or could disperse the peptide via exocytosis (Yang et al., 1995; Bernstein et al., 1996; Burdick et al., 1997). Senile plaques, it should be noted, are focal lesions, and even within regions of the brain that contain large numbers of amyloid deposits a large number of neurons remain, apparently unaffected (Yang et al., 1995). A deceased cell, for example, may leave a remnant of insoluble amyloid which provides a nucleus or “seed” for the further growth and aggregation of the SP (Burdick et al., 1997), similar to the mechanism hypothesized for amyloid fibril formation in prion disorders (Prusiner and DeArmond, 1994). Additional neurons could be killed by the excessive inflammation caused by the extracellular amyloid (McGeer and McGeer, 1995). This scenario not only accounts for the focal nature of SP, but also potentially accounts for the presence of lysosomal contents (Bernstein et al., 1996) and RNA (Ginsberg et al., 1997) within the plaque core. This hypothesis also provides a mechanism for the formation of NFT pathology, since intracellular Aβ (iAβ) can more easily be envisioned interacting with tau than can extracellular deposits of the peptide.

Several lines of evidence have recently begun to converge on this possibility. Cells overexpressing either full length βAPP (Yoshikawa et al., 1992) or its β-amyloid containing C-terminal fragment (Yankner et al., 1989; Neve et al., 1992) degenerate in
culture at an accelerated rate. Similarly, cells in which amyloidogenic fragments of βAPP accumulate intracellularly as a result of treatment with lysosomal inhibitors exhibit an accelerated rate of death (Hayashi et al, 1992). Recently, a candidate protein (ERAB) has been isolated that may mediate this toxicity from an intracellular location (Yan et al, 1997). Intracellular amyloid has been detected (Turner et al, 1996), and aggregates in a form resistant to degradation (Burdick et al, 1997; Wild-Bode et al, 1997), which stimulates the further intracellular accumulation of additional amyloidogenic βAPP fragments (Yang et al, 1995). In a transgenic mouse model of AD, large amounts of intracellular amyloid fibrils were detected in the neuronal cytoplasm using immunoelectronmicroscopy and also found (albeit to a lesser extent) in AD neurons (Masliah et al, 1996). Also, Huntington’s disease patients show abnormal intracellular aggregates of the huntingtin protein within the striatum (specifically, intranuclear; DiFiglia et al, 1997), a process which may be analogous to the formation of iAβ fibrils in AD.

A2.3.7 Which Element Matters?

The preponderance of available evidence indicates that the presence of Aβ, either intra- or extracellularly, may be the primary event in the complex pathology of AD. The specificity of SP for the disease in comparison to NFT is one factor that supports this argument. Another consideration is the appearance of diffuse deposits of Aβ prior to the onset of any other obvious pathology (see A2.2.4, above). Putting the issue of direct neurotoxicity of Aβ aside for the moment, it may still initiate a pathological cascade that leads to other manifestations of the disease process (Hardy and Higgins, 1992; Hardy, 1997). The fact that SP are only weakly correlated with dementia is exactly what would be expected if plaque formation were necessary for AD but not sufficient without other “downstream” components, such as NFT and reactive microglia, both of which may be directly responsible for the neuronal and synaptic loss (Selkoe, 1997). This argument, as it stands, is based largely on correlational data, which is a necessary limitation of neuropathological studies. In order to fully understand the pathogenic processes of AD, and the importance of Aβ, it is necessary to examine the broad base of information gathered through the study of Alzheimer’s disease molecular pathology.
A3 Molecular Pathology of Alzheimer's Disease

A3.1 Genetics

Molecular insights into the pathogenesis of AD have progressed remarkably in recent years. This advance has been due to a vast improvement in the understanding of the genetic basis of the disease process. In 1990, there were no definitively identified genes related to AD; as of 1997, there were 4: 3 causative genes, and 1 imparting increased susceptibility. Importantly, cases of familial AD are indistinguishable from the non-familial, or sporadic, cases both clinically (Karlinsky et al, 1992; Kennedy et al, 1995) and neuropathologically (Lantos et al, 1992; Lippa et al, 1996). Hence, even though familial cases of Alzheimer's disease (FAD) can account for only a small fraction of the total cases of AD (certainly less than 20%; Levy-Lahad and Bird, 1996), each genetic alteration can yield significant information.

A3.1.1 β-Amyloid Precursor Protein

The earliest hint of a genetic locus for FAD came from the study of Downs' syndrome patients (DS). Individuals afflicted with DS have an extra copy of chromosome 21, either in whole or in part. Older patients, over about 50 years of age, acquire neuropathological characteristics that are identical to both sporadic and familial AD (Ropper and Williams, 1980; Mann, 1988; Mann et al, 1989). For instance, both τ (Mann et al, 1989) and Aβ (Masters et al, 1985) are identically altered in the brains of older DS patients when compared with AD cases. Further, the SP contain identical components in addition to Aβ, such as αACT (Koo et al, 1991), ApoE (Schupf et al, 1996) and HSPG (Crutcher et al, 1993). It was eventually discovered that the coding region for Aβ was, in fact, localized to the long arm of chromosome 21, implying that these AD-like symptoms could be arising from increased expression of the peptide associated with a gene dosage effect (Robakis et al, 1987).

By extending the studies in DS patients, in situ hybridization confirmed the ubiquitous synthesis of the Aβ peptide in the brain (Bahmanyar et al, 1987), and that Aβ was, in fact, part of a larger protein, the β-amyloid precursor protein (BAPP; Kang et al, 1987), in which it comprised part of the transmembrane domain. Further, it was shown
that the C-terminal end of the Aβ peptide lay embedded within the plasma membrane (Kang et al., 1987; see figure A3-1).

With this information in hand, several groups later reported a genetic linkage between some FAD pedigrees and an unknown gene on chromosome 21 (Goldgaber et al., 1987; Tanzi et al., 1987; St. George-Hyslop et al., 1987), which was thought to be βAPP. However, this hypothesis was not confirmed until several years later. Prior to the discovery of βAPP and a possible link to hereditary AD, a possible clue had emerged from the study of another form of familial amyloidosis, HCHWA-I (hereditary cerebral hemorrhage with amyloidosis, Icelandic type). A single amino acid substitution in the endogenous protease inhibitor cystatin C (glutamine for leucine at codon 68) led to its accumulation in the walls of cerebral blood vessels, eventually leading to fatal hemorrhaging (Ghiso et al., 1986). A related disorder (HCHWA-D; hereditary cerebral hemorrhage with amyloidosis, Dutch type), differing in that it was also accompanied by what appeared to be immature plaques in the brain parenchyma, was later found to be

![Figure A3-1. Schematic structure of the βAPP molecule, showing the location of the 39-43 amino acid Aβ peptide in relation to the transmembrane domain. Also illustrated are cleavage sites for the putative α-, β- and γ-secretase enzymes (see text); note that the γ-secretase site is located within the lipid bilayer of the plasma membrane, making it presumably inaccessible to proteolytic cleavage.]
caused by a similar mutation in βAPP, 22 amino acids into the Aβ region (glutamine for glutamate at codon 693; Levy et al, 1990). It was now known that an altered form of Aβ/βAPP could lead to a type of amyloidosis.

In the following year, three studies reported additional mutations in βAPP. All three of the mutations were of the missense variety, consisting of a substitution of isoleucine (Goate et al, 1991), phenylalanine (Murrell et al, 1991) or glycine (Chartier-Harlin et al, 1991) for an evolutionarily conserved valine residue 3 amino acids past the C-terminal end of the Aβ peptide (codon 717). Soon after, an additional pedigree was identified, the so-called “Swedish double mutant”, consisting of the substitution of an aspartate-leucine pair for lysine-methionine at codons 670/671, precisely at the Aβ N-terminal cleavage site (Mullan et al, 1992). All of these mutations resulted in phenotypical Alzheimer’s disease.

In the case of all βAPP mutants, the disorder is inherited in an autosomal dominant fashion, with apparently complete penetrance (Rossor et al, 1993). That is, no carrier of the mutation has been found to be unaffected beyond the age of 67 (Levy-Lahad and Bird, 1996). However, this form of inherited AD is exceedingly rare, probably accounting for less than 2% of all familial cases (Wasco et al, 1993). Given the high proportion of unexplained pedigrees, it followed that multiple genetic loci must exist (St. George-Hyslop et al, 1990).

**A3.1.2 Apolipoprotein E**

Apolipoprotein E (ApoE) is a low-density lipoprotein transport molecule believed to be important in the pathogenesis of atherosclerosis (Zhang et al, 1992; Breslow, 1996). It is also a component of both cerebral (Namba et al, 1991; Uchihara et al, 1996a) and systemic (Wisniewski and Frangione, 1992) amyloid deposits. Structurally, ApoE is a 299 amino acid protein, the coding for which is found on chromosome 19 (Poirier, 1994). In humans, three allelic variations are present (relative frequencies in brackets): ε2 (0.08), ε3 (0.77) and ε4 (0.15)(Corder et al, 1995). The different isoforms differ by single amino acid substitutions at two positions. The most common version, ApoE3, contains a cysteine residue at position 112 and an arginine residue at position
ApoE2 has a cysteine substitution at position 158, and ApoE4 has an arginine substitution at position 112 (Corder et al., 1995). Hence, each isoform differs in relative charge and can be readily separated by isoelectric focusing (Poirier et al., 1994).

Unlike other plasma apolipoproteins, ApoE has special relevance for nervous system tissue; it appears to be important for the mobilization and transport of lipids for maintenance and repair of myelin sheaths and neuronal membranes (Mahley, 1988; Poirier, 1994). Apolipoprotein E has been shown to be an important mediator of neurite outgrowth in vitro (Nathan et al., 1994), and ApoE deficient mice exhibit greater loss of synapses in the outer molecular layer of the dentate gyrus following perforant path transection (Masliah et al., 1995). The utilization of ApoE initially involves macrophages that scavenge lipids and cholesterol from areas of damage, followed by a longer period of astrocytic involvement mediating reinnervation and recovery processes (Mahley, 1988; Poirier, 1994; Suzuki et al., 1997).

In the search for further genetic linkages to AD, it was determined that several pedigrees of late-onset familial AD (late 70s+) displayed an association with a site on the proximal long arm of chromosome 19 (Pericak-Vance et al., 1991). The suspicion that this locus might be ApoE stemmed from two primary observations. First, the gene for ApoE was known to be located in the same region that had been shown to be linked to AD (Ropers and Pericak-Vance, 1991). Second, ApoE was found localized to SP (Namba et al., 1991) and was known to bind to Aβ with very high avidity (Strittmatter et al., 1993).

From these starting points, the ApoE-ε4 allele was subsequently found to be over-represented in cases of both late-onset familial AD and sporadic AD (Saunders et al., 1993). Further, this association was accompanied by a robust gene dosage effect, wherein individuals with a single copy of the ε4 allele showed elevated risk intermediate between non-carriers and homozygous subjects, and that these changes in risk were closely related to an earlier age of onset (Corder et al., 1993; Mayeux et al., 1993; Brousseau et al., 1994; Premkumar et al., 1996). This effect is so strong that the presence of the ε4 allele may be able to account for greater than half of all cases of AD (Corder et al., 1995; Roses et al., 1995). It has also been consistently found that the ε2
allele is under-represented in AD, implying that ApoE2 may somehow protect against the development of the disorder, at least in heterozygous individuals (Corder et al, 1993; Corder et al, 1995; Gómez-Isla et al, 1996).

The ApoE genotype is correlated with a number of pathological factors in AD, but not all. The ε4 allele has been linked to more severe cerebral amyloid angiopathy and the appearance of related vascular abnormalities (Shimano et al, 1989; Premkumar et al, 1996), and to an increased density of SP/Aβ deposition (Schmechel et al, 1993; West et al, 1995; Gómez-Isla et al, 1996). Conversely, the presence of the ε2 allele results in a reduced amount of both vascular and parenchymal amyloid deposits, even in confirmed cases of AD (Lippa et al, 1997). However, although the ApoE genotype is strongly associated with the age of onset and Aβ deposition, neither the ε4 or ε2 alleles appear to influence the development of NFT (Gómez-Isla et al, 1996; Lippa et al, 1997). In fact, NFT and ApoE appear to become associated later in the disease process (most notably in the case of ghost tangles), which is probably related to the role of ApoE in CNS damage repair (Benzing and Mufson, 1995; Dickson et al, 1995).

We now know that ApoE binding to Aβ can promote fibril formation and stabilization in vitro (Ma et al, 1994), and that ApoE4 binds to Aβ more efficiently than do other isoforms (Sanan et al, 1994). Following binding, uptake of the ApoE/Aβ complex occurs via receptor-mediated endocytosis (either the LDL or type A scavenger receptor), after which it is degraded by the endosomal/lysosomal system (Ye et al, 1993; Jensen et al, 1994). Hence, binding of ApoE4 may stabilize the formation of Aβ fibrils and prevent their degradation by the endosomal/lysosomal pathway, possibly by interacting with Aβ to form a more stable complex within the acidic compartment of the lysosome. In support of this, ApoE2 is known to have approximately 50 fold less affinity for the LDL receptor (Guilluame et al, 1995). This would interfere with the complex reaching the endosomal/lysosomal compartment, and could therefore be related to its purported protective effect.

Although the ApoE-ε4 genotype accounts for a sizable number of AD cases, it must be emphasized that it is a genetic risk factor for the development of the disease. Unlike the βAPP mutations (which show complete penetrance) there exist many
documented cases of ε4 homozygotes that apparently fail to develop AD, and ε2 heterozygotes that do (ε2 homozygotes often die at a younger age from hyperlipoproteinemia and severe atherosclerosis; Mahley, 1988). Even though such cases are apparently exceptional and may develop AD given sufficient time (Roses et al, 1995), ApoE polymorphism still cannot account for all cases of sporadic AD. Furthermore, other genetic loci beyond chromosome 19 exist, in particular an especially malignant form of familial AD with age of onset as low as the late 30s (Mullan, 1992).

A3.1.3 Presenilins

At approximately the same time as molecular genetic studies were determining the contributions of βAPP and ApoE to the development of AD, other studies were determining that the vast majority of familial AD cases (>95%) were actually caused by mutations elsewhere (St. George-Hyslop et al, 1990). However, thorough searches for linkages based on other elements of SP and NFT proved fruitless. This suggested that the other genes involved were critical elements of the pathogenic process, rather than direct pathological manifestations of the disease.

In late 1992, a genetic link was identified on the distal portion of the long arm of chromosome 14 that appeared to account for the bulk of these cases, with the notable exception of a large kindred of Volga Germans (Schellenberg et al, 1992). Unfortunately, the only known coding regions on chromosome 14 that could be considered to be connected to AD (αACT and cathepsin G) were quickly ruled out as candidates in the same report. Hence, lacking specific clues as to the nature of the abnormal protein, the genetic characterization of this alteration proceeded at an extremely slow pace.

Eventually, repeated refinements of the linkage analysis paradigm were able to narrow the search region to a small enough area for direct sequencing and comparison with unaffected individuals. In mid-1995, a multi-institutional team was able to identify an entirely novel protein, then called S182 (Sherrington et al, 1995). This protein was putatively identified as an integral membrane protein with 7 transmembrane (TM) domains (although it may have as many as 9; Hardy, 1997), a large hydrophilic loop between TM regions 6 and 7, and a length of 467 amino acids. Less than one month
later, while performing a search based on S182 sequence homology, the mutation responsible for the Volga German pedigree was localized to the distal region of the long arm of chromosome 1 (Levy-Lahad et al, 1995). Virtually simultaneously, the protein was independently sequenced by two groups and tentatively named STM2 (Levy-Lahad et al, 1995; Rogaev et al, 1995). This second protein was slightly shorter (448 amino acids) and 67% identical to S182, with most of the sequence divergence occurring within the hydrophilic loop. This was taken to imply slightly different specificities for as yet unidentified function, and a uniform nomenclature was adopted: presenilins (PS) 1 and 2, based on their order of discovery and perceived relative importance to the disease process (Rogaev et al, 1995; Levy-Lahad and Bird, 1996).

Antibodies raised against various portions of PS1 and 2 revealed widespread neuronal localization within the brain (in both AD and control cases), with a predominantly perinuclear appearance and some punctate, granular staining within neurites; no consistent staining was found within SP or NFT (Cribbs et al, 1996; Murphy et al, 1996; Uchihara et al, 1996b; Giannakopoulos et al, 1997; Weber et al, 1997). Hence, the presenilins are not a physical component of the pathology.

Based on homology with two C. elegans proteins, sel-12 (Levitan and Greenwald, 1995) and spe-4 (Sherrington et al, 1995), the presenilins have been hypothesized to be involved with either Notch signaling or intracellular protein trafficking, respectively. The former of these two possibilities thus far has gained the only experimental support. Notch signaling, an important developmental pathway involved in determining cell fate, is partially regulated in C. elegans by sel-12, which is 50% identical to PS1 (Levitan and Greenwald, 1995). Wild type presenilin is also able to substitute for dysfunctional sel-12, whereas mutant PS is not (Levitan et al, 1996). Further, developmental expression of PS mRNA closely parallels expression of Notch receptor mRNA in the rodent brain (Berezovska et al, 1997). PS2 mutations have also been shown to increase susceptibility to apoptotic cell death in vitro, including that caused by exposure to Aβ (Wolozin et al, 1996). Finally, Notch3 receptor mutations in humans cause an autosomal dominant condition resulting in multiple strokes and dementia (Joutel et al, 1996), and PS1 knockouts in mice are highly lethal, resulting in death mainly via intracerebral
hemorrhaging (Wong et al, 1997). The significance of these findings for actual AD pathology are still highly controversial, with much of the work still largely speculative.

The form of familial AD caused by PS mutations is very similar to other forms of AD. As with βAPP mutations, and in contrast to ApoE polymorphism, PS mutations result in an autosomal dominant form of the disorder with complete penetrance, strongly implying causality (Levy-Lahad and Bird, 1996). Presenilin 1 pedigrees uniformly present with an extremely malignant form of AD, with a very early age of onset (Lampe et al, 1994), whereas age of onset in the PS2 pedigree closely resembles sporadic AD (Levy-Lahad et al, 1995). Consistent with this finding, SP density is much more severe in PS1 cases (Mann et al, 1996a) in comparison with both PS2 (Mann et al, 1997) and sporadic AD, in spite of similar disease duration. This observation is further supported by studies of Aβ production in cultured fibroblasts from PS1 and PS2 patients (Querfurth et al, 1995), and in mutant PS1 transgenic mice (Duff et al, 1996). The only other known difference in phenotype is a marked increase in the incidence of seizures and myoclonic jerks in PS1 patients relative to other forms of AD (Lampe et al, 1994).

There are now greater than 40 known mutations in PS1, and at least 2 in PS2 (Levy-Lahad and Bird, 1996; Hardy, 1997). Together, these genetic changes may be able to account for as much as 95% (80% PS1, 15% PS2) of all cases of FAD, and 13% of all Alzheimer's disease cases (Barinaga, 1995). Yet, at least one pedigree remains with unexplained heritability (Rogaev et al, 1995), indicating that other AD molecular elements remain to be elucidated.

A3.1.4 The Aβ Hypothesis Revisited

The specificity of extensive deposits of Aβ in AD in comparison with other aspects of pathology, the early accumulation of Aβ in diffuse deposits prior to other degenerative changes, and the apparent evolution of plaques from diffuse to neuritic (and finally to a “burnt-out” stage) are all factors indicative of a primary role of Aβ in the pathogenesis of AD. However, studies of the molecular genetics of AD and related in vitro studies have provided the strongest support for this hypothesis. For instance, βAPP mutations are causative for AD, whereas no such mutation has ever been found
in the τ gene on chromosome 17 (Selkoe, 1996). The important issue is how these genetic changes relate to the development of Alzheimer's disease, and what they can illustrate about the pathogenic mechanisms responsible.

Down's syndrome patients, which have higher levels of expression of βAPP due to a gene dose effect, invariably develop AD at an early age. Further, the earliest appearing abnormality is diffuse Aβ deposition (Mann and Esiri, 1989; Ikeda et al., 1989; Giaccone et al., 1989), apparently around neuronal perikarya (Allsop et al., 1989). This implies a causative role for increased production of the Aβ peptide and development of AD (Selkoe, 1996). A similar alteration has, in fact, been found in the case of the Swedish double mutant pedigree, in that cells transfected with this form of βAPP secrete at least 6-8 fold more Aβ into the culture medium as normal βAPP transfectants (Citron et al., 1992; Cai et al., 1993; Golde et al., 1993; Martin et al., 1995). These cells also acquire a pool of intracellular Aβ that is produced by a mechanism distinct from that used in the generation of the secreted form of the peptide (Martin et al., 1995; Tienari et al., 1997).

One problematic observation that emerged from these studies was that the other major class of AD causing βAPP mutants, the codon 717 pedigrees, did not show this increase in Aβ secretion or altered βAPP processing (Yanagisawa et al., 1992; Cai et al., 1993; Golde et al., 1993). This problem was later resolved through the discovery of a subtle alteration in Aβ production: codon 717 mutations all lead to the increased production of a longer form of Aβ (Aβ42(43)), whereas wild type βAPP generated a shorter form (Aβ40) (Suzuki et al., 1994). Since it was known that longer forms of Aβ were much more insoluble than the shorter forms (Barrow and Zagorski, 1991; Burdick et al., 1992), a propensity towards the generation of a type more prone to aggregation might be the factor accounting for the appearance of AD in these families. Recently, it has been found that the SP from this class of FAD have an unusually high percentage of Aβ42(43) in comparison to sporadic AD (Mann et al., 1996b). In fact, most of the SP related Aβ in both DS and AD is of the longer form, whereas the shorter, soluble form is more commonly associated with cerebral amyloid angiopathy (Gravina et al., 1995; Iwatsubo et al., 1995; Mann et al., 1996b). Similarly, the longer form appears to be the first to appear
in diffuse plaques in humans (Iwatsubo et al, 1996), cats and dogs (Cummings et al, 1996b), and transgenic mice (Johnson-Wood et al, 1997); Aβ40, the predominantly secreted [short] form, appears later in SP, which are first "seeded" by the longer peptide (Hardy, 1997). Long Aβ also accumulates intracellularly, especially in cells transfected with the Swedish double mutant (Tienari et al, 1997), and is highly resistant to degradation, whereas short Aβ appears only transiently in an intracellular location (Burdick et al, 1997; Wild-Bode et al, 1997).

Related changes in Aβ handling have been identified in the other genetic factors of AD. ApoE-ε4 polymorphism results in an increased density of SP and a correspondingly earlier age of onset, while having a minimal influence on the development of NFT pathology (see A3.1.2, above). Although little is known thus far about the presenilin mutations, the PS1 mutation has been shown to result in a higher SP density (Mann et al, 1996a). Both PS1 and PS2 mutations have been shown in vitro to lead to the production of the longer form of Aβ (Selkoe, 1996; 1997; Hardy, 1997).

Hence, all of the known genetic alterations connected with AD have been linked to alterations in Aβ metabolism. Thus, it has become widely accepted that Aβ is the central factor in the disease process. Logically, research has therefore focused on the processing of βAPP and the cellular mechanisms responsible for the generation of potentially amyloidogenic derivatives.

A3.2 Processing of the β-Amyloid Precursor Protein

A3.2.1 Structure

The β-amylloid precursor protein exists in multiple forms, ranging in relative size from 91.5 to 135 kDa (Dyrks et al, 1988; Selkoe et al, 1988). It is a type I integral membrane glycoprotein (a single transmembrane domain) with a ubiquitous expression in all tissue types (Selkoe et al, 1988); in the brain, βAPP mRNA accounts for 0.2% of the total mRNA (Beyreuther et al, 1993). In vitro, it has a short half-life of only 20-30 minutes, after which time the large N-terminal end, which faces the extracellular space (Dyrks et al, 1988), is exfoliated (Weidemann et al, 1989). In addition to being localized
at the cell surface, a significant proportion is also found inserted into the membrane of the Golgi apparatus (Palacios et al, 1992).

The gene for βAPP is composed of 19 exons, which are alternatively spliced to produce 3 major and 5 minor species of protein (Beyreuther et al, 1993). The Aβ region is located on parts of exons 16 and 17, and is present in all isoforms except for the exceedingly rare βAPP_{563} (König et al, 1992). Of the three major species, βAPP_{595} is the most highly expressed in the brain, followed by βAPP_{751} and βAPP_{770} (in a ratio of about 20:10:1; Tanaka et al, 1989) and a very small amount of βAPP_{714} (Golde et al, 1990). Finally, astrocytes and microglia also produce trace amounts of variants of the three major isoforms by excluding the 18 amino acids of exon 15 (König et al, 1992; Beyreuther et al, 1993).

![βAPP_{770}](image)

A3.2.2 Function

There has been little or no consensus about the function of βAPP, although several putative functional domains have been identified (see figure A3-2). Since Aβ is produced and secreted under normal conditions both in vitro and in vivo (Haass et al, 1992b; Seubert et al, 1992; Shoji et al, 1992), it may also have a physiological role. Function related research can be categorized into 4 main areas: cell contact/adhesion, extracellular protease regulation, response to injury, and synaptic function.

A3.2.2.1 Cell Contact and Adhesion

The ubiquitous CNS distribution of βAPP has been taken to suggest a role in cell contact and adhesion (Shivers et al, 1988). βAPP is known to associate with the extracellular matrix (ECM)(Small et al, 1992), and may do so via high affinity interactions with HSPG (Narindrasorasak et al, 1991). The β-amyloid peptide similarly binds to HSPG (Buée et al, 1993). This is potentially important since HSPG is a normal component of SP (Snow et al, 1988, 1990) and may influence the development of NFT (Goedert et al, 1996). Binding sites for a variety of ECM components such as heparin (Schubert et al, 1989; Small et al, 1993, 1994), collagen (Allsop and Williams, 1994), laminin (Narindrasorasak et al, 1992), and fibronectin (Narindrasorasak et al, 1995) can be found along the extracellular region of βAPP. At least one of these domains (heparin) may mediate βAPP’s ability to influence neurite extension, an effect similar to that of laminin (Small et al, 1993, 1994). Also, a significant percentage of cell surface βAPP (upwards of 50%) has been shown to exist in a proteoglycan form (Appican) with chondroitin sulfate glycosaminoglycan side chains; these are typically found attached to cell surface proteins important for adhesion (Shioi et al, 1993). Therefore, one possibility is that βAPP is involved in a fundamental adhesion process that becomes dysfunctional in AD (Kisilevsky et al, 1990).

A3.2.2.2 Protease Inhibition

A closely related area of research involves the role of βAPP as an endogenous protease inhibitor. Shortly after its discovery, the longer forms of βAPP (751 and 770)
were found to possess properties similar to serine protease inhibitors of the trypsin family (Kitaguchi et al, 1988; Tanzi et al, 1988; Ponte et al, 1988). The critical domain, a 57 amino acid insert close to the N-terminal end (encoded by exon 7), is homologous to the Kunitz-type protease inhibitors (KPI; Travis and Salversen, 1983). Based on these studies, the secreted form of βAPP was later identified as protease nexin-II (PN-2), a secreted serine protease inhibitor produced by several types of cultured cells (Olterdorf et al, 1989) and platelets in vivo (Van Nostrand et al, 1990, 1991; Bush et al, 1990). Since βAPP is a prominent platelet secretory protein, it follows that it might be involved in the coagulation cascade. This has been demonstrated (Bush et al, 1993), and dovetails with the involvement of mutant βAPP in HCHWA-D (Levy et al, 1990). Further, βAPP has been shown to contain an inhibitory domain for the metalloproteinase gelatinase A (Miyazaki et al, 1993) which has been recently shown to be involved in the process of platelet aggregation (Sawicki et al, 1997). Alternatively, PN-2 can stimulate cell growth (Roch et al, 1993), so a relationship with the ECM as outlined above is still possible. Finally, protease nexin-II is a potent antichymotrypsin (Van Nostrand et al, 1989) that operates via the same mechanism as αACT. Given the presence of αACT as an early plaque component, and considering the similar functions of both αACT and PN-2, it is conceivable that AD pathogenesis may be related to a dysregulation of extracellular proteolysis.

A3.2.2.3 Response to Injury

The ratio between the KPI-containing βAPP isoforms 751 and 770 changes (relative to 695) in AD, depending on the brain region examined (Palmert et al, 1988). In general, the relative abundance of the KPI-containing isoforms increases throughout the brain in AD (Tanaka et al, 1989). This increase is particularly large in damaged areas, and correlates with SP density (Johnson et al, 1990). In addition to supporting a possible disturbance in proteolysis, this also indicates that expression of the KPI-containing forms may be connected to increased neuronal damage. Studies in rodents and cell culture confirmed that several types of neuronal injury, such as that following a stab wound (Otsuka et al, 1991), heat shock (Abe et al, 1991a; Johnson et al, 1993), ischemia
(Kalaria et al, 1993), kainic acid administration (Kawarabayashi et al, 1991) and other lesions (Siman et al, 1989; Töpper et al, 1995), resulted in an increase in βAPP. Further, the KPI-containing βAPP species exhibited larger relative increases (Abe et al, 1991b; Willoughby et al, 1992), especially in astrocytes (Solà et al, 1991). The reason for this is not known, although may be related to a neuroprotective role of secreted βAPP (Goodman and Mattson, 1994; Masliah et al, 1997). Since Aβ has been shown to activate both microglia (Yan et al, 1996) and the inflammatory complement pathway (Rogers et al, 1992), its role in an injury response cascade cannot be entirely ruled out.

A3.2.2.4 Synaptic Function

The β-amyloid precursor protein is synthesized in the cell body and undergoes fast axoplasmic transport in neurons (Koo et al, 1990), where it is associated with vesicular elements at the synapse (Schubert et al, 1991; Shigematsu et al, 1992). Although βAPP has been shown to have synaptotrophic properties that are similar to its effects on neurite outgrowth (Mucke et al, 1994), other evidence suggests a more direct role in synaptic physiology. Electrical stimulation results in N-terminal βAPP secretion (Nitsch et al, 1993). Further, βAPP secretion can be induced via cholinergic receptor binding (Nitsch et al, 1992, 1993), an effect mediated by phosphorylation of a serine residue (probably by protein kinase C) located 7 amino acids into the C-terminal cytoplasmic tail (Gandy et al, 1988; Buxbaum et al, 1990; Caporaso et al, 1992b; Suzuki et al, 1992; Gandy et al, 1993; Knops et al, 1993). The secreted N-terminus of βAPP may act as a potassium channel agonist (Furukawa et al, 1996), and/or the Aβ peptide may act as a potassium channel antagonist (Etchebbigaray et al, 1993, 1994) to directly modulate synaptic function. Although the C-terminal tail has been shown to complex with the GTP-binding protein G0 (Nishimoto et al, 1993), it is unknown whether or not this is actually involved in a signal transduction pathway.

Many of the major putative functions of βAPP are related to the secretion of the N-terminal region into the extracellular space. Therefore, knowledge of the normal roles of βAPP in the brain, specifically related to how and under what circumstances it is processed and secreted, could lead to important insights into the pathogenesis of AD.
Also, since Aβ must be generated by some event involving the cleavage of βAPP, the elucidation of these pathways could identify important therapeutic targets.

**A3.2.3 Secretory Pathways**

The N-terminus of mature βAPP is typically secreted into the extracellular space (Weidemann et al., 1989), leaving behind a membrane associated fragment of about 9 to 11.5 kDa (Oltersdorf et al., 1990). Originally, it was thought that this constitutive cleavage event could lead to the generation of Aβ. However, it was soon demonstrated that normal βAPP cleavage occurred close to the surface of the plasma membrane (within the Aβ region), indicating that constitutive processing could not lead to SP formation in AD (Esch et al., 1990; Sisodia et al., 1990). The cleavage site was later identified as lysine^687^-leucine^688^ (or lysine^16^-leucine^17^ of Aβ), followed by immediate removal of the terminal lysine residue (Anderson et al., 1991; Wang et al., 1991; Pasternack et al., 1992).

The enzyme responsible for constitutive, nonamyloidogenic secretion of βAPP has yet to be identified, but has been named α-secretase; the enzymes responsible for the production of Aβ, following a cleavage event after methionine^671^ and valine^711^ (Aβ_40) or isoleucine^713^ (Aβ_42), have been named β- and γ-secretase, respectively (Selkoe, 1996; c.f. figure A3-1). The Aβ-generating mechanism has been termed the alternative processing pathway, and it is widely believed that a shift in relative balance towards this pathway (such as might be caused by βAPP mutations) is necessary for the occurrence of AD. The constitutive secretory pathway can be stimulated by a number of manipulations (see A3.2.2, above). In general, activation of the α-secretase pathway appears to require the phosphorylation of serine^730^, probably by protein kinase C (Gandy et al., 1993). Since protein kinase C activity appears to be reduced in AD (Van Huynh et al., 1989), and since levels of soluble βAPP similarly decline (Van Nostrand et al., 1992), this is one possible way that the amyloidogenic alternative processing pathway may be favored.

α-Secretase is a membrane bound endoprotease that appears to cleave at a site 12-13 amino acids from the surface of the plasma membrane, independent of substrate primary sequence (Sisodia, 1992). The enzyme may be an ectopeptidase, such as
endopeptidase-24.11, with an active site that faces the extracellular space (Hooper, 1993). However, although α-secretase is likely to be a metalloprotease, most of the common ectopeptidases have been eliminated as candidates (Walsh et al, 1994). Chloroquine (Caporaso et al, 1992a) or leupeptin (Sambamurti et al, 1992) will inhibit the intracellular degradation of βAPP but will not affect secretion of the N-terminus, indicating that α-secretase is not an endosomal/lysosomal enzyme. Most likely, α-secretase may be an enzyme associated with the membrane of the Golgi apparatus, since treatment with agents that disrupt the functioning of this compartment (such as brefeldin A or monensin) block βAPP secretion (Caporaso et al, 1992a, 1994).

β-Secretase could also be either an ectopeptidase or an endopeptidase associated with the Golgi apparatus. Although a cathepsin G-like protease has been isolated that can cleave at the correct site (Razzaboni et al, 1992), further evidence for this candidate has been lacking. An interesting possibility is that the β-secretase cleavage event may occur during the course of constitutive secretory processing. Normal processing results in the generation of an array of carboxyl terminal fragments, some of which begin precisely at the amino terminus of Aβ (Seubert et al, 1993), and others which likely begin at the α- and γ-secretase sites (Estus et al, 1992a, b). Further, the amount of truncated βAPP generated due to β-secretase activity in vitro is sufficient to account for secreted Aβ also present in the culture medium, although the β-secretase cleavage event appears to occur at a much lower frequency in comparison to that of α-secretase (Seubert et al, 1993; Koo and Squazzo, 1994). Slowing the rate of βAPP maturation or transport from the Golgi apparatus to the cell surface is able to shift the constitutive secretory pathway towards β-secretase cleavage (Gabuzda et al, 1994), supporting the notion that β-secretase is an enzyme associated with the Golgi apparatus. At present, it is not known whether β-secretase cleavage is carried out by a distinct enzyme or is merely the result of alternative cleavage by α-secretase.

Although it is possible that a cytosolic or extracellular protease may be responsible for β-secretase cleavage, the γ-secretase site is buried within the membrane and would presumably be inaccessible to such a mechanism (see figure A3-1; Cole et
However, βAPP is a transmembrane protein, and these are typically degraded by the lysosomal system (Hare, 1988). During this process, the protein moves from the membrane of the endosome to the lumen of the lysosome, thereby allowing full access for all degradative enzymes (Renfrew and Hubbard, 1991). One likely site, then, for γ-secretase activity is within the endosomal/lysosomal compartment.

βAPP has been found within clathrin-coated vesicles, which are known to be involved in transport to the endosomal/lysosomal system (Nordstedt et al, 1993), and treatment with substances that disrupt endocytic internalization (such as colchicine and methylamine) prevent this from occurring (Refolo et al, 1995). Furthermore, mature cell surface βAPP has been observed within lysosomes, along with an array of proteolytic by-products, many of which contain Aβ and are therefore potentially amyloidogenic (Golde et al, 1992; Haass et al, 1992a, 1993; Caporaso et al, 1994). Fibrillar Aβ is also found within clathrin-coated vesicles and punctate, electron dense bodies that are almost certainly lysosomally derived (Masliah et al, 1996). Burdick et al (1997) have also shown that lysosomal Aβ is the longer isoform.

Evidence for the intracellular site of Aβ generation has come from in vitro studies using several compounds (see table A3-1). Brefeldin A or monensin are able to block secretion of βAPP and Aβ, while chloroquine or ammonium chloride block secretion of Aβ alone (Caporaso et al, 1992a, 1994; Sambamurti et al, 1992; Shoji et al, 1992; Martin et al, 1995). However, monensin treatment gives rise to a pool of intracellular Aβ generated independently of the secretory pathway (Martin et al, 1995). Both leupeptin and E64 cause an accumulation of amyloid-bearing fragments within lysosomes but do not affect secretion (Cole et al, 1989a; Golde et al, 1992; Haass et al, 1992; Munger et al, 1995), whereas chloroquine and ammonium chloride block secretion and cause intracellular accumulation of what is likely the longer isoform of Aβ (Tsuzuki et al, 1995; Wild-Bode et al, 1997). The generation of a much broader group of proteolytic derivatives following leupeptin/E64 treatment is consistent with inhibition of a subset of lysosomal enzymes while leaving others with intact activity. These results indicate that the enzyme that generates the C-terminus of Aβ is not associated with the Golgi apparatus; an acidic compartment of the trans-Golgi network may generate secreted Aβ
(which is predominantly short Aβ; Hardy, 1997; Wild-Bode et al, 1997), but intracellular Aβ (entirely the long form; Burdick et al, 1997; Wild-Bode et al, 1997) is likely generated elsewhere. In addition to the endosomal/lysosomal system, generation within the endoplasmic reticulum is also a possibility (Masliah et al, 1996; Wild-Bode et al, 1997).

**Table A3-1. In Vitro Data For βAPP Processing**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>βAPP</th>
<th>Aβ</th>
<th>p3</th>
<th>βAPP</th>
<th>fβAPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>E64</td>
<td>Cysteine Proteases</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Cysteine Proteases + Serine Proteases</td>
<td>2-4,10</td>
<td>5,7</td>
<td>5</td>
<td>↑1,4</td>
<td>↑1,4; a</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Serine Proteases</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>General Lysosomal</td>
<td>2,3</td>
<td>↓5,7</td>
<td>↓5</td>
<td>↑1,3</td>
<td>↑1,12b</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>General Lysosomal</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>↑1,6,9</td>
<td>↑1,6,9</td>
</tr>
<tr>
<td>Monensin</td>
<td>General Lysosomal &amp; late Golgi Function</td>
<td>↓5,11</td>
<td>↓5,11</td>
<td>↓5,11</td>
<td>5,6; d</td>
<td>(↑)5,6; d,e</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>Golgi Function</td>
<td>↓5,11</td>
<td>↓5,11</td>
<td>↓5,11</td>
<td>5,6; d</td>
<td>5,6; d,e</td>
</tr>
</tbody>
</table>

Light shading highlights a decrease, whereas dark shading indicates an increase

N/A: not applicable/not studied; - : no effect

*as measured in conditioned media

**As determined for the endosomal/lysosomal fraction and whole cell lysates

*Leupeptin & E64 cause the accumulation of a much wider array of fragments than NH₄Cl/chloroquine

this is likely to be the longer form of Aβ rather than simply fβAPP

Not determined, but chloroquine and NH₄Cl have identical mechanisms

Immature form of βAPP accumulates within the ER/Golgi apparatus

secreted Aβ is blocked by both, but intracellular Aβ continues to be generated following monensin

A truncated form of Aβ, called p3 (after its relative molecular weight), is secreted in parallel with Aβ and is sensitive to the same biochemical manipulations (Haass et al, 1993a). This fragment is thought to represent the γ-secretase product derived from the C-terminal βAPP remnant that is produced following normal α-secretase activity. β-Amyloid derivatives corresponding to the p3 fragment have recently been demonstrated in senile plaques in both DS and AD, although they occur as later elements (Kida et al, 1995; Iwatsubo et al, 1996). These findings are also consistent with reports that the N-terminal cleavage event can occur during normal secretory processing (Seubert et al, 1993) and that the C-terminal amino acid residues are what predispose the peptide to aggregation (Barrow and Zagorski, 1991).

A3.3 The Source of Alzheimer's Disease?

By this point, two factors should be clear from the available evidence:

1. the Aβ peptide is the critical factor in AD pathogenesis, although later pathological events (e.g., cell loss) are required for the clinical manifestations of the disorder (sections A2 and 3). It is not known whether the extracellular deposition or the intracellular generation of Aβ is a more crucial element in the disease process.

2. a possible location for the final cleavage event leading to intracellular Aβ production lies within the endosomal/lysosomal system; in any case, substances which inhibit lysosomal function lead to the accumulation of intracellular Aβ (predominantly of the longer, amyloidogenic variety) and potentially amyloidogenic forms of βAPP.

Haass et al (1992a) have proposed that the common element in AD is a disturbance of the balance between the constitutive and alternative processing pathways, resulting in an increased utilization of the latter and a subsequent elevation in Aβ production. One intriguing possibility is that inhibitors of the lysosomal system could be a viable treatment strategy (Golde et al, 1992; Estus et al, 1992b). However, other
evidence has shown that these treatments are, in fact, cytotoxic - suggesting that slower Aβ turnover within lysosomes may be directly involved with AD pathogenesis (Hayashi et al, 1992). Intracellular Aβ, which is essentially all of the pathogenic longer form of the peptide (Turner et al, 1996; Wild-Bode et al, 1997), aggregates within lysosomes (Masliah et al, 1996; Burdick et al, 1997) and the neuronal cytoplasm (Masliah et al, 1996). It may therefore be that the toxic effects of Aβ are exerted intracellularly, prior to its secretion (Yang et al, 1995; Burdick et al, 1997; Wild-Bode et al, 1997; Yan et al, 1997). Since all these data were gathered via *in vitro* studies, how relevant are they for the vulnerable aged human brain?

To address these issues, we must turn once again to neuropathology and two hypotheses about the AD pathogenic cascade. The first is that the lysosomal system, made deficient by the normal aging process, is linked directly to the development of the disease. Hence, manipulations interfering with the function of the lysosomal system *in vivo*, which have been shown to mimic cellular aging, might result in the accumulation of iAβ (as they do *in vitro*) and subsequent neurodegeneration. The second hypothesis is that the iAβ generated as a consequence of lysosomal inhibitor treatment might not be directly neurotoxic, but might instead serve to increase neuronal vulnerability to a secondary insult or precipitating factor; one likely candidate is excitotoxic injury.
A4 Two Hypotheses of AD Pathogenesis

A4.1 The Lysosomal System in Aging and Alzheimer's Disease

During the course of normal aging, the brain (and other tissues) accumulates intracellular aggregates of lysosomally associated, electron dense, autofluorescent substances known collectively as lipofuscin (Miyagishi et al, 1967; Kemper, 1984). In fact, lipofuscin is the best known marker of neuronal aging, and its accumulation in brain tissue has been shown to be linearly related to age (Moore and Ivy, 1995; Moore et al, 1995). Although lipofuscin is a heterogeneous substance, it is believed to be largely proteinaceous in nature (Palmer et al, 1986; Ivy et al, 1990a). This is consistent with the observation that aging cells suffer a decline in their ability to degrade proteins (Lavie et al, 1982; Ishigami and Goto, 1988, 1990). Since protein synthesis also declines with age (Rattan et al, 1992; Johansen and Rattan, 1993), this results in an increasing reliance on proteins which have remained past their normal half-lives and may have acquired subtle functional alterations (Rothstein, 1985; Reznick et al, 1985).

Early studies failed to find consistent differences in lipofuscin accumulation between AD subjects and controls (Mann and Sinclair, 1978). More recent results point to an increase in larger lipofuscin granules, indicating a possible increase in rate of formation (Dowson et al, 1992), or in relative “aging” of the cells. Others have observed large increases in lipofuscin deposits specifically within populations of neurons considered to be at risk for degeneration in AD (Cataldo et al, 1991; Nixon et al, 1992; Cataldo et al, 1994). It has also been shown that lysosomal enzymes (Cataldo and Nixon, 1990; Cataldo et al, 1990; Cataldo et al, 1996) and endogenous lysosomal inhibitors (Li et al, 1993) are found within SP, and that immunoreactivity for these proteins increases markedly within neurons that are susceptible to neurodegeneration (Nakamura et al, 1991; Nixon et al, 1992; Yasuhara et al, 1993; Cataldo et al, 1994). These findings suggest that a link exists between less efficient lysosomes and the loss of neurons in AD, and that Aβ deposits may originate within the lysosomal compartment.

A4.1.1 Lysosomal Inhibitors, Aging and Neuronal Ceroid Lipofuscinoses

The neuronal ceroid lipofuscinoses (NCL) are a class of inherited disorders that present with a characteristic accumulation of intracytoplasmic storage bodies containing
a lipofuscin-like substance, usually called ceroid lipofuscin (CL)(Palmer et al, 1986; Vesa et al, 1995). In a variety of experimental subjects, administration of inhibitors of lysosomal function (such as the cysteine protease inhibitor leupeptin or the more general inhibitor chloroquine) causes the accumulation of a similar lipofuscin-like substance (Ivy et al, 1984, 1990a, b; Ivy, 1992b; Nunomura and Miyagishi, 1993). Hence, it has been suggested that administration of lysosomal inhibitors models NCL disease and lipofuscin formation, and therefore aging of the lysosomal system (Ivy et al, 1984; Ivy and Gurd, 1988; Ivy et al, 1990a, b; Ivy, 1992a).

Leupeptin (LEU) is a microbial product (leucyl-leucyl-arginal, N-blocked with an acetyl group) that is a partially reversible inhibitor of lysosomal cysteine proteases, such as cathepsins B, H and L (Kirschke and Barrett, 1987). The mechanism of inhibition involves binding of the terminal aldehyde group to the cysteine residue at the active site of these enzymes; weak inhibition of serine proteases is also possible (Marzella and Glaumann, 1987). In contrast to many other lysosomal inhibitors, leupeptin has a low toxicity and is easily soluble in water (Kirschke and Barrett, 1987; Marzella and Glaumann, 1987). Leupeptin treatment has been shown in vitro to cause an intracellular accumulation of βAPP and Aβ, the probable location of which is lysosomal (Cole et al, 1989a; Estus et al, 1992b; Golde et al, 1992; Haass et al, 1992a; Shoji et al, 1992; Sambamurti et al, 1992; Haass et al, 1993).

Chloroquine (CHL) is a commonly used antimalarial drug (Jaeger et al, 1987; White, 1988; Slater, 1993; Edwards et al, 1994) also used in the treatment of autoimmune disorders such as systemic lupus (Wallace, 1994) and rheumatoid arthritis (Fox, 1993). In malaria, chloroquine accumulates within the acidic food vacuole of the intraerythrocytic parasite and prevents hemoglobin degradation, essentially starving the organism to death (Krogstad et al, 1985; Slater, 1993). In autoimmune disorders, chloroquine is thought to act by interfering with antibody processing (Thorens and Vassalli, 1986; Fox, 1993; Wallace, 1994). Chloroquine is a lysosomotropic weak base that accumulates within lysosomes when present extracellularly (Kirschke and Barrett, 1987), and has essentially identical effects as ammonium chloride (Ohkuma and Poole, 1978; Thorens and Vassalli, 1986; Pfeifer, 1987; Marzella and Glauman, 1987). In its
unprotonated form, CHL is relatively lipophilic and is readily membrane soluble; upon entering an acidic environment such as the lysosome, it becomes protonated and too polar to escape rapidly (Mellman et al, 1986; Marzella and Glaumann, 1987). The net effect of this accumulation is to raise the vesicular pH by as much as 1 or 2 units (Okhuma and Poole, 1978), which results in a generalized inhibition of lysosomal enzymes that normally have an acidic pH optima (Koenig, 1984; Marzella and Glaumann, 1987; Pfeifer, 1987). As with LEU, CHL (and ammonium chloride) administration has been shown to cause intracellular accumulation of fβAPP and Aβ (Cole et al, 1989a; Estus et al, 1992b; Golde et al, 1992; Caporaso et al, 1992a, 1994; Tsuzuki et al, 1995; Wild-Bode et al, 1997).

Both normal lipofuscin (Bancher et al, 1989b, c) and the ceroid lipofuscin found in NCL (Wisniewski and Maslinska, 1989; Wisniewski et al, 1990a, b) are known to contain fragments of βAPP (fβAPP) that are potentially amyloidogenic, in that they contain the Aβ region. Also, fragments of βAPP are found to be highly concentrated within lysosomes, and co-localize with lipofuscin in cases of AD (Benowitz et al, 1989). Further, cases of the adult form of NCL display a small increase in the incidence of diffuse plaques (Wisniewski et al, 1990b) and τ and ubiquitin positive PHF (Love et al, 1988). A similar increase in PHF and ubiquitin immunoreactivity is seen following chronic leupeptin treatment in rats, although this may be related to a secondary effect of the drug on cytosolic enzymes (such as calpains I and II) rather than a direct result of lysosomal inhibition (Ivy et al, 1992a; Ivy, 1992a; Takauchi and Miyoshi, 1995).

**A4.1.2 Lysosomes and Amyloid**

Since the endosomal/lysosomal system either generates or contributes to the accumulation of intracellular Aβ (see A3.2, above), these results together implicate a faulty or progressively dysfunctional lysosomal system as the source of Aβ (in this case, the pathogenic Aβ42 isoform) in both normal individuals and in Alzheimer's disease (Nixon et al, 1992; Cataldo et al, 1994). However, why isn't Aβ degraded within the lysosomal compartment? More importantly, how does it become deposited extracellularly in plaques when it appears to be a component of intracellular lipofuscin?
In answer to the first question, lysosomal dysfunction in normal aging and following administration of lysosomal inhibitors results in a decrease in the efficiency of protein degradation, rather than a complete halt. In vitro, this causes the accumulation of a variety of peptide fragments (see A3.2, above), which can be considered as backlogged intermediates in a slower degradative pathway. Furthermore, as peptide fragments within the lysosomal compartment become progressively smaller in size, their rate of degradation declines (Palmer et al, 1986; Hare, 1990), presumably because of a corresponding reduction in endopeptidase access. In the case of dysfunctional lysosomes, such as in NCL, it tends to be the smaller fragments (about 4 kDa) that show the greatest accumulation (Palmer et al, 1986). The Aβ peptide has a calculated molecular weight of 4.2 kDa, and thus falls within this range. Furthermore, Aβ has been shown to be most insoluble and prone to aggregation within a pH range of 3.5-6.5 (Burdick et al, 1992), which overlaps significantly with the normal lysosomal pH of approximately 4.5-5.0 (Mellman et al, 1986), and is intrinsically resistant to proteolysis (Knauer et al, 1992). In further support of this, it has recently been shown using immunoelectronmicroscopy that lysosomal inhibition with chloroquine can cause a build up of Aβ42 within lysosomes (Tsuzuki et al, 1995).

With respect to the second problem, there are two ways that intralysosomal Aβ could escape and become deposited extracellularly. First, lysosomes are known to regurgitate (into the cytoplasm) peptide fragments that are resistant to degradation, and these can sometimes be quite large (>4 kDa; Buktenica et al, 1987; Isenman and Dice, 1989). Masliah et al (1996) have shown that fibrillar Aβ deposits (probably Aβ42; Johnson-Wood et al, 1997) exist intracellularly within both the cytoplasm and lysosomally derived electron dense bodies in a transgenic mouse overexpressing the V717F mutant βAPP (which produces proportionally more Aβ42). It could be that Aβ forms initial aggregates within a favorable lysosomal environment and then “escapes” into the cytoplasm (possibly after damaging the lysosomal membrane; Yang et al, 1997) where it could exert toxic effects. The fragments of βAPP (and Aβ) that accumulate within lysosomes following incubation with a variety of inhibitors are known to accelerate cell death in vitro (Hayashi et al, 1992), so the contents of the lysosomes could be
liberated as a by-product of neurodegeneration. Second, Aβ could simply be released into the extracellular space by exocytosis, since this process occurs under normal conditions (Wisniewski et al, 1994; Bernstein et al, 1996).

Although both possibilities could account for the presence of lysosomal contents within SP (Cataldo and Nixon, 1990; Bernstein et al, 1996), the former of the two (i.e., cell death) is more likely for several reasons. First, the focal nature of amyloid deposits within the brain parenchyma argues against the formation of SP (and the associated localized cell death) via a process of constitutive secretion (Yang et al, 1995; Burdick et al, 1997). It might be expected that such a process, coupled with possible toxicity of extracellular Aβ, would cause very large deposits of amyloid and correspondingly huge areas entirely devoid of cells. Not only are such areas nonexistent, recall that relatively large deposits of extracellular amyloid can exist in the absence of appreciable cell loss in the striatum and cerebellum (McGeer et al, 1994), and that some “normally aged” individuals can possess SP densities diagnostic of AD in the absence of synapse loss and dementia (Lue et al, 1996). Second, very little of the pathogenic longer form of Aβ, which actually forms the nucleus of the initial extracellular amyloid deposit, is actually secreted under most circumstances (Wild-Bode et al, 1997; Hardy, 1997). In contrast, Aβ_{42} is present intracellularly whereas Aβ_{40} (the predominantly secreted, “soluble” form that is found later in SP; lwatsubo et al, 1995, 1996) is present either in very small quantities (Turner et al, 1996) or is undetectable (Wild-Bode et al, 1997). Similarly, long Aβ forms intracellular aggregates (Burdick et al, 1997) which can stimulate the accumulation of additional intracellular amyloid (Yang et al, 1995), whereas short Aβ is rapidly degraded (Burdick et al, 1997). Also, secreted Aβ is likely generated by a pathway distinct from that producing intracellular Aβ (Martin et al, 1995; Turner et al, 1996; Wild-Bode et al, 1997; Tienari et al, 1997). Third, RNA is also present in the plaque core (Ginsberg et al, 1997) along with lysosomal contents; since neither is normally present extracellularly, both may comprise part of the original plaque “nucleus” formed by a deceased neuron loaded with Aβ_{42}. Fourth, DiFiglia et al (1997) have demonstrated that abnormal intracellular aggregates of the huntingtin protein may be responsible for the neurodegeneration seen in Huntington’s disease. Notably,
extracellular deposits of huntingtin are not found, implying that this "intracellular amyloidosis" may be all that is required for the occurrence of neurodegeneration in this disorder, and possibly in other disorders of protein aggregation as well.

**A4.1.3 The Direct or Causal Hypothesis of iAβ Toxicity**

Therefore, it follows from available evidence that SP may originate as a consequence of a build-up of intracellular amyloid (possibly emanating from a lysosomal source) directly leading to cell death. In a recent report, Yan et al (1997) described an intracellular protein associated with the endoplasmic reticulum (ERAB) that could mediate Aβ-induced neurotoxicity from an intracellular site. The resulting "seed" of Aβ left over as a remnant of this process could then precipitate the formation of larger plaques containing, among other things, aggregates of secreted Aβ (Burdick et al, 1997). During growth and maturation of the deposit, the triggering of inflammatory processes could account for the ensuing death of bystander neurons in the vicinity of the SP (McGeer and McGeer, 1995). I will call this the direct or causative hypothesis of intracellular Aβ toxicity.

**A4.2 Excitotoxic Injury as a Pathogenic Mechanism in AD**

The term "excitotoxicity" was coined by Olney (e.g., 1990, 1994) to refer to brain damage resulting from excitatory amino acids such as glutamate. Excitotoxicity is now thought to occur in epilepsy, ischemia and following traumatic brain injury (Erecinska and Silver, 1996). Commonly, excitotoxicity is triggered by an excitatory amino acid (EAA; such as glutamate) or analog thereof (such as aspartame; Olney, 1990). Because of their ubiquitous distribution and potential to inflict damage, EAA's have often been put forward as key elements in a variety of neurodegenerative disorders. In fact, one of the best correlates of dementia in Alzheimer's disease is the degeneration of glutamatergic association systems (Francis et al, 1993), and these neurons are susceptible to the development of NFT (Kowall and Beal, 1991). Also, the areas of characteristic AD pathology distribute along EAA neocortical association pathways (Greenamyre and
Young, 1989), and the most vulnerable areas closely parallel those of systemically administered excitotoxins (Lothman and Collins, 1981; Ben-Ari et al, 1989).

A4.2.1 Mechanisms of Excitotoxicity

The process by which cells die can be divided into two phases: an acute phase (due to a large influx of sodium ions) where the neurons swell immediately, and a delayed phase (due to activation of calcium dependent processes) marked by degeneration (Choi et al, 1989). Since activation by glutamate (Connor et al, 1988) or agonists such as kainic acid (KA; Miller et al, 1989) lead to characteristic rises in Ca\(^{2+}\) content, and since manipulations such as the removal of extracellular Ca\(^{2+}\) prevent excitotoxic cell death, increases in [Ca\(^{2+}\)] are thought to be of primary importance (Choi et al, 1989; Mattson and Mark, 1996).

Calcium is probably the foremost second messenger ion, a role which extends to all other systems beyond the brain (Erecinska and Silver, 1996). Calcium ions are responsible for the regulation of a number of intracellular enzymatic processes, and it is these that become dysfunctional during periods of disordered calcium homeostasis (Siesjö et al, 1989). In fact, despite a large number of redundant mechanisms that strive to keep intracellular calcium levels low (Erecinska and Silver, 1996), age-related impairments in these systems appear to be commonplace (Roth, 1989). This is important, since even moderate prolonged elevations of [Ca\(^{2+}\)] could inappropriately activate calcium dependent systems and result in significant damage (Landfield et al, 1989). Thus, given the amplified vulnerability to excitotoxic processes with increasing age (Auer, 1991), even the wear-and-tear of normal synaptic function may be sufficient to result in neuronal attrition in some individuals (Olney, 1990; Crutcher et al, 1993).

It is well known that cells lacking high levels of calcium binding proteins are less resistant to hyperstimulation and can be protected by calcium chelation (Scharffman and Schwartzkroin, 1989; Mattson and Mark, 1996). High intracellular concentrations of the calcium binding protein parvalbumin are associated with resistance to Ca\(^{2+}\)-mediated neurodegeneration via a number of pathways (Scharffman and Schwartzkroin, 1989; Nitsch et al, 1989; Leranth and Ribak, 1991). Furthermore, parvalbumin positive neurons are spared in Alzheimer's disease (Ferrer et al, 1991; Hof et al, 1991), and
other areas rich in calcium buffering capacity (such as the cerebellum) are similarly resistant to degenerative processes (Heizmann and Braun, 1995).

**A.4.2.2 Aβ/βAPP and Excitotoxicity**

Although high concentrations of Aβ are known to be toxic (Yankner *et al*, 1989), lower concentrations do not appear to be so (Yankner *et al*, 1990). However, a substantial amount of evidence indicates that the Aβ peptide, even when present at very low concentrations, increases neuronal vulnerability to excitotoxic and oxidative damage (Koh *et al*, 1990; Mattson *et al*, 1992; Patel *et al*, 1995). This effect may be mediated through increased intracellular calcium, which can be directly observed following Aβ treatment (Mattson *et al*, 1992, 1993a), and can be blocked by lowering extracellular Ca^{2+} (Mattson *et al*, 1992) or following the administration of K+ channel agonists (Goodman and Mattson, 1996), Ca^{2+} channel blockers (Mark *et al*, 1995) or antioxidants (Mattson and Goodman, 1995). Neuronal cultures pretreated with Aβ also show an increased incidence of abnormally phosphorylated τ and ubiquitin accumulation in response to an excitotoxic insult (Mattson *et al*, 1992; Mark *et al*, 1995). Although Aβ has been shown to directly block K⁺ currents (Etcheberirigaray *et al*, 1994), the interaction of Aβ with excitotoxic processes appears to require an aggregated, or "older" form of the peptide (Mattson *et al*, 1993b), and is only apparent following several days of incubation, suggesting a cumulative process rather than an immediate interaction with a channel or receptor (Patel, 1995). Similarly, Aβ can be taken up by cultured cells and form intracellular aggregates (Yang *et al*, 1995; Burdick *et al*, 1997), so the actual presence of Aβ intracellularly may be the critical element mediating this effect.

In contrast, βAPP may directly interact with and open K+ channels (Furukawa *et al*, 1996). As outlined above (A3.2.1), βAPP is secreted in response to neuronal activation, suggesting a role in synaptic function (Nitsch *et al*, 1992, 1993). This interaction leads to neuronal hyperpolarization and reduced calcium influx (Mattson and Barger, 1993; Furukawa *et al*, 1996). Hence, whereas Aβ destabilizes [Ca^{2+}], βAPP may function in the opposite sense (Mattson and Barger, 1993; Mattson *et al*, 1993a). In fact, βAPP has been shown to directly antagonize the effects of Aβ on excitotoxicity.
(Goodman and Mattson, 1994). Interestingly, βAPP isoforms containing the Kunitz protease inhibitor region are more effective at attenuating excitotoxic damage, as shown in transgenic mice overexpressing low levels of either βAPP<sub>695</sub> or βAPP<sub>751</sub> and subsequently exposed to kainic acid (Masliah et al, 1997), a finding consistent with the upregulation of KPI-containing species following neural trauma (A3.2.1).

A4.2.3 Excitotoxic Cell Death in AD

There are numerous other symptoms of damage in Alzheimer’s disease that could be related to disruptions in calcium regulation. These can be grouped into four classes of altered enzymatic activity (Orrenius et al, 1996): phospholipases (leading to membrane damage), endonucleases (leading to apoptotic cell death), protein kinases/phosphatases (leading to abnormal phosphorylation) and proteases (leading to cytoskeletal damage). Unfortunately, there is a dearth of direct evidence for in vivo excitotoxicity in AD, which can be largely attributed to the lack of a good animal model.

A4.2.3.1 Membrane Damage

Membrane damage is commonly observed in Alzheimer’s disease, although what proportion may be due to phospholipase activation is unknown. At the ultrastructural level, cell membranes are on average 0.4 nm thinner and appear less densely packed (Mason et al, 1993). Also, the membrane composition is slightly altered due to a decrease in the amount of free cholesterol (Mason et al, 1993). Given the role of ApoE (which is responsible for cholesterol transport; Mahley, 1988) in AD, the changes in cholesterol may be related to aberrant function of this protein. Alternatively, the participation of free radical-mediated oxidative damage cannot be ruled out, since this is known to be an important factor in Down’s syndrome (Busciglio and Yankner, 1995), aging (Stadtman, 1992) and Alzheimer’s disease (Richardson, 1993; Thomas et al, 1996; Yan et al, 1996). On the other hand, excitotoxicity and oxidative damage are tightly intertwined processes. For example, glutamate can increase free radical production, and antioxidants (Mattson and Goodman, 1995) and potassium channel agonists (Goodman and Mattson, 1996) can partially block this effect.
A4.2.3.2 Apoptosis

A sizable proportion of excitotoxic cell death occurs via an apoptotic (programmed cell death) process, rather than by simple necrosis (Charriaut-Marlangue et al, 1996; Orrenius et al, 1996). The activation of enzymatic pathways are essential for apoptosis to occur (Nicholson et al, 1995), including endonuclease activity regulated by Ca²⁺ concentration (Orrenius et al, 1996). Apoptotic cell death is known to occur in both Alzheimer's disease (Su et al, 1997), and in Down's syndrome neurons in vitro (Busciglio and Yankner, 1995). Furthermore, \( \beta \text{APP}_{717} \) mutations are known to induce apoptotic cell death in culture (Yamatsuji et al, 1996), as are presenilin 2 mutations (Wolozin et al, 1996). Hence, it is possible that familial AD cases represent instances of enhanced vulnerability to excitotoxic damage leading to apoptosis. It should be emphasized that there is no direct evidence that AD is a disease of which aberrant apoptosis is the central feature. Since apoptosis occurs under many circumstances it is likely to simply represent a mechanism of cell death in AD. As one example, transgenic mice that massively overexpress \( \beta \text{APP} \) mutants or mutant PS do not shown remarkable apoptotic cell death (Hardy, 1997).

A4.2.3.3 Abnormal Phosphorylation

Excitotoxic processes can alter the balance between protein phosphorylation and dephosphorylation in a manner which can be unpredictable (Saitoh et al, 1991). Given the established role of protein phosphorylation in \( \beta \text{APP} \) processing (A3.2.2) and NFT formation (A2.2.1), the possibility exists that an imbalance caused by Ca²⁺ toxicity may be connected with AD (Greenamyre and Young, 1989). In vitro, moderate increases in extracellular Ca²⁺ results in increased \( \tau \) phosphorylation and ubiquitin immunoreactivity, and causes granulovacuolar degeneration (Mattson et al, 1991). However, there does not appear to be extensive aberrant protein phosphorylation in AD, so the process cannot be a general one (Goedert et al, 1995).

Environmental exposure to excitotoxins in humans can lead to the appearance of NFT, as illustrated by the occurrence of the pathology in Guam ALS/PDC (which is linked to the consumption of the NMDA agonist L-BMAA; Spencer et al, 1987) and ALS (which is associated with excessive oxidative damage; Olney, 1990). Furthermore,
infusion of glutamate into the rat cortex causes abnormal \( \tau \) phosphorylation (Irving et al, 1996), and the chronic presence of EAAs in culture media can result in the appearance of PHF-like structures over a period of several weeks (De Boni and McLachlan, 1985). Also, in addition to being abnormally phosphorylated, NFT exhibit a large proportion of nitrotyrosine residues (Good et al, 1996). Nitrotyrosine is produced through the oxidation of tyrosine by peroxynitrite, which is formed by a reaction between nitric oxide (produced during glutamatergic synaptic activity) and the superoxide anion (produced as a by-product of normal mitochondrial respiration), thus providing another link between excitotoxic processes and oxidative damage (Mattson and Mark, 1996).

**A4.2.3.4 Abnormal Proteolytic Activity**

Calcium-activated proteases are involved in structural alterations of the neuronal cytoskeleton (Lynch and Seubert, 1989). Thus, widespread, persistent elevations in \( \text{Ca}^{2+} \) could conceivably lead to the appearance of cytoskeletal pathology and/or a slow deterioration of the brain's structural integrity (Landfield et al, 1989; Lynch and Seubert, 1989). Excitotoxic processes activate calcium dependent proteases (especially calpain I) that degrade structural proteins (Siman and Noszek, 1988). Evidence indicates that calpain I activity increases substantially in AD, a finding consistent with an excitotoxic mechanism of cell death (Nilsson et al, 1990; Saito et al, 1993). However, the protease inhibitor leupeptin, which inhibits calpain (Toyo-Oka et al, 1978), is known to cause the accumulation of PHF-IR in rats (Ivy et al, 1989; Ivy, 1992a; Takauchi and Miyoshi, 1995). Hence, it is at present unclear how abnormal proteolysis interacts with aberrant phosphorylation to produce NFT-like cytoskeletal abnormalities.

**A4.2.4 A Relationship To Lysosomal Dysfunction?**

Therefore, a significant number of studies indicate a role for excitotoxic processes in the pathogenesis of AD. This is not surprising, given that \( \beta \text{APP} \) is secreted during synaptic activity (Nitsch et al, 1992, 1993) and is upregulated in response to excitotoxic damage of all types (reviewed in Mattson et al, 1993a; c.f. A3.2.1). Further, this is also consistent with the epidemiological connection between incidents of head trauma, which
are known to trigger excitotoxic cascades (Erecinska and Silver, 1996), and the development of AD (Mortimer et al, 1985, 1991).

Although vulnerability to excitotoxic injury is known to increase with age (Auer, 1991), it is unknown if this is related in any way to age-associated deficits in lysosomal function. However, Bahr et al (1994) conducted an experiment in a stable hippocampal slice preparation indicating that chronic incubation with chloroquine (CHL) resulted in increased intracellular accumulation of fβAPP and a related decrease in synaptophysin-IR. Further, administration of the excitotoxin kainic acid (KA) before CHL incubation caused the appearance of a stable variant of fβAPP that was resistant to turnover following CHL washout. The consequences of this interaction with regards to excitotoxic degeneration were not assessed in this study, nor was the converse manipulation of inducing the lysosomal dysfunction and fβAPP accumulation prior to the excitotoxic challenge.

**A4.2.5 The Indirect or Permissive Hypothesis of iAβ Toxicity**

Therefore, even if intracellular Aβ has no immediate consequences for cell death (leading to subsequent plaque formation, etc.; see A4.1, above), it may be that the presence of the peptide creates a predisposition to neurodegeneration by another mechanism potentially connected to AD pathogenesis, such as excitotoxicity. I will call this the indirect or permissive hypothesis of intracellular Aβ toxicity.
A5 Animal Models of Alzheimer’s Disease

In order to evaluate these two hypotheses, it is first necessary to have a model system with which to work. Unfortunately, no animal model exists that possesses all of the characteristics of AD. In fact, the major obstacle to therapeutic advances in AD is the lack of a suitable animal model for the disorder.

A5.1 AD-Like Pathology in Animals

A5.1.1 Non-Human Primates

Most of the characteristics of the disease appear to be primate specific. Non-human primates, such as rhesus monkeys (Wisniewski et al, 1973; Wisniewski, 1979) and marmosets (Baker et al, 1993), develop both senile plaques and neurofibrillary tangles with increasing age. The SP contain an identical Aβ peptide (Podlisny et al, 1991) and some other plaque associated elements, such as αACT (Abraham et al, 1989; Cork et al, 1990) and ApoE (Poduri et al, 1994). However, there are slight differences in other aspects of the pathology. αACT does not appear to be expressed in plaque-associated astrocytes as it is in AD (Koo et al, 1991), and congophilic angiopathy is only apparent in very old subjects (Cork et al, 1990). Further, the NFT-like lesions differ ultrastructurally from those of AD in that they twist every 50 rather than every 80 nm (Wisniewski, 1979).

Nevertheless, the primate is widely considered to be the best naturally occurring model of AD pathology. Unfortunately, primates are difficult to obtain, hard to work with, and exceedingly costly. Furthermore, the animals must reach very old age (20+ years) to develop the pathology, and even then there is considerable variability in the amount of neuropathology that develops spontaneously. This makes for very expensive and slow research endeavors, thus making non-human primates better suited for later stages of investigation, after supportive data have been gathered in other paradigms.

A5.1.2 Dogs

An interesting alternative to the primate is the domestic canine. It has been known for many years that aged dogs will develop neuropathology similar to early stages
of AD (Wisniewski et al, 1970; Giaccone et al, 1990; Cummings et al, 1996a). The SP that are apparent in the dog brain resemble diffuse plaques, in that they generally lack congophilia, dystrophic neurites and associated reactive glia (Giaccone et al, 1990; Cummings et al, 1996a). Similar to primates, dogs have an Aβ sequence identical to that of humans (Johnstone et al, 1991). Unlike primates, however, dogs are widely considered to lack cytoskeletal pathology analogous to NFT (Wisniewski and Terry, 1973; Cummings et al, 1996a).

The canine has many advantages over the primate as a model of AD. Obviously, dogs are much easier to obtain in comparison to monkeys, are vastly cheaper, and far easier to work with as experimental subjects. Unlike non-human primates, dogs share the same environment as humans, and would likely be exposed to the same factors that might influence the development of AD (Cummings et al, 1996a). Importantly, they also begin to show signs of AD-like pathology at a relatively early age (approximately 8+ years), whereas even the domestic cat fails to develop diffuse amyloid deposits until the rare age of 18 years or more (Nakamura et al, 1996).

A5.1.3 Rodents

Even when taking into account the advantages of the dog, a rodent model of AD is still preferable for many reasons. Rodents are relatively inexpensive, breed quickly, and have a short lifespan. Because of these factors, experiments are possible with large numbers of subjects and manipulations. Unfortunately, both rats and mice do not develop pathology that in any way can be considered to be Alzheimer's disease. Although extremely old rats will occasionally develop plaque-like lesions (Vaughan and Peters, 1981), these are associated with spongiform changes in the brain, indicating a likely association with a prion-type pathology rather than AD.

It is unknown why rodents fail to develop any AD-like pathology with increasing age. One possibility is that plaque formation is a very slow biochemical process in vivo (Kisilevsky et al, 1992), and that rodents simply do not live long enough to develop the pathology. Another possibility is the known difference in the primary sequence of the β-amyloid peptide in rodents (Lannfelt et al, 1993), since it is identical in non-human primates and canines. Both rats (Shivers et al, 1988) and mice (Yamada et al, 1987)
have amino acid substitutions at positions 5, 10 and 13 of Aβ. Although this does not appear to alter the peptide's ability to aggregate in vitro (Shivers et al., 1988; Fraser et al., 1992), it does not rule out altered in vivo processing of the precursor (which differs by 19 amino acids in total) to produce different, nonamyloidogenic fragments.

Hence, the only alternative for the development of a rodent model of AD has been the production of transgenic animals harboring the gene for the human amyloid precursor protein. Since the mouse is genetically categorized much better than the rat, attempts at developing transgenic models have mainly focused on this species. The history of this field can be roughly split into 3 phases (see table A5-1).

The earliest attempts at AD transgenic mice involved the insertion of a coding sequence for a C-terminal fragment of βAPP, approximately 100 amino acids in length. This peptide therefore includes the entire coding sequence for the Aβ peptide, generally starting at the N-terminal end, thus eliminating the need for β-secretase cleavage of βAPP to free the peptide (although γ-secretase would still be required). The rationale for this derived from observations that the C-terminal end of βAPP was potentially neurotoxic, and that this property was likely due to its amyloidogenic potential (Yankner et al., 1989; Yoshikawa et al., 1992). The two viable models (Sandhu et al., 1991; Kammesheidt et al., 1992) did not appear to develop the characteristic plaques and tangles of AD, although the latter of the two did show some signs of developing congophilic angiopathy. Both models also showed a punctate intracellular accumulation of the transgene, possibly indicating its presence within the endosomal/lysosomal compartment (Kammesheidt et al., 1992). Alternatively, the peptide, which is essentially a "junk" protein, may have been recognized as such and degraded by the endoplasmic reticulum immediately after synthesis (Hiller et al., 1996), potentially preventing its subsequent processing into Aβ.

Once it became clear that fragmentary βAPP was unlikely to produce a model of AD, the second generation of transgenics was introduced with coding for various full length transcripts (Lannfelt et al., 1993). In response to one of the obvious difficulties with earlier efforts, greater attention was paid to obtaining higher levels of expression (Wight and Wagner, 1994). However, these newer mice either did not develop appropriate
### Table A5-1. Transgenic Mouse Models of Alzheimer's Disease

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<th>Authors</th>
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<th>Expression</th>
<th>Neuropathology?</th>
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<td>JC Viral</td>
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<td>High (5X)</td>
<td>SP, all classes; No NFT; Impaired spatial learning</td>
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</table>

The three “phases” of transgenic models are indicated by different shading.

**Authors:** All of the models listed here are multi-author works; this has been omitted for space considerations (see reference section for complete listing).

**Transgene:** C-100/4 refers to the C-terminal fragment of βAPP; the full length forms of βAPP used in the models of Games et al (1995) and Hsiao et al (1996) were both mutant forms (single and double mutants, as shown) known to cause autosomal dominant AD in humans; it is suspected (though not proven) that the mutant form of the transgene is responsible for the success of these models, although the higher levels of expression are also likely important.

**Promoter:** Abbreviated promoters are as follows: Dys - Dystrophin; NSE - Neuron Specific Enolase; MTIIA - metallothionine IIA; PDGF - Platelet Derived Growth Factor; PrP - Prion Protein.
neuropathology (Perry et al., 1995) or only developed diffuse deposits of Aβ (Quon et al., 1991; Higgins et al., 1993, 1994), raising the possibility that simple overexpression was insufficient given the short murine lifespan (Lannfelt et al., 1993).

Recently, two relatively successful murine models have been developed, each using full length βAPP, but using mutant forms known to cause autosomal dominant familial AD in humans (Games et al., 1995; Hsiao et al., 1996). Both strains have been shown to develop all forms of SP in a roughly age-dependent manner, although neither has as yet been shown to have NFT. Although the strain studied by Hsiao et al. has also been shown to have a small impairment in spatial learning and memory (Hsiao et al., 1996), it is unclear whether this is actually due to the formation of SP or to a sensorimotor deficit. It should be pointed out that Perry et al. (1995) have shown that another group of βAPP transgenic mice develop marked sensorimotor dysfunction in the apparent absence of amyloidogenesis. Nevertheless, this avenue of research is likely to provide answers to many other key questions about amyloidosis. Additional models have recently been produced using similar techniques (Sturchler-Pierrat et al., 1997), including double mutants co-transgenic for a presenilin 1 mutation (Borchelt et al., 1997). Future developments in transgenic technology, such as site-selective regulation (Morris and Morris, 1997) and the use of inducible promoters (Mayford et al., 1996), will help in dealing with some of the methodological issues raised by the earlier studies.

A5.2 The Choice of A Model

Therefore, transgenic mice are an acceptable model system in which to test the direct hypothesis of iAβ toxicity, since they are at least capable of developing SP pathology in the event of cell death caused by the peptide. Alternatively, a natural model (such as a monkey) can provide similar information, although much more limited in scope due to the difficulty in obtaining and working with such animals. Although the formation of extracellular Aβ deposits after neuronal death would lend additional credence to the hypothesis that iAβ is a pivotal element in AD pathogenesis, it is not critical for this thesis, the primary goal of which is to assess the potential toxicity of iAβ. In either the direct or indirect form, this hypothesis can be evaluated in the normal rat.
A6 Rationale and Experiments

Substantial evidence links lysosomes and iAβ to the pathogenesis of Alzheimer’s disease, at levels of both molecular and neural pathology. This includes:

(1) Alzheimer’s disease is overwhelmingly associated with aging, with risk and incidence rates increasing in parallel

(2) the lysosomal system becomes steadily dysfunctional in parallel with the normal aging process, as seen through manifestations of decreased protein turnover, such as lipofuscin accumulation

(3) the lysosomal system in Alzheimer’s disease exhibits changes suggestive of greater dysfunction than normal, and NCL patients show moderate increases in AD-like neuropathology

(4) the Aβ peptide is the central element in the disorder, and this appears to be generated and/or accumulate within the lysosomal system

(5) insoluble Aβ accumulates intracellularly, possibly after forming aggregates within a favorable lysosomal environment

(6) lysosomal enzymes (and RNA) are found within SP, possibly as a result of dying neurons in (5) releasing their contents into the extracellular space

(7) lysosomal inhibitors have been shown in vivo to model the aging lysosomal system and NCL disease, and can cause some manifestations of brain aging and Alzheimer’s disease

(8) lysosomal inhibitors cause the intracellular accumulation of potentially amyloidogenic fragments of βAPP and Aβ

My working hypothesis is that the dysfunctional lysosomal system that accompanies normal aging is a major contributing factor in the accumulation of intracellular Aβ and, hence, the disease process. Therefore, manipulation of the lysosomal system through the use of inhibitors can mimic the situation seen in normal aging, and should lead to the appearance of iAβ (which has not been demonstrated in vivo using lysosomal inhibitor treatment). The induction of iAβ in vivo is the first goal of this thesis and is examined in each of the 7 experiments.
The role of intracellular Aβ in AD is unknown, and the second goal of this thesis is to determine if the appearance of intracellular Aβ has any consequences that could be connected to the pathogenesis of the disease. The most likely possibility is that intracellular Aβ will be neurotoxic. Although not critical in this situation, neuronal death may be followed by extracellular deposition of the peptide as cellular "remains" which may serve as the nucleus for SP formation. This causative hypothesis was tested in experiments 1 and 2, using mice transgenic for human βAPP and non-human primates, both of which are considered to be at least theoretically capable of developing SP. These experiments utilized the direct brain infusion of leupeptin to induce iAβ; in a later experiment (4), this hypothesis was re-tested by administering the lysosomal inhibitor chloroquine via a systemic route in the same strain of transgenic mouse. In all cases, iAβ was induced by these manipulations but was never associated with any additional pathological consequence under the conditions used in this thesis.

As an alternative to playing a direct role in cell death, it is possible that intracellular Aβ is not directly neurotoxic, and instead creates a predisposition or vulnerability to another insult or injury. One likely mechanism, as outlined above (A4.2), is excitotoxicity. It is possible that Aβ may accumulate intracellularly and may, under most circumstances, be relatively harmless. However, it may impart an increased risk for excitotoxic neurodegeneration. In the AD brain, this could also account for the release of intracellular Aβ and the consequences that this entails. This permissive hypothesis was first tested in experiment 3 using infusion of CHL as a means to induce iAβ in normal rats, and the systemic injection of the glutamatergic agonist kainic acid (KA) as the means of excitotoxic injury. The hypothesis was also tested by performing a similar series of experiments (5 to 7) in rats using systemically applied CHL. In these experiments, iAβ induction was associated with a heightened vulnerability to neuronal loss caused by KA. In the case of the final experiment, this cell loss was also associated with a deficit in the Morris watermaze test of spatial learning and memory.

These results suggest that iAβ itself is not immediately harmful but can impart an increased risk for other potentially damaging processes that may be involved in AD pathogenesis. Thus, the reduction of iAβ should be considered a viable therapeutic goal.
SECTION B:

GENERAL METHODS
B1 Subjects

All animals maintained at the college (mice and rats only) were kept under a 12:12 light:dark cycle with food and water available ad libitum. Subjects were housed individually during experiments.

B1.1 Mice

Mice were bred at the University of Toronto (Div. of Comparative Medicine) from original stock provided by Dr. S. Zain (see Sandhu et al., 1991, for details). Genotyping was conducted at the downtown facility (courtesy of Dr. DRC McLachlan), where the animals were group housed (3-4 animals/cage) in micro-isolation units. Prior to the start of experiments, animals were transported to the suburban animal facility at Scarborough college, where all studies were to take place. Both male and female mice were used, with an approximate weight range of 25-30 grams (ages are described in the appropriate experiments). Additional wild type mice (C57/BL6), also male and female, were obtained from Charles River, Canada.

B1.2 Rats

Male Sprague-Dawley (SD) or Long-Evans Hooded (LEH) rats (both supplied by Charles River, Canada), aged 3-4 months (approximately 300-400 grams), were used.

B2 Drugs

B2.1 Lysosomal Inhibitors

The experiments in this thesis utilized either leupeptin or chloroquine. Both of these substances are well known inhibitors of lysosomal function (Koenig, 1984; Mellman et al., 1986; Pfeifer, 1987; Kirschke and Barrett, 1987; Marzella and Glaumann, 1987). They have both been shown to cause an increase in an intralysosomal lipofuscin-like substance, which has been hypothesized to represent relative cellular aging (Ivy et al., 1984, 1990a, b; Ivy and Gurd, 1988; Ivy, 1992a, b; Nunomura and Miyagishi, 1993). Both were dissolved in phosphate buffered saline (PBS; 0.9% NaCl in 0.1 M sodium phosphate buffer, pH=7.4) for direct brain infusion or intraperitoneal (i.p.) injection (in the
case of chloroquine). For oral administration of chloroquine, double-distilled water was used as the vehicle instead of PBS. Doses and rationale(s) for dose selection are described in the individual experiments.

**B2.2 Kainic Acid**

The glutamatergic agonist kainic acid (KA) was used in order to examine excitotoxic interactions with intracellular Aβ. Kainate is a commonly used excitotoxin that produces well characterized patterns of damage (e.g., O'Shaugnessy and Gerber, 1986; Gayoso et al, 1994). Further, KA has been repeatedly shown to induce βAPP as a consequence of its neurotoxic effects (i.e., neuronal death), an effect very similar to that caused by other types of neuronal injury (Siman et al, 1989; Kawarabayashi et al, 1991; Willoughby et al, 1992; Solà et al, 1993; Töpper et al, 1995).

Kainic acid was prepared fresh daily in PBS, and pH adjusted (to 7.2) with concentrated sodium hydroxide. Subjects were injected i.p. with either KA or PBS vehicle. When possible, KA/PBS administration was performed on pairs of animals. For rats, a dose of 12 mg/kg was used. Seizures were scored using the Racine scale (Racine, 1972): [1] repetitive mouth movements, usually accompanied by drooling; [2] stereotypical head nodding; [3] forelimb clonus; [4] rearing; [5] rearing and falling. Note that higher level seizures usually contain elements of lower ones. The scale was modified with the addition of a class 6 (multiple rearing and falling incidents) and class 7 (running and jumping) seizure (Cammisuli et al, 1997). Onset of status epilepticus (SE) was defined as 5 minutes of continuous motor seizure activity, and typically began between 20-30 minutes following the first seizure (FS) incident in the rat. Seizures were monitored in order to verify the effectiveness of the drug, which is seizure dependent (Petito et al, 1977; Zucker et al, 1983; Ruth, 1984; Nadler et al, 1986; Nitsch and Hubauer, 1986; Ruth and Feinerman, 1988). Status epilepticus was terminated after 30 minutes (total) with an injection of 65 mg/kg sodium pentobarbital and 5 mg/kg of atropine sulfate; PBS injected controls were treated in an identical manner. Mice were given an initial injection of 12 mg/kg, followed by an additional 24 mg/kg 2 hours later.
Mice were scored using a similar seizure scale to the rats, and were otherwise treated the same with the exception of being allowed a 45 minute period of SE.

B3 Tissue Preparation

All animals were sacrificed with an overdose of ketamine/xylazine and perfused transcardially with phosphate buffered saline at 4°C, followed immediately by 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB; pH=7.4). The brains were removed and post-fixed for a minimum of 24 hours prior to any further processing.

Following the post-fixation period, tissue was either sectioned using a vibratome or further prepared for freezing or paraffin embedding. For freezing, tissue was cryoprotected in 25% sucrose in PB overnight, and then immersed in -40°C isopentane, followed by storage at -70°C until sectioned on a sliding microtome. Prior to paraffin embedding, tissue was washed for 30 minutes in PB, followed by 1 hour each in an ascending series of ethanol solutions (2 x 70%, 80%, 90%, 95%, 2 x 100%), histoclear (1:1 histoclear/absolute ethanol, 2 x histoclear) and paraffin (2x); tissue was sectioned on a rotary microtome. These different procedures were performed as required to obtain different section thickness and as dictated by antibody reactivity under differing conditions of tissue preparation.

B4 Histology

Tissue was mounted on glass slides coated with 0.05% chrome-alum/0.65% gelatin. For cresyl violet, tissue was rehydrated in a descending series of xylene and ethanol solutions (3-5 minutes each: 2 x xylene, 2 x 100% ethanol, 2 x 95%, 70%, 50%, 25%, water), immersed in 1% cresyl violet acetate for 5 minutes, dehydrated in an ascending ethanol series (as above), and coverslips applied. For the periodic acid-Schiff reaction (PAS), tissue was rehydrated (as above), treated with 0.5% periodic acid for 20 minutes and washed in running tap water for 10 minutes. Incubation in Schiff’s reagent (576 ml double-distilled water, 24 ml concentrated HCl, 11.4 g sodium metabisulfite, 6 g pararosaniline) for 10 minutes was then followed by a 10 minute wash in running tap water, dehydration and coverslipping.
B5 Immunohistochemistry

B5.1 Method

For immunohistochemistry, sections were pre-incubated in 5% normal serum (horse or goat, for monoclonal and polyclonal primary antisera, respectively) and 0.3% Triton X-100 in PB for 1 hour prior to primary antibody incubation (vibratome and frozen sections were reacted free-floating). Pilot tests were run to determine endogenous peroxidase activity, and in all cases indicated negligible reactivity. Therefore, standard peroxidase blocking was omitted. Incubation in primary antibodies was carried out overnight at 4°C. Incubation in biotinylated secondary antibodies (horse anti-mouse IgG or goat anti-rabbit IgG where appropriate; 7.5 µg/ml PB, 1 hour; Vector) and ABC conjugation (Vectastain ABC Standard, 45 minutes; Vector) were performed at room temperature. Reactions were visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.8 mg/ml; Sigma) in 0.01% H2O2 /PB. Additional sections were processed in absence of the primary antibody (or preabsorbed with the antigen when possible) to determine nonspecific reactivity. More detailed information on immunohistochemistry theory and methodology are available elsewhere (Larsson, 1988).

B5.2 Specificity

All antibodies employed in this thesis are commonly used, certified commercial antibodies or are otherwise well characterized. The critical elements of this work rely on the detection of Aβ and its precursor, βAPP. Antibodies used to detect Aβ are specific for the peptide under most circumstances. However, since Aβ is derived from βAPP, any epitope on Aβ will exist within βAPP. Therefore, under some conditions of denaturation in vitro and in vivo (such as might occur during βAPP processing) some cross-reactivity may occur. Currently, no method has been derived to definitively distinguish Aβ from βAPP and fβAPP in vivo by any means, although demonstration of a staining dissociation between Aβ and βAPP antibodies is a reasonable approach. Antibodies against βAPP do not detect Aβ, although they may sometimes detect APLPs (amyloid precursor-like proteins), which are highly homologous proteins of unknown function that do not, however, contain Aβ (Slunt et al, 1994).
Antibodies to βAPP included commercial monoclonals directed against the N- and C-terminal ends (22C11 and βAPP_643-695/Jonas, Boehringer-Mannheim, 10 μg/ml; Weidemann et al, 1989; Koo et al, 1990; Bush et al, 1990; Small et al, 1992; Nitsch et al, 1993; Caporaso et al, 1994; Hsiao et al, 1996). Polyclonal antibodies were provided either by Drs. Dale Schenk (Athena Neurosciences, San Francisco; α3 raised against the N-terminus, and α6 raised against the C-terminal 100 amino acids, both at 1:100 dilutions; Oltersdorf et al, 1990; Refolo et al, 1995; Furukawa et al, 1996; Games et al, 1996) or Yasuo Ihara (University of Tokyo; R1 against N-terminal residues 45-62 and R2 against C-terminal residues 666-695, both at 1:250 dilutions; Takio et al, 1989; Otsuka et al, 1991; Yanagisawa et al, 1992; Saito et al, 1994; Murakami et al, 1995).

Antibodies to Aβ included the commercially available monoclonal 4G8 (raised against Aβ17-24, Senetek, St. Louis, 4μg/ml; Kim et al, 1988, 1990; Shoji et al, 1992; Anderson et al, 1992; Hsiao et al, 1996) and an Aβ1-40 polyclonal (Boehringer-Mannheim, 10 μg/ml; Griffin et al, 1995; Nakamura et al, 1996; Cataldo et al, 1996). Additional antibodies were provided by Drs. Dale Schenk (Athena Neurosciences, San Francisco; the monoclonals 10D5 and 6C6, both of which recognize an epitope within Aβ1-16, both at 10 μg/ml; Arriagada et al, 1992; Seubert et al, 1992; Haass et al, 1992; Citron et al, 1992; Sparks et al, 1993; Seubert et al, 1993; Poduri et al, 1994; Games et al, 1995; Sparks, 1996; Verbeek et al, 1996; Gómez-Isla et al, 1997) and Yasuo Ihara (University of Tokyo; polyclonal Aβ1-28, 1:250 dilution; Yamaguchi et al, 1988a; Kanemaru et al, 1990; Saito et al, 1994; Murakami et al, 1995).

Astrocytes were detected with a commonly used antibody against GFAP (Boehringer-Mannheim, 5 μg/ml; Khurgel et al, 1995, 1996). GFAP-IR was examined as a means to locate areas that may have sustained damage from any of the experimental treatments, or may have been subjected to seizure activity (Khurgel et al, 1995, 1996). Polyclonal antibodies directed against either rat or mouse IgG (where appropriate; Sigma, 1:100) or serum (Sigma, 1:3000) were used to detect extravasation of serum proteins into the CNS, a positive indicator of blood-brain barrier dysfunction following seizure-induced damage (Ruth, 1986; Ruth and Feinerman, 1988; Schmidt-Kastner et al, 1990, 1993; Suzuki et al, 1995; Hoshino et al, 1996).
For negative staining controls, additional sections were processed in the absence of the primary antisera or, where possible, preabsorbed with the antigen. For the demonstration of extravasated serum proteins, anti-rat IgG was preabsorbed with purified rat IgG (Sigma) as a first level control, and anti-rat serum was preabsorbed with an excess of whole rat serum (Sigma) or rat IgG (Sigma; to remove IgG-IR). Antibodies against Aβ were preabsorbed with an excess of synthetic Aβ1-28 or Aβ1-40 (Sigma).

N.B.: In the case of figures shown throughout the thesis containing both histological and/or immunohistochemical data, the micrographs shown are representative of the effect discussed (that is, approximately the median), except where otherwise noted.
SECTION C:

EXPERIMENTS
C1 Experiment 1: Leupeptin Infusion in Transgenic Mice Results in the Intracellular Accumulation of Aβ

C2.1 Introduction

The purpose of this first experiment was twofold: (1) to assess the possibility that mimicking aging of lysosomes through the intracerebroventricular administration of the inhibitor leupeptin (B2.1) would lead to the appearance of intracellular Aβ, and (2) to determine if iAβ induced under these conditions leads to the development of degenerative neuropathology. For this first experiment, transgenic mice were used, since they were considered to be capable of developing SP as one possible consequence of this manipulation.

C1.2 Methods

C1.2.1 Subjects

A total of 18 animals were used, 6 each of wild type (WT), heterozygous and homozygous (see B1.1; all aged between 9 and 12 months). Half of the animals were assigned to the vehicle treatment group, and the other half to the drug condition. Each group of 3 subjects was comprised of 2 male and 1 female animal. When available, littermates were used.

C1.2.2 Surgery and Drug Treatment

Animals were anesthetized with a 1:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) at a dosage of 0.1 cc/30 g body weight and placed in a modified small animal stereotaxic instrument. The scalp was swabbed with 70% alcohol, shaved and a small incision made along the midline. All stereotaxic intracerebroventricular (i.c.v.) injections were made into the left lateral ventricle (M/L ±1.3 mm, A/P -0.2 mm, D/V -1.5 to -2.0 mm). Injections were made at a rate of 1 μl/minute (2 μl total volume), followed by a diffusion time of 3-5 minutes, and then a slow withdrawal over an additional 2 minutes. At the conclusion of the injection, the wound was closed with a surgical wound clip and an antibiotic cream applied (1% clorhexidine acetate; Hibitane). All animals were sacrificed approximately 24 hours following the final injection.
Phosphate buffered saline (PBS) served as the vehicle (veh) for leupeptin (LEU; Boehringer-Mannheim; 60 μg/μl), with concentrations calculated based on the drug salt. Three injections were made with a separation of approximately 48 hours (Monday, Wednesday, and Friday) through the same injection site by removing the wound clip (under ketamine/xylazine anesthesia). This dose and schedule of drug administration was selected based on pilot trials which estimated this regimen as the approximate upper limit of tolerance for the mouse.

C1.2.3 Tissue Preparation and Histology

Sagittal vibratome (1000 Series; TPI, St. Louis) sections were taken of the fixed tissue (50 μm) and floated into PB. Some sections were mounted on chrome-alum/gelatin coated slides for histological processing (cresyl violet; B4). Additional tissue was embedded in paraffin blocks and sectioned sagittally at 4 or 10 μm. Subjects were selected for an individual run of histology and processed for immunohistochemistry in a counterbalanced manner (c.f. C1.2.4, below).

Monoclonal antibodies to GFAP (5 μg/ml; Boehringer-Mannheim) and the N-(βAPPN; 22C11, 10 μg/ml; Boehringer-Mannheim) and C-terminal (βAPPC; 643-695/Jonas, 10 μg/ml; Boehringer-Mannheim) ends of βAPP were used to demonstrate astrocytes and βAPP, respectively. Monoclonal antibodies against Aβ1-16 (6C6 and 10D5, 10 μg/ml; Athena Neurosciences, San Francisco) and Aβ17-24 (4G8, 1:250; Senetek, St. Louis) were used to demonstrate fβAPP/Aβ accumulation. Similarly, a polyclonal antiserum raised against Aβ1-28 (IαAβ) was also used (see B5).

C1.2.4 Data Analysis

Slides were examined independently by 3 observers blind to treatment conditions, and ranked from highest to lowest on a 6 point scale (1=weak reaction). Three full counterbalanced sets were constructed for this purpose.

C1.3 Results

The main finding of this experiment was that LEU infusion caused an increase in
iAβ in the absence of other additional pathology. Early examination revealed that any
drug effects (as determined by GFAP, Aβ and βAPP-IR) were restricted almost entirely
to the hippocampus and septum, with the former structure being the most affected.
Hence, results obtained by subjective ranking were based entirely on assessment of the
hippocampus. Male and female animals did not differ in any case, and homozygous and
heterozygous animals were, in most cases, very similar.

C1.3.1 Histology

Cresyl violet stained sections did not reveal any areas of marked cell loss, even
within hippocampal cell fields where Aβ-IR was observed in response to the drug
treatment (Fig. C1-1). Hippocampal morphology in general appeared normal, although
there was some evidence of slight compression in some cases from repeated injections.

Figure C1-1. Leupeptin treatment does not cause cell loss in hippocampus. (A) Wild type, PBS; (B)
Heterozygous, PBS; (C) Wild type, LEU; (D) Heterozygous, LEU. Shown are nearby sections from the
same subjects depicted in figure C1-2. Original magnification, 20x (scale bar in D: 200 μm).
C1.3.2 Immunohistochemistry

Leupeptin treatment resulted in an elevation of Aβ-IR. Ranking of the immunostaining indicated that 7/9 of the drug treated animals fell into the higher category of moderate to severe, and 7/9 of the PBS controls were ranked in the lower category (p=0.0154, by direct calculation of binomial expansion; Fig. C1-2). A tendency towards a gene dosage effect was detected (Kruskal-Wallis ANOVA; $\chi^2(2)=3.2671$, p<0.2). For instance, only 1/3 WT animals treated with LEU were scored as 5 or greater, whereas 2/3 of the heterozygous and all of the homozygous animals were (c.f. Fig. C1-3). Significant reactivity in the case of the TG animals appeared to be localized to cells that were putatively identified as astrocytes, based on their stellate morphology and location within the hippocampus (Fig. C1-4); the sole high-ranked WT animal showed increased labeling almost entirely within the pyramidal cell layer. In any case, the reactivity was exclusively intracellular; no extracellular amyloid deposits were detected in any of the animals (see Fig. C1-4). Similar results were obtained in LEU treated mice with other antibodies directed against Aβ, but not βAPP (Figs. C1-5 and 6).

Figure C1-2. Semiquantitative analysis of Aβ-IR in the hippocampus of LEU treated mice. There was a small trend towards a gene-dose effect, with homozygous showing more IR than heterozygous mice (p<0.2). The effects of LEU were generally indistinguishable between both types of TG, although the effect was more marked in comparison to the WT. Overall, the effect of LEU was significant (p<0.02).
Figure C1-3. Leupeptin treatment for 1 week resulted in increased Aβ-IR (A: WT, vehicle; B: heterozygous, vehicle; C: WT, leupeptin; D: heterozygous, leupeptin; E: AD cortex; F: subject from (D), antigen absorbed), shown here using the monoclonal antibody 10D5 (hippocampus CA3, magnification: 20x, scale bar in A: 200 μm; labeling in D: SO - stratum oriens, SP - stratum pyramidale, SR - stratum radiatum, SL-M - stratum lacunosum-moleculare). Homozygous animals were similar to the heterozygous transgenics (but c.f. Fig. C1-2, above). The results for 6C6 were the same (c.f. Fig. C1-5). Most of the label can be seen within the pyramidal cell layer, although numerous faintly labeled cells (most likely astrocytes) can be identified within the boxed area in (D)(c.f. Fig C1-4).
Figure C1-4. Intracellular Aβ-IR in LEU treated mice versus extracellular Aβ in AD (10D5 antibody, 10 μm paraffin sections; all mouse photomicrographs taken from the hippocampus, original magnification: 100x, scale bar: 40 μm). (A) Heterozygous, PBS; (B) AD Cortex, counterstained with cresyl violet; (C) Heterozygous, LEU; (D) Heterozygous, LEU, antigen absorbed. Compare the punctate/granular staining associated with the cells in the pyramidal cell layer of the LEU treated mouse (C) with the unlabelled cells closely associated with the SP in (B). Note also that several cells in (C) exhibit clear areas (indicated by arrowheads), indicating a section taken through the cell body (the clearer area may represent part of the nucleus). (E) and (F) show several cells of astrocytic morphology from the same LEU treated animal immunostained with either 10D5 or βAPPN, respectively (c.f. Figures C1-5 & C1-6).
Figure C1-5 (following 2 pages). Aβ antibodies do not show the same pattern of IR as those directed against βAPP. (A) AD Cortex, 10D5; (B) AD Cortex, 4G8; (C) PBS treated TG, 10D5; (D) LEU treated TG, 10D5; (E) PBS treated TG, 4G8; (F) LEU treated TG, 4G8; (G) PBS treated TG, 22C11; (H) LEU treated TG, 22C11. All mouse photomicrographs are taken from the hippocampal pyramidal cell layer and counterstained with cresyl violet (4 μm paraffin sections; original magnification: 100x, scale bar: 40 μm). Again note the distinction between clear cell associated Aβ-IR (D and F) in comparison with extracellular Aβ (A and B; arrow in A indicates what may be intracellular Aβ-IR in AD brain). The pattern of cellular IR obtained with Aβ antibodies (10D5, 4G8 and 10Aβ) is nearly identical. (I) AD cortex, 10Aβ (scale bar: 40 μm); (J) LEU treated TG, 10Aβ, antigen absorbed; (K) PBS treated TG, 10Aβ; (L) LEU treated TG, 10Aβ; (M) PBS treated TG, anti-βAPP643-695; (N) LEU treated TG, anti-βAPP643-695. In contrast, βAPPN and βAPPC antibodies demonstrate a distinctly different pattern of immunostaining, primarily localized around the cell perimeter. This pattern of βAPP-IR is also different from that observed outside of the pyramidal cell layer, on cells which are most likely astrocytes (note the labeled cellular process in the upper left-hand corner of N; c.f. Fig. C1-4E and F and Fig. C1-6, below).
Figure 4.6: Antibodies directed against 
PP6P detect cells of the mamilary astrocytic morphology outside of 
the principal cell layers of the hippocampus in LEU treated mice (shown: stratum lacunosum-moleculare of 
PAPP, LEU, PBS).
The Aβ antibodies label a much larger portion of the cell body than do the βAPP antibodies, which mainly label the perimeter of the cells (compare C1-5D, F and L to H and N). These labeled cells are neurons, given their location within the pyramidal cell layer and general morphology (large, round and/or pyramidal shape with a large nucleus). Notably, this is different than the pattern of cell associated labeling outside of the pyramidal cell layer, where the immunostained cells are of general astrocytic morphology with all visible parts of the cell labeled (Fig. C1-4E and F, and Fig. C1-6A and B; Note also the labeled cellular process in Fig. C1-5N).

Leupeptin also caused a large increase in GFAP-IR, further indicating an astrocytic response to the drug (Fig. C1-7A and B). In total, 7/9 of the leupeptin treated

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**Figure C1-7.** Animals treated with LEU for 1 week (B) showed a pronounced astrocytic reaction (as seen by GFAP-IR) in contrast to those treated with PBS vehicle (A) (shown: septum, heterozygous mouse, original magnification: 10x; scale bar: 400 μm). The effect was identical in the hippocampus. (C) Semiquantitative analysis of GFAP-IR in hippocampus; 7/9 of the LEU treated animals were classified as “reactive”, whereas 8/9 PBS treated animals were placed in the lower category (sign test, p<0.004). There was no clear trend towards any effect of the presence of the transgene.
animals received a median ranking of moderate-severe, whereas 8/9 of the PBS treated mice were placed in the lower category (see C1-7C; p=0.0038, by binomial expansion). The presence of the transgene did not appear to anyway influence this reaction.

**C1.4 Discussion**

Infusion of leupeptin caused an increase in intracellular fβAPP/Aβ-IR, but did not result in the formation of any extracellular deposits resembling SP. No evidence was found of any cell loss, even within the hippocampus, the area where fβAPP/Aβ-IR was found to increase markedly in response to the drug treatment.

Leupeptin administration resulted in the appearance of fβAPP/Aβ-IR, which can be clearly seen as associated with the cell body of pyramidal neurons. It is unlikely that the immunoreactivity seen in this experiment represents βAPP, since a clear dissociation of staining patterns can be seen when comparing βAPP immunolabeling to that of Aβ. The Aβ immunostaining likely represents intracellular fβAPP/Aβ for several reasons. First, cell associated Aβ has been shown to exist intracellularly, with little or none being associated with the cell membrane (Yang et al, 1995; Turner et al, 1996; Burdick et al, 1997; Wild-Bode et al, 1997; Tienari et al, 1997). Second, leupeptin treatment in vitro causes Aβ and fβAPP to increase (primarily within the lysosomal compartment), as shown by subcellular fractionation of cell lysates (Cole et al, 1989a; Caporaso et al, 1992a; Golde et al, 1992; Haass et al, 1992a). Ivy et al (1984) showed using electron microscopy that material accumulating in response to leupeptin infusion is localized intracellularly within lysosomes. Similar recent studies have shown the existence of fβAPP (Tsuzuki et al, 1994; Murakami et al, 1995) and Aβ_{42} (Tsuzuki et al, 1995) intracellularly (following treatment with the lysosomal inhibitor chloroquine). Also, βAPP-C100 transgenic mice (similar to those used in this study) accumulate Aβ positive granules within lysosomes (Kammesheidt et al, 1992; Neve et al, 1996), and amyloid fibrils have been demonstrated intracellularly in another transgenic mouse model (Masliah et al, 1996).

Significant Aβ- and βAPP-IR was localized to cells that were putatively identified as astrocytes in the animals treated with LEU, primarily in the transgenic subjects.
Although it is possible that the astrocytes are taking up Aβ positive peptide fragments from neurons, the observation that the WT mice only showed neuronal Aβ-IR (albeit weak) would argue against this. Since identical staining of these cells was also obtained with βAPP antibodies it seems more likely that the labeling represents full length βAPP rather than Aβ or fβAPP. It is interesting to note that the transgene promoter in this strain of mouse is from the JC papovavirus (Sandhu et al, 1991). The JC virus is responsible for progressive multifocal leukoencephalopathy in humans, a disorder of both oligodendrocytes and astrocytes (Dubois-Dalcq, 1995). Since the JC viral promoter has a tendency to direct mRNA synthesis in glial cells (Small et al, 1986), it may be that the astrocytes are producing elevated βAPP as a generalized response to the drug. Since similar immunostaining was also seen with an antibody directed against the N-terminal region of βAPP, which is not overexpressed in these animals, the staining probably represents a combination of the transgene and endogenous murine βAPP.

Leupeptin also caused an increase in GFAP-IR, which has previously been reported (Ivy, 1992a). A possible mechanism for this effect is not obvious. One possibility may be the presence of increased fβAPP. Although Aβ is known to affect microglia (Davis et al, 1992; Khoury et al, 1996; Yan et al, 1996), it has never been reported to directly cause an astrocytic reaction. Furthermore, the lack of any detectable extracellular Aβ-containing peptides would also appear to weaken this argument, since aggregated Aβ appears necessary for astrocytic activation (Pike et al, 1995). More likely, the astrocytic reaction may be indirectly caused by an imbalance in cellular function in drug exposed areas, which would affect both neurons and astrocytes.

Regardless, this treatment schedule did not result in the appearance of any plaque-like pathology in the transgenic animals, nor did it result in pronounced cell loss, which might otherwise be expected from elevated intracellular Aβ (Hayashi et al, 1992; Yang et al, 1995). Since cell counting procedures were not used in this study, a more subtle loss cannot be ruled out. It may be that the intracellular accumulation of Aβ results in neurodegeneration and deposition of the peptide very slowly in vivo. Hence, it remains possible that a longer period of drug administration, as well as administration to areas other than those observed in this study, could cause more significant pathology.
C2 Experiment 2: Infusion of Lysosomal Inhibitors in Primates
Does Not Cause SP Formation

C2.1 Introduction

The experiment with the transgenic animals may have failed to produce AD-like pathology for three reasons. First, the transgenic mice used expressed a fragment of human βAPP, rather than the full protein. It is possible that the entire gene, including its normal endogenous regulatory region, is a necessary prerequisite for the induction of pathology using this paradigm. The second possibility is that the treatment time with leupeptin was too short to cause pathological changes beyond that of intracellular Aβ-IR. Technically, it is very difficult to achieve reliable long-term administration in the mouse (although pilot experiments indicated that the results of experiment 1 were similar at 2 weeks of treatment). Finally, the primary areas of drug effect (hippocampal cell field CA3/4 and the septum) may not have been sufficiently susceptible to this treatment.

Hence, to address these issues the decision was made to perform a similar experiment in non-human primates, which are the best known naturally occurring model of Alzheimer’s disease (see A5.1). Since non-human primates have an identical Aβ sequence, and a very similar βAPP gene, this corrects the problem with fragmentary βAPP and lack of a potentially important normal regulatory region. Furthermore, the infusion was carried out continuously for 8 weeks, and conducted via a porous local access fibre implanted in the perirhinal/entorhinal cortex. This results in dispersion of the drug along the entire length of the cannula (Dubach et al, 1997) for a much longer period of time, and targets a different area prone to the development of SP pathology in AD.

C2.2 Methods

C2.2.1 Subjects

Two aged macaques (Macaca nemestrina) were used for the drug administration, and two untreated animals (age=21 years) served as additional controls. All subjects were female. The first experimental subject (age=22.45 years) was assigned to a LEU treatment condition, and the second (age=16.2 years) to CHL (B2.1). The latter compound was used in order to compare effects between two different inhibitors. All
surgery and subsequent maintenance and housing of the subjects was carried out at the University of Washington Regional Primate Center (Seattle), in collaboration with Dr. M. Dubach (Dubach et al, 1997).

C2.2.2 Surgery and Drug Treatment

Briefly, subjects to be implanted were maintained under halothane anesthesia in a sterile surgical suite, under veterinary supervision. Iohexol, a radio-opaque contrast agent used clinically in human neurosurgery, was injected stereotaxically into the lateral ventricles as per a published atlas of the pigtailed macaque brain (Winters et al, 1969). Optimal placement for the access fibre was then determined using x-ray assistance. The polyacrylonitrile-polyvinyl chloride dialysis tubing was threaded through the perirhinal/entorhinal cortex via the use of a telescoping push tool, and the leading edge cemented in place (after emerging through a nearby exit hole) with a cyanoacrylic adhesive (Vetbond Tissue Adhesive; Animal Care Products, St. Paul, MN). Following insertion, the drill holes were sealed with gelfoam, and the trailing edge of the tubing (non-porous) passed subcutaneously to a midscapular point. The tubing was then connected to an osmotic minipump (Alzet, 2ML4; Alza Corporation, Palo Alto, CA), filled with either CHL or LEU (each at 60 mg/ml in a physiological saline solution, supplemented with 0.2 mg/ml garamycin to prevent infection). These doses were determined based on prior work using these inhibitors in non-human primates (Ivy, 1992a). The 2ML4 minipump operates for 28 days at a continuous flow rate of 2.5 µL/hour. Hence, for 8 weeks of treatment the pump required replacement once, under light anesthesia. In the case of the CHL treated animal, an additional minipump was implanted (containing the vehicle solution) in a similar manner on the opposite side of the brain.

C2.2.3 Tissue Preparation and Histology

Following the end of the treatment period, the subjects (including controls) were sacrificed with an overdose of sodium pentobarbital (Nembutal) and perfused transcardially with physiological saline followed by 3% paraformaldehyde. The CHL treated animal was not perfused, but was otherwise treated the same. The brains were
removed and cut into 8 5-mm slices perpendicular to the intercommisural line, and stored in 10% formalin until photographic records were made. Following recording, some tissue slabs were sectioned frozen (30 μm) or in paraffin blocks (8-10 μm) (B3).

Some sections were mounted on glass slides and processed for cresyl violet and/or PAS (B4). The PAS stain has been used to demonstrate the effectiveness of both LEU and CHL in vivo (Ivy, 1984; Ivy et al, 1990a,b). Other sections were processed for immunohistochemistry (B5). Monoclonal antibodies to Aβ1-16 (10d5, 10 μg/ml; Athena Neurosciences), Aβ17-24 (4G8, 1:250; Senetek) and GFAP (5 μg/ml; Boehringer-Mannheim) were used, as well as additional polyclonal antibodies to Aβ1-28 (1oAβ1-28, 1:250; Dr. Y. Ihara), the N-terminus of βAPP (α3, βAPP20-304, 1:100; Athena Neurosciences), the C-terminus of βAPP (α6, βAPP590-695, 1:100; Athena Neurosciences), and monkey IgG (Sigma Immunochemicals, 1:100). In the case of paraffin embedded tissue, the sections were pretreated with concentrated formic acid for 2-3 minutes to unmask cross-linked Aβ epitopes (Kitamoto et al, 1987; Klunk and Pettegrew, 1990). The antibody against monkey IgG was used since pilot data from CHL infused mice had indicated possible serum protein extravasation as one drug effect.

C2.3 Results

The CHL treated monkey was observed having a seizure 6 days post-surgery, and died of fatal convulsions 2 days later. The LEU treated monkey exhibited no signs of illness, and survived for the full 8 weeks of treatment. The CHL treated monkey displayed diffuse, elevated IgG immunoreactivity on the drug treated side (figure C2-1). The reactivity extended over nearly all of this hemisphere, and was abolished by preabsorbing the antibody with purified monkey IgG prior to incubation. No IgG-IR was detected in the LEU treated monkey.

Drug treatment resulted in an increase in PAS+ cells, an effect which could be observed at a distance of half a centimeter or more from the cannula tubing (see figure C2-2). There were no areas of gross cell loss (Fig. C2-3), except for the area immediately adjacent to the access fibre in the CHL treated animal, the tissue from which was in generally poor condition.
Figure C2-1. IgG-IR in CHL treated monkey. Note the general diffuse staining on the treated side (left), whereas the SAL infused side (right) is only lightly reactive (paraffin embedded, no magnification). The sections were processed simultaneously, and the reaction was repeated several times (with identical results) to rule out variations in section thickness. Antiserum preabsorbed with purified IgG was unreactive.

Figure C2-2. PAS reactivity in LEU treated monkey; comparable areas in the untreated (A) versus treated (B) side (paraffin embedded, original magnification: 5x, scale bar: 800 μm). The edge of the cannula is visible in the upper left-hand corner of (B). Note the numerous PAS positive cells scattered throughout the cortex, often located quite distant from the cannula. (C), inset: alternative view (same magnification) of (B), contrast enhanced, with elevated background threshold.

No SP of any class were observed in response to either drug, although numerous SP were found in AD tissue run concurrently as a positive control (Fig. C2-4). Moderately stronger intracellular Aβ/βAPP-IR was seen on the treated side versus the untreated side following LEU treatment. However, the signal was very faint (Fig. C2-5) and was not detected with βAPP antibodies (not shown). An increase in GFAP-IR was observed only in the immediate vicinity of the access fibre, which may have been due to tissue damage (not shown). Although some sporadic labeling was detected in the CHL treated subject, the fact that this animal died of severe seizures shortly after the beginning of the experiment casts doubt on this as a valid finding, although the lack of SP pathology is still certain.
Figure C2-3. LEU treatment did not result in large scale cell loss. (A) Untreated side; (B) Infused side (shown: cortex directly overlying access fibre and the corresponding area on the uninfused side; original magnification: 10x, scale bar: 350 μm). The results of CHL infusion were the same, except in the immediate vicinity of the access fibre (not shown).

Figure C2-4. Infusion of LEU did not cause SP pathology. (A) LEU infused monkey, cortex directly over access fibre; (B) AD cortex (shown: 4G8 antibody, original magnification: 5x, scale bar: 900 μm). CHL infusion also did not cause SP formation (not shown), and the results for other Aβ antibodies were identical.

C2.4 Discussion

Infusion of the lysosomal inhibitors chloroquine or leupeptin in non-human primates gave qualitatively the same results as a similar experiment in transgenic mice. Neither drug caused the formation of SP or overt cell loss, although a small increase in cell associated Aβ/fβAPP-IR was found with LEU. Also, CHL treatment caused the appearance of extensive extravasated IgG-IR, which may signal the presence of a BBB abnormality. Although the number of subjects was small, the results presented here are consistent with experiment 1 (C1).
Figure C2-5. LEU infusion caused a small increase in iAβ-IR. (A) LEU treated monkey, uninfused side; (B) LEU treated monkey, infused side (edge of access fibre visible on the left); (C) AD cortex; (D) Adjacent section to B, primary antibody omitted (shown: 4G8 antibody, original magnification: 20x, scale bar: 190 μm). Numerous faintly labeled cells can be seen on the drug infused side (examples shown to the left of asterisks); no SP were detected. Results were similar with other Aβ antibodies tested; no labeling was obtained with the βAPP antibodies.

Even though the AD tissue samples run concurrently as positive controls for the staining procedure exhibited numerous SP of all types, no such deposits were seen in any of the monkeys. One possible explanation for this finding is that the perfusion procedure used resulted in an extraction or wash-out of any diffuse Aβ in the tissue. However, the CHL treated animal also did not show any extracellular Aβ, and this animal was not perfused. It is also clear that both drugs were active and entering the CNS, since (1) PAS+ cells were visible even at relatively large distances from the LEU infusion area, and (2) the CHL treated animal developed severe seizure activity. In the
latter case, the length of drug treatment (8 days) may have been insufficient to allow any SP-like deposits to occur. However, 2 months of LEU infusion did not induce SP formation, even though some iAβ-IR was detected. If intracellular Aβ is directly responsible for SP pathology, we might expect to see the appearance of some amyloid deposits after this length of time, at least in the area directly adjacent to the access fibre.

The occurrence of fatal convulsions in the CHL treated subject complicates the interpretation of the results. Seizures are a common sign of CHL overdose in humans (Torrey, 1968; Fish and Espir, 1988), rats (Osifo and DiStefano, 1978) and mice (Amabeoku, 1992; N’Gouemo et al, 1994), and seizures are known to cause BBB dysfunction (Johansson and Nilsson, 1977; Petito et al, 1977; Ginsberg et al, 1985). It may be that the seizures caused by the drug are in some way related to the BBB damage in this case. Alternatively, the continuous infusion of the drug could cause vascular toxicity and result in seizures by allowing access to the CNS of an epileptogenic and/or excitotoxic substance. Damage to the BBB as a consequence of CHL usage has not been reported, although it is usually taken orally as an antimalarial compound and was directly infused in this experiment. However, many fatal cases of malaria result from a blockage of cerebral blood vessels by parasitized erythrocytes, resulting in severe anoxia, coma and a variety of other neurological manifestations (Warrell, 1992; Hamer and Wyler, 1993). In these cases, the brain is often examined at autopsy, and hence may provide somewhat indirect evidence for a similar effect of CHL. Although very few such studies exist, one contrast is potentially informative. One study reported the presence of perivascular ring hemorrhages and the deposition of IgG complexes in and around capillary basement membranes, implying significant vascular system damage in cerebral malaria (Oo et al, 1987), whereas another study did not (MacPherson et al, 1985). Interestingly, subjects in the latter study were treated with quinine, whereas subjects in the former underwent standard antimalarial therapy involving chloroquine. Although the mechanism of the two compounds is believed to be similar (Krogstad et al, 1985), it is tempting to conclude that the difference in findings is due to the presence of CHL in one group of patients. Since only a single subject was examined in the current experiment, and since severe seizures also occurred, the finding of possible BBB damage requires additional confirmation.
Therefore, the increased presence of intracellular Aβ/βAPP is not alone sufficient to cause the subsequent development of SP, whether as a consequence of cell death or by another means (such as exocytosis of iAβ). Long term treatment with LEU in a non-human primate did not produce results markedly different from that of 1 week of treatment in transgenic mice (experiment 1), even though monkeys are considerably more susceptible to the development of SP-like pathology than this strain of mouse. Although longer treatments and/or another animal model (such as a different strain of transgenic mouse) may yield different results, these findings argue against such an approach. First, very long treatment with these substances would likely cause nonspecific degenerative changes due to defective protein turnover (Ivy et al, 1984; Takauchi and Miyoshi, 1989), and is technically very difficult to achieve reliably in mice. Second, since the animal models used in experiments 1 and 2 express a form of Aβ with an identical sequence to that of humans, and Aβ-IR is found intracellularly following these treatments, it would seem that other animal models would have little to offer that could alter the results substantially. Even in the case of mutant βAPP transgenics (Games et al, 1995; Hsiao et al, 1996) the Aβ peptide is identical to that found in normal humans.
C3 Experiment 3: Chloroquine Infusion in Rats Results in Intracellular Aβ and an Increased Susceptibility to Kainic Acid-Induced Neurodegeneration

C3.1 Introduction

Experiments 1 and 2 demonstrated that treatment with LEU caused the appearance of intracellular Aβ/fβAPP, but is not obviously neurotoxic and does not lead to the extracellular deposition of the peptide. This can be taken as evidence against the direct involvement of intracellular Aβ in the development of AD-like pathology (such as SP or neuronal death). The alternative hypothesis, that iAβ is indirectly responsible for the development of pathology (i.e., iAβ is necessary but not sufficient), remains untested. What if the lysosomal dysfunction that parallels the aging process (and modeled in experiments 1 and 2 with LEU) leads to iAβ accumulation that is not directly responsible for the occurrence of AD, but is merely a risk factor? Hence, the increasing risk of AD with greater age may be tied to a steadily increasing concentration of intracellular Aβ. This hypothesis implies that factors in addition to greater age/lysosomal dysfunction/iAβ are actually responsible for precipitating the pathology. One such factor is excitotoxicity, and the goal of this experiment was to determine if a synergism exists between this process and iAβ in vivo (c.f. A4.2).

The purpose of this experiment was to demonstrate that CHL infusion was capable of inducing an increase in iAβ, and to then determine if the presence of iAβ was associated with an increased susceptibility to excitotoxic neurodegeneration. Leupeptin does not act solely on lysosomal proteases, and is known to also inhibit cytoplasmic serine (Barrett, 1980) and cysteine (Toyo-Oka et al, 1978) proteases. Since Ca²⁺-activated neutral cysteine proteases (the calpains, or CANPs) are involved in degeneration following excitotoxic injury (Siman and Noszek, 1988), the inhibitory effect of leupeptin on these enzymes may interfere with the neurodegenerative process. Hence, this and subsequent experiments used chloroquine alone, since this does not interact with cytosolic enzymes (Ohkuma and Poole, 1978; Mellman, 1986; Kirschke and
Barrett, 1987) that might interfere with excitotoxic effects. Chloroquine treatment causes the intracellular appearance of Aβ in peripheral tissues (Murakami et al, 1995; Tsuzuki et al, 1994, 1995), and the related compound ammonium chloride has been convincingly shown in vitro to lead to intracellular Aβ accumulation (Wild-Bode et al, 1997).

C3.2 Methods
C3.2.1 Subjects

Male Sprague-Dawley rats (n=28) were used for this experiment (B1.2).

C3.2.2 Surgery and Drug Treatment

Animals were anesthetized with an intraperitoneal (i.p.) injection of a 1:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) at a dosage of 0.1 cc/100 g body weight. Approximately 10 minutes later, an i.p. injection of sodium pentobarbital (30 mg/kg body weight) and atropine sulfate (5 mg/kg) was administered, just prior to placement of the animal in the stereotaxic instrument. The scalp was swabbed with 70% alcohol, shaved, and a small incision made along the midline. A stainless steel cannula was fixed in place using acrylic dental cement, and 2 jewelers screws were inserted into the skull as anchors for the cannula assembly. All cannula implantations were made into the left lateral ventricle (1.7 mm lateral, A/P -0.4 mm, D/V -4.5 mm). The cannula was connected to a 3-4 cm length of polyethylene tubing, which was passed subcutaneously to a midscapular Alzet 2002 osmotic minipump (Alza Corporation, CA). This model operates at a continuous rate of 0.5 μL/hour for 2 weeks. At the conclusion of the procedure, the wound was closed over the cannula assembly with surgical wound clips and an antibiotic cream applied (1% chlorhexidine acetate; Hibitane).

Phosphate buffered saline (PBS; 0.9% NaCl in 0.1 M sodium phosphate buffer, pH=7.4) served as the vehicle (veh) for chloroquine diphosphate (CHL; Sigma, 30 mg/ml), with concentrations calculated based on the drug salt. This dose was selected based on prior work with CHL infusion in the rat (Ivy et al, 1984). Once the minipump was filled with the infusion solution, it was attached to the catheter tubing/cannula assembly and incubated overnight in PBS at 37°C, as per the manufacturer's
recommendations for brain infusion. Approximately half (n=15) of the subjects were assigned to the CHL treatment condition, and the remainder to the control group. Of these (n=13), 4 were unimplanted controls included as a comparison for the effects of pump implantation. One week following the surgical procedures, half of the animals in each group were randomly assigned to the KA treatment condition (B2.2). The short period of SE used normally causes little damage; hence, shorter, controlled seizure activity prevents the occurrence of a ceiling effect and allows for a cleaner assessment of increased susceptibility to KA-induced damage.

C3.2.3 Tissue Preparation and Histology

Half of the subjects were sacrificed immediately (B3) following the 30 minute period of SE. These subjects served as the comparison group for the subsequent effects of KA-induced neurodegeneration. That is, these animals illustrate the effects of CHL on the CNS prior to any degenerative influence, and establish what changes can later be associated with the effects of KA. Sacrifice immediately following SE rather than before KA administration is a better comparison group since it permits a dissociation of the immediate (KA+CHL: no survival time) and long-term (KA+CHL: 1 week survival time) neuroanatomical consequences of KA-induced SE. The remaining animals were sacrificed at the latter time point and the brains prepared for freezing (sectioned at 20-30 μm) and paraffin embedding (sectioned at 6 μm) as described (B3).

Slide mounted sections were processed for PAS or cresyl violet (B4). Immunohistochemistry was performed (B5) using the following antibodies: polyclonal 1αAβ1-28, BαAβ1-40, α3 (anti-βAPP20-304), α6 (anti-βAPP590-695), anti-rat IgG (Sigma Immunochemicals, 1:100), and anti-rat serum (Sigma Immunochemicals, 1:3000); monoclonal GFAP (5 μg/ml; Boehringer-Mannheim), 10D5 (anti-Aβ1-16), 22C11 (N-terminus of βAPP; anti-βAPPN) and anti-βAPP643-695 (anti-βAPPC).

Some sections were processed in the absence of the primary antibody to determine nonspecific reactivity, or were preabsorbed with the antigen where possible. Additional tissue from KA-treated rats (n=6, 28 day survival) and an AD case was examined as positive controls.
C3.3 Results

Infusion of CHL resulted in increased iAβ-IR in CA3, and a corresponding potentiation of neuronal loss in this area in response to KA treatment.

Animals receiving chloroquine infusion developed seizures in response to KA faster than either PBS infused or unimplanted controls [FS: F(2,11)=13.91, p<0.001; SE: F(2,11)=4.33, p<0.05]. Seizure time for PBS infused animals showed a similar trend, though not significant (Fig. C3-1). No differences were found by chi-square analysis in maximum level of seizure attained or in the frequency of different seizure types elicited.

C3.3.1 Histology

Only 1/3 animals treated with KA alone (1 week survival) showed cell loss, and this was bilateral in hippocampal area CA3/4. In contrast, those treated with CHL+KA (4/4) showed cell loss in hippocampal area CA3/4 which was extensive on the treated side (2 showed some neuronal loss on the contralateral side; Fig. C3-2). CHL alone caused a small increase in PAS positive staining restricted almost entirely to hippocampal areas CA3/4 (Fig. C3-2D, inset; likely indicating the area of drug effect) and never resulted in a visible loss of neurons within this region. However, 1 week after KA administration, dense PAS+ staining was observed to be co-localized with neuronal loss (Fig. C3-2, E-H; also true of the PBS+KA animal found to have CA3 damage).

C3.3.2 Immunohistochemistry

Chloroquine treatment produced an increase in Aβ immunoreactivity in areas CA3/4 of the hippocampus (Fig. C3-3). The Aβ-IR was nearly completely confined to small, cell associated granules after both 1 (5/7) and 2 (6/8) weeks of treatment, and was not different at the two time points studied. In contrast, the staining pattern of the βAPP antibodies was much different (Fig. C3-4). βAPP antibodies labeled cells of stellate morphology in CHL treated subjects (most likely astrocytes), whereas they only weakly labeled the perimeter of CA3/4 pyramidal neurons (pyramidal cell layer, large cell body and distinctive shape). βAPP antibodies did not detect astrocyte-like cells in the PBS infused animals, except near the cannula tract (not shown).
Figure C3-1. Effects of CHL on latency to develop KA-induced seizures. Chloroquine significantly shortened the time to the development of seizures (mean ± standard error). Note that animals infused with PBS also showed a slight decrease in latency, although this was not statistically significant. *Scheffe's test, p<0.05 (both FS and SE, compared to both the no pump and vehicle infused conditions).

Figure C3-2 (Following Page). CHL increases PAS reactivity and increases neurodegeneration in response to KA. Left side - untreated, right side - infused; PBS + KA: cresyl violet (A,B), PAS (C,D); CHL + KA: cresyl violet (E,F), PAS (G,H). Sections processed for PAS and cresyl violet were adjacent (magnification: 10x, scale bar: 450 μm). Compare the effect of CHL infusion alone (without KA) for 1 week (D, inset; same original magnification, scale bar: 450 μm) to PBS for two weeks (with KA); PAS staining is increased in CA3 (marked with *). This is also the area that is damaged following KA treatment in CHL infused animals. Kainic acid did not produce noticeable neuronal loss in the PBS infused animal on either side. In contrast, note the marked pyramidal cell loss in areas CA3/4 of the CHL treated animal and the nearly perfect correspondence with PAS reactivity (arrows and large boxed area of CA3); substantially more damage is present on the drug treated side.
Figure C3-3. CHL induces Aβ-immunoreactivity in hippocampus (shown: B2Aβ1-40, cresyl violet counterstain, CA3/4, drug infused side; left panels - original magnification: 40x, scale bar: 97 μm; right panels - original magnification: 100x, scale bars: 40 μm). (A,B) PBS infused animal (B [inset], AD cortex); (C,D) CHL infused animal; (E,F) Same subject in C/D, antigen absorbed primary antibody (nearby sections). Note the cell associated Aβ-immunoreactive granules in the CHL treated animal (cells containing these are indicated by arrowheads in D) and the relative paucity of such granules in the PBS infused control (single arrowhead in B). No granules are visible when the antibody is preabsorbed with an excess of Aβ1-40 (compare B and D with F). This effect was similar with other Aβ antibodies, and was not noticeably different between animals treated for either 1 or 2 weeks. Also of note is the normal appearance of the Nissl stained cells in E/F - further evidence of iAβ not directly causing neuronal loss.
**Figure C3-4.** βAPP antibodies show a different labeling pattern than antibodies directed against Aβ (shown: A, PBS infused animal; B-F, CHL infused animal; A/B, CA3 pyramidal cells; C-F, CA3 stratum radiatum; original magnification: 100x, scale bars: 40 μm). (A) anti-βAPPN; (B) anti-βAPPN (inset [B], primary antibody omitted); (C) anti-βAPPN; (D) anti-βAPPN, primary antibody omitted; (E) anti-βAPPC; (F) anti-βAPPC, primary antibody omitted. In spite of staining most of the cellular processes of cells of distinct astrocytic morphology, βAPP antibodies label the perinuclear area of pyramidal neurons, a different pattern than that obtained with Aβ antibodies (c.f. Fig. C3-3). No cells resembling astrocytes were immunostained with βAPP antibodies in PBS infused animals (not shown).
As expected from the βAPP immunostaining, an astrocytic reaction was seen for both CHL and KA (Fig. C3-5). Both CHL and KA caused an increase in GFAP-IR, indicative of an astrocytic response (although the response to KA appears after the 1 week survival period). Morphologically, the astrocytes were hypertrophied, although proliferation cannot be ruled out. The combined treatment produced an additive effect. Examination of adjacent sections immunostained with antibodies to either Aβ or βAPP indicated that activated astrocytes (following KA) contained significant Aβ/βAPP on the CHL treated side only, even though an astrocytic response was clearly visible on the uninfused side. This was also true of hypertrophied astrocytes in the PBS+KA treated animals not showing hippocampal damage, in that both sides showed astrocyte hypertrophy, but did not show Aβ/βAPP-IR. A few Aβ/βAPP+ astrocytes were found in the immediate area of cell loss in the single PBS+KA treated animal showing this effect. Those animals that received the combined treatment of CHL+KA showed virtually no Aβ/βAPP-IR in CA3 pyramidal cells (which were largely destroyed; c.f. Fig C3-2).

Many of the effects of kainic acid are relatively permanent (Fig. C3-6). Rats subjected to 2-3 hours of seizures and allowed to survive for 28 days were run as positive controls for all of the staining procedures. Once again, PAS staining can be seen associated specifically with the area of damage (C3-6A and B). Also, a strong astrocyte presence can be detected, more so in the damaged region (C3-6C). Similar to

**Figure C3-5 (Following Page).** Astrocytic reaction to CHL and KA. Left side - untreated, right side - infused; (A-F) GFAP, (G,H) BαAβ1-40. (A,B) PBS+PBS, (C,D) CHL+PBS, (E-H) CHL+KA. Sections processed for GFAP- and Aβ-IR were adjacent (all animals from 1 week survival group; original magnification: 10x, scale bar: 450 μm). CHL caused an astrocytic response (D vs. B and C), as did KA (E/F vs. A-D); the KA effect shown in E (CHL+KA, untreated side) is indistinguishable from PBS+KA (both sides; not shown). Also, the effects of KA and CHL appear additive (D+E approximately = F). However, note that the astrocytes on the CHL treated side exhibit increased Aβ-IR, whereas those on the untreated side do not, in spite of being in an activated state. Similar reactivity was observed with βAPP antibodies (not shown, but see Figs. C3-4 and 6), and was the same for PBS+KA treated subjects (in comparison with E).
the effects observed for KA in this experiment (and its interaction with CHL), astrocytes in the vicinity of the damaged area are βAPP positive (C3-6D).

Examination of animals sacrificed after one or two weeks of infusion revealed that CHL caused significant extravasation of serum proteins (4/5 and 7/8, respectively), occasionally very severe (3 rats, all at 1 week treatment; Fig. C3-7, compare A and B). Subjects infused with PBS showed serum protein extravasation along the cannula tract only (inset panels in A). The lateral ventricles were dilated, indicating accompanying vasogenic edema. Kainic acid also caused a similar extravasation of serum protein.

Figure C3-6. Persistence of KA-induced effects; KA-treated animal, 2 hours SE, 28 day survival (hippocampus, horizontal serial sections, original magnification: 5x, scale bar: 875 μm). (A) cresyl violet, (B) PAS, (C) GFAP, (D) βAPP (R1; a similar effect is seen with Aβ antibodies). Note the continued association between cell loss, PAS staining, GFAP- and βAPP-IR. The area demarcated by the square is particularly intense, although the area of effect extends below this region in the image.
Tissue obtained from subjects immediately after 30 minutes of SE displayed extensive incursions of extravasated serum proteins in the hippocampus, paleocortical regions (piriform, perirhinal, and entorhinal cortices), amygdala and septum (incursions were usually not found in all areas in a single animal). Occasional incursions were also observed in the striatum and (rarely) in the neocortex. The KA-induced incursions could appear as small, focal infarcts, or as larger diffuse deposits; in both cases, a blood vessel could always be found in close association, usually near the center. In contrast, the CHL-induced disruption was much more dispersed. At one week following KA treatment, these incursions had almost entirely vanished, and were never seen in KA treated animals that had survived for 28 days. The exception to this were those animals treated with CHL, wherein large extravasated protein deposits could still be found, often in areas quite distant from other effects of the drug infusion (Fig. C3-7C-F).

C3.4 Discussion

Continuous infusion of CHL in rats produces effects similar to those of LEU infusion in non-human primates (C2) and in mice given a series of bolus injections of LEU over one week (C1). Infusion of CHL resulted in an increase in iAβ-IR, and this increase was associated with later increased susceptibility to KA-induced degeneration.

Figure C3-7 (Following Page). Chloroquine and kainic acid cause serum protein extravasation. Top Panels: Low magnification (scale bar: approximately 3 mm) photomicrographs of PBS (A) and CHL (B) treated rat brain (1 week), with infusion side to the right (inset A [top]: IgG-IR localized along the cannula tract; [bottom]: adjacent section to cannula, antigen absorbed primary antibody; original magnification, 2.5x, scale bar: approximately 1 mm). CHL caused a massive extravasation of serum protein, shown here with IgG-IR (the effect was identical for anti-rat serum antibodies, with and without IgG absorption). Note also the prominent ventricular dilation in the CHL treated animal, indicative of vasogenic edema. Bottom Panels: Higher magnification (10x, scale bar: 400 μm) photomicrographs of the piriform/entorhinal area of PBS (C,D) and CHL (E,F) treated animals (infused side on the right). Both animals were given KA and allowed to survive for 1 week. Although a few examples of protein extravasation can be seen in the PBS treated subject, several large, intense incursions can be observed in the CHL treated rat on the side of the cannula implant.
Increased Aβ-immunoreactivity is found within CA3/4 pyramidal cells, along with weak PAS reactivity (both of which presumably indicate the range of the drug effect). This population of neurons degenerates during the 1 week survival period following KA-
induced seizures. As with experiments 1 and 2, the appearance of iAβ-IR did not directly lead to cell death in the affected region. Also similar to previous experiments, immunostaining with βAPP antibodies did not reveal the same pattern of cellular labeling as Aβ antibodies, which identified small punctate granules within the confines of the cell body of pyramidal neurons. Hence, the Aβ-IR likely represents iAβ/fβAPP and not βAPP. The small granules may represent lysosomally localized iAβ (which would be expected following treatment with a lysosomal inhibitor), or may represent aggregates of iAβ located elsewhere in the cytoplasm. It has recently been reported that intralysosomal Aβ compromises the integrity of the lysosomal membrane, allowing leakage of the contents into the cytoplasm (Yang et al, 1997).

These findings are consistent with several studies indicating a potentiating effect of subtoxic concentrations of Aβ on excitotoxic processes (Koh et al, 1990; Mattson et al, 1992; Patel et al, 1995). However, in the case of this experiment, the Aβ is induced intracellularly rather than added to the culture medium where it is unclear if it exerts its effects extracellularly or not. Although infused Aβ may be neurotoxic (Yankner et al, 1989, 1990), transgenic mice which display massive deposits of extracellular Aβ (covering up to 50% of cortical area, a far greater amount than ever seen in AD) show no evidence of substantial cell loss (Irizarry et al, 1997). Hence, the main finding of this experiment illustrates that Aβ could potentially exert a toxic influence from an intracellular location, as suggested by others (Yang et al, 1995; Wild-Bode et al, 1997; Burdick et al, 1997; Yan et al, 1997).

The Aβ/βAPP-IR observed associated with reactive astrocytes following KA likely represents βAPP. It is unlikely that the related APLP proteins are responsible for this staining, since these do not contain an Aβ region and would thus not cross-react with Aβ antibodies (Slunt et al, 1994; Crain et al, 1996). Reactive βAPP+ astrocytes are known to be closely associated with damaged areas and remain βAPP-IR for at least one month following the lesion (Siman et al, 1989; Töpper et al, 1995), in agreement with the findings of this experiment. It is unlikely that the astrocytic reactivity is due entirely to the uptake of the fβAPP from the dying CA3 neurons (although some may be), since
astrocytes have been shown to manufacture βAPP mRNA in similar situations (Abe et al., 1991; Kawarabayashi et al., 1991; Willoughby et al., 1992; Solà et al., 1993).

The persistent residual PAS staining present following cell loss is not Aβ. This is not surprising, since normal rats do not develop senile plaques and these were not an expected finding in this experiment in any case (cf. A5.2 and C1). Periodic acid-Schiff positive staining is a common finding following neuronal damage, and its composition is unknown (Bennett et al., 1995). It may simply be cellular debris left over following neuronal destruction. However, since the PAS staining can remain for as long as 28 days following a KA-induced lesion (as shown in this experiment), it more likely represents a “permanent” marker of cell loss. Since astrocytes are intimately associated with this deposit, it may be composed of highly glycosylated extracellular matrix (ECM) components synthesized as part of the process of glial scar formation (Bennett et al., 1995). Preliminary efforts to determine the identity of this substance (by attempting to remove the stain by pretreating the tissue with either heparinase or chondroitinase) were unsuccessful, which may be due to the formaldehyde fixation (unpublished observations). In any case, the PAS staining procedure may be a useful tool for the rapid identification of damaged areas in the brain.

Chloroquine caused the appearance of a massive amount of IgG/serum protein-IR on the infused side. Serum proteins, such as IgG, cannot normally cross the blood-brain barrier (Triguero et al., 1989), but can often be found within the brain following a variety of injuries (Suzuki et al., 1995; Hoshino et al., 1996). As such, immunohistochemical methods aimed at detecting extravasated serum proteins within the CNS parenchyma are a well accepted method of determining BBB disturbances (Schmidt-Kastner et al., 1990, 1993), and correlate extremely well with traditional methods using systemic injections of exogenous tracer compounds such as HRP and Evan’s blue (Ruth, 1986; Ruth and Feinerman, 1988). Hence, the IgG/serum protein-IR observed in this experiment (and in C2; see figure C2-1) may represent BBB dysfunction caused by CHL infusion (although this cannot be concluded with certainty without the use of direct tracers and/or electron microscopic examination). Although the mechanism for this effect
is unknown, severe BBB damage creates some difficulties in interpreting the results of this experiment.

The blood-brain barrier is relatively impermeable to KA, and only nanomolar quantities actually reach the brain following systemic administration (Berger et al, 1986; Lefauconnier et al, 1986). Since the rate of onset and severity of KA-induced seizures is dose dependent (Lothman and Collins, 1981), this could indicate that a compromised BBB is the reason for the reduced latency to chemical seizure induction found in this study and others using CHL (Amabeoku, 1992; N'Guoemo et al, 1994). Even in the case of PBS infused animals, a slight reduction in latency is observed relative to unimplanted controls (this could be related to the slight damage to the BBB resulting from the cannula implantation; Fig. C3-7). Hippocampal CA3 neurons express, at high densities, a receptor with the highest binding affinity for kainic acid in the brain (Berger and Ben-Ari, 1983; Werner et al, 1991). This translates both in vivo (Wuerthele et al, 1978; Nadler et al, 1986) and in vitro (Rimvall et al, 1987) to a heightened vulnerability to KA for these neurons. Hence, the increased loss of CA3/4 neurons in response to the KA/CHL combination may not be entirely due to increased iAβ/iβAPP, but may be related to some extent on more KA reaching the brain. Also, the increased residual KA-induced BBB damage following CHL can occur in distant areas (such as the entorhinal cortex; Fig. C3-7), in which no other effects of CHL were observed and are likely well beyond the region of effective drug diffusion. This may be indicative of more KA reaching the brain and/or more severe seizures in CHL treated animals.

Therefore, although the current results are consistent with iAβ increasing susceptibility to excitotoxic injury, the possibility that a BBB compromise may have occurred raises some concerns. However, CHL has an advantage over other lysosomal inhibitors, in that it has the potential to be administered systemically. This approach might circumvent complications arising from BBB compromise (which may be due to vascular toxicity caused by high local concentrations of CHL).
C4 Experiment 4: Intraperitoneal Chloroquine in Mice Increases iAβ-IR in the Brain Without Accompanying Manifestations of Blood-Brain Barrier Dysfunction

C4.1 Introduction

Experiment 3 established that infusion of CHL directly into the brain produces elevated intracellular Aβ/fβAPP similar to that of infused CHL. Unfortunately, the method used to induce iAβ/fβAPP accumulation in this experiment may have caused BBB damage. Hence, the purpose of this study was to determine if chronic systemic chloroquine treatment could be used to circumvent this problem and still induce iAβ. This experiment also represents a fundamental shift in drug delivery strategy, making it necessary to first re-evaluate earlier results obtained by infusion (C1). That is, it is possible that iAβ induced by systemic CHL might lead to SP formation, whereas infusion of LEU (affecting limited areas) did not. Therefore, transgenic mice (as per experiment 1) were used as subjects in this experiment to assess this possibility.

C4.2 Methods
C4.2.1 Subjects and Drug Treatment

A total of 42 male and female wild type (WT) and transgenic (TG) mice, aged 16-31 months (mean age=22.24; s.d.=5.49), served in the experiment (B1.1). The animals received i.p. injections of either CHL (45mg/kg; n=18) or vehicle (PBS; n=24) once/day for six consecutive days. This dose of CHL was selected based on published data on the toxic effects of CHL (Amabeoku, 1992); 45 mg/kg is the highest dose that can be given to mice systemically without inducing signs of seizure activity and morbidity. On the day following the final dose, half of the subjects from each treatment group were randomly assigned to the KA challenge group (included as a positive control for possible BBB damage), while the remaining animals were injected with PBS. Sex, strain and age were counterbalanced for all treatment combinations. The members of the KA group received an initial i.p. injection of 12 mg/kg, which was followed by a supplementary injection of 24 mg/kg two hours later. Status
epilepticus (SE) was defined as in B2.2. Following 45 minutes of status epilepticus (SE), the animals were sacrificed with an overdose of sodium pentobarbital, perfused transcardially and prepared for frozen sectioning at 30 μm (B3).

Two additional animals (both heterozygous) were implanted with osmotic minipumps (Alzet 2002; Alza, CA; 2 weeks operation, 0.5 μl/hour) containing 60 μg/μl CHL. The pumps were placed subcutaneously (s.c.), between the shoulder blades, and connected to a fixed cannula via a short length of polyethylene tubing (also s.c.). The procedure was essentially identical to that of the rats (C3), and the implantation coordinates were the same as for mice given i.c.v. injections (C1). After one week of infusion, the animals were given a single injection of 12 mg/kg KA. Seizures were terminated after 45 minutes of SE.

For the chronic systemic experiment, some animals were excluded from the histological analysis due to failure to develop SE (other mild seizures were seen; n=2) or use in another study (n=8). Mice implanted with CHL pumps were also used for a separate experiment and are included to illustrate effects of infusion on seizure latency.

**C4.2.2 Tissue Preparation and Histology**

Coronal sections were taken (30μm) from the septum to the hippocampus. Every eighth section was processed for immunohistochemical localization of endogenous IgG and serum proteins (B5). Adjacent sections were immunostained using antibody preabsorbed with an excess of reagent grade mouse IgG or serum, respectively, or with cresyl violet. Also, some sets were examined using anti-mouse serum preabsorbed with an excess of IgG (to demonstrate the presence of extravasated serum proteins other than IgG). Following processing, IgG incursions present in the tissue were counted (regardless of size or location) by three experimenters, each blind to the treatment conditions. Data were analyzed by stepwise multiple regression (Statistical Analysis System; SAS Institute). Anti-serum antibodies revealed the same staining pattern, and were thus not quantitated. Finally, additional antibodies were used for localization of astrocytes (Boehringer-Mannheim anti-GFAP) and Aβ (BoAβ1-40 and lαAβ1-28)(B5).
C4.3 Results

Chloroquine caused a marked increase in Aβ immunoreactivity throughout the brain, likely reflecting the presence of iAβ (Fig. C4-1). There were no signs of any plaque-like pathology in either the WT or TG mice under these treatment conditions. Further, CHL caused a widespread astrocytic response (not shown), even though cresyl violet stained sections did not show noticeable cell loss. In no case could TG mice be reliably distinguished from WT in any treatment condition. Examination at higher magnification revealed Aβ-IR to be cell associated, largely as small punctate granules (Fig. C4-1).

Analysis of the convulsant response to the KA challenge indicated the presence of a marginally significant accelerated time to first seizure (FS) for the members of the CHL treatment group [F(1,25)=3.81, R^2=0.1322, p<0.06; Fig. C4-2]. Time to SE was not affected, and calculations based on time relative to the second KA injection gave the same results. In contrast, the two animals infused i.c.v. with CHL reached SE in under 30 minutes when given 12 mg/kg KA (not shown).

Kainic acid also caused an increase in the number of IgG incursions in the brain [F(1, 17)=6.07, p<0.025]. However, animals pretreated with CHL had fewer instances of extravasated protein [F(1,17)=7.41, p<0.015]. These effects were restricted to limbic areas, such as the hippocampus, septum, amygdala and the piriform/entorhinal cortices. Similar results were obtained with the polyclonal antibody against mouse serum proteins, with and without preabsorbing with purified IgG (Fig. C4-3). Staining was abolished by preabsorbing the antisera with purified mouse IgG (for anti-mouse IgG) or mouse whole serum (for anti-mouse serum).

C4.4 Discussion

The administration of chloroquine chronically via the systemic route yields results that are very similar to those found following infusion of CHL into the lateral ventricles of rats. In the case of this experiment, the increased cell associated Aβ-IR was much more widely distributed, being found in many areas other than the hippocampus. Also, systemically applied CHL caused a pronounced increase in GFAP-IR, indicating an astrocytic response to the drug similar to that obtained following direct brain infusion. In
Figure C4-1 (Following Page). Systemically administered CHL causes an increase in cell associated Aβ-IR (shown: perirhinal cortex from 2 TG mice, lαAβ1-28; left panels - original magnification: 40x, scale bars: 100 μm; right panels - original magnification: 100x, scale bar: 40 μm). (A,B) PBS treated; (C,D) CHL treated (inset [C]: antigen absorbed primary antibody, nearby section); (E,F) AD cortex (cresyl violet counterstain). Note the small, cell associated granules present in many of the cells from this region (C and D); the effect is identical at higher magnifications in the hippocampus and other regions (not shown). No structures resembling extracellular amyloid deposits were ever detected in either WT or TG mice in response to CHL treatment.
Figure C4-2. Proconvulsant effect of CHL. Chloroquine pretreatment caused a marginally significant ($p<0.06$) decrease in time to develop the FS, but not SE. Sex, strain or age did not influence the results in any case.
Figure C4-3. Chloroquine pretreatment prevents KA-induced serum protein extravasation. Shown: amygdala/piriform area (original magnification: 5x, scale bars: 800 μm) from a representative set of animals, illustrating typical sections reacted simultaneously for immunohistochemical localization of serum proteins (preabsorbed with excess IgG) at each treatment condition (A: control, B: KA, C: CHL, D: KA+CHL). Inset (B): adjacent section preabsorbed with mouse serum (same original magnification). The prominent serum protein extravasation found following KA-induced seizures is eliminated by CHL pretreatment; an identical effect is observed for IgG-IR alone.
contrast with the results of experiment 3, repeated intraperitoneal injections of CHL did not cause the appearance of extravasated serum proteins in the brain. Further, although seizures caused by kainic acid induced characteristic serum protein extravasation, this was largely prevented by CHL pretreatment, even though CHL may have had a mild proconvulsant influence on its own.

The most important finding of the current experiment is the increase in Aβ-IR, which reflects the same Aβ/βAPP accumulation found following direct brain infusion of CHL or LEU. As with cell associated Aβ-IR in previous experiments, this is most likely localized intracellularly (c.f. C1.4). No sign of SP-like pathology was found, even in the case of the oldest TG animals (30+ months old), and no overt neuronal loss occurred (although a small loss cannot be ruled out without careful study). Since lysosomal dysfunction increases markedly with age (as measured by lipofuscin accumulation in mice; Moore and Ivy, 1995; Moore et al, 1995), and since elderly C100-βAPP TG mice are also known to accumulate amyloidogenic fragments of the transgene within lysosomes (Kammesheidt et al, 1992; Neve et al, 1996), the finding that these older mice also do not develop the pathology can be taken as further evidence against a direct role of iAβ arising as a consequence of lysosomal dysfunction (that is, without the involvement of a precipitating factor) in AD pathogenesis.

Kainic acid treatment caused serum protein extravasation, primarily in limbic areas known to be vulnerable to KA-induced seizures (Ben-Ari et al, 1986; Ruth and Feinerman, 1988). Similar to animals receiving CHL infusion (C3), systemic CHL created a proconvulsant effect (although the reduction in seizure development latency was not nearly as pronounced in this experiment). However, since CHL attenuated the BBB effect of KA, the mild proconvulsant response is unlikely due to a compromised BBB. In fact, this finding casts doubt on the proconvulsant effect of CHL in experiment 3 being due to more KA reaching the brain. Further, duration of SE was the same for both CHL and PBS treated animals and severity of the evoked motor seizures in no way appeared different, so seizure severity is not likely to be responsible for the difference between the two treatments.

The apparent protective influence of CHL on the BBB is not likely to be related to
a loss of the endogenous IgG tracer used to evaluate this effect. Chloroquine has poorly understood immunosuppressive properties, but is used in the treatment of rheumatoid arthritis (Fox, 1993) and systemic lupus (Wallace, 1994). The endosomal/lysosomal system is a critical intracellular compartment in antigen presenting cells (such as macrophages), which require the activity of acidic hydrolases to process target proteins into fragments to be presented at the cell surface (Brockman and Murphy, 1993). One possibility is that chloroquine may interfere with antibody production by creating a disturbance in this aspect of immune system function (Antoine et al, 1985; Thorens and Vassalli, 1986; Fox, 1993). However, the same result (BBB protection) is obtained by using antisera to mouse whole serum (which recognizes dozens of different serum proteins) from which all IgG immunoreactivity has been removed (Fig. C4-3), indicating that the loss of the IgG marker could not be responsible for the observed effects.

Therefore, systemic administration of CHL caused an increase in intracellular Aβ-IR similar to that found following i.c.v. CHL infusion. Importantly, this increase was not paralleled by massive serum protein extravasation indicative of a possible BBB compromise. Therefore, this method of drug administration allows the effects of the induction of iAβ to be dissociated in vivo from the possible influence of BBB damage, and creates an opportunity to re-evaluate and confirm the interactions between iAβ and excitotoxicity observed in experiment 3.
**C5 Experiment 5: Dose Response of Oral Chloroquine and its Interaction with Kainate in Rats**

**C5.1 Introduction**

Experiment 4 demonstrated that it is possible to induce effects of CHL similar to those of direct brain infusion while using a systemic treatment paradigm. The most important finding of this study was that iAβ-IR could be induced in absence of the BBB abnormalities that may occur following CNS administration of the drug. Hence, the hypothesis that the accumulation of iAβ/fβAPP is a risk factor for increased excitotoxic damage can now be assessed in absence of this confounding factor.

In order to confirm the results of experiment 3, SD rats were once again used in a similar experimental design (KA+CHL: no survival [this experiment], KA+CHL: 1 week survival [experiment 7]), with the exception that CHL was now given systemically. Since early results indicated that rats did not tolerate repeated intraperitoneal injections of CHL well as did mice (2/3 animals died of fatal tonic convulsions), an oral route of administration was selected instead. Since pilot data also indicated that the effects of systemic CHL in the rat may be complex, this experiment elucidated a dose-response for the CHL/KA interaction in order to determine the full range of these effects. This determination will allow post-KA effects (i.e., neurodegeneration) to be associated with the induction of iAβ at the appropriate CHL dose [in experiment 7].

**C5.2 Methods**

**C5.2.1 Subjects and Drug Treatment**

A total of 42 SD rats (B1.2) were used in this experiment. Six doses of CHL were used (0, 7.5, 15, 22.5, 30 and 45 mg/kg), with 7 animals at each dose. The maximum dose was selected based on published data on CHL LD$_{50}$ (140 mg/kg) and CD$_{50}$ (89.2 mg/kg) in rats, and the finding that CHL alone will not induce seizures below 50 mg/kg (Osifo and DiStefano, 1978). Chloroquine was prepared fresh daily in double-distilled water, and administered orally (by gavage; g.v.) on each of 7 consecutive days. Animals were carefully monitored in Plexiglas boxes for 2-3 hours following drug administration to determine any behavioral manifestations of seizures (B2.2). Based on preliminary data
from the chronic experiment, 3 doses were selected (0, 15 and 45 mg/kg; n=6/dose) for an acute (single dose) study.

Kainic acid was prepared and administered as described (B2.2). Injections of KA (chronic: n=4/dose, acute: n=3/dose) or PBS vehicle (n=3/dose) were conducted exactly 2 hours after the final CHL dose (or 2 hours after the single CHL dose in the case of the acute experiment). Seizure latency data from 6 CHL-untreated animals was also pooled with the 3 acute, CHL vehicle subjects for a better estimate of control parameters.

**C5.2.2 Tissue Preparation and Histology**

Animals were sacrificed immediately following termination of seizures with an overdose of sodium pentobarbital, perfused transcardially, and prepared for frozen sectioning at 20-30 μm (B3). Some sections were processed for cresyl violet. Subjects were selected for an individual run of histology and processed for immunohistochemistry in a counter-balanced manner, with all cells of the experimental design represented when possible. Immunohistochemistry (B5) was performed using the same antibodies to GFAP, Aβ, βAPP and serum proteins as experiment 3; some procedures were followed by cupric sulfate intensification (0.5%, 0.05 M Tris-HCl buffer, pH=7.60, for 10 minutes).

**C5.2.3 Data Analysis**

Slides were coded and analyzed by experimenters blind to treatment conditions. In the case of GFAP, Aβ and βAPP, slides were analyzed based on a 3 point scale (0, + or ++), and a representative value obtained for each treatment condition. For IgG-IR, serum protein incursions were counted for 1 set of animals (as per experiment 4), and were also evaluated on a 2 dimensional 4 point scale for all subjects based on examination of the amygdala/piriform/entorhinal area, which showed the most consistent effects (table C5-1). A score was assigned (one/side), for 12 serial sections separated by 240 μm each. Scores were summed for each rater (3 in total), and a median value determined; these values were plotted to arrive at a representative score. This more involved procedure was necessary for IgG incursions due to the greater variability of the effects at lower doses of CHL. Seizure data were analyzed by ANOVA, followed by
Table C5-1. Rating Scale for IgG Extravasation

<table>
<thead>
<tr>
<th>Number</th>
<th>0-2</th>
<th>&lt;10</th>
<th>10-100</th>
<th>&gt;100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spread</td>
<td>None &amp;/</td>
<td>Focal &amp;/</td>
<td>Diffuse &amp;/</td>
<td>Widespread</td>
</tr>
<tr>
<td>Intensity</td>
<td>or Faint</td>
<td>or Mild</td>
<td>or Moderate</td>
<td>&amp;/or intense</td>
</tr>
</tbody>
</table>

Scheffe’s test for multiple pairwise comparisons and Dunnett’s test for multiple comparisons with a control group (Statistical Analysis System; SAS Institute, Cary, NC). Six animals which were given KA but failed to reach SE (1 per dose) were included in the experiment for comparison, but were treated as a separate group when considering drug effects.

C5.3 Results

C5.3.1 Chronic Chloroquine Treatment

Subjects were never observed as having seizures following any dose of CHL alone. ANOVA results and subsequent post-hoc tests did not detect any effect of CHL on KA-induced seizure onset latency. However, lower doses of CHL did appear to slightly delay the onset of seizures, an effect which peaked at 15 mg/kg but did not reach statistical significance [FS: t(17)=1.0938, p<0.29; SE: t(17)=1.4629, p<0.17; compared to all other animals]. At doses above 15 mg/kg, the direction of the effect reversed, such that the highest CHL dose was significantly proconvulsant in comparison to all other doses [FS: t(17)=2.1814, p<0.05; SE: t(17)=2.5243, p<0.025](Fig. C5-1).

A widespread increase in cell associated punctate, granular Aβ-IR was detected at the second highest dose of CHL used (30 mg/kg; Fig. C5-2). Extracellular Aβ-IR in the form of SP-like structures were never detected at this dose, and all Aβ antibodies yielded essentially the same results. The results at the highest dose of CHL (45 mg/kg)
Figure C5-1. CHL slightly alters the time to KA-induced seizure development. Lower doses of CHL had a slight tendency to slow seizure development (FS: p<0.29, SE: p<0.17, at 15 mg/kg). The highest dose did produce a significant proconvulsant effect (FS: p<0.05; SE: p<0.025), but only when compared to all other doses.

were markedly different. Although 2 of the animals showed an effect similar to that of 30 mg/kg, most of the remaining animals (4/7) at 45 mg/kg showed a large increase in Aβ-IR within the neuropil, an effect which was also widely distributed (i.e., most of the brain studied) but was particularly striking within the hippocampus (Figs. C5-3 and C5-4). In these animals, the Aβ-IR was almost entirely eliminated from the cell body. Antibodies directed against various portions of βAPP confirmed that at least some portion of the immunostaining at 45 mg/kg was likely due to a redistribution of the precursor and not solely Aβ (Fig. C5-3). Kainic acid used within the parameters of this study had no detectable effect on Aβ/βAPP-IR.

Figure C5-2 [Following Page]. Oral CHL increases Aβ-IR in the rat (shown: perirhinal/piriform cortex at 3 key doses, loAβ1-28; left panels - original magnification: 20x, scale bar: 200 μm; right panels - original magnification: 100x, scale bar: 40 μm). (A,B) Vehicle; (C,D) 15 mg/kg; (E,F) 30 mg/kg; (G,H) Same subject as E/F, antigen absorbed primary antibody. The Aβ-IR is clearly cell associated, and the increased labeling appears almost entirely in cells of neuronal morphology. Higher magnification (right panels) shows a punctate distribution almost entirely within the confines of the cell body. Other Aβ (and not βAPP) antibodies revealed a similar effect.
Figure C5-3. The highest dose of CHL caused Aβ/βAPP redistribution (A, B: vehicle; C, D: 45 mg/kg CHL; hippocampus, original magnification: 5x, scale bar: 780 μm). Left side - 10D5 αAβ1-16, intensified with 0.5% cupric sulfate; right side - R1αβAPP, unintensified (N-terminus 45-62 of βAPP). Labeling is redistributed from the cell body (which is normally faintly stained) to a diffuse, granular neuropil localization. Note the clear profile of the dentate gyrus granule cell layer in the CHL treated animal (arrows in C and D) in comparison to the vehicle treated control.

Chloroquine treatment altered IgG/serum protein-IR in several ways. Initially, semiquantitative examination (i.e., by ranking) of 2 sets of subjects comprised of 4 animals from each of 4 equally spaced doses (0, 15, 30 and 45 mg/kg, half with KA) indicated that the effects of the drug were ubiquitous (table C5-2). The lowest CHL dose in this comparison, corresponding to the peak anticonvulsant dose, resulted in the increased presence of serum protein immunoreactivity in the brain. In parallel with the convulsant dose-response curve, higher doses reversed this effect, such that the proconvulsant dose of 45 mg/kg attenuated the effect of KA. This was unlikely due to an
immunosuppressive effect of CHL, since similar results were found with anti-serum antibodies preabsorbed with IgG, and since no change was observed in areas known to have weak barrier properties (Ruth and Feinerman, 1988; Wolburg and Risau, 1995) such as the hypothalamus (table C5-2) and subfornical organ (Fig. C5-5). Attempts at quantitative determination of the number of incursions indicated that the effect was highly variable, especially with regard to incursion size; in the case of KA treated animals, a single incursion could encompass several hundred \( \mu \text{m}^2 \). Since the effects of both CHL and KA appeared best represented by the piriform/entorhinal cortex and the amygdaloid nucleus, these regions were selected for further study.
### Table C5-2. Distribution of IgG/Serum Protein-IR*

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<th>CHL Dose</th>
<th>FRC</th>
<th>PAR</th>
<th>PRH</th>
<th>PIR</th>
<th>HYP</th>
<th>STR</th>
<th>AM</th>
<th>HIP</th>
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</tr>
</thead>
<tbody>
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<td>Vehicle</td>
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<td>0</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vehicle + KA</td>
<td>0</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>•</td>
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<td>0</td>
</tr>
<tr>
<td>15 mg/kg</td>
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<td>+</td>
<td>+</td>
<td>++</td>
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<td>+</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>15 mg/kg + KA</td>
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<td>++</td>
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<td>++</td>
<td>++</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>30 mg/kg</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45 mg/kg + KA</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Scores determined using the criteria defined in table C5-1.

**Legend for Neuroanatomical Areas:**
- FRC - frontal cortex
- PAR - parietal cortex
- PRH - perirhinal cortex
- PIR - piriform/entorhinal cortex
- HYP - hypothalamus
- STR - striatum
- AM - amygdala
- HIP - hippocampus
- Oth - other areas, such as septum, thalamus, white matter, etc.

Evaluation of all subjects in the experiment based on a 2 dimensional 4 point scale (table C5-1) confirmed the results of the initial examination. Scatter plots made of the median ratings of 3 experimenters revealed skewed distributions of the IgG effect for both incursion frequency (Fig. C5-6A) and intensity and spread (Fig. C5-6B), with the peak centered at 15 mg/kg. Plotting group medians for each treatment combination (Fig. C5-6C) indicated that KA administration did not increase the number of incursions, but did result in a marked increase in their distribution and intensity throughout the area. The total number of IgG extravasation events determined for all brain areas for one set of representative animals confirmed the results of the overall semiquantitative analysis, and also indicated that kainate treatment increased the total number of incursions throughout the brain (Fig. C5-7). Further collapsing the data into CHL treatment groups and ignoring KA treatment also indicated that the peak effect of CHL occurred at 15 mg/kg for both number of incursions and their severity (Fig. C5-8; c.f. Fig. C5-9).
Figure C5-5. CHL Treatment has little effect on baseline IgG levels. The highest dose of CHL (B) did not consistently reduce IgG-IR in areas with a weak BBB in comparison to vehicle (A), indicating that the reduced immunostaining could not be accounted for by a loss of the endogenous IgG marker (shown: subfornical organ, original magnification: 5x, scale bar: 800 μm).

Stronger GFAP-IR was observed only at the highest dose of CHL administered, an effect independent of KA treatment condition (Fig. C5-10). The effect could be distinguished in most brain areas examined, including those that were not affected by the apparent BBB abnormalities. Many of the hypertrophied astrocytes appeared to have processes in contact with blood vessels. Additional direct examination of astrocytes and IgG incursions at 15 mg/kg CHL (using an anti-mouse IgG antibody with significant rat IgG cross-reactivity as the secondary antibody for anti-GFAP) did not indicate an association between the two events (not shown).

Figure C5-6 (Following Page). Semiquantitative evaluation of CHL/KA effects on IgG-IR; determination based on examination of amygdala + piriform/entorhinal cortex. Scatter plots of median rating scores of 3 experimenters for number (A) and intensity and spread (B) of IgG incursions. The distribution is skewed, with a peak at 15 mg/kg CHL. Inspection of the scatter plots showed that KA did not alter the number of incursions in this region, but did increase their severity - note that the higher data points in (B) are nearly all KA treated animals. Plotting the median for each treatment combination confirmed these observations (C). Note also that the intensity and spread function for KA+CHL is very similar to the convulsant dose-response curve (Fig. C5-1).
Figure C5-7. Total IgG incursions quantified for all areas examined. When all areas are taken into account, KA-induced seizures do appear to increase the number of IgG incursions. Note the correspondence between the actual number of incursions and semiquantitative estimates.

C5.3.2 Acute Chloroquine Treatment

Acutely administered CHL had no discernible effects at either dose on the time required to elicit the first motor seizure incident or to the development of status epilepticus (Fig. C5-11). No immunohistochemical effects of acute CHL were found on Aβ-IR (Fig. C5-12), serum protein extravasation (Fig. C5-13) or GFAP-IR (not shown).

Figure C5-9 (Following Page). Illustration of effects of CHL/KA on IgG-IR (amygdala/piriform area, original magnification: 2.5x, scale bar: 1.5 mm). Left - CHL alone, right - with KA; drug combinations are annotated directly on the figure. At lower doses, CHL potentiates the effect of KA (D), whereas higher doses are attenuative (F and H). The same pattern of effects was seen for antibodies against rat whole serum made unreactive for IgG.
Figure C5-10. Astrocytic reaction to the highest dose of CHL. Shown (original magnification: 10x, scale bar: 480 μm): medial septum (a, b) and dorsal caudate nucleus (c, d). Left side - vehicle treated, right side - CHL (45 mg/kg). Only animals at this dose showed an astrocyte response, and the effect was not associated with serum protein extravasation; although not specifically shown here, a secondary antibody used (Vector biotinylated anti-mouse IgG) is highly crossreactive with rat IgG, and no IgG positive incursions can be detected in association with reactive astrocytes in the regions shown here.

Figure C5-11. Acutely administered CHL has no effect on the latency to the development of either the first post-KA seizure or status epilepticus. The control group shown also contains data from an additional 6 KA treated SD rats.
Figure CS-12. Acute CHL does not affect Aβ-IR. Shown: hippocampus of a vehicle (A) and CHL (B; 45 mg/kg) treated subject (original magnification: 5x, scale bar: 800 μm). This dose of CHL normally produces detectable changes in Aβ-IR (c.f. Figs. C5-3 and 4) when administered orally each day for 1 week.

Figure CS-13. Acute CHL does not alter IgG-IR. Shown: amygdala/piriform area of a vehicle (A) and CHL (B; 15 mg/kg) treated subject (SD rats, original mag.: 2.5x, scale bar: 1.5 mm). CHL at this dose normally produces perivascular serum protein deposits (such as IgG) when given orally each day for 1 week (c.f. Fig. C5-9).

C5.4 Discussion

Chronic oral administration of chloroquine in rats is capable of reproducing the effects of repeated intraperitoneal injections in mice (experiment 4). Importantly, an oral dose of 30 mg/kg was found to induce cell associated Aβ-IR without the appearance of other effects of CHL, making this dose ideal for future study of the interaction with KA-induced neurodegeneration. While the highest dose of CHL (45 mg/kg) produced some effects in rats similar to that of mice (slightly faster seizure development, attenuation of KA-induced serum protein extravasation and an astrocytic reaction), Aβ/βAPP-IR was
### Table C5-3. Summary of Primary CHL/KA Interactions*

<table>
<thead>
<tr>
<th>Effect</th>
<th>Vehicle</th>
<th>15 mg/kg</th>
<th>30 mg/kg</th>
<th>45 mg/kg</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>KA</td>
<td>PBS</td>
<td>KA</td>
</tr>
<tr>
<td><em>IgG-IR</em></td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>GFAP</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aβ-IR</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aβ/βAPP-IR</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Seizure Time</em></td>
<td>Normal</td>
<td>Slower</td>
<td>Normal</td>
<td>Faster</td>
</tr>
</tbody>
</table>

*Combinations that result in no change from control values are left blank

**A small reduction relative to control subjects may have occurred

1Punctate cellular localization, possibly endosomal/lysosomal

2Diffuse (granular) neuropil redistribution away from cell bodies

redistributed away from the cell body of affected neurons at this dose. Lower doses of CHL caused the manifestation of some effects (increased serum protein extravasation and slightly slower seizure development) opposite to those found at the highest dose (see table C5-3 for summary). Acute CHL had no effect at either of the two CHL doses examined in this experiment.
It is interesting that the possible BBB compromise (as determined by IgG/serum protein-IR) subsequent to i.c.v. infusion of CHL is associated with a decreased latency to develop KA-induced seizures, whereas the BBB dysfunction found following orally administered CHL is not (and may actually be linked to slower seizure development). This may reflect differences in magnitude of the BBB dysfunction (compare figure C3-7 to figure C5-9), such that the huge disruption found in the case of direct brain infusion masks other physiological effects of the drug. Regardless of the nature of these physiological effects, there is a remarkable similarity between the dose-response functions for chloroquine’s anti- and proconvulsant effects and its effect on IgG extravasation with and without KA. This implies that similar, if not identical, CHL-induced physiological processes may be behind each of these events.

This study therefore indicates that CHL given orally to rats for 1 week at a dose of 30 mg/kg will create the necessary preconditions (i.e., increased iAβ-IR) for the evaluation of the permissive hypothesis of iAβ neurotoxicity. Importantly, animals given this dose of CHL do not exhibit any of the other effects of CHL that might complicate the interpretation of the results, such as increased IgG/serum protein immunoreactivity in the brain (indicative of a possible BBB compromise) or altered latency to seizure development (which may indicate the occurrence of KA-induced seizures that are more or less severe, thus influencing subsequent neurodegeneration).
C6 Experiment 6: Electroencephalographic Correlates of Chloroquine Treatment Indicate Changes in Seizure Onset Latency But Not Severity

C6.1 Introduction

A potentially important issue has arose in experiments 4 and 5: does chronic CHL treatment produce changes in the latency to develop KA-induced seizures? A decrease in seizure development latency might indicate an increase in seizure severity. If such an effect exists, it should possess an EEG correlate, a possibility addressed in this experiment. Also, does the EEG evidence indicate that CHL increases or decreases the severity of KA-induced seizures, such that subsequent neurodegenerative changes might be altered?

C6.2 Methods

C6.2.1 Subjects and Drug Treatment

Long Evans Hooded (LEH) rats (n=17) were obtained and housed as described (B1.2). Subjects were randomly assigned to drug treatment conditions prior to the start of any manipulations. Doses were selected based on the peak anti- (15 mg/kg, n=5) and proconvulsant (45 mg/kg, n=5) effects of CHL as demonstrated in experiment 5 (the remaining 7 animals received double-distilled water vehicle).

Rats were prepared surgically courtesy of Dr. N.W. Milgram and C.J. Ikeda-Douglas (U. of Toronto) as described in detail elsewhere (Milgram et al, 1995). Briefly, twisted wire bipolar electrodes were prepared from Teflon-coated stainless steel wires, 190 μm in diameter, with a tip separation of 500 μm. The animals were implanted under similar surgical conditions as experiment 3. All rats were implanted with electrodes in both the perforant path and dentate gyrus (A/P: -3.5 mm, M/L: +2.2 mm). For this experiment, the perforant path electrode was used solely for electrophysiological guidance during the implantation procedure. The electrodes were inserted into a 9-pin connector plug that was mounted on the skull surface with acrylic dental cement and anchored with stainless steel jeweler's screws, one of which served as the ground electrode and the reference for unipolar recording.
As described previously (B2), drugs were prepared fresh on the day of use in their appropriate vehicle solutions. As with experiment 5, CHL was administered for 7 consecutive days and KA or PBS vehicle (one animal per dose, for histological comparison only) was injected 2 hours following the final dose. Animals were sacrificed a maximum of 2 hours after this injection (or after 30 minutes of SE, whichever came first). Drug treatment began following a 3-4 week recovery period.

**C6.2.2 Tissue Preparation and Histology**

All subjects were sacrificed with an overdose of sodium pentobarbital and perfused transcardially, followed by post-fixing, cryoprotection and freezing prior to coronal sectioning at 30 μm (B3). Immunohistochemistry (B5) was performed using antibodies to GFAP, Aβ (1αAβ1-28) and rat IgG.

**C6.2.3 Data Collection and Analysis**

Electroencephalographic recordings on awake and freely moving animals were made using a Grass polygraph, and were amplified and stored digitally on an IBM compatible computer. Prior to a recording session, animals were allowed 10-15 minutes to adapt to the testing chamber. Data were collected in three sessions on days 1 (acute), 4 (subchronic) and 7 (chronic) at regular intervals (baseline and 4 subsequent time points post-CHL, each separated by 30 minutes). Recordings were also obtained for similar time points subsequent to KA injection, and a continuous printed polygraph record was kept for this entire period.

A single data collection time point consisted of 10, 4 second EEG sweeps (digitally sampled at a rate of 256 hertz), each separated by 1 minute. A measure of EEG signal strength was calculated as an index of neuronal activity (essentially a measure of amplitude) at each time point by integrating the absolute value of all 10 sweeps individually, followed by dropping the highest and lowest values and averaging the remainder. This number was then converted to a percentage of day 1 baseline value, prior to any drug treatment. This measure is very similar to total power; a comparison of baseline data points sampled from the first 8 animals indicated a very
strong positive correlation between signal strength and total power \[ r = 0.9343; t(60) = 20.3088, p < 0.0001 \].

Rates of seizure development and EEG data were analyzed by regression ANOVA (SAS general linear model procedure), followed by multiple comparisons with the control group using Dunnett's procedure.

**C6.3 Results**

**C6.3.1 Time to Seizure Development**

The higher dose of chronically applied CHL caused a significant overall shortening of the time required to develop signs of epileptiform discharge on the EEG record compared to vehicle treated controls \[ F(1,8) = 12.33, p < 0.008 \]. There was also a clear tendency towards a slowing of the development of these events at 15 mg/kg \[ F(1,8) = 2.73, p < 0.14 \] (Fig. C6-1). Chloroquine alone never caused any type of EEG discharge event at any time.

![Figure C6-1](image-url)

Figure C6-1. Time to the occurrence of major post-KA landmarks. CHL given daily for 1 week caused an overall proconvulsant response at 45 mg/kg \( p < 0.008 \), and signs of a potential anticonvulsant response at 15 mg/kg \( p < 0.14 \), as determined directly from EEG recording. *s= p<0.05 relative to control, Dunnett's test.
C6.3.2 Changes in EEG Signal Strength

Overall, the only significant change in pre-KA EEG signal strength was a drug*day interaction \[F(4.18)=4.10, \ p<0.02\]. No significant changes in signal strength were detected following a single administration of CHL at either dose (Fig. C6-2A). However, subchronic administration (Fig. C6-2B) resulted in a significant drug effect \[F(2.9)=4.58, \ p<0.043\], which was manifested by an increase at 45 mg/kg \[F(1.6)=7.83, \ p<0.032\] and an intermediate, marginally significant potentiation at 15 mg/kg \[F(1.7)=4.38, \ p<0.075\]. These changes appeared to largely vanish with chronic treatment, such that the animals given the highest dose were similar to vehicle treated subjects (with perhaps slightly lower signal strength).

Kainic acid caused a large increase in signal strength for all groups \[F(1.9)=46.50, \ p<0.0001\] that was time dependent \[F(2.18)=16.51, \ p<0.0001\](Fig. C6-2D). There was a marginally significant interaction between CHL and KA \[F(4.18)=2.86, \ p<0.054\], with the higher dose CHL group climbing to a higher level of signal strength while the lower dose attained a level slightly lower than that of the vehicle treated subjects. Thus, there was a definite association between the increase in signal strength and time to the development of seizure activity (compare Figs. C6-1 with the final point in C6-2D). Unfortunately, analysis of time points beyond 90 minutes was not possible due to the occurrence of SE in most animals, which created huge variability in this measure by 120 minutes post-KA. However, two findings bear mentioning. First, the only subject from the 15 mg/kg CHL to reach SE by this time had a signal strength that was

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**Figure C6-2 (Following Page).** Changes in EEG signal strength with CHL and KA. (A) acute; (B) subchronic; (C) chronic; (D) chronic, including post-KA period. Scales are the same for all figure parts with the exception of (D), where the magnitude of the KA effect necessitates rescaling of the Y-axis. Chloroquine treatment did not alter EEG signal strength after a single dose (A), but did produce a dose dependent increase after subchronic administration (B; \(p<0.043\) *\(p<0.05\) compared to controls, Dunnett's test). By the seventh day of treatment (C), this effect had largely disappeared, with the 15 mg/kg group of CHL treated animals showing a nonsignificant potentiation. (continued on next page....)
Kainic acid injection (arrow on Fig. C6-2D) resulted in a rapid potentiation of signal strength ($p<0.0001$). For comparison, the last 3 time points from C6-2C are plotted on the rescaled axis. Further, CHL treatment interacted with KA to produce a larger potentiation of signal strength in the 45 mg/kg CHL dose group, whereas the lower CHL dose caused a slight attenuation of the KA effect.
579% of baseline, well above that of any of the 45 mg/kg CHL treated rats. Second, examination of individual sweeps through several classes of seizure discharge did not reveal any differences. Such an event is illustrated in figure C6-3, comparing sweeps through a class 6 motor seizure between a vehicle and a 45 mg/kg CHL treated subject, each with similar baseline EEG activity. In this case, which was similar to other such comparisons, the signal strength during the seizure episodes differed by less than 0.5%.

C6.3.3 Immunohistochemistry

Under these conditions neither KA nor CHL produced signs of BBB dysfunction in the LEH rats, as indicated by IgG-IR in the brain. As expected, positive IgG-immunostaining was observed in the subfornical organ and median eminence of the hypothalamus. Examination of sections immunostained with Aβ antibodies revealed that the higher dose of CHL was capable of causing increased cell associated Aβ-IR in this strain (Fig. C6-4). However, none of these animals were found to possess the distinct neuropil immunostaining found in SD rats treated at this dose for 1 week (c.f. Figs. C5-3 and C5-4). In addition, 45 mg/kg CHL did not produce increased GFAP-IR in this strain (nor did KA under these brief seizure conditions; not shown).

C6.4 Discussion

Chronic CHL treatment was able to replicate the key findings from the SD rats in experiment 5, as well as detecting possible EEG correlates, in LEH rats. The changes in EEG signal strength over the course of 7 days, and not on the first day of treatment, suggest that the physiological effects of CHL in this context occur only following chronic administration of the drug. Although an EEG correlate for altered latency to seizure development was uncovered, this experiment did not find evidence for chronically applied CHL causing an increase in KA-induced seizure severity.

Once an animal reaches SE, the EEG signal strength measure does not appear to vary consistently with CHL dose. In fact, the only 15 mg/kg treated subject to reach SE by the final time point (in spite of similarities to other animals in this group in all other
Figure C6-3. Illustration of typical seizure activity. Shown: a comparison between an animal pretreated with the vehicle solution (top trace) or 45 mg/kg CHL (bottom trace) for 1 week prior to KA administration. Baseline activity for each of these subjects is very similar (left side traces). Single sweeps collected during a class 6 seizure episode (multiple rearing and falling; right side traces) illustrate the typical lack of difference in signal strength between even the most severe seizures; in this case, the signal strength differs in magnitude by less than 0.5% (179.545% versus 180.437% of baseline for the vehicle and CHL treated animals, respectively; the sweeps were collected at 120 minutes post-KA).
Figure C6-4. Confirmation of effects of chronic CHL on Aβ-IR in LEH rats. Posterior hippocampus (original magnification: 5x, scale bar: 860 μm) from vehicle (A) and CHL (B; 45 mg/kg) treated animals. Note that the cell layers of the hippocampus and dentate gyrus are uniformly darker in the treated animal. This effect is widespread, as shown here in photomicrographs of the perirhinal/piriform cortex (original mag.: 10x; scale bar: 430 μm) of a different set of subjects (C: vehicle+PBS, D: vehicle + KA, E: 45 mg/kg CHL + KA); numerous darkly labeled cells can be seen scattered throughout the different cortical layers in (E), with the highest density in layers 2 and 3; KA alone has no effect. (F) High magnification (100x, scale bar: 40 μm) of (E), showing dense immunostaining of layer 2 neurons. The labeling is distinctly granular, filling most of the cytoplasm, consistent with an intracellular location.
aspects) exhibited a larger increase than any other subject studied at any dose. This therefore implies that subjects treated with the CHL dose that slows seizure development will attain levels of seizure severity that are at least as severe as animals in the other treatment conditions. Also, no difference in the incidence rate of seizure events prior to SE were detected in an earlier experiment (experiment 3), nor were any differences found by comparing EEG samples collected during SE in this study. Hence, CHL appears to affect the rate of seizure development without necessarily affecting the severity. However, a disproportionate change in EEG frequencies with CHL cannot as yet be ruled out. Although preliminary examination of Fourier transformed EEG sweeps (power spectra; unpublished observations) did not show any obvious change, the statistical power of this study is insufficient to assess altered frequency profiles.

Following subchronic treatment, a dose dependent effect of CHL appears on EEG signal strength (0 < 15 < 45 mg/kg CHL). However, chronic treatment results in an alteration in EEG signal strength, such that most of this effect has vanished (45 ≤ 0 < 15 mg/kg CHL). Whatever the underlying cause of this change, it appears to be related to the rate of seizure development in these subjects. That is, the slightly higher neuronal activity in the low dose-CHL group appears to impart a small degree of resistance to seizure development, whereas the slightly lower activity in the animals treated with 45 mg/kg results in the opposite tendency. Perhaps a nonspecific increase in neuronal activity interferes with the synchronization required for the development of seizure activity, whereas the slight suppression seen at 45 mg/kg leads to a partial removal of this barrier. Since administration of KA was only undertaken at the acute and chronic time points, it would be interesting to determine if this effect holds at subchronic treatment (where 45 mg/kg CHL would be expected to slow seizure development more than the 15 mg/kg dose). Also, since the potentiation in signal strength seen at 45 mg/kg reverses by 7 days of treatment, it’s possible that a similar effect would be found for the lower dose of CHL, given a sufficient administration period (at which point even this dose would become proconvulsant).

Although the LEH rats used for chronic CHL administration show the same increase in iAβ/fβAPP-IR and anti/proconvulsant responses to CHL as do SD rats
(experiments 3 and 5; note also the overall similarities in the time to the development of FS and SE), they were resistant to the BBB effects of CHL and KA, and did not show the characteristic astrocytic response to CHL (or the diffuse neuropil redistribution of Aβ/βAPP-IR). Even though the effect of CHL on IgG-IR (and BBB) in SD rats parallels the effects on seizure development (experiment 5), this experiment shows that the two are not necessarily connected by the same underlying physiological perturbation. A similar argument extends to the effect of CHL on astrocytes, indicating that astrocytic activation is not necessarily related to the other effects of CHL at 45 mg/kg.

In any case, these effects are all clearly mediated by a process related to chronic exposure to the drug. Chloroquine is known to have a long half-life, which is several months in humans (White, 1988). Hence, it may be that CHL must simply accumulate to some critical level to cause the effects observed here. Alternatively, the effects may be related to dysfunctional protein turnover brought about by lysosomal inhibition. One manifestation of this, of course, is the increase in cell associated Aβ/βAPP-IR.
C7 Experiment 7: Chloroquine Doses Sufficient to Induce Intracellular Aβ Potentiate KA-Induced Neurodegeneration and Result In a Corresponding Deficit In Spatial Learning and Memory

C7.1 Introduction

Experiment 5 demonstrated that a dose of 30 mg/kg of CHL, orally administered for 1 week to the rat, could induce cell associated Aβ-IR. This experiment also demonstrated that a possible BBB compromise did not occur at this dose, making it ideally suited for the evaluation of the permissive hypothesis of iAβ. Furthermore, animals treated with this dose of CHL do not show any signs of altered seizure susceptibility (which may be reduced at lower doses and increased at higher doses)(experiments 4, 5 and 6). This is an important consideration; even though no clear indication of increased seizure severity was found in experiment 6, this still remains a possibility at the highest dose evaluated. The highest dose of CHL also alters the distribution of Aβ/βAPP-IR, such that a shift away from the neuronal cell body occurs. Hence, the use of 3 key doses (15, 30 and 45 mg/kg) will allow a full dissociation of chloroquine’s effects to be determined with regards to subsequent excitotoxic degeneration induced by kainic acid. This experiment is therefore the logical second part of experiment 5, in which animals given CHL for 1 week will now be allowed to survive for an additional week after the administration of KA (similar to experiment 3). Since we now know what the brain looks like after 1 week of CHL treatment, we can use this information to interpret the outcome of this final study.

Since AD patients possess severe learning and memory deficits, an important early step towards the development of an appropriate animal model (or suitable experimental manipulation) is that it should possess (or produce) deficits in these processes. For example, the appearance of AD-like pathology in at least two strains of transgenic mice (Hsiao et al, 1996; Nalbantoglu et al, 1997) roughly parallels the development of an impairment on the Morris watermaze (Morris et al, 1982), a well known test of spatial learning and memory that is sensitive to limbic system damage (which is prominent in AD; see section A2), particularly the hippocampus. Since damage to the hippocampus and other limbic structures is a likely outcome of this experiment
(c.f., experiment 3), the Morris watermaze is an appropriate task in this regard. It should be noted that in both of these published reports the relationship claimed was between extracellular deposits of Aβ and deficits in learning and memory in the watermaze. The two goals of the behavioral assessment in this experiment are different: (1) does iAβ induced by CHL administration result in the development of a detectable impairment in spatial learning and memory (which might be expected if iAβ perturbs neuronal physiology as a possible precursor to cell death), and (2) since systemic KA is known to produce profound watermaze deficits as a result of excitotoxic limbic system damage (Milgram et al., 1988; Gayoso et al., 1994), does iAβ augment this deficit (possibly in parallel with increased neurodegeneration)?

Chloroquine can have numerous effects, of which only those involving the CNS are of concern here (c.f., Jaeger et al., 1987; White, 1988; Garg et al., 1990). However, in order to evaluate any behavioral manifestations of CHL treatment, the other major peripheral and central effects of the drug must be ruled out as possible confounding factors. This necessitates the use of additional behavioral procedures (see table C7-1).

**Table C7-1. Control Procedures for CHL/KA Behavioral Evaluation**

<table>
<thead>
<tr>
<th>Confounding Variable</th>
<th>Control Procedure(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Retinal toxicity</td>
<td>a. Visible Platform Test [vision]</td>
</tr>
</tbody>
</table>
| 3. Increased psychomotor activity, diffuse neuropsychiatric disturbances, depression and/or drowsiness | a. Open-Field Activity [locomotor activity] b. Rearing [exploratory behavior] c. Anxiety Related Measures:  
  
  i. Grooming [fear of novelty]  
  ii. Side Preference [fear of light]  
  iii. Wall Preference [fear of open spaces] |

*See methods section for details; specific variable measured is indicated in square brackets.*
C7.2 Methods

C7.2.1 Subjects and Drug Treatment

A total of 64 male Sprague-Dawley (SD) rats were used in this experiment (B1.2). Subjects were randomly assigned to drug treatment condition (0, 15, 30 or 45 mg/kg CHL; n=16/dose) prior to the experiment and KA treatment (n=7/dose) immediately prior to injection. Chloroquine (B2.1) was orally administered between 5 and 6 pm (after completion of behavioral testing). Kainate injections (B2.2) were conducted 2 hours after the final CHL dose on day 7. Seizures were scored and terminated as described (B2.2), with the exception that animals were allowed a maximum of 3 hours post-KA to develop SE. Fourteen animals (7 PBS, 7 KA) at each dose were allowed to survive for 1 week; 2 PBS treated animals were sacrificed after one week of CHL (as per experiment 5).

C7.2.2 Behavioral Procedures

Animals were first sorted into eight squads. The eighth squad, consisting of two extra animals at each dose (sacrificed prior to KA injection) were handled separately, and were only tested in the watermaze. Each of the other squads contained one animal from each of the 8 experimental combinations, and were coded such that all experimenters involved in conducting the testing procedures were blind to treatment conditions. Test groups were counterbalanced such that all drug combinations were represented equally at each test position in the order (e.g., subject #1 was, at some point in the experiment, every drug combination exactly once). On each day, sensorimotor (SMT; wire suspension [WS] and inclined plane[IP]) and open-field (OFT) testing occurred in the morning, between 9 am and noon. Half of the animals received SMT first (alternating days of WS and IP first), and the other half OFT. Watermaze (WM) testing started at approximately 1 pm. The entire test battery was carried out three times: immediately prior to drug administration (baseline), between doses 6 and 7 of CHL (prior to KA administration; post-CHL), and 7 days after KA injection (post-KA).

C7.2.2.1 Sensorimotor Tests

Both the wire suspension and inclined plane tests have been described and analyzed in detail elsewhere (Murphy et al, 1995). During testing, subjects were
maintained in plastic Nalgene cages to prevent the repeated stress of removal from a wire mesh cage, the floor of which the animal will always grasp when picked up (potentially resulting in claw injury). Testing was conducted in the same darkened room as OFT (see below), following an adaptation period of 10-15 minutes. When switching between the two tasks was required, a 10 minute rest period was first observed.

For the wire suspension task, an animal was allowed to grasp, with its forepaws, a rigid plastic coated wire suspended 64 cm above a cushioned surface. The latency to fall was measured on 3 consecutive trials (2-3 minute intertrial interval; ITI), and the maximum score was 120 seconds. The first trial and the best trial (of three) were used in the analysis, as well as the calculated mean and median latencies for each subject.

For the inclined plane test, each animal was placed onto the surface of a rectangular Plexiglas plane (60 x 122 cm) inclined at a fixed angle (beginning at 25°). Subjects were placed at the upper edge of the plane at a distance of approximately 10 cm from the top and released after a 5 second delay to allow for stable footing. If the rat did not freely slide backwards within 5 seconds of release the trial was scored as a success and the animal was returned to its holding cage. An animal was given a maximum of 4 trials at any angle. The angle of inclination was then increased by 1° and the procedure repeated following an ITI of 2-3 minutes. Testing was discontinued if no successes were scored on two consecutive angles of inclination (i.e., eight consecutive falls). Angle of first fall, total number of falls, and threshold angle (defined as the last angle at which the animal succeeded at least once) were determined for each subject.

C7.2.2.2 Open-Field Test

Locomotor/exploratory behavior was evaluated in an open-field arena (75 x 75 x 30 cm) divided into 25 equal squares. Testing was conducted in a quiet, darkened room illuminated with 40 watt, frosted red light bulbs. Two light bulbs were located one at each of the other test stations (WS and IP), and two others were placed at adjacent corners on one side of the open-field arena, providing uneven illumination. Subjects were allowed to adapt to the testing room for 10-15 minutes prior to observation, and were allowed 20 minutes rest when switching between SMT and OFT.
Prior to each trial, the arena was thoroughly wiped with a disinfectant solution (Deosan) and dried. To begin a 10 minute trial, the animal was placed in the center square of the arena, facing the lights. Animals were indirectly observed on a video monitor connected to an overhead camera, and all sessions were recorded on videotape for later analysis.

A square was considered entered when an animal crossed through with all 4 paws. An estimate of relative preference for the peripheral compared to central squares was obtained by taking a ratio (inner squares/outer squares). The ratio was adjusted to equalize the areas of each region. A similar procedure was carried out to determine side preference for the illuminated vs. the unilluminated side of the box. In addition, the number of rearing incidents were tabulated and the amount of time spent in grooming activity was recorded.

C7.2.2.3 Watermaze Test

The watermaze used for this experiment was constructed of galvanized steel, with a diameter of about 2 meters. A single 60 watt light bulb affixed to the edge of the tank provided the only illumination, and also served as one of the major extramaze cues. Other cues, which were always fixed, included the two experimenters, a coat rack, a blackboard, and the tripod for the video camera. The escape platform, constructed from transparent Plexiglas (25 cm in diameter, with a scored surface to allow for better traction), was submerged 2.5 cm below the surface of the water, which was maintained at 24-26° C. Subjects were moved to and from the tank with a pool net, and were towel dried after each trial. During the ITI (which was always a fixed 20 minutes), the animals were held in Nalgene cages under a heat lamp to prevent hypothermia.

One experimenter timed the latency to swim from the start position to the target platform, while the other sketched the swim path on a standardized diagram. All trials were video taped for possible later examination. After finding the platform, the rat was allowed to remain for 30 seconds. The maximum length of any trial was 90 seconds, and subjects which failed to find the platform within the allotted time were gently guided to it with the pool net. Three types of trial were administered, the mix of which varied based on the time point evaluated.
The first trial type was a probe trial or free swim, during which a subject was placed in the tank without a platform and allowed to swim for the maximum amount of time. In this case, the time spent in the different quadrants was recorded, as well as the number of times a subject crossed a target region within a quadrant (defined as the specific area where a hidden platform had been or would be located).

The second type of trial was a flagged or cued platform test, in which the escape platform was marked with a prominent cue (in this case, a plastic bottle). The location of the flagged platform varied from trial to trial between 3 separate locations, the order of which was counterbalanced. The rats always started from a fixed position within the tank, 1.2 meters from the target.

The final trial type was the hidden platform variant, in which subjects were forced to rely on extramaze cues to find the escape route. The submerged platform was located in a fixed position within one quadrant, and the animals were released from one of 4 counterbalanced start positions, spaced evenly along an arc 1.2 meters from the platform.

The baseline test procedure consisted of a free swim trial, followed by 3 flagged platform trials. Post-CHL testing was composed of 3 flagged platform trials, 8 hidden platform trials, and a free swim. Testing conducted at the post-KA time point was similar to post-CHL, excepting that, in the case of the hidden platform trials, the location of the platform and the start positions were reversed relative to the extramaze cues.

C7.2.3 Tissue Preparation and Histology

Animals were sacrificed one week post-KA and prepared for frozen sectioning at 30 μm (B3). Some sections were mounted for histological processing for PAS or cresyl violet. In the case of PAS staining, the dimedone modification (PAS-D; Bennett et al, 1995) was also used for some slide sets; this procedure both enhances contrast and increases the specificity of the stain for glycoproteins. Briefly, slide-mounted sections are rehydrated in a graded ethanol series and incubated in 0.5% periodic acid for 20 minutes, washed in running tap water, then dehydrated to absolute ethanol. Following this, slides are incubated in 5% dimedone (5,5-dimethyl-1,3-cyclohexanedi; Sigma) in absolute ethanol for 1 hour at 60° C. Subsequent to dimedone treatment, tissue
samples are rehydrated, incubated in Schiff's reagent for 20 minutes, washed in running tap water, dehydrated and coverslipped. For this experiment, the purpose of the PAS procedure was to assist in the location of damaged areas (Bennett *et al*, 1995).

Subjects were selected for an individual run of histology and processed for immunohistochemistry (B5) in sets comprised of the eight test squads. Immunohistochemistry was performed using the same antibodies to GFAP, Aβ, βAPP and serum proteins as in experiment 3.

**C7.2.4 Data Analysis**

For histology and immunohistochemistry, slides were examined and scored as per section experiment 5. Since completely counterbalanced slide sets were constructed, reactivity was also ranked on an eight point scale, similar to experiment 1.

Behavioral data were analyzed by regression ANOVA (SAS general linear model procedure), with multiple comparisons conducted using both Scheffe's test for all pairwise comparisons and Dunnett's procedure for multiple comparisons with a control. Flagged platform trials were collapsed into three scores (one per time point), and both sets of hidden platform trials were analyzed as blocks of 4. Swim distances were determined by collecting digital measurements of the swim paths using SigmaScan image analysis software (version 3.0; Jandel Scientific Software).

For the watermaze probe trials, additional nonparametric analyses were carried out (by ranking time spent in the target quadrant) using the Kruskal-Wallis ANOVA procedure. Further, a learning criterion was developed based on deviation of probe trial scores from the distribution of target platform dwell times (single subject *t* scores) during the free swim trial, when all animals were naive. By using the estimated population variance derived from the free swim trial, individual *t*-scores were determined for each animal based on deviations from this population; this allowed the determination, with 95% confidence, of which individual subjects had learned the platform location. By then tabulating the number of subjects that "learned" in each condition, a direct calculation of the group learning probabilities could be determined by using the binomial expansion formula.
C7.3 Results

C7.3.1 Seizure Development

Chloroquine pretreatment had a significant overall effect on the time to develop both the FS and SE [F(3,24)=4.86, p<0.009]; no individual comparison was significant with either Dunnett’s or Scheffe’s test. However, comparing the 45 mg/kg group with all others revealed significant acceleration in both time to FS [t(26)=3.2156, p<0.004] and SE [t(26)=3.0724, p<0.005]. A similar comparison between the 15 mg/kg group and all others indicated that a significant slowing in time to develop FS had occurred [t(26)=2.2715, p<0.04], although not in SE [t(26)=1.3391, p<0.1921], which may be due to the imposed ceiling time of 180 minutes post-KA (Fig. C7-1).

Two animals did not recover from the sodium pentobarbital injection (one 0 mg/kg CHL + KA and one 15 mg/kg CHL + PBS) and were excluded from any analysis of post-KA data. Also, one animal (15 mg/kg CHL + KA) became impossible to handle and was not used in any further behavioral tests.

C7.3.2 Sensorimotor Ability

There were no significant effects or interactions detected on the wire suspension test. The only significant effect detected for the inclined plane task was a steady improvement across trials [e.g., first fall angle, F(2,92)=8.59, p<0.0004; Fig. C7-2].

Figure C7-1. Effects of CHL on KA-induced seizure development. When contrasted with other treatment groups, the highest dose of CHL accelerates the development of both FS and SE. In a similar contrast, the 15 mg/kg dose significantly slows the development of the first seizure incident. *=p<0.05, **=p<0.005.
Figure C7-2. KA/CHL does not affect performance on either the wire suspension (A) or inclined plane (B) tests. Data shown are representative of all other variables analyzed for these tasks.

**C7.3.3 Exploratory Behavior**

Locomotor and exploratory behavior, as assessed by square entries and rearing incidents, respectively, did not change noticeably when animals were retested at the various time points. However, animals given kainate one week prior to the final open-field test were markedly hyperactive, regardless of CHL dose \([F(2,90)=14.08, p<0.0001; \text{Fig. C7-3A}]\). Although rearing behavior is often highly correlated with locomotor activity in the open-field, number of rearing incidents did not show a parallel increase in the KA treated animals \([F(2,90)=1.74, p<0.19; \text{Fig. C7-3B}]\).

All three anxiety related measures showed habituation effects with repeated testing. The amount of time spent in novelty-induced grooming behavior declined \([F(2,90)=5.92, p<0.004; \text{Fig. C7-3C}]\), and the relative preferences for both the lighted side of the arena \([F(2,88)=12.75, p<0.0001; \text{Fig. C7-3D}]\) and the central open area \([F(2,90)=17.94, p<0.0001; \text{not shown}]\) increased. Although there was a marginally significant reduction in KA treated animals' preference for the lighted side \([F(2,88)=3.32, p<0.05]\), a corresponding effect was not detected either in novelty-induced grooming behavior or in a relative reduction in thigmotaxic behavior.
Figure C7-3. Effects of KA/CHL on open-field variables such as number of squares entered (A), number of rearing incidents (B), novelty-induced grooming behavior (C) and relative preference for the lighted side of the box (D). Chloroquine alone did not have any effects on any of the variables measured in the open-field arena, nor did it interact with KA. However, KA did induce marked hyperactivity (p<0.0001; A) without affecting rearing, and may have had a small effect on side preference (P<0.05; D), although a corresponding effect was not detected on other anxiety related measures (compare C and D).
C7.3.4 Spatial Learning and Memory

Neither CHL nor KA affected performance on the flagged platform test. All test groups exhibited progressive improvement on ability to swim to the cued platform with repeated trials [F(2,90)=49.74, p<0.0001; Fig. C7-4].

Examination of performance on the first series of hidden platform trials (those administered post-CHL but prior to KA) did not reveal any effect of CHL. For this analysis, subjects were pooled across CHL dose for maximum power (n=16 per group). All groups demonstrated a large reduction in escape latency between the first and last four trials [F(1,60)=69.47, p<0.0001; Fig. C7-5A]. The lack of CHL effect can best be illustrated by viewing the percentage improvement of each group on this measure (Fig. C7-5B). All CHL treatment groups showed a large, dose-independent increase in time spent in the platform quadrant during the probe trial relative to free swim behavior [F(1,52)=121.99, p<0.0001; Fig. C7-5C], an effect also seen for the number of platform crossings [F(1,52)=158.62, p<0.0001; Fig. C7-5D].

Figure C7-4. CHL/KA treatment has no effect on flagged platform performance. No treatment combination had any influence on learning to swim to a visibly cued escape platform.
Figure C7-5. CHL had no effect on learning the location of a hidden platform. All treatment groups show large decreases in escape latency between the first and last four trials (A; p<0.0001), an effect which is dose-independent, as shown by the lack of between-group differences in percentage improvement (B). All groups were able to demonstrate equal knowledge of the platform location, as shown by the amount of time spent in the target quadrant (C) and number of times the platform area was crossed (D).
One week after KA treatment, the subjects were retested in the watermaze with the platform in a new location. Overall, there was a significant improvement in escape latencies between the first and last block of trials \( F(1,45)=51.96, p<0.0001 \). Also, there was a significant CHL*trial block interaction \( F(3,45)=3.16, p<0.035 \). Although no individual contrasts were informative, it appeared that the significant interaction term might be due to poorer improvements in the higher doses of CHL (30 and 45 mg/kg) combined with KA (Fig. C7-6A). Analysis of the percentage improvement scores for each group (Fig. C7-6B) revealed a significant effect of CHL treatment \( F(3,44)=7.98, p<0.0002 \), an effect due mainly to the 30 mg/kg group (Dunnett’s test). However, there were no significant effects of KA or a KA*CHL interaction. Further, analysis based on swim distance rather than escape latency did not change the results.

The results of the probe trial were similar in several respects. Time spent in the target quadrant showed a marginally significant KA*CHL interaction \( F(3,45)=2.39, p<0.082 \; \text{Fig. C7-6C} \), which in this case could be attributed to the relatively poor performance of the 30 mg/kg CHL + KA group. Comparison of platform crossings during the probe trial with chance crossings of the same area during the free swim revealed a significant increase \( F(1,45)=172.09, p<0.0001; \) Fig. C7-6D), as well as a significant KA*CHL*trial block interaction \( F(3,45)=3.33, p<0.03 \) and a marginally significant KA*CHL interaction \( F(3,45)=2.38, p<0.085 \). Again, this appeared to be due to smaller increases at the higher KA/CHL combinations. Examination of all possible pairwise comparisons with Scheffe’s test determined that the only significant group contrast was between the 30 mg/kg CHL group and its KA treated counterpart (for time spent in the target quadrant during the probe trial).

As an alternative approach, the data for the time spent in the target quadrant during the probe trial were rescoring as performance rankings split between PBS/CHL and KA/CHL conditions, giving two single factor (CHL dose) treatments. A Kruskal-Wallis one-way ANOVA indicated that there was no difference in rank-order clustering within the PBS/CHL treatment combinations \( \chi^2(3)=0.94, p<0.99 \). In contrast, subject scores did not distribute by chance within the KA/CHL combinations \( \chi^2(3)=8.08, p<0.05 \). The number of animals that “learned” the platform location were determined and tabulated, by treatment combination, and the exact probability of finding this number
Figure C7-6 (Starting on Previous Page). Effects of KA/CHL on post-KA watermaze performance. (A) KA combined with CHL results in an attenuated improvement in reaching the escape platform; note how the higher CHL treatment groups with KA (arrows) do not improve as much as all other drug combinations. (B) A similar effect can be seen when the escape latency data are expressed as a percentage improvement score; the 30 mg/kg CHL dose group does poorly in general (*=p<0.05, Dunnett’s test), although the treatment combinations of both 30 and 45 mg/kg CHL + KA appears to result in greater impairment. (C) Examination of the time spent in the target quadrant during the probe trial shows that the 30 mg/kg CHL + KA group did not learn the location of the platform as well as the other groups (*=p<0.05, Scheffe’s test). (D) Analysis of the number of times the exact platform area was crossed during the free swim and the probe trial revealed that there was a significant KA*CHL interaction (p<0.03). Although individual group contrasts were not informative in this case, it appears that animals receiving higher doses of CHL (and KA, for 30 mg/kg), do not improve as much as other subjects (percentage improvement scores are noted directly above each group; note close parallel with C7-6C).
Table C7-2. Nonparametric Analysis of Target Quadrant Dwell Times

<table>
<thead>
<tr>
<th>CHL (mg/kg):</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>r</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>7</td>
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<tr>
<td></td>
<td>73.5</td>
<td>77</td>
<td>110.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;0.004</td>
<td>p&lt;0.003</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KA</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>92.5</td>
<td>103.5</td>
<td>109</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-Wallis Group Rank Score; probability is for at least r successful out of n subjects (for the binomial expansion formula).

of “successful” subjects was calculated using binomial expansion (see methods). As expected from figure C7-6C, the only group of animals that failed to learn the location of the platform was the 30 mg/kg CHL combined with KA (table C7-2).

C7.3.5 Histology

The PAS procedure most commonly detected the presence of positive staining material in the posteriomedial cortical nucleus of the amygdala (PMCo) in animals treated with KA and CHL; the staining was rare (1/6, and faint) in animals given KA alone. A small number of animals (2/7) at 15 mg/kg CHL+KA showed this effect, compared to 10/14 of the 30 and 45 mg/kg (both with KA) groups. Staining was more extensive in 30 mg/kg treated animals, sometimes extending into the amygdala/piriform (APir) and posterior amygdala/hippocampal (AHiP) transition areas (Fig. C7-7). Some

Figure C7-7 (Following 2 Pages). Relationship between KA/CHL and PAS staining in the amygdala/piriform region. First page: low light, high contrast image of PAS-D staining (original magnification: 5x, scale bar: 850 µm), with KA/CHL dose combinations annotated directly on the figure (important regions are marked on G). Notice that in this representative set of animals that only the two highest doses of CHL in combination with KA (F and H) produce an effect. Second page: different set of animals, this time shown with the PAS procedure minus the dimedone modification (original magnification: 5x, scale bar: 850 µm). In both sets, the PAS staining in the 30 mg/kg CHL + KA animal is much more extensive than the animal treated with the higher dose, in which the staining is restricted to the PMCo.
KA-treated animals also showed isolated PAS+ cells within the CA3 pyramidal cell layer of the ventral hippocampus and the piriform and entorhinal cortices. The dorsal hippocampus was affected in 1 case (15 mg/kg CHL+KA), and other areas of the amygdala were not affected (see table C7-3). Although most of these animals were given 30 mg/kg CHL + KA, the staining was not extensive. Animals not given KA, regardless of CHL dose, did not exhibit noticeable PAS+ staining.

Careful study of adjacent sections stained with PAS or cresyl violet indicated that the intense PAS+ areas were associated with cell loss regardless of CHL dose (Fig. C7-8). The PMCo was the most commonly affected area, followed by the AHiP (usually only in the 30 mg/kg CHL+KA group). As expected from the PAS staining, animals pretreated with 30 mg/kg CHL showed more extensive cell loss in this area (Fig. C7-9) and not in areas lacking PAS+ material (dorsal hippocampus, other amygdaloid nuclei; Fig. C7-10).

**Table C7-3. Areas Affected by KA (by CHL Dose)**

<table>
<thead>
<tr>
<th>CHL Dose(+KA)</th>
<th>PMCo¹</th>
<th>Transition Areas²</th>
<th>Pir/Ent/Hip/Endo³</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>45</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Numbers shown are the number of subjects showing PAS positive staining in this area out of a total of 7 (6 in the case of the vehicle treated group).

¹Posteriomedial cortical nucleus of the amygdala
²the amygdala/hippocampal transition area (AHi) and/or the amygdala/piriform transition area (APir)
³Piriform (Pir) and entorhinal (Ent) cortices, hippocampus (Hip; CA3 only) and the dorsal endopiriform nucleus (Endo)
Figure C7-8. Relationship between PAS staining and cell loss in the posteriomedial cortical nucleus of the amygdala (PMCo). Shown are adjacent PAS (A, B) and cresyl violet (C-F) stained sections (M&P from Fig. C7-7, closely matched for section angle and location; original magnification: A-D, 5x, scale bar: 850 μm) from an animal with a normal appearing PMCo area (left panels) and from a subject with PMCo degeneration (right panels). Note the correspondence between the PAS+ area outlined in (B) and the largely destroyed PMCo in (D), and compare with similar areas in (A) and (C). Photomicrographs (E) and (F) are higher magnification (20x, scale bar: 200 μm) of the boxed areas in (C) and (D), respectively. The more densely packed neurons of the AHiP transition area are visible along the top part of the image, whereas the PMCo is visible in the lower portion. In this case, most of the large neurons in the PMCo have been lost, and the area has been infiltrated with large numbers of cells of microglial morphology.
Figure C7-9. Relationship between CHL/KA and cell loss in the amygdala (AHiP at top of photomicrograph, PMCo at bottom; original magnification: 20x, scale bar: 200 μm). The PMCo is destroyed in both F and H, but note the severe damage to the adjacent transition area in F, compared to the relative sparing of this area in H.

Figure C7-10 (Following page). Lack of significant damage to other areas of the amygdala and dorsal hippocampus (shown: cresyl violet and PAS [alternating], animals from C7-9F [left panels] and G [right panels]; G has severe damage to the PMCo and AHiP; original magnification: 5x, scale bar: 800 μm).
C7.3.6 Immunohistochemistry

The 2 animals examined at each dose following one week of CHL treatment (sacrificed prior to KA, between CHL doses 6 and 7; 24 hrs. after final dose) showed increased intracellular Aβ-IR (30 mg/kg+) and slightly elevated GFAP-IR (one of the 45 mg/kg animals). There was no indication of the redistributed Aβ/βAPP-IR found in the 45 mg/kg CHL treated animals in experiment 5, nor was any increase in IgG-IR found (not shown). Since these animals were sacrificed sooner than the animals in experiment 5, this may indicate that these effects dissipate in under 24 hours.

Several of the effects of CHL (as found in experiment 5) were absent when examined at one week post-KA (this experiment). Although KA-treated subjects frequently exhibited numerous hypertrophied astrocytes in seizure prone areas, there was no detectable influence of CHL at this time point (Fig. C7-11). Also, there were virtually no extravasated IgG or serum protein deposits in any of the animals examined at any drug combination, although areas such as the median eminence and subfornical organ still showed dark immunostaining (not shown). Finally, none of the 14 animals examined that were treated with 45 mg/kg CHL showed any signs of the redistributed Aβ/βAPP-IR, although 5/14 showed a very small residual increase in Aβ/fβAPP-IR (compared to 1/14 at each of 15 and 30 mg/kg, and none of the controls; Fig. C7-12). Thus, the effects of chronic CHL treatment are largely reversible, with most effects eliminated 1 week after termination of drug administration.

In regions where neurodegeneration had occurred, such as the PMCo and AHIP, reactive, βAPP+ astrocytes colocalized with areas of PAS reactivity (Fig. C7-13). Some PAS+ cells in damaged areas appeared to be astrocytes, based on their process morphology as seen with this stain.

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Figure C7-11 (Following Page). Effects of KA/CHL on astrocytes one week post-KA (shown: perirhinal/piriform cortex border in one set of animals, with drug combinations annotated directly on the figure; cresyl violet counterstain, original magnification: 10x, scale bar: 400 μm). No consistent effect of CHL could be detected across all sets (alone or in combination with KA) although KA treatment usually caused increased GFAP-IR in brain areas known to be sensitive to KA-induced seizures.
Figure C7-12. Increased Aβ-IR at the highest CHL dose (shown: anterior hippocampus, lαAβ, original magnification: 5x, scale bar: 800 μm). Five of 14 animals at 45 mg/kg CHL showed a small, residual increase in cell associated Aβ-IR at one week post-KA (i.e., one week after the end of CHL treatment), compared to only 1/14 at each of 15 and 30 mg/kg, and none at 0 mg/kg. Similar effects were seen with other Aβ antibodies.

C7.4 Discussion

Systemic administration of CHL, followed by an excitotoxic challenge with KA, results in a potentiation of neurodegeneration (similar to that seen in experiment 3), accompanied by a corresponding deficit in spatial learning and memory. Importantly, the dose at which these results are primarily found (30 mg/kg) is the dose that has been shown to cause the accumulation of Aβ/βAPP-IR in the absence of other confounding effects of CHL (experiment 5)(see table C7-4 for a summary of results; c.f. table C5-3). Hence, cell associated Aβ (in this case induced by the lysosomal inhibitor CHL) is clearly associated with an increased susceptibility to excitotoxic damage, and may therefore be an important contributor to AD pathogenesis.
Figure C7-13. βAPP positive astrocytes are found in areas of cell loss, such as in the vicinity of the posteriomedial cortical nucleus of the amygdala (shown: medial PMCo border, original magnification: 20x, scale bar: 200 μm). Left side panels: PBS injected, right side panels: KA injected (A,B: PAS, C,D: GFAP-IR, E,F: R1αβAPP). The diffuse PAS+ substance was not associated with any dose of CHL, only with the occurrence of degeneration (compare A and B; c.f. Fig. C7-8). Numerous reactive astrocytes are present in the region (D), which are weakly βAPP+ (boxed area in F; labeling also seen with Aβ antibodies). The βAPP+ astrocytes were frequently associated with peripheral areas of the lesion (as shown here) rather than within the damaged area, which usually contained numerous microglia-like cells (c.f. Fig. C7-8F).
Table C7-4. Summary of CHL/KA Interactions After 1 Week Survival

<table>
<thead>
<tr>
<th>Chloroquine Dose*</th>
<th>Vehicle PBS</th>
<th>15 mg/kg PBS</th>
<th>30 mg/kg PBS</th>
<th>45 mg/kg PBS</th>
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<tbody>
<tr>
<td>Effect</td>
<td>KA</td>
<td>KA</td>
<td>KA</td>
<td>KA</td>
</tr>
<tr>
<td>IgG-IR</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GFAP(^1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iAβ/iAPP-IR(^2)</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAS/Cell Loss(^3)</td>
<td></td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Watermaze(^4)</td>
<td></td>
<td>(↓)</td>
<td>↓</td>
<td>(↓)</td>
</tr>
<tr>
<td>SMT</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>OFT(^5)</td>
<td></td>
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</tr>
<tr>
<td>Seizure Time</td>
<td>Normal</td>
<td>Slower</td>
<td>Normal</td>
<td>Faster</td>
</tr>
</tbody>
</table>

*Combinations that result in no change from control values are left blank

\(^1\)Hypertrophied, βAPP+ astrocytes are found closely associated with areas of cell loss

\(^2\)The increase is fairly weak, and can be detected in less than half of the subjects at this dose

\(^3\)As in experiment 3, the PAS staining (with and without dimerone) marks damaged areas

\(^4\)The 30 mg/kg CHL+PBS and 45 mg/kg CHL+KA subjects showed a slight impairment during acquisition of the hidden platform task, but minimal deficits (if any) were detected on the cued platform task; although the latter group commonly showed damage to the PMCo, no damage of any kind was detected in the former

\(^5\)Increased locomotor activity only; no other variables were significant
Cell loss was most consistently detected in animals given 30 or 45 mg/kg CHL plus KA (rats given PBS did not show any signs of pathology at any CHL dose). The damage (as seen by both PAS and cresyl violet stains), was focused within the amygdaloid nucleus (usually the PMCo, although AHbP and APir were also affected in more severe cases). The lack of more extensive damage, particularly within the hippocampal formation (although 4 of 7 of the 30 mg/kg CHL + KA group showed some sporadic PAS+ staining in the ventral hippocampus, piriform/entorhinal cortices and/or the dorsal endopiriform nucleus), was probably due to the intentionally mild SE induced in this experiment (in order to avoid ceiling effects).

Seizure activity induced by KA is generally thought to begin within the hippocampal formation (Lothman and Collins, 1981), followed by propagation to other sites (Handforth and Ackermann, 1995), and it is a well established fact that seizure activity is required for damage to occur (Nadler et al, 1986). The amygdaloid complex is endowed with heavy reciprocal connections with the piriform and entorhinal cortices, hippocampus, septum, hypothalamus, and brain stem, as well as highly complex internal connections between different subnuclei (de Olmos et al, 1985), so damage via propagating seizure activity is a definite possibility. The major areas of damage with this treatment paradigm (the PMCo, and AHbP and APir to a lesser degree) have all been shown to be severely damaged by systemic KA administration (O'Shaugnessy and Gerber, 1986; Gayoso et al, 1994). However, the specificity seen in this experiment has never been reported, and neuropathology following systemic KA administration has only been studied in detail for relatively long periods of SE in which damage to all limbic areas is extensive.

It is unlikely that the 2 other main effects of CHL, as determined in experiment 5, are responsible for the neurodegeneration found in this experiment. First, if CHL is indeed causing BBB damage, as suggested by the presence of serum protein immunoreactivity in the brain, this is found at 15 mg/kg CHL, a dose where damage (i.e., cell loss) was uncommon in this experiment and at which no watermaze deficit was found. Second, the higher dose of CHL, which leads to the faster onset of KA-induced seizures, shows less damage (and less of a watermaze deficit) than the lower dose of
30 mg/kg. Thus, it also seems unlikely that the results of this experiment are related to seizure severity, which may be related to seizure development latency.

The apparent partial reversal of the effects of CHL at the dose of 45 mg/kg CHL was an unusual finding. Some animals in experiment 5 were found to exhibit a redistribution of Aβ/βAPP-IR away from the neuronal cell body and into the surrounding neuropil. Two possibilities emerge from this. The first possibility is that the loss of Aβ from the actual neuronal cell body is responsible for the reduction in damage, which is directly in line with the main hypothesis of this thesis (that intracellular Aβ is related to the neurodegenerative process). Second, it has been shown that βAPP is capable of attenuating the effects of Aβ on excitotoxicity (Goodman and Mattson, 1994) and reducing excitotoxic damage in general (Masliah et al, 1997), influences probably mediated by an ability to stabilize [Ca^{2+}]; (Mattson and Barger, 1993; Furukawa et al, 1996). It’s possible that βAPP (a component of the redistributed neuropil immunostaining previously observed at 45 mg/kg CHL) may be responsible for this treatment group being less affected than the next lower dose.

It is believed that damage to the hippocampal formation is responsible for deficits in watermaze performance following systemic KA (Gayoso et al, 1994). Although most of the damage found in this study appears to be centered on the PMCo, a marked deficit in both acquisition and retention is observed only following KA-induced damage to the adjacent amygdala/hippocampal and amygdala/piriform transition areas, and possibly additional sporadic damage to other areas of the hippocampal formation (at 30 mg/kg CHL + KA). Although there are no published reports dealing with a role of the PMCo in spatial learning and memory, it is possible that damage mainly to the PMCo (as seen with 45 mg/kg CHL + KA) causes a mild deficit in acquisition of the watermaze task. However, this appears unlikely given that a remarkably similar deficit is seen in the animals given 30 mg/kg CHL without KA - a group which showed no signs of any neuropathology in this experiment. It is also clear that damage to the PMCo does not result in any changes in anxiety or emotion related behavior assessed in this experiment. Such changes are seen following amygdala kindling (Niemenen et al, 1992; Cammisuli et al, 1997) or lesions (Morris et al, 1996; Scott et al, 1997), so this portion of
the amygdala may also not be directly involved in the processing of responses connected to fear, anxiety and emotionality in the rat.

The WM impairment caused by interactions between higher doses of CHL and KA cannot be explained by any factor other than a deficit in spatial learning and memory caused by corresponding neurodegenerative changes. Chloroquine alone did not have any effect on the ability to learn the location of the hidden platform, as seen in both rates of platform acquisition and during the probe trial administered post-training. This indicates that iAβ, which is mainly evident at a dose of 30 mg/kg CHL after 1 week of drug treatment, does not significantly perturb performance on this task and it is the actual loss of neurons that is responsible. Further, the behavioral control procedures did not indicate that the deficit found in the watermaze was likely to be related to other influences of either KA or CHL.

The sensorimotor tests did not yield any evidence consistent with CHL-induced myopathy or other sensorimotor dysfunction, such as balance (Jaeger et al, 1987; White, 1988). Both the WS (Miquel and Blasco, 1978; Jänicke et al, 1983) and IP tests (Murphy et al, 1995) have been shown to be accurate indicators of sensorimotor dysfunction, with the latter test being extremely sensitive (Murphy et al, 1995).

Since CHL can produce a variety of diffuse neuropsychiatric symptoms, such as paroxysms of psychomotor activity (Garg et al, 1990), drowsiness and depression (Jaeger et al, 1987), and altered blood pressure (Looareesuwan et al, 1986), several anxiety related variables were examined to exclude the influence of these factors on WM performance (in which the motivation to escape is, at least in part, driven by fear). Three measures were chosen (each based on a different anxiety evoking stimulus), since individual variables of this nature have a variety of problems when assessed in the OFT. However, neither side preference (designed to assess the animals' fear of lighted areas; Crawley and Goodwin, 1980; Costall et al, 1989), grooming behavior (a displacement response to a variety of mild stressors, such as novelty; McFarland, 1966; Gispen and Isaacson, 1981), nor proportion of square entries within the open region (fear of open spaces, analogous to time spent on the open arms of the elevated plus maze; Lister, 1990) showed a consistent influence of either KA or CHL. Each of these variables are known to be affected by anxiolytic (e.g., benzodiazepines) and anxiogenic
(e.g., FG7142) compounds in ways consistent with decreased and increased anxiety, respectively (Dunn et al., 1988; File et al., 1988; Moody et al., 1988; Costall et al., 1988; Lister, 1990). Further, habituation effects were detected for each of these measures (increased preference for the lighted side, increased central square entries, and decreased grooming) with repeated testing, consistent with the reduced anxiety expected to accompany repeated exposure to the test environment (Gispen and Isaacson, 1981; Lister, 1990). Thus, an altered anxiety profile is unlikely to be a contributing factor to the watermaze results. Changes in locomotor activity are not usually reported following damage to the amygdala (White and McDonald, 1993; Decker et al., 1995), although both decreases (Nieminen et al., 1992) and increases (Cammisuli et al., 1997) have been reported following kindling-induced seizures in the amygdala, an effect which is likely transient. Since damage to the amygdala is not clearly associated with increased locomotor activity in this experiment, it is therefore likely that the increased open field activity seen at all doses of KA is a by product of seizure activity, either in this structure or elsewhere in the brain.

All groups were able to learn the cued platform task without any difficulty. This task involves learning the association between a readily visible cue and a platform which is not located in a fixed position. Hence, neither drug caused an impairment in the rats' ability to see cues and perform this simpler associative task. The visual aspect of this task is an important consideration, since CHL is capable of causing retinal toxicity (White, 1988). Furthermore, recent evaluation of a small number of animals treated with the highest dose of CHL or vehicle (for one week) in a visual discrimination T-maze did not reveal any differences between the two groups (unpublished observations). Also, unaltered performance of the cued platform task rules out reductions in motivation to escape from the water, since such an impairment would also likely be observed on this task as well as the hidden platform. Finally, this test also demonstrates the lack of a generalized deficit in swimming ability, since this would affect performance on this task as well as the hidden platform variant.
SECTION D:

GENERAL DISCUSSION AND PERSPECTIVE
D1 Summary and General Conclusions

It is well accepted that risk and incidence rates of AD increase substantially with age (Breteler et al, 1992). Since lysosomal dysfunction (Lavie et al, 1982; Isigami and Goto, 1988, 1991) and lipofuscin accumulation (Moore et al, 1995; Moore and Ivy, 1995) also parallel the aging process, and given the substantial evidence for the involvement of the former in processing, deposition and/or turnover of the β-amyloid peptide (sections A3.2 and A4.1), it was hypothesized that Alzheimer's disease may be connected to lysosomal dysfunction leading to the appearance of iAβ and additional downstream pathology (the direct or causal hypothesis). Since treatment with lysosomal inhibitors mimics the age-related decline in lysosomal function (Ivy et al, 1984; Ivy and Gurd, 1988; Ivy et al, 1990a, b; Ivy, 1992a), experiments were conducted using this paradigm (experiments 1 and 2) to investigate the possibility of a causal link between a faulty lysosomal apparatus and AD pathogenesis.

Although subject populations (transgenic mice and non-human primates) were used that were considered to have the potential to develop elements of AD pathology (e.g., neuronal loss and possibly SP), induced lysosomal dysfunction was insufficient to cause any manifestation as such. The main effect of lysosomal inhibition was to cause the intracellular accumulation of Aβ/fβAPP. A similar effect is known to occur following in vitro treatment with the same compounds used in experiments 1 and 2. Therefore, the simple hypothesis that iAβ leads to the development of AD appears unlikely, although the possibility cannot be ruled out that different model systems, inhibitors, or treatment parameters may yield different results.

Even if iAβ is not a causative factor in AD pathogenesis, it may still have an indirect or permissive role. That is, it may represent an age-associated risk factor that creates a predisposition to a second, triggering event. It is well known that Aβ increases neuronal vulnerability to excitotoxic injury (Koh et al, 1990; Mattson et al, 1992; Patel, 1995), that vulnerability to excitotoxicity increases with age (Auer, 1991), and that a relationship exists between patterns of excitotoxic damage and AD (Greenamyre and Young, 1989; Francis et al, 1993). Excitotoxic processes are thought to occur in epilepsy, ischemia and following traumatic brain injury (Erecinska and Silver, 1996), and
at least the latter has been convincingly linked to AD (Mortimer et al, 1985, 1991). Therefore, additional studies were undertaken (the remainder of section C) to evaluate the possibility that iAβ (induced as a consequence of lysosomal inhibitor treatment) imparts a greater risk for excitotoxic damage.

Even though CHL infusion combined with excitotoxin (KA) treatment resulted in a potentiation of neurodegeneration in the population of neurons showing increased Aβ/fβAPP-IR (hippocampal cell field CA3/4), these neurons are also known to be exceptionally vulnerable to KA. This made a clear interpretation of the results of experiment 3 difficult, since the observed cell loss could have simply been due to more KA accessing the CNS via an unusually permeable BBB, an alternative hypothesis supported by the finding that subjects infused with CHL showed both increased serum protein/IgG-IR in the brain and heightened sensitivity to KA-induced seizures.

Chloroquine, however, can be given systemically. This route of administration was attempted with two main goals: (1) to determine if systemically applied CHL would cause the accumulation of Aβ/fβAPP within the brain, and (2) avoid the confounding influence of possible BBB damage. These goals were attained, first with mice (experiment 4), and then rats (experiment 5). Due to a variety of complex effects of CHL (experiments 5 and 6), a simple experiment with a single dose of CHL combined with KA (similar to experiment 3) was not a feasible means of evaluating the permissive factor hypothesis. Therefore, the interaction of KA with the dose response function of CHL was examined instead, with emphasis placed on the CHL dose of 30 mg/kg (the dose where maximum iAβ-IR was seen largely in absence of other effects of the drug).

The key dose of systemic CHL (30 mg/kg) rendered neurons (primarily in parts of the amygdala, although other areas were mildly affected) vulnerable to a subsequent challenge with KA and caused deficits in spatial learning and memory (experiment 7), effects which were independent of CHL's other influences. The increased neuronal loss associated with elevated cell associated Aβ/fβAPP-IR was similar to that found earlier (experiment 3), with the exception of the localization of the degeneration. Even though the results of experiment 3 were confounded by the possibility of a large BBB disruption caused by CHL infusion, the results of experiment 7 indicate that at least some of the
degeneration is likely related to CHL-induced iAβ-IR rather than solely due to more KA reaching the brain.

The implication of these general findings is important to our understanding of AD pathogenesis. Under no circumstances during the course of this work did iAβ-IR lead to the appearance of any neuropathology. The presence of iAβ did not in itself lead to the manifestation of impairments on any of the behavioral tasks examined, indicating that, at least under these conditions, the presence of iAβ likely also had limited physiological consequences. However, these data do indicate that a iAβ may combine with a precipitating traumatic event to cause cell death. In the animal system used in these experiments, no SP pathology was ever observed. This was not totally unexpected, since SP pathology has never been found in a normal rodent under any experimental or natural conditions. In AD, a similar neurodegenerative pathway may result in the extracellular deposition of Aβ as a remnant of deceased cells (as suggested by others; Yang et al, 1995; Burdick et al, 1997; Wild-Bode et al, 1997), which (for whatever reason) does not occur in the rodent. This deposition could then lead to further destructive processes that have been attributed to extracellular Aβ (such as the chronic activation of inflammatory pathways, for example), further accentuating neuronal loss and leading to the profound cognitive impairments that are characteristic of the disorder.

Although rats do not develop extracellular deposits of Aβ, additional clues relating to the significance of these findings lie embedded within the characteristics of neurodegeneration found in these experiments, including the PAS+ material found within areas of cell loss, the localization of damage to the amygdala, and the possible effects of CHL on the BBB.
D2 Characteristics of Neurodegeneration

D2.1 Periodic Acid-Schiff Positive Staining

One of the more intriguing aspects of this work is the consistent appearance of a largely extracellular PAS+ material is areas of neurodegeneration. This pattern of reactivity has been frequently reported as a correlate of excitotoxic lesions, and most likely represents glycoprotein and/or proteoglycan staining, particularly since PAS+ material cannot be removed by pretreatment with lipases or amylases (e.g., Ivy et al., 1984; Bennett et al., 1995), and has been shown biochemically to be largely proteinaceous (Ivy and Gurd, 1988). However, although thought to represent changes in the ECM, the identity of this substance is at present a mystery. There are two interesting possibilities that relate to AD pathogenesis: the PAS+ material may actually be a secreted proteoglycan form of βAPP associated with the ECM, or may be the ECM protein HSPG.

One of the leading theories regarding the function of βAPP is its purported role in cell contact and adhesion (section A3.2.1). For instance, βAPP readily associates with the ECM (Small et al., 1992), possibly via high affinity interactions with HSPG (Narindrasorasak et al., 1991) or other major ECM components (Narindrasorasak et al., 1992, 1995; Allsop and Williams, 1994). More importantly, the astrocytic form of βAPP (mostly of the KPI containing variety) expressed at the cell surface has been shown to exist largely as a proteoglycan (Appican; βAPP + at least 2 chondroitan sulfate glycosaminoglycan [GAG] sidechains; Shioi et al., 1993, 1995). Also, Appican is secreted in significant quantities, at least in vitro (Shioi et al., 1992). Hence, given the association of reactive βAPP+ astrocytes with damaged regions (Siman et al., 1989; Töpper et al., 1995; also shown in experiments 3 and 7), and since βAPP produced by reactive astrocytes is largely the KPI isoform (Kawarabayashi et al., 1991; Willoughby et al., 1992; Solà et al., 1993), the PAS+ substance may represent Appican produced as part of the glial scar formation process.

Although this speaks to the issue of the functional role of βAPP, it is not entirely clear what this means for the development of AD pathology. Although reactive astrocytes surround the periphery of the SP, they are not usually thought of as being the
source of Aβ (Itagaki et al., 1989; section A2.2.2). Also, it should again be noted that very few actual cells were stained with PAS/PAS-D in the lesion area, although some did appear to be astrocytes (experiment 7). This may be due to the mainly secretory nature of Appican, explaining the preponderance of diffuse extracellular staining. However, the large areas of diffuse PAS reactivity were never observed as being immunostained with βAPP antibodies. Although this could be due to reduced antibody binding caused by the large GAG sidechains, three factors argue against this interpretation. First, none of the six βAPP antibodies, directed against diverse regions of the protein, labeled the PAS+ substance. Similarly, no immunoreactivity was observed with any of the Aβ antibodies, some of which likely crossreact with secreted βAPP. Second, diffuse βAPP/Aβ-IR has never been reported in rat KA-induced lesions, in spite of the utilization of numerous different βAPP and Aβ antibodies (e.g., Siman et al., 1989). Last, these results were obtained while both Aβ and βAPP antibodies successfully labeled reactive astrocytes in the vicinity of the lesion (Siman et al., 1989; Töpper et al., 1995; this work). Given these staining dissociations, it seems unlikely that the PAS+ substance is βAPP.

Heparan sulfate proteoglycan is a major component of the extracellular matrix and vascular basement membrane involved in cell adhesion and neurite extension (Snow and Wight, 1989). It has been shown to be an early component of SP in both AD (Snow et al., 1988, 1990) and DS (Crutcher et al., 1993), and is also associated with AD-related microangiopathy and vascular amyloid deposits (Perlmutter et al., 1990). This relationship extends to other pathologies involving amyloidoses other than AD, both within the CNS (Snow and Wight, 1989) and in other tissues (Young et al., 1989). In fact, HSPG and the amyloid peptide appear to be deposited almost simultaneously (Young et al., 1989), and an upregulation of HSPG mRNA transcription occurs prior to the formation of the deposit in experimental models of amyloidoses (Ailles et al., 1993).

Extracellular Aβ is tightly associated with the ECM (Buée et al., 1993), and ultrastructural studies using cationic dyes (cuprolinic blue and ruthenium red) have demonstrated that this is likely mediated by interactions with GAGs such as heparan sulfate (Young et al., 1989). Importantly, HSPG has been shown to accelerate SP
formation when co-infused with Aβ (Snow and Malouf, 1993), and heparan sulfate glycosaminoglycan is able to induce formation of both SSF and PHF in vitro (Goedert et al, 1996). Although both HSPG and heparan sulfate have been found in NFT and SP, the GAG component may actually be a more critical factor for the development of these lesions (Su et al, 1992; Goedert et al, 1996).

Although a suitable antibody was unavailable during the course of this research to assess this possibility (MAB1948, the only commercially available HSPG antibody that might have been able to detect rat HSPG, was unreactive in preliminary trials), this remains an interesting hypothesis. For instance, the characteristics of the amygdala lesion induced by the combination of KA and CHL in experiment 7 are very consistent: a central, PAS+ lesion area containing significant microglial infiltration (as seen with cresyl violet and known to occur with KA-induced damage; Akiyama et al, 1994), encircled by reactive βAPP+ astrocytes. This bears a remarkable similarity to the spatial structure of a senile plaque, where a core region of amyloid (which can be also be stained with a modification of the PAS procedure; Glenner et al, 1981) is engulfed by reactive microglia and surrounded by a ring of peripheral, reactive astrocytes (Dickson et al, 1988; Itagaki et al, 1989).

Since normal rodents, for whatever reason, do not develop SP (Shivers et al, 1988; experiment 1), the fact that the PAS+ area does not contain Aβ is not critical in this case. The reactive microglia within the lesion would be expected to be utilizing large amounts of ApoE to scavenge lipids and cholesterol from the damaged neurons (Mahley, 1988; Poirier, 1994; Suzuki et al, 1997). Also, the inflammatory cascade and reactive astrocytosis associated with the lesion could result in the presence of other early SP elements, such as αACT (McGeer and McGeer, 1995). Therefore, if the PAS+ substance is HSPG, then other [non-Aβ] elements believed to be involved in early SP formation are also likely to be present. Thus, an experimental strategy similar to that used here could precipitate SP formation in a more suitable model system, such as primates (Wisniewski et al, 1973), dogs (Cummings et al, 1996a), cats (Cummings et al, 1996b; Nakamura et al, 1996) or transgenic mice capable of developing extracellular Aβ deposits (Games et al, 1995; Hsiao et al, 1996).
D2.2 Localization to the Amygdala

It was a rather surprising finding that the area most sensitive to degeneration was a collection of amygdaloid subnuclei. Systemic KA-induced seizures often cause severe degeneration in this part of the amygdala (O'Shaughnessy and Gerber, 1986; Gayoso et al., 1994), but this is thought to be a region of secondary damage. In this case, it appeared to be the primary target of the degenerative processes triggered by combining lysosomal inhibition and excitotoxicity. The amygdala, however, may have a significant role in the development of AD.

The amygdala (particularly the cortical nuclei) exhibits an extraordinarily high density of SP in AD (Arriagada et al., 1992), and may actually possess the highest plaque count of any brain area (Kemper, 1984; Brady and Mufson, 1990). In DS patients, the amygdala also shows the earliest signs of amyloid deposition (Mann et al., 1989). Similarly, in aged individuals, the amygdala is also one of the first regions to develop plaques, earlier than the entorhinal cortex, and much earlier than the hippocampus (Braak and Braak, 1991, 1993). Unlike other areas of the brain, neurons within the amygdala also collect both NFT and Lewy bodies (Schmidt et al., 1996b), a finding which could be related to the massive cell loss found in this region (Vereecken et al., 1994). Studies using brain imaging techniques have also shown that a sizable reduction in amygdala volume is one of the earliest changes in the AD brain (Cuénod et al., 1993; Maunoury et al., 1996), an effect augmented by the presence of the ApoE4-ε4 polymorphism (Lehtovirta et al., 1995). Consistent with an early involvement of the amygdala, initial clinical indicators of AD often have an emotional component, such as anxiety, depression and paranoia, that precede the symptoms of spatial disorientation and profound memory loss that likely signal entorhinal/hippocampal dysfunction (Gustafson et al., 1995). It is interesting that the earliest signs of NFT pathology appear in the transentorhinal region, while the amygdala (again, cortical and amygdalocortical transition areas) is severely affected later in the disease process (Hyman et al., 1990; Braak and Braak, 1991). This may explain why no abnormal τ-IR was detected in preliminary studies, even though changes suggestive of abnormal τ phosphorylation have been found in rats subsequent to glutamate infusion (Irving et al., 1996).
Alzheimer's disease is often thought of as being the end result of an Aβ-induced cascade (Hardy and Higgins, 1992; Selkoe, 1997; Hardy, 1997). Therefore, the finding that a portion of the amygdaloid nucleus is the most vulnerable area in this paradigm is not inconsistent with what is known about the role of this region in the early stages of AD. In fact, one of the most dysfunctional amygdaloid subnuclei is the ventromedial cortical nucleus (or VMCo; Brady and Mufson, 1990), which may be analogous to the PMCo in the rat. It may therefore be that, in AD, the cascade of Aβ accumulation, neuronal death and SP formation originates in the amygdala and then spreads to other areas, a hypothesis supported by the findings of this thesis.

D2.3 A Dementia Continuum?

Thus, the characteristics and locations of the lesions observed during the course of these experiments (especially experiment 7) could be considered to be an example of what occurs in the initial stages of AD. Obviously, this hypothesis needs examination in an animal model that is capable of developing SP pathology. However, are any other effects of CHL significant?

A general age-related decline in protein turnover capacity is well known (Lavie et al, 1982; Ishigami and Goto, 1988, 1990). Further, this decline is associated with a relatively steady increase in lipofuscin accumulation in post-mitotic cells (Moore et al, 1995; Moore and Ivy, 1995) and can be modeled by the administration of lysosomal inhibitors (Ivy et al, 1984; Ivy and Gurd, 1988; Ivy et al, 1990a, b; Ivy, 1992a). Patients suffering from NCL disease have a dysfunctional lysosomal system (Palmer et al, 1986; Vesa et al, 1995) and typically show a large increase in ceroid lipofuscin containing Aβ/fβAPP (Wisniewski and Maslinska, 1989; Wisniewski et al, 1990a, b). In adult cases, a small increase in AD-like pathology is also observed (Wisniewski et al, 1990b; Love et al, 1988). Conversely, there is also some evidence indicative of an increased rate of lipofuscin formation in AD (Dowson et al, 1992), especially in populations of neurons considered to be “at risk” for eventual degeneration (Cataldo et al, 1991; Nixon et al, 1992; Cataldo et al, 1994).
Alzheimer's disease is overwhelmingly associated with the aging process, with risk and incidence rates increasing in parallel (Breteler et al., 1992). Although the original hypothesis of this thesis was that a dysfunctional lysosomal system (also linked to the aging process) may play a causative role in the formation of AD-like pathology via the accumulation of iAβ, this was shown to be unlikely (experiments 1 and 2). However, it was later shown (in the remainder of section C) that lysosomal inhibition sufficient to cause increased cell associated Aβ/fβAPP-IR could impart significant risk for neurodegeneration if a suitable additional factor were encountered. Although in the case of this study an excitotoxin was used, in reality other traumatic events are likely, such as head injury (Mortimer et al., 1985, 1991; Roberts et al., 1991, 1994; Newman et al., 1995).

Hence, in an aged individual, the Aβ peptide could accumulate intralysosomally (Tsuzuki et al., 1995; Knauer et al., 1992), and begin to aggregate in an acidic environment (Burdick et al., 1992) rendered inefficient for normal protein degradation. The peptide could then escape from this compartment into the cytoplasm (or elsewhere) by damaging the lysosomal membrane (Yang et al., 1997) or through some other mechanism. Subsequent neural trauma could then “liberate” the Aβ into the extracellular space, along with lysosomal enzymes (Friede, 1965; Cataldo and Nixon, 1990; Cataldo et al., 1990). Once released, the now extracellular Aβ could seed SP formation, and thus potentially begin a pathological cascade (Hardy and Higgins, 1992; Hardy, 1997). This could involve a further increase in vulnerability to excitotoxicity (Crutcher et al., 1993), inflammatory damage (McGeer and McGeer, 1995; Lue et al., 1996), or direct Aβ-induced neurotoxicity (Yankner et al., 1990) should the concentration become sufficiently high (however, recall that transgenic mice exhibiting massive extracellular amyloid deposition show little, if any, evidence of cell death; Irizarry et al., 1997). In younger individuals, or in older ones with a more efficient lysosomal apparatus, the Aβ peptide may not exist in sufficient quantities to begin this process following injury, or may only deposit in a diffuse, non-aggregated form that can be readily cleared in most cases. This could also explain the presence of some SP in otherwise normal elderly persons, since they could thus originate through normal, use-related excitotoxic neuronal attrition (Olney, 1990; Crutcher et al., 1993).
Lysosomal inefficiency and lipofuscin formation are highly variable processes which may not affect all neurons equally, even within a relatively homogenous cell population (Moore and Ivy, 1995). Similarly, individuals will also show considerable variability, which can be attributed to many factors, both environmental and genetic. Therefore, individuals with an endosomal/lysosomal system in a suitably inefficient state may be prone to the development of AD. But what of other aged persons that should also have some degree of lysosomal dysfunction?

Alzheimer’s disease is the most common cause of dementia in the elderly, accounting for about 50% of all cases (Katzman, 1986). After AD, multi-infarct or vascular dementia (MID) is the next most common, comprising approximately 2/3 of the remaining cases (Alafuzoff et al, 1987b; c.f. Fig. A2-1). Therefore, together these two classifications cover greater than 80% of all demented patients. Although significant overlap exists (Alafuzoff et al, 1987b), a connection between the two has rarely been postulated. This is puzzling, given that the vascular system likely plays a significant role in AD. Consider:

1. \(\beta\)APP is a major serum protein, probably involved in blood clotting and/or platelet aggregation (Bush et al, 1993; Miyazaki et al, 1993)
2. At least one \(\beta\)APP mutation causes a disorder characterized by massive cerebral hemorrhage (HCHWA-D; Levy et al, 1990)
3. HSPG, which is a major SP component, is also a major component of the vascular basement membrane (Snow and Wight, 1988)
4. ApoE dysfunction is known to be a causative factor in the development of atherosclerosis (Mahley, 1988; Breslow, 1996), and there is an increased frequency of the E4 allele in MID (Shimano et al, 1989)
5. Presenilin 1 knock-outs are lethal, resulting in death by massive cerebral hemorrhage (Wong et al, 1997)

Does a relationship exist, and could it be elucidated by CHL-induced lysosomal dysfunction?

One of the major characteristics of MID is a highly dysfunctional BBB (Alafuzoff et al, 1983; Leonardi et al, 1985; Wallin et al, 1990). This can also be demonstrated by
immunostaining for extravasated serum proteins (Alafuzoff et al, 1985; Kalaria et al, 1991). Not only is this the same methodology used to demonstrate BBB compromise in this thesis, but the deposits of extravasated serum proteins seen following lower doses of chronic CHL bear a remarkable morphological similarity to those of MID. Although BBB damage is difficult to directly demonstrate in AD (Mooradian et al, 1988; Dysken et al, 1990), Aβ is known to directly damage endothelial cells and cause vascular constriction (Thomas et al, 1996), and some serum proteins are found in SP that are not of CNS origin (Kalaria and Grahovac, 1990).

Could lysosomal dysfunction be connected to the development of both disorders? The consequences of CHL treatment as seen in this study are caused by chronic administration only (experiment 5), which implicates faulty protein turnover and lysosomal inhibition as the primary mediators of the various drug effects. Although the neurodegenerative changes detected at the higher dose combinations of CHL with KA are consistent with the hypothesis of lysosomal inhibition leading to the accumulation of iAβ/βAPP and a corresponding increase in vulnerability to neural trauma (thus precipitating AD under the right set of circumstances), it is unclear how milder lysosomal inhibition could damage the BBB. It is perhaps worth noting that an abnormality in an endogenous protease inhibitor (cystatin C) leads to BBB damage, although in this case it is rather extreme (HCHWA-I; Ghiso et al, 1986), and it is unknown if the consequences of this mutation directly relate to faulty protein turnover.

Nevertheless, consider the following set of circumstances, which will be referred to as scenario B (to distinguish it from the AD-inducing possibility outlined above). An aged individual with less lysosomal dysfunction than in scenario A encounters a precipitating event, such as a small stroke (Erecinska and Silver, 1996). The data from this thesis suggest that this individual might then be predisposed to the development of MID rather than AD, with combined cases (Alafuzoff et al, 1987b) another possibility. It's also possible that the presence of extravasated serum proteins, such as circulating antibodies, may be related to the dementing characteristics of this disorder. Otherwise normal individuals can possess antineuronal antibodies in their blood serum (Stefansson et al, 1985), the concentration of which have been shown to increase with age in rodents
(Kulmala et al., 1987). Antineuronal antibodies are believed to be involved in the neuropsychiatric manifestations of systemic lupus (West, 1994), and have also been shown to be associated with behavioral abnormalities in a mouse model of this disease (Šakic et al., 1993).

Therefore, it is possible that lysosomal dysfunction could represent not only an underlying risk factor for AD, but also for MID. A continuum of dementing disorders could thus be envisioned, wherein the particular type of dementing illness developed is dependent upon an interaction of a precipitating or triggering event with the particular level of lysosomal inefficiency present in an individual.
D3 Future Directions

Given what has been established about the effects of CHL on the CNS, both with and without KA, the question arises as to what implications this might have for human use of this common anti-malarial drug. Could CHL usage lead to an elevation in risk for the development of AD or MID? There are, as yet, no population or case control studies on the CNS effects of CHL. However, there are many epidemiological studies of both types evaluating the incidence of dementia in rheumatoid arthritis (RA) patients (see McGeer and McGeer, 1995, for review). Since persons afflicted with RA are often treated with CHL or its derivatives (Fox, 1993), perhaps a detailed re-examination of these subjects could provide illumination on this matter.

In addition to its direct role as a lysosomal inhibitor, CHL has several side effects which have been addressed throughout this work. An important step in the verification of this thesis should therefore involve the use of a different type of inhibitor. As an interesting alternative, the antibiotic compound bafilomycin A₁ could be considered. This drug, which functions by blocking the activity of the lysosomal proton pump, has been shown to have effects similar to CHL, at least in vitro (Bahr et al., 1994). This substance might, for instance, reproduce the effects of CHL on Aβ/βAPP accumulation without the accompanying puzzling alterations in BBB function and seizure development. Also, potentially informative modifications in the basic experimental design could be performed, such as continuing inhibitor treatment post-KA or using older subjects, both of which may better mimic post-traumatic conditions in the aged human brain.

Finally, the major shortcoming of this approach is its inability to directly assess the contribution of iAβ/βAPP to subsequent excitotoxicity; CHL is a general inhibitor of lysosomal function, the use of which leads to the build-up of a wide array of intralysosomal substances in addition to Aβ/βAPP. Although research on naturally occurring model systems such as primates and dogs could answer the question as to whether this treatment paradigm actually leads to SP development, it cannot provide a complete solution. Similarly, transgenic animals known to overexpress βAPP and produce Aβ in large quantities (Games et al., 1995; Hsiao et al., 1996) could also be used. Since these animals do not show any AD-like pathology until they reach
approximately 6-8 months of age, then combined lysosomal inhibition and excitotoxicity at a younger age could accelerate the pathogenic process. It would be interesting to determine if under these conditions SP formation and neurodegeneration would be first observed in the amygdala, as was found in the normal rats used in this study.

This strategy does not, however, exclude the influence of developmental abnormalities brought about by the transgenic manipulation. Future studies using the next generation of transgenic animals should better address this question. Inducible promoters (Mayford et al., 1996) could be used to turn βAPP-transgene synthesis on or off at will, which would circumvent altered CNS development. If the conclusions of this thesis are correct, then blocking production of βAPP prior to the administration of CHL/KA should attenuate the neurodegeneration caused by this manipulation.

Thus, these future transgenic studies could confirm the involvement of the endosomal/lysosomal system and cell associated Aβ in the pathogenesis of AD. Such findings would also reaffirm lysosomes as a major target for future therapeutic intervention in the disease pathway, and thereby help focus future research efforts towards a viable treatment strategy.


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