Behavioral and histochemical characterization of a novel BACE Knockout x PDAPP mouse model of Alzheimer's Disease: Examination of potential effects of BACE inhibition on Alzheimer's Disease and the role of APP, Aβ and BACE in normal and pathological memory function.

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Declaration

In accordance with the University of Edinburgh's postgraduate regulation 3.8.7, I declare that the works described in this thesis are my own, except for where otherwise stated below, and that the composition of this thesis is also my own work.

Elizabeth Brigham performed testing for 4h on Day 6 of hidden platform water maze testing from Study 001 13mo cohort #3 (June 2002) as part of a training exercise.

Ferdie Soriano performed testing on Days 2-3 of visual cued navigation water maze testing from Study 001 18mo cohort #1 (May 2003) due to a family emergency.

Elizabeth Ludington performed high-level statistical tests in parallel to mine as an expert comparator to my analyses.

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Abstract

This dissertation describes the phenotypic characterization of a BACE knockout (KO) x PDAPP transgenic mouse line, utilizing behavioral, histochemical, and pharmacologic methods. Overproduction and accumulation of the amyloid-β (Aβ) peptide in the brain has been implicated as one of the causal factors in the development of Alzheimer's Disease (AD). Based on this concept, several transgenic mouse models have been created that overexpress human mutant Amyloid Precursor Protein (hAPP) that reproduces many of the cognitive and histopathological features of AD. Recently, the β-site cleaving enzyme (BACE) responsible for the first proteolytic cleavage of APP has been characterized, and subsequent research has led to the propagation of BACE inhibition as a prime experimental strategy for AD therapy.

Currently, there are many academic and pharmaceutical company laboratories actively engaged in developing therapeutic inhibitors of BACE for AD. However, the theoretical repercussions of BACE activity reduction have not yet been fully addressed in an *in vivo* model. Indeed, although overproduction of Aβ leads to neuroanatomical and cognitive pathology in human patients and animal models, lack of Aβ may also result in deleterious cognitive effects. Examining the behavioral and histological phenotypes of BACE KO animals on normal and hAPP overexpressing backgrounds is an effective way to assess whether the inhibition of BACE is a reasonable strategy for the treatment of AD.

To examine this issue a series of behavioral studies were conducted using homozygous and hemizygous BACE KO mice, PDAPP mice, and BACE KO; PDAPP lines together with relevant controls. The studies included various protocols in a cued and spatial watermaze task and detailed analysis of the occurrence of epileptiform seizures. Objective methods were used to analyse the changes in learning ability and the frequency of seizures.

The results from the characterization of the BACE KO x PDAPP mouse line indicate that the absolute loss of BACE and Aβ caused profound spatial memory

deficits, sometimes greater even than that of hAPP mice alone. In addition, absolute BACE KO was associated with spontaneous seizures as well as greater seizure activity in drug-induced seizure experiments. However, the partial hemizygous deletion of the BACE gene on a hAPP background appeared to improve spatial memory performance on certain measures and protect against drug-induced seizure responses relative to hAPP mice. The research described in this dissertation is consistent with the notion that, under certain circumstances, therapeutic inhibition of BACE may prove to be a valuable strategy for treatment of AD. In addition, these studies also support an important role for the β-amyloid processing pathway in "normal" learning and memory processes, possibly by regulating neuronal activity levels.

Chapter 1 Introduction

In the past 50 years advances in medicine have helped to contribute 10 years to the average lifespan of citizens in industrialized countries, and 20 years to the average world citizen (Goulding et al., 2003). Worldwide there is a boom in the 50-80 year range, as people are now more likely to survive childhood diseases and accidental trauma, and receive effective treatments for previously terminal conditions. While the rapidly expanding elderly population is a testament to the success of conventional medicine, it also underscores the need for accelerated progress in geriatric medicine to maintain a high quality of life for this burgeoning age group.

Among the diseases that often await those who reach their golden years, Alzheimer's Disease (AD) is prominent as a severely debilitating and terminal disease, characterized by progressive neuronal loss in regions of the brain involved in cognition. Patients with AD generally present with memory loss and confusion, which deteriorates to dementia, complete loss of day-to-day function and death (Rogan and Lippa, 2002).While AD is a terrible disease clinically, it also inflicts a severe emotional and financial toll on the caregivers of the afflicted. They must not only provide constant professional care for the patient, but also watch them as they mentally lose themselves and everyone else in their lives. It is estimated that the economic costs of AD patients in the United States receiving federal medical benefits currently exceed $50B per year (Brookmeyer et al., 1998). The economic burden of AD is expected to expand to $80B by year 2010 when the United States' AD population is projected to swell from 4M to 14M patients (Sloane et al., 2002). In comparison, worldwide populations of diagnosed AD are conservatively projected to triple to 34M by 2025, a number which does not reflect the evolution of the disease (Corporation, 2001; Kalaria, 2003).

The basic biology of AD has grown immensely over the past three decades, and one hypothesis to explain the neuronal and cognitive pathologies present in AD is the Amyloid Cascade Hypothesis. In this hypothesis, accumulations of pathogenic

amyloid-β peptide trigger a disease pathway in which cognitive impairments are the clinical sequelae of a complex neurodegenerative process (Glenner and Wong, 1984a, b; Selkoe, 1991; Hardy and Higgins, 1992). Among the many putative targets aimed at modifying deleterious amyloid levels in the AD patient, one stands out as a singularly hopeful candidate for effective AD therapy -- the Beta-site Amyloid Precursor Protein Cleaving Enzyme (BACE), linchpin of the catalytic process that produces the pathogenic amyloid-β peptides (Citron, 2002).

This dissertation is devoted to presenting a working background of the pathogenesis of AD, particularly in reference to the amyloid cascade hypothesis, describing what is currently known about BACE with regard to AD, and framing this information relative to a BACE-centered AD therapeutic concept. The behavioral and histochemical experiments presented here will characterize novel genetically modified BACE knockout mouse lines that overexpresses human mutant amyloid. This phenotypic information about the BACE knockout x PDAPP mouse will examine the theoretical risks and values of therapeutic BACE inhibition strategy for AD, as well as illuminate the role of the amyloid processing pathway in normal cognition.

1.1 History of the Alzheimer's Disease

The origins of Alzheimer's Disease as a strictly defined medical disorder lie in early 20th century Europe. Throughout the western world medical thinking was entering a new era, aided by the widespread use of the microscope, cell theory and classical anatomy, in which the pathology of disease was being investigated in terms of histological findings. Alois Alzheimer, and Franz Nissl were respected medical doctors practicing at the Municipal Hospital for Lunatic and Epileptics in Frankfurt (Stadtische Irrenanstalt), and were two of the founding fathers of neuropathology (Kreutzberg and Gudden, 1988). Alzheimer was a histologist with a gift for pathological description, whose earlier independent work consisted of examinations of ceruminary glands. In partnership with Nissl, Alzheimer focused on the anatomy of the cerebral cortex, and when Nissl left Irrenanstalt in 1895, Alzheimer became

the Institute's Director. With the vacuum left by Nissl's departure, Alzheimer expanded his medical research to schizophrenic and manic-depressive patients, which would eventually bring him into contact with one of the most famous patients in the history of clinical neuroscience, Auguste D (Graeber et al., 1998).

In 1903 Alzheimer left Irrenanstalt to rejoin Nissl in the Heidelberg laboratory of Emil Kraepelin, the preeminent psychiatrist of the time. The following year in 1904, they moved to the University of Munich, and began working with the patients of the Munich asylum as well as the University Psychiatric Institute. In 1905 Auguste D. entered the Psychiatric Institute at the University of Munich, at the age of 56 after experiencing a 5-year period of progressive confusion and memory loss, pronouncing herself lost mentally. Auguste D.'s condition deteriorated rapidly to dementia, and died in the Frankfurt asylum in 1906. Upon examination of her brain tissue using Bodian's silver staining technique, Alzheimer noticed several "tangle-like baskets" in Auguste D.'s cortex, as well as a smaller overall cortical volume compared to normal brains.

In late 1906 Alzheimer reported his findings from Auguste D.'s brain at a meeting of the South-West German Society of Alienists, and in 1907 provided a deeper account of pre-senile dementia and cortical tangles. Over the next decade Alzheimer treated several more patients who presented with similar symptoms and shared this aberrant cortical pathology. By 1911 other pathologists were reporting similar findings, and impressed with his original descriptive work, Alzheimer's colleagues moved to name the disease after him (Graeber et al., 1997).

1.1.1 Alzheimer's Disease Diagnosis, Early Therapies, and the Development of the Amyloid Cascade Hypothesis

AD today is diagnosed and pathologically confirmed today much as it was in Alois Alzheimer's day, with a few technical improvements. Patients presenting with senility and disorientation are tested with a battery of examinations, like the Alzheimer's Disease Assessment Scale - Cognitive Subscale (ADAS-COG, driven

by verbal and memory tasks), Mini Mental State Examination (MMSE, a quick portable examination with a functional bent). These tests as well as others are used to assess the specific cognitive and daily functional deficits to form a differential diagnosis of AD (Folstein et al., 1975; Rosen et al., 1984; Mega, 2002). Disease severity and progression is scored typically with longitudinal ADAS-COG scores and purely functional Clinical Dementia Rating (CDR) tests (Price et al., 1991). As there is no definitive diagnostic test yet for AD, confirmation of the disease ultimately comes with positive histological staining of amyloid plaques upon the patient's death. Aside from the original neuronal plaques and fibrillary tangles, dystrophic neurites and gliosis of areas surrounding neuritic plaques are now considered to be consistent neuropathological features of AD (Figure 1.1). Research with advanced neuroimaging techniques like MRI and SPECT, finer clinical cognitive assessments, as well as investigation of meaningful disease biomarkers from the periphery like CSF measurements of amyloid and tau proteins continues to evolve, in hopes of providing earlier and more accurate diagnoses of AD (Boss, 2000; Teunissen et al., 2002; Sunderland et al., 2003; Zakzanis et al., 2003).

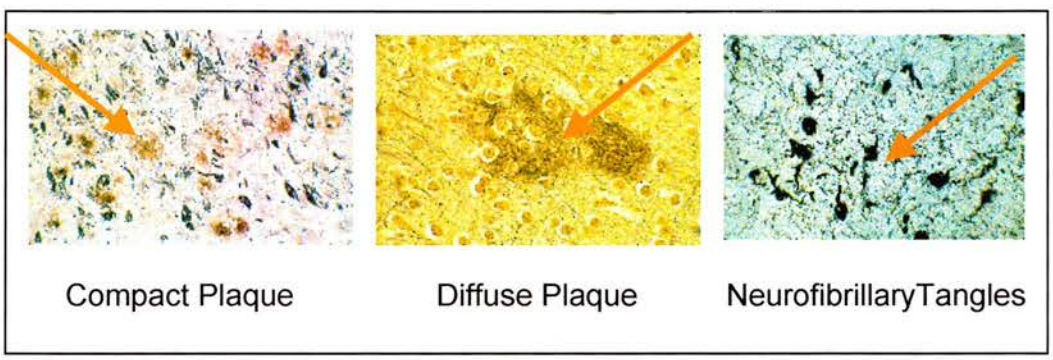


Figure 1.1 Histological Features of Alzheimer's Disease. All tissues are human cortical tissues stained with Bodian's Silver stain unless otherwise stated. Left panel: Silver stain ("quenched" with Haematoxylin & Eosin pre-stain), red arrow pointing to compact senile amyloid plaque formation; there are several in the image. Middle panel: Red arrow pointing to a single diffuse amyloid plaque, which unlike compact plaques lacks an amyloid core and swollen neurites. Right panel: Several neurofibrillary tangles are shown; the red arrow points to a globoid neuron rich in intracellular accumulations of the microtubule protein tau.

Despite these many theoretical advancements in the field of AD, the major care regimen for AD patients remains institutionalization (Manton, 2003). As more became known about the brains of AD patients, it was noted that aside from the hallmark neurofibril tangles and neuronal plaques, AD brains show a marked neuronal loss in the hippocampus, cortex, and other regions of the forebrain (Jellinger and Bancher, 1998). In addition to what was first pharmacologically viewed as essentially a cholinergic loss, AD brains display a broad decrease in neurotransmission (Sirvio, 1999; Arendt, 2001). From this information came the first attempts at treatment aimed at neurochemical modulation in AD.

Between 1950 and the present day several classes of palliative AD therapies were tested that shared a common neurobiological premise: to increase neurotransmission in hopes of generating large enough signals to overcome effects of neuronal loss in the regions of the brain involved with cognition (Davies and Maloney, 1976; Perry et al., 1978a; Perry et al., 1978b; Smith and Swash, 1978; Smith et al., 1978; Davies, 1979a, b; Perry et al., 1980; Atack et al., 1983; Davies, 1985). Acetylcholine (ACh) agonists and AChEsterase inhibitors (Tacrine, Donepezil, Rivastigmine, Galantamine), GABA antagonists, Ca^{++} modulators and metal ion chelators have all played upon boosting neurotransmission, with varied results (Knopman, 2003; Werber et al., 2003). More recently drug companies have antagonistically targeted the N-Methyl D-Aspartate (NMDA) receptor, which is intimately involved in memory processes, resulting in the drug Memantine for moderate to severe AD, which proffers modest cognitive improvements over a time period which has yet to be determined (Ferris, 2003).

Other therapeutic approaches like non-steroidal anti-inflammatories (Ibuprofen) and antioxidants (Vitamin E) have been empirically found to reduce amyloid production and have been used in combination with AChEsterase inhibitor drugs to treat AD (Doraiswamy, 2002; Helmuth, 2002). In contrast to AD treatments based in the premise of increasing neurotransmission, some developing AD research efforts is currently focused on direct neurodegeneration caused by accumulated dyshomeostasis of metal ions, traumatic brain injury or neurological viral infections

(Itzhaki, 1994). Other drugs are given to treat the other symptoms of AD, including depression, anxiety, and/or psychosis, which are generally more responsive to therapy than memory and basic functional impairments (Devanand, 1997).

All clinical trials for potential AD treatments are challenging and extremely costly as they involve elderly patients who may be on a number of other medications, and have a range of mental impairments that affect regimen compliance. In addition, AD clinical trials typically must have study durations of 12 months or longer for reasonable measurement of changes in disease progression (Fillit et al., 2002; Karlawish and Clark, 2002). Unfortunately, the many difficult clinical trials for AD treatments have produced too few drugs. Currently, AChEsterase inhibitors are the most successful and widely-used AD palliatives improving cognition in a limited population of AD patients for short period of time, but leaving the growing need for robustly efficacious AD treatment largely unmitigated (Trinh et al., 2003). Fueled in part by this unmet medical need and also by the revolution in molecular biology, AD researchers had breakthrough discoveries in AD biology in the late 1980s.

The classic plaques and tangles described nearly a century before were now gaining new definition through biochemistry. The neuronal plaques found throughout the hippocampus, frontal cortex, and entorhinal cortex are largely comprised of extracellular deposits of amyloid-beta peptide (Aβ) (Glenner and Wong, 1984b; Masters et al., 1985). Aβ is the 40-42 amino acid product of complex proteolysis of the Amyloid Precursor Protein, APP, with three predominant isoforms in the brain, APP695, APP751, and APP770 (Tanaka et al., 1988). Aβ is a 4 kDa protein ubiquitously expressed and is normally generated by neurons, glia, lymphocytes, and endothelial cells throughout the body (Selkoe et al., 1988). While Aβ is the major component of plaques, the classic neurofibrillary tangles (NFTs) were in turn found to be majorly comprised of intracellular assemblies of a microtubule-associated protein call tau (Goedert et al., 1988).

1.1.2 The Amyloid Cascade hypothesis

The discovery of these defining molecular components in AD brains caused much excitement within the scientific and medical communities, and in time ignited a debate over the role of each protein and histological feature in the progression of AD from primary and secondary disease to end-state pathology. While the amyloid versus tau controversy raged hotly for years, two vital pieces of evidence led to the rise of the amyloid cascade hypothesis as the central framework upon which many therapeutic drug efforts are now based.

The first finding was related to the levels of amyloid plaques and tau-positive NFTs in normal and aged brains. Neurologists at Washington University reported that while all aged brains accumulated NFTs and tau proteins over time, the numbers of NFTs did not correlate to Clinical Dementia Rating (CDR) scores in AD patients and their brains upon autopsy. While this result argues that tau-positive NFTs may be a consistent feature of normal aged brains, there is a modest positive correlation between plaque counts and CDR in AD patients (Price et al., 1991). Later research expanded on the basis of this modest initial finding, showing that presynaptic terminal loss is itself coincident with neuritic plaques and that this loss is tightly associated with cognitive decline (Lassmann et al., 1993; Masliah et al., 1994; Masliah et al., 2001b). The relationship between amyloid burdens in the brain and clinical staging of AD has been controversial, as various groups have both claimed and refuted any direct correlation between staging of cognitive deficits in AD and amyloid burden (Price et.al. 1991; Nagy et al., 1995; Nagy et al., 1996). However, these once tenuous and sporadic correlations have been bolstered recently by improvements on MRI methods and stereologic measurements of amyloid histopathology (Nagy et al., 1995; Nagy et al., 1996; Naslund et al., 2000; Bussiere et al., 2002). The further discovery that specific forms of amyloid, like soluble and oligomeric amyloid peptides (including Aβ-derived diffusible ligands or ADDLs), are strongly correlated to clinically diagnosed AD further strengthened the case for the functional amyloid cascade hypothesis (Lue et al., 1999; Gong et al., 2003).

The second major piece of evidence in support of the amyloid cascade hypothesis was the discovery that monogenetic mutations in the amyloid precursor protein gene (APP) are robustly associated with AD. Down's Syndrome is a genetic disease in which Chromosome 21 is triplicated, and the afflicted have mental retardation and invariably develop AD at an early age. In 1985 APP was found to be the major neuronal plaque constituent in both AD and Down's Syndrome (Masters et al., 1985; Robakis et al., 1987). After a series of scientific forays examining Chromosome 21, several mutations were found in the three isoforms of APP in the plaques of patients with familial AD (Levy et al., 1990; Goate et al., 1991; Murrell et al., 1991; Hendriks et al., 1992; Mullan et al., 1992). Eventually these familial AD (FAD) mutations of the APP gene would gain more familiar monikers, Swedish mutation (K670N, M671L), Flemish mutation (A692K), London mutation (V717I), Indiana mutation (V717F), and the original characterized Dutch mutation (Q692E) all of which cause presenile deposition of amyloid in the brain (Figure 1.2).

1.1.3 Biology and processing of the Amyloid Precursor Protein

Upon identification of FAD mutations, it was apparent that there are genetic hotspots of mutation (codons 670, 692, and 717), resulting in FAD patients with symptoms in their mid-30s (Van Broeckhoven et al., 1987; Van Broeckhoven C, 1987; Siman et al., 1993). APP is broadly expressed across the tissues of the human body, and exists as a large type I transmembrane protein of about 110-130kDa with much of the protein located within the extracellular regions of the protein (Masters et al., 1985; Robakis et al., 1987; Siman et al., 1993). Immunochemical analysis with AD brain tissues provided evidence that APP is highly processed, undergoing a series of endoproteolytic cleavages leading to amyloid-β fragments varying from 38-43 amino acids in length (Sisodia et al., 1990) (Figure 1.3). The previously discovered APP mutations were clustered around regions of APP that are cleavage sites of the processing enzymes. In the presence of the various APP mutations, metabolic processing becomes altered, shifting the ratios of the various metabolite fragments, with more Aβ1-42 than Aβ1-40 production (Suzuki et al., 1994).

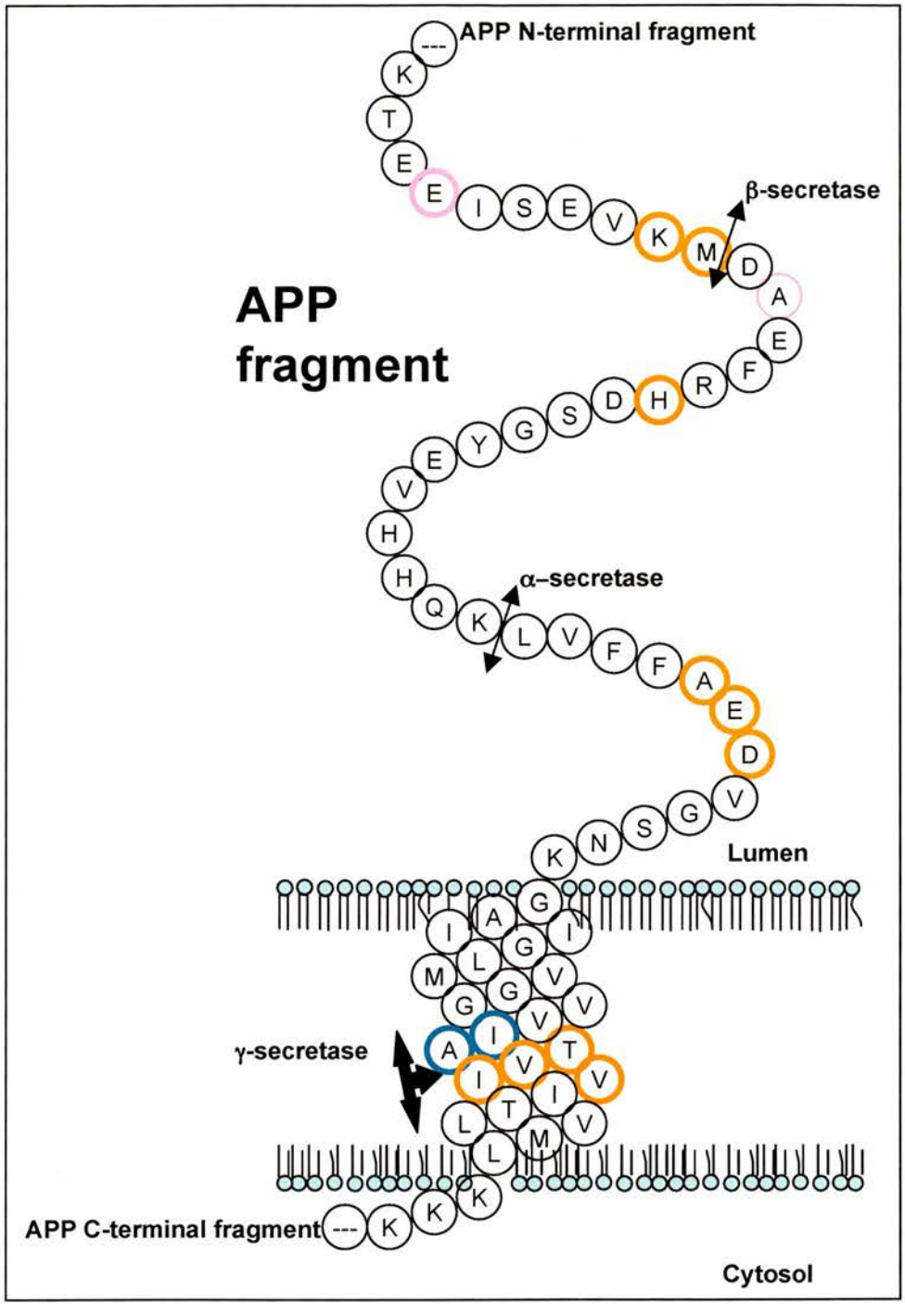


Figure 1.2 APP sequence and processing sites. This schematic of the human wildtype APP amino acid sequence shows the processing sites for α-, β- and γ-secretase (heterogeneous intramembrane cleavage). Individual amino acid residues outlined in red, blue (Aβ1-42/43 cleavage site itself) and purple (also linked to vascular amyloidosis) indicate sites that have been identified as mutational hotspots that result in accumulations of pathogenic amyloid Aβ1-42.

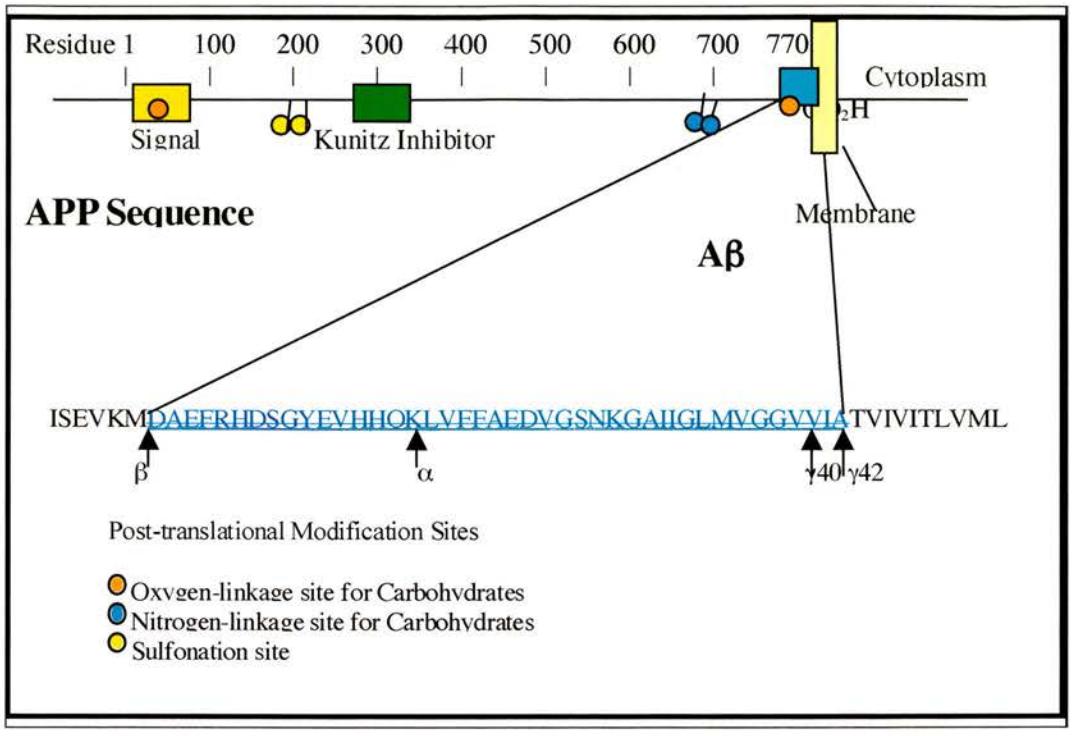


Figure 1.3 The Amyloid Precursor Protein sequence. The amino acid sequence of the human APP is displayed at the top of the figure, ranging from residue 1 to 800. Red, blue and yellow circles delineate sites undergoing post-translational modifications for carbohydrate linkage or sulfonation. The yellow box indicates the region of the signal sequence which directs the protein to various organelles for processing. The green box indicates a putative Kunitz Inhibitor region, which may protect the protein precursor from proteolysis by serine proteases like trypsin. The aqua box indicates the 40 or 42 amino acid sequence containing the amyloid peptide itself. The specific sequence of the amyloid peptide is expanded, with the Aβ1-42 sequence shown in blue text, with arrows pointing to processing sites for α-, β, and γ-secretase cleavage shown below.

Aβ1-42 is more abundant in plaques as opposed to normal aged brain tissues, and was thus pronounced the amyloidogenic fragment. Later in vitro experiments with human AD cortical tissue confirmed that Aβ1-42 is indeed the APP metabolite responsible for the formation of plaques, as it was also found to have the ability to form fibrils and oligomers, the structural basis for promotion of amyloid deposits (Mattson, 1997; Lambert et al., 1998; Kirkitadze et al., 2002). Additionally it was found that the Aβ1-42 peptide is neurotoxic to neuronal cells in certain cell culturing conditions, promoting cell death through activation of apoptotic pathways (Estus et al., 1997; Troy et al., 2000; Allen et al., 2001; Kienlen-Campard et al., 2002).

While accumulations of Aβ1-42 can theoretically be viewed as a causal factor in the etiology of FAD disease, which currently represents only ~5% of all AD cases, the peptide also accumulates in the brains of patients with non-genetic AD, making it a likely player in the early etiology of sporadic AD (AD Education and Referral Center, 2004). The number of total AD cases linked to FAD has slowly increased over the past 20 years, as more mutations and genetic polymorphisms have been found, with the preponderance of cases affecting Aβ1-42 levels in some manner. While the neurotoxic properties of Aβ1-42 can possibly explain the dramatic neuronal loss seen in AD brains, this is a pathological feature that appears late in the disease long after the first signs of cognitive decline. It is possible that Aβ1-42 is still directly responsible for pre-apoptotic cognitive dysfunction. However, confirmation of this hypothesis requires the development of more sophisticated experimental models of AD and deeper understanding of the processing enzymes that create Aβ as well as the normal cellular role of APP and its metabolites.

1.1.4 Modulation and mechanistic regulation of Aβ

Although the metabolic processing of APP is a normal highly regulated process for the majority of people, disruptions to this processing can lead to deleterious accumulation of pathological amyloidogenic peptides. APP is a large integral membrane protein trafficked through the constitutive secretory pathway, and from fractionation experiments distinct sets of amyloid peptide can be recovered from

different subcellular locations, indicating that multiple enzymes in diverse organelles processed the peptide. Also, it is clear that from experiments using homogenized and cultured human AD brain tissue that there are soluble metabolized fragments of APP that are not the major Aβ1-40 or amyloidogenic Aβ1-42 peptides (Sisodia et al., 1990; Haass et al., 1992; Seubert et al., 1992; Iwatsubo et al., 1994). After a number of biochemical experiments, the processing of APP is found to occur in two ways, producing longer fragments in one mechanistic pathway (38-43 amino acids in length, which includes the amyloidogenic fragment Aβ1-42) and truncated peptides in another (19-22 amino acids in length) (Figure 1.4).

In the 1990s considerable AD research was focused on identifying the proteolytic activities that cleave the amyloidogenic Aβ1-42 fragment. At the same time, more information about Aβ1-42 itself was becoming available, shedding light on a protein lifecycle that is constantly growing in complexity. Investigation has revealed the existence of distinct pools of extracellular or secreted Aβ1-42 and intracellular Aβ1-42, which are either destined for secretion or intracellular localization (Koo and Squazzo, 1994; Cook et al., 1997; Wild-Bode et al., 1997; Greenfield et al., 1999). Three pathways of Aβ production have been discovered, of which there are differing pathways for secreted Aβ1-42 (traversing the trans-golgi network) and for intracellular Aβ1-42 (which is processed at the endoplasmic reticulum). In addition, a minor amount of secreted Aβ is processed after reinternalization at the plasma membrane by endo- and lysosomes. Clearly, with these many varied locations, the amyloidogenic processing activity is present in several subcellular compartments.

Genetic evaluation of a new group of FAD patients in 1995 led to the elucidation of an enzymatic complex protein involved in both amyloidogenic and non-amyloidogenic APP processing. A gene was cloned from Chromosome 14 that contained missense mutations that produced an autosomal dominant for of AD that presented well before sporadic AD onset (Sherrington et al., 1995). This gene, called Presenilin 1 (PS1) is 60kB in length with 13 exons, and is the first component identified of the g-secretase enzyme, an activity that acts downstream of α- and β-secretase, which were as yet unidentified in the mid-1990s.

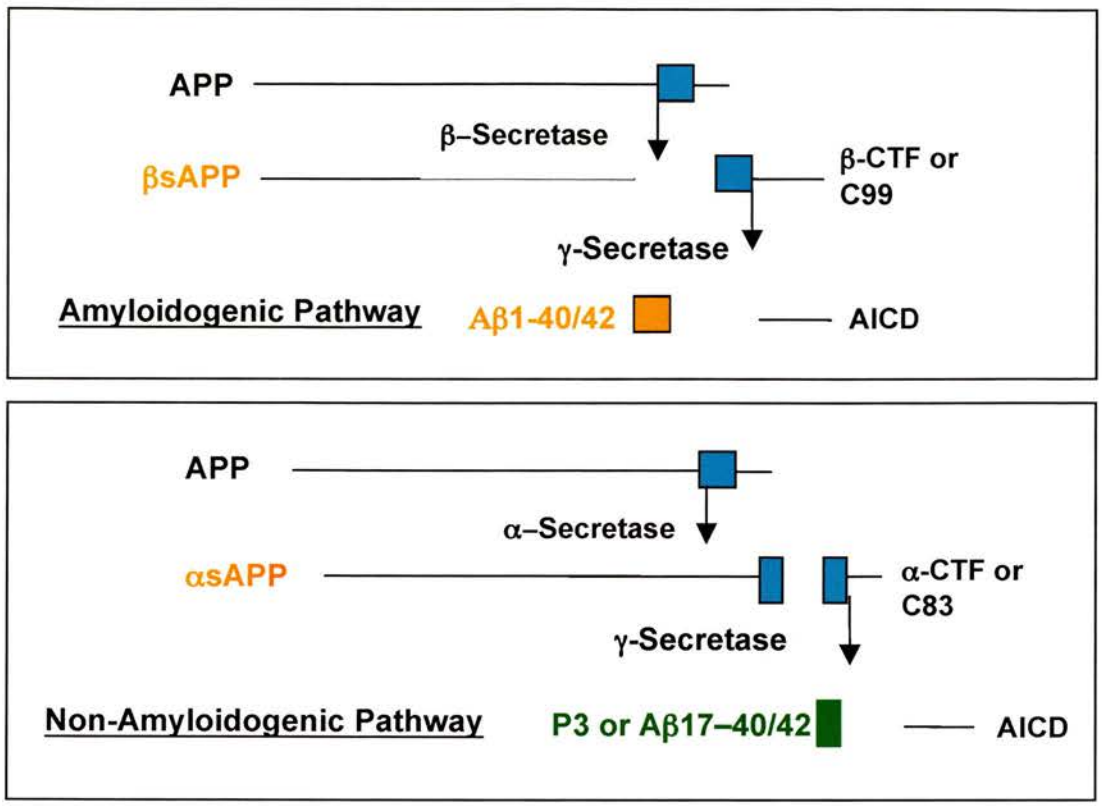


Figure 1.4 Amyloidogenic and non-amyloidogenic processing of APP. Upper panel: The amyloidogenic APP processing pathway. APP is cleaved by β-secretase at +1 to produce the βsAPP fragment that is trafficked out of the cell, and the C-terminal β-fragment called β-CTF or C99. The amyloid peptide is produced by subsequent γ-secretase of C99, resulting in Aβ1-40/42 and the amyloid intracellular domain (AICD) which is translocated to the nucleus. Aβ1-42 is considered the amyloidogenic peptide, as it is capable of toxic aggregation. Lower panel: The non-amyloidogenic APP processing pathway. APP is cleaved by α-secretase at +17 to produce the αsAPP secreted fragment and α-CTF or C83. Following C83 cleavage by γ–secretase, the AICD fragment and the Aβ17-40/42 fragment. Endoproteolysis by α- and β-secretase is a competitive enzyme process, in which the vast majority of APP is processed by the α-secretase pathway.

1.1.5 γ-Secretase

The cleavage sites of the γ–secretase enzyme are unusual in that APP cleavage is an intramembrane event, and cleavage by γ-secretase can result in a number of lengths of amyloid peptide fragments (Gu et al., 2001). These findings clarify the basis of the hotspots of gene mutations in APP, PS1 and PS2, a second novel gene similar to PS1 found on Chromosome 1 whose missense mutations also lead to FAD. Mutations in these regions give rise to increased production of Aβ1-42, as these mutations cause preferential cleavage of γ-secretase at the site that generates Aβ1-42 (Citron et al., 1997). Further study led to the discovery that γ-secretase has other substrates, including the Notch receptor, which is critical for neural development as well as adult differentiation of cells (De Strooper et al., 1999). Development of drugs to reduce Aβ1-42 production via inhibition of γ–secretase now require a complex degree of specificity, as the first γ-secretase inhibitory compounds also reduced production of thymocytes (Hadland et al., 2001). Research involving γ-secretase continues, in hopes of identifying specific components that would proteolyze membrane-bound amyloid fragments while sparing the Notch receptor.

1.1.6 α-Secretase

The processing of the non-amyloidogenic species is initiated by an enzyme called the α-secretase, which cleaves APP at the cell surface between residues 16 and 17 of the amyloid peptide (Anderson et al., 1992). The resulting truncated cleavage products are αAPP, a soluble ectodomain fragment that is secreted from the cell, and the membrane-bound C-terminal fragment C83, a substrate for γ-secretase (Nunan and Small, 2000). Following intramembrane cleavage by γ-secretase, C83 is further processed to the p3 peptide (Aβ17-40 or Aβ17-42) and the Amyloid Intracellular Domain AICD or CT57-59. This enzymatic activity is called the α-secretase, and since the discovery of its actions on APP, several different α-secretases have been identified from previously known gene products (Asai et al., 2003). These α-secretases include a number of A Disintegrin And Metalloproteinase

(ADAM) family members, including ADAM9, ADAM10, and ADAM17, also called Tumor Necrosis Factor Converting Enzyme or TACE. Experimental evidence shows that several different ADAM enzymes are required to produce α–secretase activity on APP, and that a number of other proteins like TGF-α and L-Selectin Adhesion Molecule are also α-secretase substrates (Condon et al., 2001). However, α–secretase cleavage of Aβ is the major APP processing enzyme of non-neuronal cells, with little of this activity in neurons (Saitoh and Mook-Jung, 1999).

1.1.7 β-Secretase

While work related to α-secretase has been successful and contributes to the understanding of the APP lifecycle, most research efforts are committed to elucidating processes that clearly yield the amyloidogenic fragment of Aβ generated by β-site cleavage. The β-secretase activity processes APP mostly in the lumen of the ER, cutting at the +1 Asp to liberate a soluble βAPP ectodomain and a membrane-bound C99 fragment (Siman et al., 1993; Hussain et al., 2003). After cleavage by the γ-secretase complex, C99 proteolysis largely yields the amyloid peptides Aβ1-40 and Aβ1-42 as well as the AICD fragment. Alternately, β-secretase cleavage occurs in the TGN, preferentially cleaving at Glu 11 of the amyloid sequence, resulting in a C89 membrane-bound fragment that is further truncated to Aβ11-40 and Aβ11-42 by γ-secretase (Huse et al., 2002; Liu et al., 2002).

Scientific pursuit of the β-secretase entity was intense in the late 1990s, aided by several clues gleaned from prior research (Vassar and Citron, 2000). Isolated Aβ1-42 from plaques suggests that the site for β-cleavage is the Asp +1 amino acid of the Aβ peptide. It is certain from experiments that established the subcellular localization of Aβ1-42 that the β-secretase activity exists in the ER and TGN. β-secretase is also localized to trafficking organelles with an acidic environment, as treatments that altered pH in cells also disrupt β-site cleavage of APP. Other experiments suggest that β-secretase activity is expressed across many tissues of the body, with higher levels in neurons in regions of the brain like the neocortex,

hippocampus and entorhinal cortex (ERC) where amyloid deposits were prominent (Haass et al., 1992; Haass et al., 1993; Roher et al., 1993; Seubert et al., 1993; Koo and Squazzo, 1994; Citron et al., 1995; Knops et al., 1995; Zhao et al., 1996). Finally, the β–secretase activity had to be either a membrane-bound protease itself or one closely associated with a membrane protein, as APP lacking any transmembrane domain is not a substrate for β-site cleavage in cells (Citron et al., 1995). Components of the α- and γ-secretase complexes had been identified in 1994 and 1995, but the β-secretase remained tantalizingly elusive throughout the 1990s.

In the meantime, the rapid expansion of knowledge about the biology of AD allowed for a number of targets for disease-modifying therapeutic intervention to be proposed and investigated, to varying degrees. At the same time however, the AD research field was conducted was being fundamentally changed by the introduction of several transgenic animal models of AD. These useful AD animal models allowed for biochemical, histological as well as cognitive analysis of in vivo manipulation of APP, its processing enzymes, and metabolites and provided a basis for developing and testing innovative new AD therapeutics. Prior to describing these transgenic models, it is critical to lay the groundwork for the various behavioral tasks used to test the cognitive deficits in the AD transgenic models, and to discuss the relationship between these tasks and the neuropsychological tests used to diagnose and assess AD.

1.2 Behavioral tasks used to assess animal models of AD and their relevance to human cognitive tests

Alzheimer's Disease (AD) is associated with a broad spectrum of cognitive impairments that have a negative impact of the daily living functions of patients and severe practical repercussions for their families and other caregivers. The most commonly described cognitive deficits are those related to episodic and declarative memory, abstract thinking, as well as attentional impairments (Devanand et al., 1992; Perry and Hodges, 1996; Devanand, 1997; Petersen, 1998). Other clinical features that complicate the dementia profile of AD patients may include aggression,

depression, and anxiety disorders (Cooper et al., 1990).

Ideally, animal models of AD should display behavioral deficiencies that have a relationship to the clinical disease. However, many of the neuropsychological tests like the CDR, ADAS-COG, and MMSE used to diagnose and assess the severity of AD in a patient rely on the use of verbal abilities to delineate a patient's cognitive status (Folstein et al., 1975; Rosen et al., 1984; Price et al., 1991; Mega, 2001). In addition, even the non-verbal testing paradigms between the clinical and laboratory settings are also highly divergent. This represents a major challenge for experimenters involved in animal modeling of AD, as many tasks must be validated with respect to the region of the brain involved or a general behavioral concept and not a primarily clinical similarity. The tasks discussed in this section are those that are commonly employed to assess the cognitive status of transgenic mouse models of AD, and their relationships, if any, to AD deficits in neuropsychological examinations will also be presented.

A rough division can be drawn for the tasks used to assess rodent memory function, which are either aversive or appetitive in reinforcement. Aversive tasks involve exposure to a noxious stimulus or set of stimuli, with reinforcement being applied via bodily escape from the effects of the stimuli. Performance in appetitive tasks does not typically involve escape, but rather draws on reinforcement based on extinguishing of an internally motivation (Golob and Taube, 2002). While many animal behavioral tasks are aversively reinforced, more clinical tests for AD are appetitive. Indeed, in some cases, tasks that are aversively reinforced in animals have clinical test correlates that are appetitive (Table 1.1):

Animal Task	Stimulus	Human Task	Motivation	Verbal or Instrumental Component
Morris water maze	Water, swimming	VR water maze	**Internal**	Yes
Fear Conditioning	Electric Shock	Fear Conditioning	Electric Shock	No
Eyeblink Conditioning	Corneally-directed air	Eyeblink Conditioning	Corneally-directed air	No
Startle Inhibition	Loud Noise	Double-Click Task	Loud Noise	No
T/Y-Maze	Exploration/Novelty*	Eye Movement	Exploration/Novelty	No
Social Recognition	Social/Internal	Facial Recognition	Social/Internal	Yes
Object Recognition	Exploration/Novelty	Object Recognition	Exploration/Novelty	Yes

Table 1.1 Cognitive tasks used in assessing AD models and their human neuropsychological task correlates, with externally motivated or aversive animal tasks in upper table, and internally-motivated animal tasks in the lower table. When any human task has an inherent element of language, reading, device operation

1.2.1 Spatial memory tasks

There have been several prominent theories of memory processes proposed in the past few decades, like the Cognitive Mapping Theory, Working Memory Theory, Declarative Memory, Configural Association Theory, Relational Memory Theory, but all of them have a significant functional component based in the hippocampal formation ((Olton et al., 1978; Walker and Olton, 1979a, b; Squire, 1986; Sutherland et al., 1989; Rudy and Sutherland, 1995; Cohen et al., 1997). In AD, the hippocampus is one of the earliest affected and most damaged areas of the brain, leading to subsequent memory impairment (O'Keefe, 1976; Cooper et al., 1990; Hubbard et al., 1990; Adelstein et al., 1992; Convit et al., 1993; Killiany et al., 1993; Bouras et al., 1994). Anecdotally AD patients are prone to wandering; this has a defined clinical basis as AD patients are impaired in spatial working memory due to the particular affliction of the hippocampus (Cooper et al., 1990; Adelstein et al., 1992) The hippocampus is home to the "place cells" that are responsible for the encoding of map information that is accessed by working memory when spatial tasks must be solved (O'Keefe, 1976; Wiener et al., 1989; Wilson and McNaughton, 1994).

Morris water maze

Developed in the early 1980s by Richard Morris, the water maze has become one of the most commonly used behavioral paradigms to assess memory in transgenic AD models (Morris, 1984). Drawing on rodents' innate ability to swim and the evolutionary drive to escape from swimming by finding land, the water maze task assesses the spatial navigation from memory of rodents who must locate a submerged platform using various cues and strategies. The water maze apparatus is simple, consisting typically of a circular pool filled with water tinted with an occludant, a visible or non-visible landing platform submerged beneath the surface of the water, and a video tracking system to monitor and analyse the movements of the animal (Figure 1.5, and 2.5-2.9). The water maze paradigm is highly modifiable, as visual cues can be employed both within and without the perimeter of the pool. Experimental designs that employ single platform locations can provide information about spatial reference memory, memory retention when the platform is removed, and also perseveration when the platform location is moved. Alternatively, experimental designs that feature a series of platform locations and require learning locations to a specified criterion can provide greater information about the status of working spatial memory and learning capacities.

Aside from the benefits of being able to access various types of spatial memory with simple study design changes, the ability to perform in the water maze has been examined to a high degree in lesion and pharmacological studies. Competence for performance in the water maze is dependent on the hippocampus, although other brain regions compensate for injury or drug-induced hippocampal (Schenk and Morris, 1985; Morris et al., 1986; Morris et al., 1990). In later sections, several transgenic models are presented that replicate pathological features of hippocampal deposition of amyloid with concomitant loss of hippocampally-mediated spatial memory function in the water maze (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Chen et al., 2000; Chishti et al., 2001).

In human clinical assessments, a virtual reality version of the water maze has been reported (Astur et al., 2002). Patients with hippocampal resections are severely impaired in the ability to use extramaze cues to locate the virtual platform, compared to age-

matched control patients with either no brain surgery or surgery on regions other than the hippocampus. While there are major differences between the animal version and the human virtual versions of the water maze, as the human task is appetitive and requires instrumental learning and coordination to perform, it is clear that across species the reliance on the hippocampus is preserved (Bunsey and Eichenbaum, 1996).

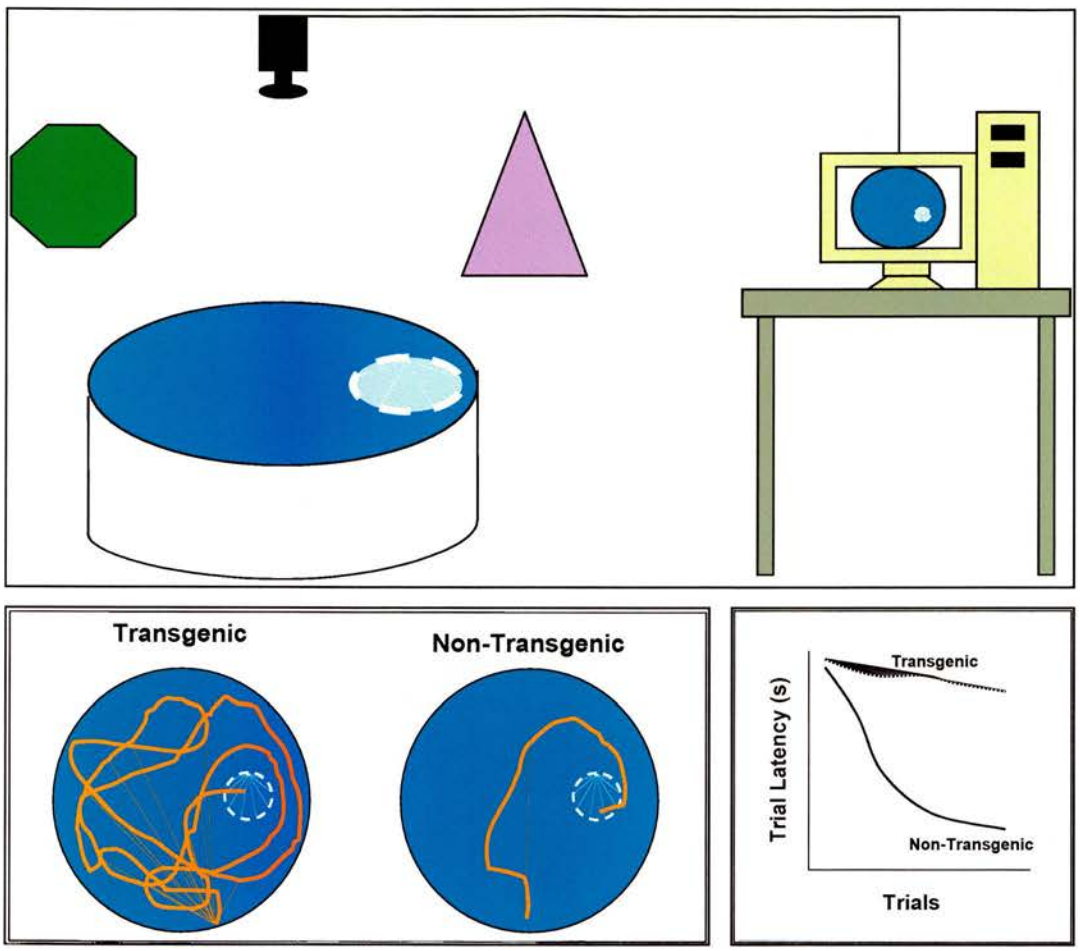


Figure 1.5 The Morris water maze. Upper panel: Typical water maze laboratory, in which a water-filled pool with a submerged landing platform is surrounded by external cues. Automated water mazes feature trial recording by camera, with data collected by computer. Lower left panel: Sample water maze swim paths (in red) of animals with spatial memory impairments (transgenic), and unimpaired animals (non-transgenic). Note the length and random nature of an impaired animal's swim path versus the shorter, more direct swim path of an unimpaired animal. Lower right panel: Sample graph of water maze swim trial latencies. Spatial learning and memory in non-transgenic animals is represented by rapid reduction of swim trial latencies over time, while transgenic animals have a markedly slower reduction in swim trial latency.

T and Y-Mazes

In the early 1900s dry land rodent mazes were commonly used to examine the rudimentary psychology of learning, and were often highly complex structures (Olton, 1979). Mazes were large platforms with walled pathways leading to exits and/or food rewards. In the modern study of learning and memory mazes have become simpler, with only a few possible passageways. The most common of these are the T and Y-mazes, which have a starting arm (the stem of the T or Y), 2 choice arms, which may have connecting passages back to the start arm (Blodgett et al., 1949). Rodents are measured for their ability to explore one arm and either naturally explore the next arm (alternation) or to explore the "correct" arm based on memory of the presence of food rewards.

These mazes can be used to study spatial working memory, as error rates of for entering a wrong arm to collect a food reward can be measured. Alternatively these mazes can be used unbaited as a paradigm to assess spatial memory as a function of spontaneous explorative entries into each arm (alternations) and the repeated visitation of any arms (perseveration) (Lester, 1968). The clinical relationship to a human deficit with T- or Y-mazes is more tenuous than with the water maze, as the closest correlates are visual exploration tests. Patients are shown a series of images while their eye movements are tracked electronically; reduced novelty-seeking and overall visual exploration are evident in AD patients (Daffner et al., 1992; Daffner et al., 1994; Daffner et al., 1999). While animal maze tasks rely on spatial working memory as well as exploration, the human task depends more heavily on visual attention and may be influenced by amyloid pathology in the visual cortex, in addition to the hippocampus.

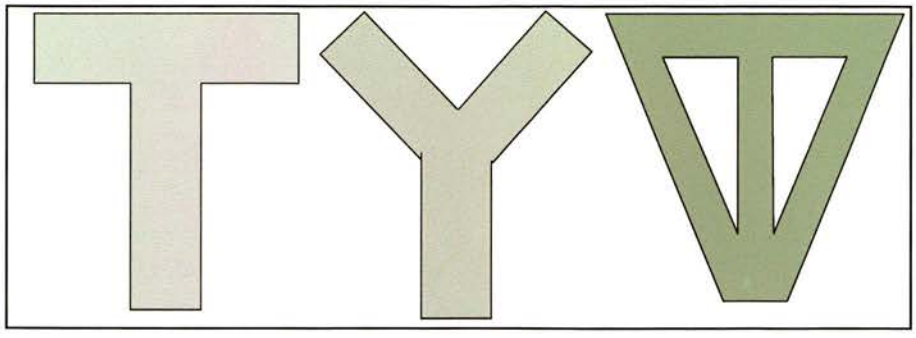


Figure 1.6 T-, Y- and Modified T-mazes. Rodents are placed in a passageway junction or an arm terminus and allowed to freely explore. Alternations between arms and number of arms entered are motivated by a rodent's instinct to explore and working spatial memory.

Barnes circular holeboard maze

It is useful to describe another spatial memory task that has no direct clinical correlate that has nonetheless been used to assess transgenic mouse models of AD. Developed in the 1970s by Carol Barnes, the Barnes maze is an elevated circular platform with a ring of small holes near the perimeter of the circle (Figure 1.7) (Barnes, 1979; Pompl et al., 1999). These smaller holes are fitted with shallow cups or an escape tubnnel. Rodents in the Barnes maze are stimulated to seek to a tunnelhole via exposure to loud noise or bright light. Over several explorative trials animals will learn to escape efficiently to the escape hole, using external spatial cues.

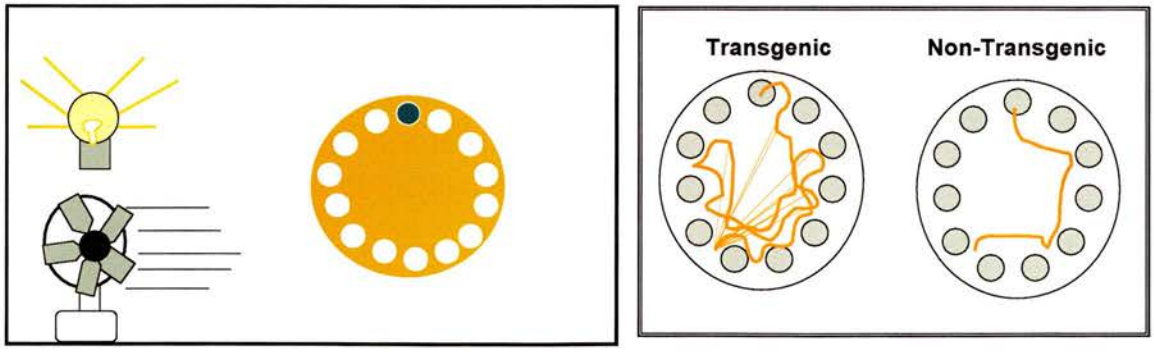


Figure 1.7 Barnes maze apparatus. Left panel: Circular holeboard with one escape tunnelhole, with adjacent noxious noise or light devices. Right panel: Example escape paths of spatially impaired and unimpaired animals.

1.2.2 Recognition tasks

Related to the spatial memory tasks, recognition tasks have aspects of spatial learning removed from their design by balancing the presentation of objects and images in space. In both human and animal tasks recognition memory tests are appetitive without a noxious component to the sampling or match-to-sample phases. While there is greater overlap between AD patients and animals in the anatomical regions of the brain involved in processing recognition memory, there is still an element of difference due to deviations between species due to the greater use of olfaction in guiding exploration in rodents.

Object recognition

In the spontaneous object recognition tasks, rodents are placed in an arena in which there are one or more objects (Ennaceur and Delacour, 1988; Rothblat and Kromer, 1991). While exploring the arena the experimental animal will also examine these objects and the object exploration time is recorded (sampling) (Figure 1.8). After a delay, the rodent is returned to the arena, in which a facsimile of the original object plus a novel object has been placed. The amount of time the animal spends inspecting the novel object in comparison to the familiar object is recorded and expressed as recognition index. In the human task, patient subjects may either be given physical objects to examine or a series of visual images (Flicker et al., 1987; Purdy et al., 2002). This task is highly correlated between human and animals as it utilizes the same anatomical processing regions (inasmuch as rodent and human processes are alike) and shares similarities in study design parameters.

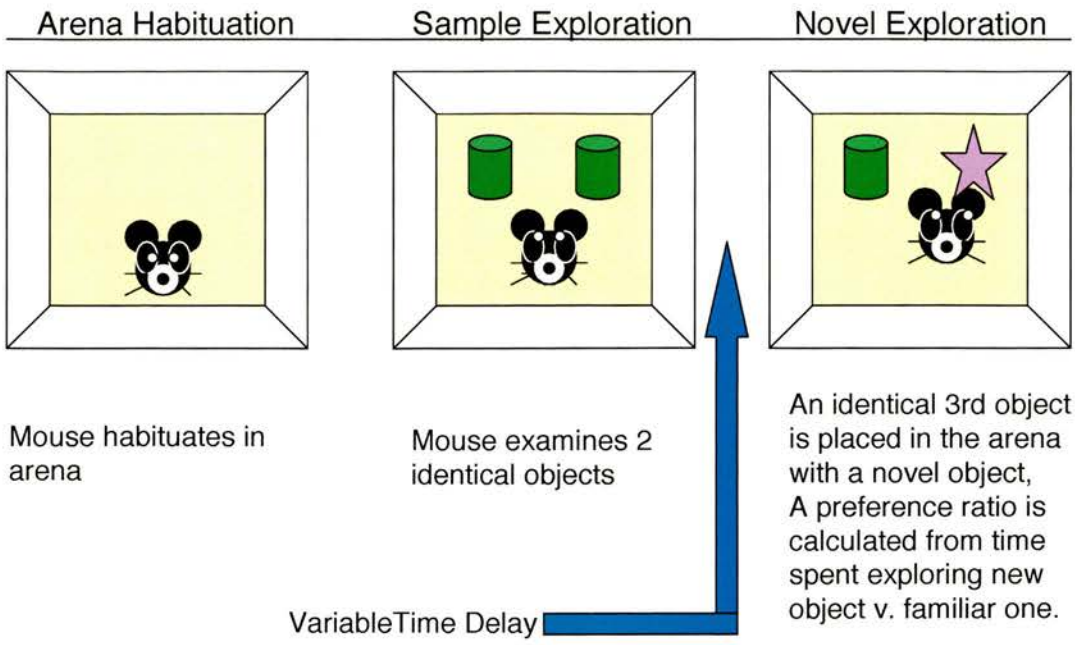


Figure 1.8 Object recognition task

Social recognition

Another recognition task that accesses a very complex type of memory is social recognition (Guan and Dluzen, 1994; Kogan et al., 2000). This task exploits the social hierarchies developed when rodents are exposed to each other or housed together. Animals are housed together or alternatively briefly exposed to each other to allow familiarity to be established. After a separation of variable time the animals are brought back together, with social exploration time (directed sniffing, visual examination) recorded. In addition any aggressive behavior is noted. In some versions of this task rodents are also brought into contact with stranger animals with which they have had no prior contact. From this interaction an index of social examination can be made from the stranger versus familiar exploration times. As complex a behavior as social recognition in rodents is, it relies on several anatomical structures within the brain, resulting in lesser dependence on the hippocampus for intact social memory function (Bannerman et al., 2001; Ferguson et al., 2001; Shang and Dluzen, 2001; Petrulis and Eichenbaum, 2003).

Social recognition tests in humans diagnosed with AD are not typically done by assessing recognition of long-time familiar persons, although the inability to recognize family and friends is often a feature of moderate to severe AD. Social recognition is typically tested by memory for a series of face images (Wilson et al., 1982; Flicker et al., 1990). As faces of other people have significant social implications, the ability to recognize faces is distinct from simple visual recognition, and relies on function of the fusiform gyrus (Sergent et al., 1992; Kanwisher et al., 1997). In addition, AD patients have a deficit in the ability to correctly name facial emotion, a complication that is relatively unexplored in animal models (Lavenu et al., 1999; Hargrave et al., 2002). While these tasks across species both rely on complex social memory processing, the differences in test parameters, functional anatomic regions, and the unknown relationship between human and rodent emotional processing also make this task difficult to evaluate for direct relevance between the laboratory and clinical settings.

1.2.3 Conditioned memory tasks

Conditioned learning and memory involves the association of a novel stimulus (unconditioned stimulus) with no inherent initial response value to a stimulus that has a characteristic response (conditioned stimulus and response). Over several pairings of the unconditioned and conditioned stimulus, the presentation of the conditioned stimulus alone will elicit the unconditioned response. This concept was made famous by the experiments of Pavlov, who paired a ringing bell to the presentation of food to canine test subjects, which respond to food by salivation. After several of these pairings contingent in time, the dogs would salivate to the ringing of the bell alone (Pavlov, 1951).

While conditioned learning paradigms have been developed for numerous physiological reflexes in animals and has clinical correlations to conditioned learning deficits in AD patients, these tasks are not as commonly used as spatial learning and memory tests described in the previous section (Solomon et al., 1991; Solomon et al., 1995). Indeed, while tasks like the water maze are generally viewed as "hippocampal" and often focuses on a few critical hippocampal synaptic pathways, conditioned learning involves

several regions of the brain and with less well-defined cortical connections. The relative lack of transgenic animal models of AD testing in conditioned learning and memory tasks does a disservice to the field as these tests in animals are highly similar to clinical tests performed on AD patients and others with memory deficits (Woodruff-Pak et al., 1990; Arendash and King, 2002; Corcoran et al., 2002; Gerlai et al., 2002; Hamann et al., 2002; Weiss et al., 2002; McCool et al., 2003; Hejl et al., 2004).

Eyeblink conditioning

The most documented type of conditioned learning deficits are in eyeblink conditioning (Woodruff-Pak et al., 1990; Solomon et al., 1991; Solomon et al., 1995). In this simple associative memory paradigm, auditory tones are presented prior to administration of a puff of air towards the cornea. In successive pairings of the tone and air stimuli, the latency between the presentation of the tone and an eyeblink response is reduced. While this eyeblink conditioning task is classically performed in rabbits, it has been successfully been validated in rats and mice and can be performed in some cases in free-moving animals (Schneiderman et al., 1962; Schmajuk and Christiansen, 1990; Weiss and Thompson, 1991; Chen et al., 1995). This paradigm is experimentally very similar in both animal and human studies (especially in physically unrestrained animals), and although the neural substrate for this activity involves the cerebellum and brainstem in addition to the hippocampus, these are also areas that are affected by amyloid burdens in AD (Pro et al., 1980; Steinmetz et al., 1992; Anderson and Steinmetz, 1994; Gabriel et al., 1996; Miller and Steinmetz, 1997; Steinmetz, 2000).

Fear conditioning

Emotional memory responses, as quantified by fear conditioning, have highly complex neural circuitry. In animal behavioral models there are typically two types of fear conditioning most commonly used, contextual fear conditioning, which relies on spatial environment as the unconditioned stimuli, and cued fear conditioning, in which the unconditioned stimulus are auditory tones (Brown et al., 1951; Kurtz and Siegel, 1966; Dexter and Merrill, 1969). While the contextual version of the fear conditioning task has

greater relationship to visuospatial memory tasks based in the hippocampus, cued fear conditioning involves a high degree of processing in the auditory cortex prior to hippocampal activation, and greater dependence on the amygdaloid nuclei (Phillips and LeDoux, 1992; LaBar and LeDoux, 1996).

While there is a high degree of variation in fear conditioning paradigms for measuring emotional memory in rodents, a typical scheme involves using mild electrical foot shock as the unconditioned stimulus and measurement of subsequent fearful crouching or freezing body positions (Blanchard and Blanchard, 1969; Davis and Astrachan, 1978). In a cued fear experiment, a rodent is placed in a box with an electrified grid floor and allowed to acclimate and explore for a period of time on the order of a few minutes, after which the animals are presented with an auditory tone followed by a footshock. After at least two cue-shock pairings, the animal is returned to its homecage. Training for contextual fear conditioning is often conducted in concert with cued fear training. After a variable time delay, the rodent is placed in the familiar shock chamber and time spent in a freezing posture is recorded. After a variable delay in which the animal is in its homecage, cued memory testing proceeds with the rodent being placed in a novel context. The novel context is typically a shockbox in which the context is changed, often by addition of chamber dividers and a flat material to cover the electric grid floor. Freezing time in this novel context is also measured to provide a contextual fear memory ratio. After a period of time in the novel context, the familiar auditory cued is presented, with freezing times recorded (Figure 1.9).

As complex as the neural basis for memory is, the animal task has a clinical correlate in human tasks. AD patients have been found to have fear conditioning deficits when given visual stimuli paired with a loud aversive auditory stimulus, and fear being measured via palmar galvanic skin response. This task has a relationship to the both elements of the cued and contextual fear conditioning task in animals, as both species of subjects must use visuospatial processing to examine the unconditioned stimuli, although in the case of the human task the auditory tone is used as the unconditioned stimulus, not the conditioned stimulus. While the ethics of using a tasks more akin to the animal test in demented patients is highly questionable and precludes the development of more related

human conditioned fear tests, there is a neural basis for emotional memory deficits in AD, as there is significant neurodegeneration in the human amygdala that makes the comparison of tests valuable (Haroutunian et al., 1998; Haroutunian et al., 1999).

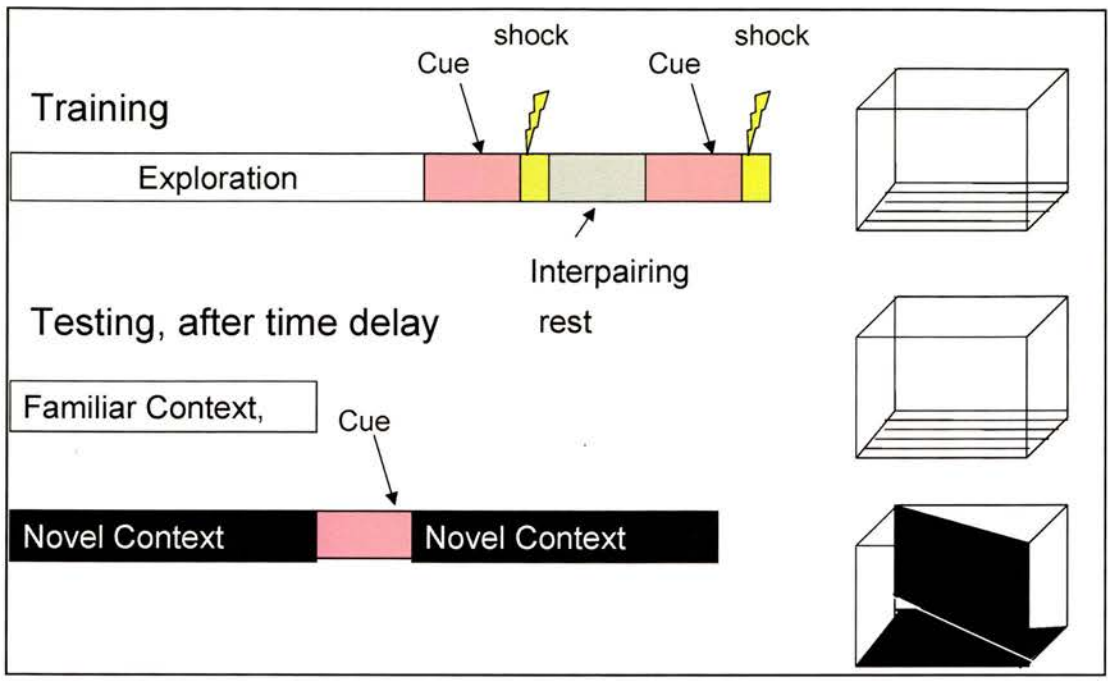


Figure 1.9 Cued and contextual fear conditioning. During training, the rodent is placed in a box with an electrified grid floor and allowed to explore freely for a short period. A cue and shock follows, in which a loud auditory tone precedes a noxious footshock; cue and shock blocks are separated by a rest period. After a delay of hours to days, spatial contextual memory is assessed by placing the rodent in the same box in which it experienced the footshock (familiar context). Fear-based recognition of the familiar environment in which the aversive stimuli was experienced is quantified by the time in which the animal crouches unmoving in a "fearful" posture. Cued memory is assessed by placing the animal in a novel environment and quantifying fearful responses to the sounding of the auditory cue that preceded a footshock previously.

Startle inhibition

The ability to habituate in startle response to a loud, unexpected noise is central to startle inhibition (Groves et al., 1974). In this paradigm a series of acoustic tones are presented at variable frequencies and durations before a very loud tone is presented. If the magnitude of the startle response is less than that recorded after the loud tonal pulse is presented alone, prepulse startle inhibition (PPI) has occurred. PPI is a task that involves

sensory gating and is not specific to memory function, but also attention and sensorimotor processing. Although deficits in PPI have been described in transgenic mouse models of AD, patients with mild AD have not been found to have this deficit (Kurtz and Siegel, 1966; Groves et al., 1974; McCool et al., 2003; Hejl et al., 2004). AD patients have been found to have sensory gating deficits in habituation to evoked encephalogram (EEG) P50 responses to loud auditory stimuli (Jessen et al., 2001), but this physiological finding has no current behavioral representation. Although the PPI task has been used to describe other cognitive deficits in transgenic mouse models of AD, the correlation to the human disease is unclear due to differences in the interspecies task and the lack of PPI impairment at least in mild AD.

1.2.4 Anxiety tasks

AD patients have been described with a constellation of psychological afflictions, including anxiety (Mega et al., 1996; Devanand, 1997; Teri et al., 1999; Ferretti et al., 2001; McCurry et al., 2004). Although many transgenic animal models of AD have been reported to show behaviors that relate to anxiety, these cognitive impairments are not given the importance that memory deficits receive as crucial behavioral elements for an *in vivo* AD model (Arendash et al., 2001; Lalonde et al., 2002). However, as these tasks are frequently used in assessing the cognitive dysfunction of transgenic models for AD, they will be described here.

Elevated plus maze

This exploration task has been successfully utilized for nearly 40 years as a screen for anxiety behaviors in rodents (Halliday, 1967; Pellow et al., 1985). The maze consists of 4 maze arms that intersect at right angles to form a plus sign on an elevated platform. On one parallel, two arms are enclosed, with a dark wall on both sides of the arm passageway (Figure 1.10). The area of arm intersection as well as the remaining two arms is open, without walls. A normal rodent in its exploration of an elevated plus maze will spend some time in the open arms, including examination of the edges of the open arms, whereas an animal considered "anxious" will spend little time in these open areas. Most elevated plus mazes used in the present are video-based with animal movement

time in open and closed arms analysed by a tracking system, allowing for relatively objective quantification of a highly subjective behavior.

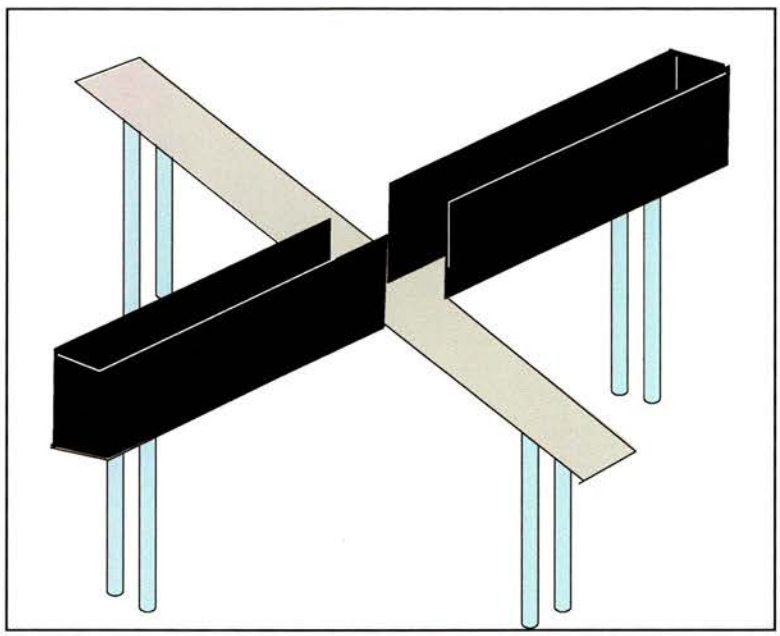


Figure 1.10 Elevated plus maze. Rodents placed in the center of the plus maze arms will have a natural tendency to explore all arms, while animals with anxiety-like phenotypes will spend less time in the exposed open arms of the maze.

Open field activity

Another rodent behavioral task that evaluates spontaneous exploration as a measure of anxiety is the open field activity task. Rodents are placed on a closed arena that is often brightly lit and allowed to freely explore. Rodent behavior is tracked in a quantitative manner, and with video tacking software can include such measures as total exploration path lengths, number of vertical rearings, time spent near the center and grooming (Delbarre et al., 1970; Britton and Britton, 1981). Anxious rodents would presumably be indisposed to movement near the center of the arena and a reduced number of exploratory movements or autoattentive behaviors compared to a non-anxious animal.

1.2.5 Locomotor activity tasks

Motoric abnormalities are part of the clinical spectrum of symptoms seen in AD patients, often described as motor agitation (Devanand et al., 1992; Mega et al., 1996; Devanand, 1997). The anatomical origins of this excessive and undirected motor activity is unresolved, possibly due to motor cortex disinhibition impairments or hyperexcitability (Alagona et al., 2001; Liepert et al., 2001; Pennisi et al., 2002). Motor hyperactivity has been noted in numerous transgenic mouse models of AD, mirroring another important non-memory cognitive impairment in AD (Dodart et al., 1999; Arendash et al., 2001; Gerlai et al., 2002; Lalonde et al., 2002; Lalonde et al., 2003).

Spontaneous locomotor activity monitoring

Highly sophisticated automated computer-based systems are used to measure locomotor activity in rodents, capable of capturing information on increasingly complex behaviors (Sanberg et al., 1985). Activity monitoring chambers are often framed by infrared beam emitter and receiver arrays that create a grid of beams that collect quantitative and qualitative information for each beam break. Detailed information can be collected on dozens of parameters as spatial maps can also be created within the confines of the activity chambers to collect even more specialized information about spatially-constrained behaviors, e.g. rearing at the chamber periphery versus near the center of the chamber (Dow-Edwards, 1998). Depending on the protocol designs employed, activity readouts can detect motor impairments, anxiety-like behaviors, seizures, and perhaps even memory perturbations with lack of habituation over several trials.

Rotorod motor coordination test

Originally developed to assess cardiovascular capacity and used heavily by pharmaceutical companies, the rotorod can be adapted to measure motor coordination and learning (Watzman et al., 1964). The rotorod apparatus consists of a motorized rod suspended above an electrified grid (Figure 2.4). The rod can be programmed to turn at constant or accelerating speeds, and set to run for specified time intervals. Rodents

placed on the rod must learn how to ambulate on the rod as well as to keep pace with any increases in rotational speed. The electrified grid below the rod serves to motivate rodents to perform the task, and latency to fall is the major measure in rotorod testing. Rotorod testing for motor coordination usually involves a period of training with a few trials per day at slow and/or constant speeds, followed by a testing day of rapidly increasing speeds (Forster and Lal, 1999). In protocols where motor learning is assessed the protocols are similar to those intended to test motor coordination, but tend to use more trials over more days to establish rates of improvement over time between animals with motor impairment and motor competency (McFadyen et al., 2003).

There are a number of behavioral tasks that are used to assess cognitive and sensorimotor function in models of AD, and their relationships to clinical tests are variable. It was important to understand the correlations between human and transgenic animal tasks and the deficits they describe before moving to an examination of the specific transgenic mouse lines themselves, as this will help us to evaluate their individual strengths as disease models.

1.3 Transgenic models of Alzheimer's Disease

During this very productive time for AD research in the 1980s and 1990s, researchers were limited by availability of experimental tissues. Banked human AD brain tissues were the primary source of research material, but are logistically difficult to obtain for many investigators and are also subject to regional variability in FAD patterns and/or other epidemiological factors (Jendroska et al., 1993). Aged animals provided another source of tissue for AD research, but maintaining large numbers of rodents to ages at which there is sufficient brain amyloid is costly and even aged animals did not reproduce many of the important pathological features of the disease. Developments in the field of transgenic technology made it possible to develop living cells, organisms, and even animals that could express gene products from other species in quantities that could be manipulated in time, spatial location and level of expression (Brinster et al., 1981; Bernstein and Breitman, 1989; Sass, 1990; Hennighausen et al., 1995; Albanese et al., 2002). Using these transgenic implantation techniques, the first successful transgenic animal models of plaque-

related neurodegeneration were created, by introducing high levels of mutant human APP genes into mice. Although no single animal model devised can replicate all the features of a complex human disease, many transgenic mice that have modifications of genes implicated in AD are today often referred to as "AD models."

1.3.1 PDAPP

The first successful transgenic AD mouse model was reported in early 1995 by researchers at Athena Neurosciences (Games et al., 1995). Taking the human Indiana mutation V717F of APP, Games and colleagues inserted the gene sequence into a βhAPP695 minigene under the control of the human Platelet-Derived Growth Factor promoter (PDGF) on a C57Bl6 background outbred to DBA2J mice (Figure 1.11). Detailed histological analysis showed that there is widespread AD-like pathology in the brains of these PDAPP mice, in the cerebral cortex and hippocampus (Masliah et al., 1996; Johnson-Wood et al., 1997; Chen et al., 1998; Gonzalez-Lima et al., 2001). At 6-9 months of age, PDAPP mouse brains began to exhibit amyloid accumulations like AD neuronal plaques, increasing in density over time until the pattern of deposition tightly resemble that of human AD. In addition to neuronal plaques, aged PDAPP mouse brains have astrocytosis and gliosis in the region of dense core plaques, hyperphosphorylated tau and have widespread synaptic loss. At earlier ages prior to visible amyloid accumulation PDAPP brains have marked hippocampal atrophy, and perturbations in synaptic transmission, with increased paired-pulse facilitation and inability to maintain long-term potentiation (LTP) (Larson et al., 1999; Dodart et al., 2000). Later studies characterized the behavioral impairments present in PDAPP mice, some of which are correlated to accumulation of plaques, like in spatial paradigms such as radial arm mazes and Morris Water Maze tasks, and other impairments, which are more plaque-independent, e.g. object recognition tasks (Morris, 1984; Dodart et al., 1999; Chen et al., 2000). PDAPP mice lack paired helical filaments and neurofibrillary tangles, but in other respects closely mirror the range of cognitive and cellular AD pathology, and this useful disease model did much to underscore the role of amyloid in the etiology of AD (Masliah and Rockenstein, 2000; Masliah et al., 2001a). Many

of the AD-like features of PDAPP mice are summarized in Figure 1.11.

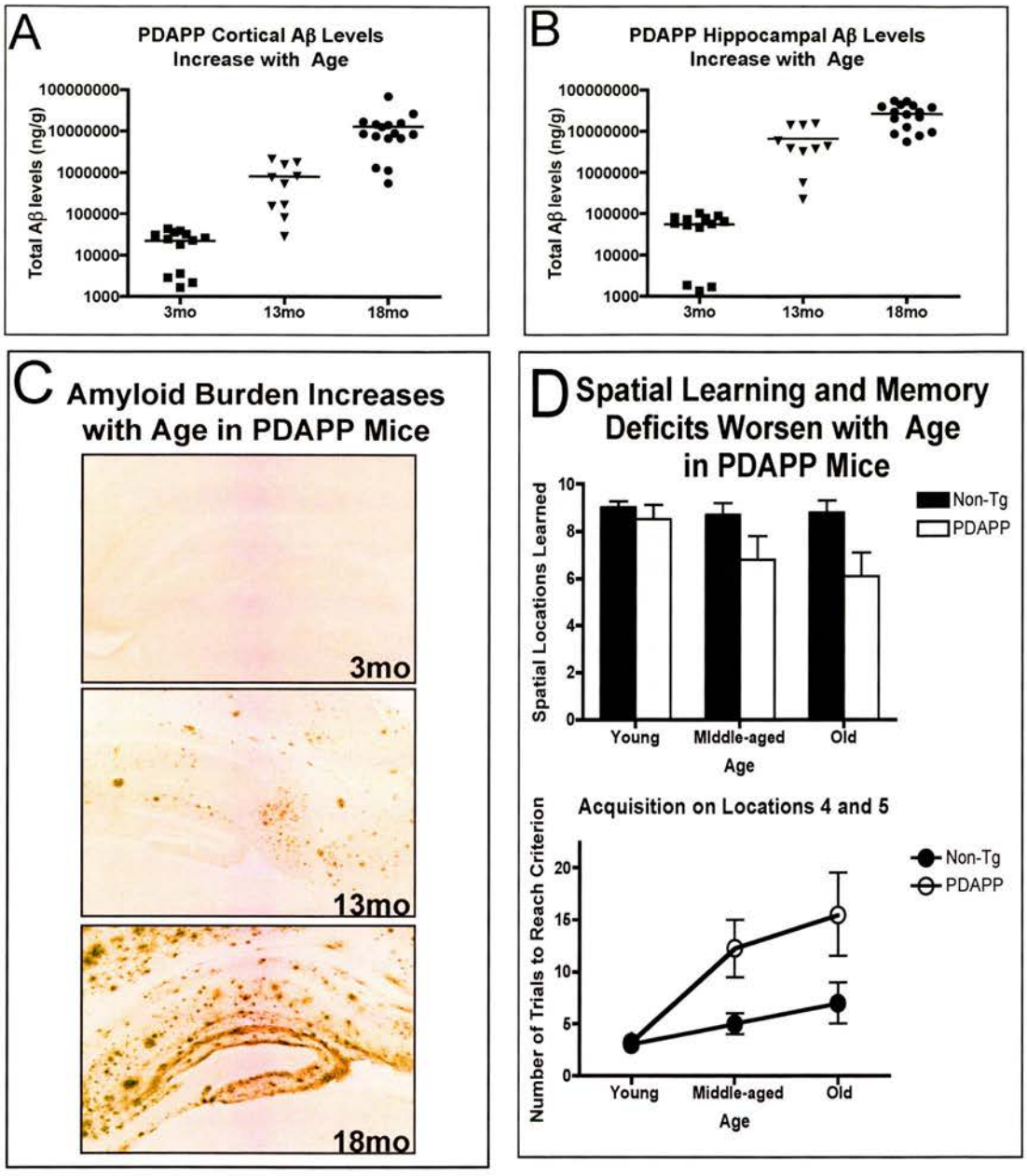


Figure 1.11 AD-like Characteristics of PDAPP mice. A,B: ELISA immunoassays reveal that with age, there is an exponential increase in total brain Aβ (which includes soluble and insoluble peptides) in both the cortex and the hippocampus. C: In parallel to the increases in total brain Aβ content, PDAPP brains have an increase in amyloid plaque-like deposition of Aβ. At 3mo there is no deposition, while by 13mo there is widespread deposition that becomes even more pronounced by 18mo. D: Serial spatial memory phenotypes of PDAPP mice to non-transgenic controls over age. Upper panel compares measures of learning capacity over age between genotypes, bottom panel displays similar comparison for memory acquisition rates (adapted from Chen et al.; 2000 in Nature).

1.3.2 Tg2576

Later in 1996 a multicenter research effort led by Karen Hsiao published their works in developing the first transgenic mouse of AD that overexpressed the human Swedish mutation (K670N, M671L) of the APP gene (Hsiao et al., 1996). By inserting this mutant hAPP into the open reading frame of a hamster prion protein (PrP) cosmid vector and injecting it into mouse blastocysts and implanted in psuedopregnant surrogate C57Bl6 dams, transgenic mice were developed and bred to uniform heterozygosity with SJL mice. The ensuing Tg(HuAPP695.K670N-M671L)2576 or Tg2576 mice produce about hAPP 5.5x the level of endogenous mouse APP. Immunochemical analyses show that these mice produce Aβ1-40 at 5x and Aβ1-42 at 14x the endogenous mouse levels, and that mice with elevated Aβ had numerous dense core and diffuse amyloid plaques. These plaques are mostly immunoreactive for Thioflavin-S (dense core plaques with β-sheet amyloid formations as opposed to general fibrillar amyloid β–sheet binding by Congo Red), and are found throughout the frontal and temporal cortices, hippocampus, entorhinal cortex, the subiculum and cerebellum. Astrocytes and glia ringed these plaques, and dystrophic neurites are evident around denser amyloid cores. These mice were initially reported to have cognitive deficits in spatial and reference memory with impairments at 10 months age, but not at 3 months of age, leaving the exact temporal details of this deficit unresolved. While other behavioral deficits in Tg2576 mice have since been validated, these results suggest that the hippocampal deficits are connected to the appearance of plaque formations over time.

The Tg2576 and PDAPP mouse models are both of great utility to researchers focused on AD, although there are important distinctions between the two models. While the PDAPP mice over time do not display frank neuronal loss but have significant synaptic loss, Tg2576 mice have no pattern of cell loss but have higher synaptic density compared to similarly aged non-transgenic controls(Chen et al., 1998; King and Arendash, 2002). Neither model has shown the presence of NFTs or paired helical assemblies, although PDAPP mice have linear hyper-phosphorylated tau filaments. Tg2576 mouse brains also stain positively for proteins involved in

intracellular protein degradation, ubiquitin and α-synuclein, making it more like the Lewy-Body Variant type of AD (Yang et al., 2000). Other researchers developed other transgenic lines with mutated human APP, with similarly varying results in the TgCRND, J20 and APP23 mouse models (Hsia et al., 1999; Chishti et al., 2001; Kelly et al., 2003).

1.3.3 APP23

Researchers at Novartis published their works in 1997 with the same Swedish mutation as the Tg2576 mice, but under the control of different promoter, murine Thy-1, with pronuclear injection to B6D2F1 mice (Sturchler-Pierrat et al., 1997). While the gross regions of expression are similar to the prior models in the neocortex and hippocampus, the resulting line of mice that expressed high levels of Aβ1-42 (APP23), and seem to model a fundamentally different kind of AD than either of the two original models. APP23s display comparatively massive glial response, more diffuse than dense core plaques, have higher levels of Aβ in the cerebrospinal fluid, and have plaques that are much more soluble in nature than those of typical AD (Bornemann et al., 2001; Kuo et al., 2001). APP23 mice have similar levels of memory and learning impairments compared to other mutant hAPP-over expressing mice in passive-avoidance and spatial maze paradigms (Kelly et al., 2003). Most notably, these APP23 mice have several focal deposits of amyloid in the endothelial lining of brain blood vessels, a hallmark of Central Amyloid Angiopathy (CAA), which 20-80% of AD patients develop (depending in the individual study's definition of CAA, which may or may not require vascular symptoms), making APP23 a model for AD/CAA or even hemorrhagic stroke rather than typical sporadic AD or FAD (Calhoun et al., 1999; Winkler et al., 2001; Castellani et al., 2004).

With these AD animal models in hand, AD research entered a new era, in which putative treatments could be tested in transgenic disease models to establish proof of concept and efficacy of therapy. Mechanisms of aggregation, synapse and cell loss could now be observed in vivo, as well as other critical events in the evolution of

AD. Indeed, transgenic mouse models of AD help to provide intriguing information about the normal function of APP and its metabolites, and how disruption of these functions contribute to cognitive deficits typical of AD (Table s1.2, 10.1-10.2).

Transgenic Model	Gene, Mutation, Promoter	Behavioral Phenotypes	Age of Phenotype	Reference
PDAPP	hAPP	Hyperactivity	3, 6, 9 mo	Dodart et.al. (1999), Behavioral Neurosci
	V717F	Object Recognition (Lilly)	6, 9-10 mo	Dodart et.al. (1999), Behavioral Neurosci
	PDGF promoter	Radial Arm Maze	3 mo	Dodart et.al. (1999), Behavioral Neurosci
		Operant Learning (bar pressing)	3, 6 mo	Dodart et.al. (1999), Behavioral Neurosci
		Spatial Reference Memory,	3-4, 10, 13, 18 mo (non-progressive)	Chen et.al. (2000), Nature
		Serial Spatial Memory	13, 18 mo (progressive)	Chen et.al. (2000), Nature
		Cued Fear Conditioning	11mo	Gerlai et.al. (2002), Behav Brain Res
		Sleep/Wake Patterns	3-5 mo, 20-26 mo (progressive)	Huitron-Resendiz et.al (2002), Brain Res
		Holeboard Spatial Working Memory	3-5 mo, 20-26 mo (progressive)	Huitron-Resendiz et.al (2002), Brain Res
		Eyeblink Conditioning	6, 10mo (non-progressive)	Weiss et.al. (2002), Neurobiol Dis
Tg2576	hAPP	Hyperactivity, Open Field	17 mo	Lalonde et.al. (2003), Brain Res
	K670N,M671L	Y-Maze Alternation	10 , 16-18 mo	Chapman et.al. (1999), Nat Neurosci
	Hamster PrP	String Agility Test	3, 9 mo, female specific	King et.al. (1999), Brain Res
		Spatial Memory Retention	6-11, 12-15, 12-18, 2-25mo (progressive)	Westerman et.al. (2002), J Neurosci
		WM Spatial Memory Acquisition	6-11, 12-18, 20-25mo (progressive)	Westerman et.al. (2002), J Neurosci
		Holeboard Spatial Reference Memory	3, 7, 9 mo (progressive)	Pompl et.al. (1999), J Neurosci Meth
		Visual Cued Water Maze	3, 9, 19 mo (progressive)	King et.al. (2002), Physio Behav
		Open Field	10, 16 mo	Chapman et.al. (1999), Nat Neurosci
		T-Maze Alternation (forced)	10, 16 mo (progressive)	Chapman et.al. (1999), Nat Neurosci
		Contextual Fear Conditioning	16-18 mo	Corcoran et.al. (2002), Learn Mem
APP23	hAPP	Passive Avoidance Memory	25mo (progressive)	Kelly et.al. (2003), Neurobio Aging
	K670N,M671L	WM Spatial Memory	3, 6, 18, 25 mo	Kelly et.al. (2003), Neurobio Aging
	Thy-1 promoter	Retention, Acquisition	(progressive)	
		Hyperactivity	6-8 w, 3, 6 mo	Van Dam et.al. (2003), Eur J Neurosci
		Rotorod Performance	3, 6 mo	Van Dam et.al. (2003), Eur J Neurosci
		Open Field	3, 6 mo	Van Dam et.al. (2003), Eur J Neurosci
		Vascular Amyloidosis, Spontaneous Microhemorrhages	12 mo	Winkler et.al. (2001), Brain Res

Table 1.2 Behavioral phenotypes of transgenic mouse models related to AD. WM is an abbreviation for water maze, while the term "progressive" indicates a behavioral phenotype that is age-related, developing over time.

1.4 Relating cognitive deficits in Alzheimer's Disease to normal functions of APP and Aβ

The behavioral deficits seen in the hAPP transgenic mice are good correlates to the

cognitive impairments observed in AD patients, but it is yet unclear how the development of AD pathology mechanistically translated to cognitive impairments. From comparisons of the hAPP transgenic mice, it is evident that there are likely multiple mechanisms that could lead to AD-like behavioral deficits. In the Tg2576 animals there is a maintained synaptic density with age that is itself correlated to poor performance in spatial memory tasks like the Morris Water Maze, while decreased synapse numbers in PDAPP mice correlate to impairment in the same tasks. One explanation for these seemingly opposing results was that increases in Aβ impaired efficient synaptic transmission, which could arise from situations with either too many ineffective synaptic connections or too few synapses to make effective synaptic connections. To understand the basis of this cognitive impairment, researchers began to experiment with Aβ in a number of different experimental paradigms.

1.4.1 Neuronal and synaptic toxicity

At one level, Aβ could be considered to have a deleterious or toxic effect on neurons, and that cognitive impairment was due to the effects of accumulations of Aβ damaging neurons. Many studies have since demonstrated toxic effects of Aβ on cortical and hippocampal cells *in vitro*, via a number of pathways including apoptosis by activation of caspases, disruption of cellular Ca^{++} homeostasis, and generation of free radicals due to oxidative stress (Mattson et al., 1993; Yatin et al., 1999; Troy et al., 2000; Allen et al., 2001; Rowan et al., 2003). This neurotoxic property of Aβ is not straightforward, as neurotoxicity is ascribed to differing lengths of Aβ peptide, various structural assemblies related to amyloid fibril elongation, as well as cellular locations. It soon became clear that Aβ neurotoxicity is not alone among the mechanisms that promote cognitive impairment, as researchers found that adding diffusible Aβ to organotypic hippocampal slice cultures altered synaptic transmission prior to cellular degeneration, and that removal of Aβ itself causes neuronal aberrations (Freir et al., 2001).

It is known that the electrophysiological profile of young PDAPP mice in the

hippocampal region CA1 is perturbed prior to any amyloid deposition, showing inability to maintain LTP, with potentiated responses to high frequency bursts, and unusually high levels of paired-pulse facilitation. These physiological aberrances closely reflect cognitive spatial learning and memory deficits seen in young pre-plaque PDAPP animals, suggesting that there is an extended period of increasing synaptic dysfunction and subtle cognitive changes before any overt neurodegeneration or synaptic loss. Accordingly, Lambert and colleagues found that direct addition of small oligomeric Aβ-derived diffusible ligands (ADDLs) to rat hippocampal slice cultures immediately inhibited LTP, hours before cellular degeneration (Wang et al., 2002). Taken together these findings argued for a biphasic effect of Aβ on cognitive loss, from impaired synaptic plasticity in the early stages of AD followed by severe neuronal loss.

1.4.2 Neurotransmission

The rapid way in which Aβ addition alters neurotransmission hinted at Aβ acting directly on some critical aspect of synaptic plasticity. Decreased cholinergic transmission has long been a hallmark of AD and there are also changes in the numbers of cholinergic receptors and their binding profiles (Bartus, 2000). In young pre-plaque APPSwe mice, increased binding of the nicotinic AChR-specific neurotoxin α–bungarotoxin is highly increased compared to non-transgenic controls (Wang et al., 2000). This α-bungarotoxin binding enhancement is present until old age, and was specific to the nicotinic receptor binding upregulation, as binding to muscarinic AChRs decreases at older ages. Aβ1-42 is found to bind tightly to the α7 subunit of nAChR with picomolar affinity, and the same Aβ peptide is found to inhibit single-channel nAChR currents in rat hippocampal neurons (Pettit et al., 2001; Bednar et al., 2002). AChR signaling appears to be intimately linked to APP and Aβ, and they are described as modulators of each other's functions. Aβ at nanomolar amounts will suppress the expression of nAChRs, while nicotine itself has been found to have the ability to inhibit amyloidogenic fibrillization (Guan et al., 2001; Ono et al., 2002). Overall these results argue at a minimum that APP and Aβ have a role in modulation of AChR transmission.

AD is not simply a cholinergic disorder, as glutamatergic signaling dysfunction has also been recently described in AD models. Using Tg2576 mice, Cha and colleagues found that there is a subtle change in the binding properties of hippocampal AMPA receptor binding in aged mice (Cha et al., 2001). This increased binding affinity of AMPA is specific and restricted to regions with amyloid deposits, and does not extend to other glutamatergic receptors. Aβ is also found to affect the ability of astrocytes to clear glutamate in culture, as they were able to uptake glutamate at much higher levels due to upregulation of the cell-surface concentration of glial glutamate transporters, leading to a concomitant decrease in neuronal glutamatergic transmission (Ikegaya et al., 2002).

1.4.3 Neuritogenesis

Throughout these studies, efforts had been largely directed to discerning the scope of the deleterious functions of Aβ in AD, but now there was interest in learning more about the normative functions of APP and Aβ. Developmental experiments have shown that APP is highly expressed in the growth cones of the developing nervous system, and that APP induced neurite outgrowth both in developing and mature hippocampal cells (Storey et al., 1996; Small et al., 1999; Salinero et al., 2000; Neill et al., 2001). In mouse neuroblastoma cells, a group from Newcastle has found that transfection of APP751 caused a rapid differentiation and neuritic outgrowth, with more neurites per cell and overall shorter process extension period than untransfected cells (Neill et al., 2001). This effect is mediated solely by membrane-associated and not soluble APP751, although other authors have shown that purified substrate APP from sporadic AD brains is sufficient to promote neurite extension and considerable branching in cultured hippocampal cells.

1.4.4 Neurogenesis

Neurogenesis has been confirmed within areas of the brain that are highly plastic, namely the cortex and hippocampus. Neural progenitor cells (NPCs) are thought to

exist as a regenerative pool, providing replacements for cells that die due to normal turnover or injury, and are an important contributor to the neural plasticity underlying learning and memory (Parent et al., 1997; Doetsch et al., 1999; Nilsson et al., 1999; Schinder and Gage, 2004; Schmidt-Hieber et al., 2004). Several lines of evidence implicate Aβ as a disruptor of this regenerative process, both in vivo and in vitro (Haughey et al., 2002a; Haughey et al., 2002b; Ikegaya et al., 2002). NPCs normally proliferate in the hippocampus, however in APPSwe mice, NPC counts are lower than non-transgenic controls. Infusion of Aβ to the ventricles of these mice also results in greatly decreased numbers of NPCs migrating to the subventricular zone (SVZ) of the olfactory bulb (OB). Cultured rat and human hippocampal NPCs have reduced proliferation and differentiation upon exposure to Aβ compared to control protein infusions, due to disruption of the Ca^{++}-regulated homeostasis of these cells, which leads to apoptosis.

Taken together, the decrease in specific types of neurotransmission in the presence of Aβ, the ability of amyloid to promote neurite outgrowth and interfere with cortical and hippocampal neurogenesis suggest that Aβ broadly disrupts synaptic plasticity at a cellular level. These excess or gain-of-function properties can be interpreted such that the normative role of APP and Aβ is to modulate synaptic transmission, the cellular basis of learning and memory, and that AD amnesia is a consequence of amyloid disrupting these processes. The line of thinking that proposes amyloid as a normal-state modulator of plasticity is experimentally well motivated, but had to be further validated in loss-of-function paradigms in which APP and Aβ is absent.

1.4.5 APP-null mice

Using similar technologies that created the first hAPP transgenic mice, APP deficient mice were created. In conjunction with Merck, a group from the University of Chicago reported in 1996 that APP-null mice created by homologous recombination in embryonic stem cells are viable and fertile, without gross brain differences from controls (Zheng et al., 1996). Further publications about the

homozygous APP-null mice report decreased spontaneous locomotion, forelimb grip strength and weighed 15-20% less than age-matched wild-type mice (Zheng et al., 1995). In addition, APP-null mice have reactive brain gliosis in CA1 starting at 14 weeks of age. These findings indicate that complete deficiency of APP permits the viable development of the nervous system, but there are neuromuscular impairments of an unknown basis. Finer analysis of the APP-null mice revealed specific disruptions of hippocampal anatomy and plasticity (Dawson et al., 1999; Seabrook et al., 1999). Dendrites and synaptic terminals are decreased in the areas of CA1 with gliosis, and APP-null mice also have impairments in the generation of LTP trains, including glutamatergic inhibitory post-synaptic currents. Additionally, it was found that APP-deficient mice are hypersensitive to seizure induction, having earlier onset and higher mortality, that is correlated to decreased neurogenesis in the corpus callosum (Steinbach et al., 1998; Moechars et al., 1999).

1.4.6 Regulation of synaptic activity

In vivo APP-null mouse data supports a synaptic plasticity modulatory role for APP, which is strengthened by subsequent in vitro experiments. Researchers at Cold Spring Harbor Laboratories used an experimental design in which an APP695 minigene is acutely overexpressed in rat hippocampal slices with a resulting in depression of excitatory synaptic transmission (Kamenetz et al., 2003). This depression is specific to decreased excitatory currents in AMPA and NMDA transmitters but not GABA, with a reduction in firing frequency but not response amplitudes. In addition, the production and secretion of Ab peptides has been measured in the presence of pharmacological agents that increase or decrease neuronal activity. Production and secretion of Ab mirror the affects of these agents on neuronal activity, implying that Ab secretion and generation is controlled by neuronal activity. Further experiments in which the C-Terminal Fragment (b-CTF) that includes Ab and the cytoplasmic domains of APP is transfected into rat hippocampal slices show that this fragment was sufficient to depress synaptic transmission (Figure 1.12). A truncated form of b-CTF lacking the cytoplasmic tail is also efficient in depressing transmission. This APP-induced synaptic depression

requires neuronal activity, as agents that block neuronal activity like tetrodotoxin (TTX) prevent this decrease in neurotransmission, which returns once TTX is washed out. These negative feedback synaptic depression effects by APP are diffusible, and are seen to spread beyond individual cells infected with APP, causing local synaptic depression.

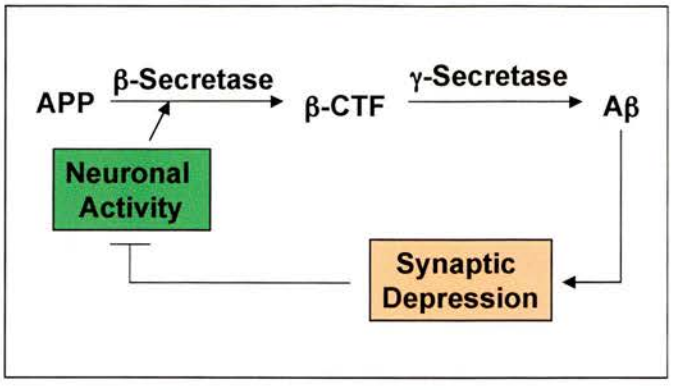


Figure 1.12 APP-Based regulation of Synaptic Activity, adapted from Kamenetz et.al., (2003). APP processing to Aβ forms a negative feedback loop which results in decreased neuronal activity. Neuronal activity stimulates the β-secretase activity, which cleaves APP to β-CTF. Processing by γ-secretase yields Aβ, which causes local synaptic depression. In turn synaptic depression inhibits neuronal activity, which can stimulate b-secretase.

APP is highly conserved throughout evolution, and it has been shown in an in vivo chick model to be a requisite for the formation of new memories. Administration of anti-APP antibodies prior to training on an inhibitory avoidance task prevents chicks from performing correct memory-driven response (Mileusnic et al., 2000). This amnesia lasts for over 24h, and is similar in another APP-removal paradigm, in which APP antisense sequences are injected prior to training. By restoring APP via injection of a synthetic APP pentapeptide, memory is rescued, suggesting that APP itself is required for early-phase memory formation and amnesia based on its absence is reversible.

Investigations focusing on uncovering the normal function of APP and Aβ demonstrate that these peptides have a wide range of effects on development, neurotransmission and synaptic plasticity, and at the same time Aβ production and

secretion are driven by neuronal activity (Panegyres, 2001). This suggests that APP and Aβ are synaptic modulators, and that they are involved in a negative feedback loop that acts to regulate neuronal hyperactivity. Unchecked accumulation of APP is neurotoxic and disrupts neural plasticity in plaque-dependent and plaque-independent ways, and understanding the regulation of Aβ has become a crucial focus point of therapeutics-minded research. Intact cognitive function lies somewhere between excess and deficient levels of Aβ, and efforts to understand the processes that mediate generation of Aβ intensified in the mid- to late 1990s. During this period critical discoveries about the entities responsible for the processing of APP were made, providing important new information about the mechanisms of APP enzymatic processing, as well as potential strategies for modulating Aβ burden in the brain.

1.5 Current treatment strategies for Alzheimer's Disease

Most of these new AD therapeutic targets are motivated by some form of the Amyloid Cascade Hypothesis, and employ various strategies to reduce Aβ burdens by addressing deposited plaques and/or soluble products of APP metabolism. These major strategies are summarized as follows: vaccination, amyloid capture, anti-inflammatory agents, cholesterol reduction, and inhibition of secretases involved in amyloidogenic processing.

1.5.1 Active immunization

Vaccination therapy for AD involves peripheral administration of the amyloidogenic peptide Aβ1-42 wholly or in part. Administration of Aβ1-42 combined with an immunoadjuvant is believed to stimulate the immune system to develop antibodies that recognize this fragment and clear it more effectively from amyloid deposits in the brain. This principle has been validated in hAPP transgenic mice, as PDAPP mice given Aβ1-42 over a period of time have a striking reduction in the number of plaques in aged mice and prevents the development of AD-like pathology when given to young pre-plaque mice (Schenk et al., 1999). Aβ immunization is also

effective in reducing amyloid burden in systems where APP generation is highly excessive and rapid, like in the CRND8 transgenic mice harboring APPSwe/Ind mutations (Janus et al., 2000). Using this animal model of early amyloid deposition, Aβ immunization results in a reduction in plaques as well as significant behavioral improvements in spatial learning and memory tasks.

The success of Aβ immunization in animal models of AD led to the initiation of clinical trials to test a similar vaccine in humans (Thatte, 2001). The initial Phase I clinical trials by Elan and Wyeth-Ayerst showed the vaccine to be safe in small numbers of healthy volunteers, but difficulties arose in the expanded Phase II trials in which the Aβ vaccine was given to 300 AD patients. 19 of these 300 patients developed an encephalomeningitis, which halted the trial (Check, 2003; Robinson et al., 2004). A subsequent report of similar vaccinations causing cerebral hemorrhaging in APP23 mice brought into focus the dangers of active immunization-mediated clearance of Aβ (Pfeifer et al., 2002). A subset of AD patients also have Central Amyloid Angiopathy (CAA), with Aβ deposits lining the endothelial cells of the brain vasculature, and removal of these vascular deposits could weaken these vessels walls, leading to hemorrhaging. While it is unclear what component of CAA–mediated hemorrhaging led to the encephalomeningitis, animal data suggests that it is also possible that the immune response itself caused an activation and migration of T-cells to the brain, causing central inflammation (Munch and Robinson, 2002; Furlan et al., 2003).

Examination of the patients from this clinical trial that did not incur this severe side effect have since shown that there is significant slowing of cognitive decline in patients who had generated antibody titres to Aβ, and further, that significant cognitive improvements were seen in patients with greater amyloid levels (Hock et al., 2003; Gilman et al., 2005). This is supported by autopsy data from a single patient's brain, in which there was a high level of amyloid plaque clearance and low number of dystrophic neurites (Nicoll et al., 2003). It is likely that there is some beneficial response of the patients due to the Aβ vaccination, however this positive finding is countenanced by the possibility of vascular side effects. While the proof

of concept driving Aβ vaccination therapy for AD is well founded, the risks of treatment in the presence of unknown levels of CAA are very high. Generally, vaccination in the elderly is a slow process requiring the use of a strong immunoadjuvant to elicit sufficient immune response (Martin, 1997; Castle, 2000). Concern about the risks of Aβ vaccination therapy may in part be mitigated with the use of a more sophisticated adjuvant that is incapable of causing T-cell migrations in future Aβ vaccination trials, and with exclusion of patients with suspected CAA. At this time Elan and Wyeth-Ayerst have initiated a second vaccine clinical trial that utilizes a new immunoadjuvant, and their progress in this new immunotherapy foray will be highly scrutinized..

1.5.2 Passive immunization

Passive treatment of AD by administration of antibodies for Aβ is another regime that has been successful in animal model research. While some of these antibodies act by binding to deposited amyloid, others bind to soluble Aβ, capturing the peptide before it is able to aggregate into plaques. This capture therapy is epitomized with by experiments with the antibody m266, which preferentially binds to the central domain of free amyloid and crosses the blood-brain barrier (BBB) at a low level. By peripheral administration of this monoclonal antibody, experimenters at Eli Lilly and Washington University were able to dramatically reduce amyloid deposits in PDAPP mice, while observing a 1000-fold increase in plasma concentrations of Aβ (DeMattos et al., 2001). Subsequent studies have shown that short-term treatments of m266 are able to reverse cognitive deficits in PDAPP object recognition without any visible changes to plaques (Dodart et al., 2002). The authors have sought to explain these observations by propagating an Amyloid Sink Hypothesis, in which removal of peripheral amyloid causes release of Aβ in central locations, diminishing plaque loads. This working hypothesis remains highly controversial. However, regardless of the debate surrounding the mechanism, it appears that the plaque-independent cognitive deficits seen in transgenic mice are now matched by a treatment that also does not directly address plaques. These reversals are possibly caused by rapid changes in synaptic plasticity. Indeed these

amyloid capture principles appears to operate on a general level as well, as peripheral administration of an agent with a simple affinity for Aβ that is incapable of passage through the BBB, gelsolin, is able to reduce amyloid burdens with behavioral improvements in Tg2576 mice (Matsuoka et al., 2003).

While peripheral administration of antibodies appears to be as successful as vaccination, it too has tough practical issues to face. Long–term delivery of antibody treatments remains susceptible to severe systemic immune reactions, even if such antibodies could be humanized without loss of potency (Stockwin and Holmes, 2003). In addition, widespread removal of soluble Aβ may have deleterious effects as the peptide becomes unavailable to perform any of its putative normative functions. Titration of the amount of capture agent will become critical in human tests. As these reports are yet only a few years old, more study is required to provide a better evaluation of these promising Aβ capture strategies in animal models before moving to clinical trials for AD.

1.5.3 Anti-inflammatory drugs

In some senses, AD can be viewed as an inflammatory disease, as there is widespread gliosis and activated microglia in AD brains, and these perturbed inflammatory processes promote the aggregation of amyloid deposits. At a certain level, some of these processes may in fact be protective or beneficial, like the activation of microglia that act as scavengers of Aβ. Other inflammatory processes in AD are deleterious, such as increased expression of cytokines that cause release of neuron-damaging free radicals; indeed there are working theories that feature AD simply as distal sequelae of prior inflammatory brain injury. In AD brains, there is an upregulation of cyclooxygenase-2 (COX-2), a potent mediator of inflammatory molecule production (Kitamura et al., 1999; Xiang et al., 2002). There are already a number of effective drugs used to reduce levels of COX-2, for the indications of inflammation and inflammatory pain, including a class called the Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Administration of NSAIDs has been experimentally found to reduce the production of Aβ both in cells and in hAPP

transgenic mice, and incrementally improve memory performance in APP/PS1 overexpressing mice receiving Aβ immunizations (Lim et al., 2001; Stephan et al., 2003). Epidemiological evidence exists that links long-term use of NSAIDs like ibuprofen and indomethacin with protection against AD, but not vascular dementia (in t' Veld et al., 2001).

Use of NSAIDs to protect against AD is an intriguing clinical strategy, as NSAIDs are widely available and inexpensive as a long-term therapy. However, given the wide range of NSAIDs with different actions available, selecting one or a few of them for a convincing clinical trial will be critical. Also, long-term treatment of AD by NSAIDs may require the development of new drugs altogether as many of them cause gastric bleeding with continued usage (Butt et al., 1988). Typically, the other medications used to treat these side effects are strong contraindications for high blood pressure, a common problem in the elderly. Finally, transgenic AD mouse model data suggests that NSAID treatments would be more efficacious in preventing development of AD only in conjunction with Aβ immunization, and may be less able to reverse established cognitive deficits altogether (Jantzen et al., 2001).

1.5.4 Cholesterol reduction

The cholesterol connection to AD remains a developing story, but one with extended clinical trial information. Cholesterol has been implicated in the metabolism of APP, glial cholesterol is required for the formation of synapses, and inhibition of cholesterol is associated with reduced dendritic outgrowth and apoptosis (Michikawa and Yanagisawa, 1999; Refolo et al., 2001; Ullian et al., 2001; Fan et al., 2002). AD brains are found to have an elevated level of the cholesterol degradation product 24S-hydro cholesterol, and subsequent treatment of AD patients with a drug that reduces cholesterol levels, Simvastatin, reduces Aβ1-40 in the patients' cerebrospinal fluid (CSF) (Lutjohann et al., 2000; Vega et al., 2003). However, this effect is seen only in moderate AD patients given exceedingly high doses. A follow-up clinical trial in which Simvastatin was given to AD patients at doses typical for simple reduction of cholesterol revealed no changes in plasma or

CSF Aβ (Simons et al., 2002). While these initial clinical trial results do not support a strong role for cholesterol inhibition as an effective strategy for treatment of AD, it is possible that it will be useful in treating those FAD patients with genetic mutations in the cerebral transport of cholesterol.

1.5.5 γ-secretase inhibition

As the endoprotease involved in the final cleavage step in the generation of Aβ, γ-secretase is itself a target for therapeutic inhibition in the treatment of AD (Esler and Wolfe, 2001). It has been proposed that the PS1 and PS2 components of the γ-secretase holoenzyme contain the specific cleavage activity needed to generate Aβ, and several groups have investigated this hypothesis with transgenic mice. Transgenic mice that overexpress PS1 are found to have spontaneous seizure activity, neurodegeneration, and increased Aβ1-42 production (Siman et al., 2000; Schneider et al., 2001; Huang et al., 2003; Jankowsky et al., 2004; Wen et al., 2004). PS2 mutations and transgenic mice are similarly associated with behavioral impairments and increased Aβ1-42 production (Oyama et al., 1998; Sawamura et al., 2000; Hwang et al., 2002). In contrast, PS1-null mice die shortly after birth, have deformed skeletons, and are deficient in neurogenesis (Shen et al., 1997).

It is believed that γ–secretase deficiency affects Notch signaling in cellular differentiation and causes cell death, as this is one of the substrates for γ-secretase. In 2001, a series of γ-secretase functional inhibitors were reported to reduce Aβ secretion up to 80% in cultures expressing APP751 as well as PDAPP mouse brains, without overt cellular toxicity (Dovey et al., 2001). In addition, conditional PS1 knockout mice in which the inactivation of the gene is restricted to the postnatal forebrain are viable, with only subtle spatial memory deficits (Yu et al., 2001). Furthermore, recent studies featuring mice with conditional knockout of PS1 and overexpression of mutant hAPP display a phenotypic rescue from their expected cognitive deficits (Chen et al., 2003; Saura et al., 2005). While this effect is more prominent in younger mice, this data serves as a strong in vivo proof-of-principle for γ-secretase inhibition as an AD target. These studies provided hope for development

of safe γ-secretase inhibitors that effectively reduce Aβ generation while leaving Notch substrate cleavage intact, a critical feature in the design of any future γ-secretase inhibitor therapeutics.

In reviewing these many proposed therapeutic approaches to AD, we now understand the depth and breadth of the difficulties facing so many of these targets. This is an opportune time to turn our attention back to the β-secretase, which has remained throughout these developments an attractive target precisely because it does not share many of the other therapeutic candidates' practical and theoretical limitations.

1.6 Identification of the β-Secretase

After the discovery in 1994 that the Aβ peptide is processed by 3 different proteases in 2 distinct pathways, focused research efforts identified and characterized the α- and γ-secretase activities, while the β-secretase remained unknown. In late 1999, there was a sudden flurry of publications heralding the identification of the protease, which was independently found by four different groups of investigators using four different experimental strategies. The multiple ways that the β-secretase enzyme was independently discovered served to definitively prove that unlike the α- and γ-secretase enzymes, β-secretase is a single enzyme with the ability to generate the amyloidogenic fragment Aβ1-42.

The first report identifying the β–secretase came from investigators at SmithKline Beecham in September of 1999, who submitted abstracts to the International Aspartic Proteinase Conference in Portugal (Hussain et al., 1999). Using a proprietary database, Hussain and colleagues isolated a partial aspartyl protease cDNA sequence, using it to probe against the full-length cDNA in a melanoma cell line. The protease is an aspartyl protease-like enzyme dubbed Asp2, and is found to exist in cell lines known to produce Aβ. In a series of critical cell line transfection experiments, Hussain and colleagues demonstrated that transient expression of Asp2

increases production of Aβ. In addition, mutations directed at the catalytic aspartyl sites of Asp2 reduces β–site cleavage of APP. Asp2 was found to co localize with APP751, and fulfills other requirements of a putative β–secretase, as it was localized to the ER and TGN.

Researchers at Amgen quickly published their findings in October 1999 with an enzyme that they called β–site APP Cleaving Enzyme, or BACE (Vassar et al., 1999). Using a high-throughput library of cDNA clones to transfect cells carrying the APP Swedish mutation (APPSwe), overexpression of the BACE clone increases the output of β–site cleavage products. BACE acts at the known β–secretase cutting positions, and antisense inhibition endogenous BACE mRNAs results in reduction of β–site cleavage products. BACE also exists in the TGN and ER compartments, and is present in all tissues of the body, but is highly expressed in AD brain.

By utilization of a public database for predicted aspartyl proteases in the worm *Caenorhabiditis elegans* scientists at Pharmacia tested human orthologues in cell lines carrying the Swedish APP mutation (Yan et al., 1999). By applying antisense constructs, a reduction of the products of β–site APP cleavage was found using a sequence from a gene they called Asp2. Soluble Asp2 cleaves synthetic APP substrates at the expected β–sites, with a greater rate of cleavage in substrates with the Swedish mutation. Examination of Asp2 showed its distribution across tissues, most notably in the brain, and reveal Asp2 to be a membrane-bound protease, a predicted requirement of the β–secretase activity.

The final group to independently identify β–secretase in 1999 was from Elan, the only group who used biochemical methodologies (Sinha et al., 1999). Employing a substrate analogue inhibitor of the β–secretase activity, Sinha and colleagues were able to purify a single protein. This purified enzyme was sequenced from the N-terminus, and is a membrane-bound protease able to cleave APP into the full-length β–site cleavage products. Unlike α– and γ–secretase activities, BACE is the sole β–secretase activity for neurons, making it a prime target for strategic inhibition to reduce amyloidogenic fragment processing.

These independent characterizations were the beginning of the explosion of research into the β–secretase enzyme, which became a focal point of research in the ensuing years. Other groups continued to independently describe the β–secretase, and it now has many names, like Asp2, Memapsin2, and BACE. While the β–secretase protease will be called BACE for the remainder of this paper, all of the variously named β–secretase enzymes share the same subcellular and regional distributions, all have a single transmembrane-spanning region, and are similar in activity and sequence to an aspartyl protease.

1.6.1 Investigating BACE

After the isolation and identification of BACE, new information about this APP protease came at a rapid pace. BACE is mapped to Chromosome 11 by using expression sequence tags matched against the GenBank database (Saunders et.al., 1999). By using the BACE amino acid sequence to screen against a cDNA database, a second novel aspartyl protease was found, BACE2 (184). BACE exists as a 51 or 70kDa protein, depending on the level of N-linked glycosylation, and is derived from one of 3 transcripts of 2.6, 4.4 or 7.0kB in length. BACE has about 52% amino acid homology to BACE2, and both enzymes share many features of a putative β–secretase activity. However, BACE2 is not likely to be a major β–secretase in neurons, as antisense constructs targeting BACE reduce β–site cleavage of Aβ to near zero levels in the presence of functional BACE2 (Vassar et al., 1999). Further human genetic database examinations have thus far been unable to find any gain-of-function mutations of the BACE gene that strongly correspond to FAD, although certain polymorphisms of BACE and APOE4 genes increase the risk for AD (Gold et al., 2003; Liu et al., 2003).

Experiments designed to elucidate the localization and expression of BACE in AD brain provided an enhanced theoretical rationale for BACE inhibitor therapies for AD. Levels of BACE are elevated in AD brain compared to aged controls, and are higher in cortex and hippocampus, as well as the subiculum and entorhinal cortex

(ERC) (Sun et al., 2002). While BACE levels were high in the CA2-4 and ERC and moderate in the dentate gyrus (DG) and CA1 regions of the hippocampus, BACE levels do not directly correlate to senile amyloid plaques and NFTs have low levels of BACE. Other authors found significant increases in both BACE expression and activity in the neocortex of AD brains, as well as the thalamus and amygdala (Fukumoto et al., 2002; Tyler et al., 2002).

Once it is synthesized at the ER, BACE contains a short signaling and propeptide domain that is cleaved prior to trafficking outside the golgi. Interestingly enough, this proprotein domain is not inhibitory, as purified pro-BACE is still capable of cleaving the N-terminus of APP substrates (Shi et al., 2001). Instead, it appears that this form of proBACE acts like a chaperone to the protease domain of BACE by promoting proper folding of the active enzyme. The cleavage of the BACE propeptide domain to the mature enzyme is mediated by a family of proprotein convertases including Furin, which cleave propeptide domains at RLPR|E amino acid sequences in the TGN, ER and endosomes (Bennett et al., 2000; Creemers et al., 2001). This processing occurs largely at the golgi, as experimental agents that interfere with the golgi, like brefeldin A or monensin also prevent propeptide removal.

After BACE is processed to maturation, it is post-translationally modified with N-linked glycosylation at the golgi at any of four potential sites (Huse et al., 2000). This carbohydrate modification is important to the function of BACE, as site-directed mutagenesis removal of two of the four sites of glycosylation reduces BACE activity on APP. While BACE appears to have no O-linked carbohydrate alterations, N-glycosylated sites are sulfated and cysteine residues in the cytoplasmic end of BACE are palmitoylated (Benjannet et al., 2001). Most cellular BACE enzyme is thus restricted to the membrane, as the increased polar anchoring due to palmitoylation acts to prevent ectodomain shedding of transmembrane proteins. Recent experiments show that ectodomain shedding of BACE is stimulated by Protein Kinase C (PKC) and the metalloproteinase ADAM 10 is the likely BACE sheddase (Hussain et al., 2003). Interestingly enough, inhibition of BACE shedding

has no effect on β–site cleavage of APP. The small fraction of BACE that is released from the membrane and becomes soluble promotes generation of Aβ, due to reinternalization to endosomes where the majority of APP processing occurs, and this remains a possible mechanism for pathogenic increases of amyloidogenic fragment levels.

The trafficking of BACE is more complicated than what was originally predicted for the putative β–secretase. BACE localizes to the TGN and ER, but the long amyloid fragment Aβ1-40/42 is produced by BACE in the ER whereas a truncated Aβ11-40/42 fragment is produced in the TGN (Huse et al., 2002). The presence of various signaling moieties determines the location of the BACE enzyme. The same single transmembrane domain that is requisite for BACE cleavage activity access to APP and the subsequent generation of the C99 peptide contains a cytoplasmic golgi trafficking signal that causes retention of this form of BACE in the TGN (Yan et al., 2001b). The fraction of BACE that is sequestered at the ER lumen is kept in place by a C-terminal dilysine motif that prevents complex carbohydrate processing for proteins bound for the golgi. BACE was also found in the endosomes and at the plasma membrane, which requires a cytoplasmic cysteine motif for trafficking to the cell surface (Capell et al., 2000).

1.6.2 BACE regulation, interactions and other substrates

Information regarding other substrates and modulators of BACE activity is emerging, and other proteins involved in the generation of amyloid may activate BACE. A recently characterized component of the γ–secretase holoenzyme, Nicastrin, is mapped to Chromosome 1 and mutations in this gene are responsible for subset FAD patients of a village in Italy (Feldman et al., 1963; Yu et al., 2000). Nicastrin forms complexes with PS1 and PS2 and is requisite for the γ–secretase activity, and recent immunoprecipitation data showed that BACE and Nicastrin bind in cells (Hattori et al., 2002). Nicastrin was also found to be able to activate BACE in COS-7 cells. Similar studies focusing on PS1 show binding to BACE as well, as they coprecipitate in human cortical cell immunoblots (Hebert et al., 2003). PS1

binds preferentially to immature BACE, suggesting a possible role for PS1 in BACE maturation or activation.

Evidence also exists that suggests a certain golgi-resident sialyltransferase is also a substrate for BACE cleavage (Kitazume et al., 2001). BACE and ST6Gal co localize together in the golgi, and when BACE is over expressed in COS cells the post-cleavage secretion of ST6Gal increases, and is reduced via proposed inhibitory competition in the presence of APPSwe. Subsequent work by the same group has definitively shown that the ST6Gal protease is cleaved by BACE between L37 and G38 (Kitazume et al., 2003). The only other known non APP-processing pathway substrate for BACE is the P-Selectin Glycoprotein Ligand-1 (PSGL-1), which is involved in the highly pleiotropic inflammatory leukocyte adhesion process (Lichtenthaler et al., 2003). Transfection studies of BACE transcripts to human monocytes and hek293 cells show PSGL-1 cleavage, which is absent in cells lacking BACE.

While BACE2 shares much gene (52%) and amino acid sequence (68%) homology with BACE, its activity is markedly different, as it is capable at cleaving at the β–site, but cleaves APP preferentially at sites that resemble α–secretase cleavage, F19 and F20 (Yan et al., 2001a). BACE2 has been implicated in the development of AD in Down's Syndrome patients as there is trisomy of the BACE2 gene at Chromosome 21. The Flemish mutation of APP (A21G) is adjacent to the preferred site of action of the BACE2, suggesting that BACE2 may have a role in the pathogenesis of Flemish FAD (Farzan et al., 2000). While BACE2 is autocatalytic and active at a range of pH value unlike BACE, it is also subject to maturational cleavage by BACE (Shi et al., 2001; Kim et al., 2002). This may be an important regulatory mechanism, as high expression levels of BACE will activate the α–secretase-like BACE2 and reduce amyloidogenic Aβ via competition for APP substrates. In addition RNA inhibition studies indicate an antagonistic relationship between BACE and BACE2, in which BACE2 acts to suppress BACE activity on APP (Basi et al., 2003).

BACE may also be acting on the APP substrate both before and after PS1-dependent γ–secretase cleavage. This new activity of BACE is thought to be responsible for the appearance of truncated Aβ species Aβ1-34, as overexpression of BACE in Hek293 cells showed a reduction of the long form Aβ1-40/42 (Fluhrer et al., 2003). While an alternative γ-secretase cleavage site could also explain the appearance of truncated Aβ, in vitro experiments in conditioned media proved otherwise. Purified BACE incubated with Aβ1-40 generated Aβ1-34 species, even when BACE and Aβ1-40/42 were incubated in cells with a loss of function mutation of PS1. This γ–secretase-like activity of BACE was unexpected, and suggests that BACE may play a role in the clearance of Aβ, as this second cleavage removes the most hydrophobic residues of the Aβ peptide. It is postulated that this shortened Aβ species may be a better substrate for the proteases that normally facilitate clearance of Aβ, the Insulin-Converting Enzyme (ICE) and Neprilysin. This is not the first example of BACE having other APP fragment substrates, as BACE is also capable of using C99 to cleave and generate the C89 fragment (Liu et al., 2002).

1.7 Rationale for BACE inhibition as a treatment strategy for Alzheimer's Disease

The human β–secretase has been hotly pursued for both basic science and therapeutic purposes, based on the rationale that if β-secretase activity could be inhibited sufficiently to reduce production of Aβ, the cognitive impairments related to Aβ could be alleviated. Subsequent discoveries about the identity, function, and amyloid-promoting properties of β-secretase have intensified its value as a prime candidate for therapeutic inhibition for AD. Unlike γ–secretase, BACE appears to be comprised of a single protease, and thus far has a much more limited number of substrates than γ–secretase. Also, there appears to be no mutations of the BACE gene that lead to early- or late-onset FAD, unlike APP, PS1, and PS2, implying that various allomorphs of BACE and presumably BACE activity allow for normal generation of APP without predisposition to AD.

Recent experiments examining the expression and activity levels of BACE in AD brain have shown that the expression is increased by 15% and activity is increased greatly from 63-185%. These are dramatic upregulations given the long half-life of proBACE and BACE, which is greater than 9h and 16h respectively (Vassar, 2002). Given that BACE is highly expressed in neurons of the brain compared to other tissues, a BACE inhibitor that effectively reduces Aβ levels in the brain may have the benefit of inherent tissue specificity, if activity of the primarily non-neuronal BACE2 is spared.

Peptidergic inhibitors of the active site of BACE have been utilized in resolution of the BACE crystal structure (Hong et al., 2000; Hong et al., 2002). These structures will prove vital to the rational design of BACE inhibitors, which can also draw on lessons from the successful development of other drugs aimed at inhibiting aspartyl proteases, namely the HIV protease (Beck et al., 2002). Like the HIV protease, the first substrate-based BACE inhibitors designed were organic compounds, as peptides are unlikely to penetrate the BBB and accumulate in the acidic cellular compartments preferred by BACE (Beck et al., 2002; Tung et al., 2002; Hom et al., 2003). While the design of an efficacious BACE inhibitor will be a challenging task, by many respects BACE remains the most promising target for AD therapeutic intervention, and must be pursued.

1.7.1 Current genetically modified BACE mouse lines: proof of principle for BACE inhibition strategies for Alzheimer's Disease

With the rapidly acquired wealth of knowledge about BACE and its activities, there is also an important drive to investigate the role of BACE in living systems, and so the first experiments examining BACE in vivo were commissioned. The first publications regarding BACE in living systems are experiments dissecting the spatial patterns of expression and activity in various tissues. BACE is as ubiquitously expressed as its major substrate APP, with the most notable expression in regions of the brain, pancreas and muscle fibre. In AD brain, BACE expression is highest in the frontal and temporal cortex (15% of aged controls), with expression

patterns in the hippocampus that are at present controversial, with reports of AD brain BACE levels modestly higher than aged controls in some studies and equivocal in others (Fukumoto et al., 2002; Gatta et al., 2002). BACE activity levels are also elevated by 63-185% in the temporal cortex and 15% in the frontal cortex (Fukumoto et al., 2002; Tyler et al., 2002).

Transgenic animal models overexpressing mutated hAPP were also assayed for BACE expression and results from the Tg2576 and PDAPP lines have been published. In both transgenic lines, BACE mRNA is detected at high levels in the cortical and limbic regions that develop amyloid plaque burdens and express transgenic hAPP, but BACE is also detected in regions not known to develop plaques, like the cerebellum (Irizarry et al., 2001; Rossner et al., 2001; Fukumoto et al., 2004). The temporal expression patterns of BACE in the brains of these transgenic animals are static, with no age-related changes in BACE protein levels even at ages when significant amyloid deposition is ongoing. While these results are descriptive of expression of the BACE gene product, it does not directly relate to BACE activity levels, which could be different from the expression patterns (Fukumoto et al., 2002; Fukumoto et al., 2004). Still, this data, along with the results indicating that BACE enzyme is not specifically localized to amyloid plaques, suggests that the role of BACE in the production of Aβ is important but subtle (Bigl et al., 2000; Rossner et al., 2001; Hartlage-Rubsamen et al., 2003).

1.7.2 hBACE1/BACE1 KO mice

Further information about the absolute effects of BACE1 function came from the report of Harrison et al. (2003), who described their characterization of BACE1 KO mice created by targeting the BACE1 gene with a LacZ reporter construct (Harrison et al., 2003). The authors also produced a transgenic BACE1 mouse line with a human BACE1 cDNA linked to a LacZ reporter gene, driven to overexpression by the CaMKIIα promoter. Visualization of the LacZ β-galactosidase expression revealed that the hBACE1 of the transgenic mouse was localized to the hippocampus, cortex, caudate putamen and caudate striatum, while the BACE1 KO

had LacZ expression largely in the hippocampus. Expression of transgenic hBACE1 protein was 4-10x that of endogenous murine BACE1, while BACE1 KO mice completely lacked the carboxy terminal fragments expected of β-secretase cleavage of APP.

Behavioral phenotyping of 6-7 week old BACE1 KO and transgenic mice was performed with the full observational battery, holeboard exploration, and the plus maze, with a number of significantly different results between the two lines. Transgenic hBACE1 mice weighed less, and were more explorative in the open arms of the elevated plus maze and visited more holeboard holes than control mice. In contrast BACE1 KO mice were more timid, with greater fecal output during observation, spent less time and moved shorter distances in the inner regions of an open field, and were inclined to examine fewer holes in the holeboard task. The BACE1 KO mice displayed stronger limb tone in resistance to pushing and an improved righting reflex compared to control animals.

The bold and timid phenotypes associated with the BACE1 transgenic and KO mice respectively suggest involvement of BACE1 activity in anxiety-related phenotypes. Harrison et.al. (2003) conducted a broad array of neurochemical analyses in a number of brain regions between the hBACE1 transgenic and control mice in addition to their behavioral phenotyping. Alterations in the levels of 5HT, 5-HIAA, homovanillic acid (HVA), and dopamine (DA) were noted across several brain structures. Transgenic hBACE1 mice had increased turnover of 5HT in the cerebellum, hippocampus, hypothalamus, nucleus accumbens (NA), and caudate striatum (CS), accompanied by increased levels of 5-HIAA in the same regions. DA levels in hBACE1 mice were reduced in the hypothalamus and NA, with increased DA turnover in the hypothalamus and CS, with a decreased DA turnover in the hippocampus. Limited neurochemical analysis of the BACE1 KO mice revealed decreases of 5HT and DA turnover in the hippocampus, as well as an increase of total DA in the striatum.

These serotonergic and dopaminergic changes as well as the bold and timid phenotypes of the hBACE1 and BACE1 KO mice argue respectively for a role in anxiety behaviors for BACE1. This report by Harrison et al. (2003) is the first published report that casts a cautionary light on BACE1 inhibition as a therapeutic strategy for AD. However, the authors are careful to point out the caveats of translating their results directly to possible effects of AD therapeutics, as their results are derived directly from manipulation of BACE1 gene expression, whereas human disease intervention is likely to result from inhibition of protein activity.

In a similar experimental concept to that of the hAPP x PS1 mice, Mohajeri et.al. developed mice that were crossbred from Tg2576 and a line of hBACE mice under the control of the Thy1.2 promoter (Mohajeri et al., 2004). While hBACE mice alone had intracellular amyloid accumulations and did not develop plaques, the APP+BACE mice had accelerated amyloid pathology, with high levels of total Aβ and Aβ1-42, and greater numbers of plaques than the hAPP mice alone. APP+BACE mice have widespread amyloid plaques, visualized in the motor, sensory and somatosensory cortices, as well as the hippocampus. Indeed, even at 4mo of age the doubly transgenic APP+BACE mice had amyloid plaques and amyloid deposits in blood vessels. Behavioral testing of this recent AD transgenic model will be illuminating, as it will present new information on the role of BACE in cognitive processes that are altered in AD.

Finally, Willem et al. (2004) reported the development of a mouse line that featured both hBACE1 and the London V717I hAPP mutation under control of the neuron-specific thy-1 promoter (Willem et al., 2004). The resulting BACE x APP[V7171I] mice, like the APP+BACE mice reported by Mohajeri et al (2004), had increased deposition of hippocampal plaques. Interestingly, the use of a parental hAPP line with the V717I mutation also yielded a hitherto unreported increase in truncated and C-terminal Aβ fragments which in turn were correlated to a dramatic 2-4fold decrease in vascular amyloid deposition in 16 and 22mo old mice. The features of this AD model underline the array of pathologies that can arise from divergent

hAPP mutations, and provide a novel framework from which to assess factors that impact parenchymal versus vascular amyloid deposition.

1.7.3 BACE1 KO x transgenic hAPP mice

Other BACE1 KO mice were developed and subsequently bred to transgenic lines that overexpress human mutant APP. One such line featured BACE1 KO on a Tg2576 background (BACE1 KO/APPtg) (Luo et al., 2001). The double transgenic mice of this line do not develop amyloid plaques, a finding that is replicated with another model, the BACE1 KO/PDAPP mouse, which is the experimental focus of this thesis. One exciting experimental hypothesis that could be explored in double transgenic mice was whether removal of BACE1 not only prevented development of amyloid plaques but also prevented the development of cognitive deficits typical of hAPP transgenic mice.

At the SfN meeting in 2003, Ohno and colleagues presented their work using the BACE1 KO mouse line developed by Luo et al (2001) bred to Tg2576 mice, and which was later published in 2004 (Luo et al., 2001; Ohno et al., 2004). The resulting BACE1-/-Tg2576+ mice were tested in social recognition and y-maze alternation memory tasks at the age of 4-6 months. In addition, hippocampal slices from these and other control mice were tested for neuronal excitability profiles specific to cholinergic function.

While Tg2576 mice at 4-6mo do not have significant amyloid deposits, they do have high levels of total brain amyloid, which are related to their pre-plaque cognitive deficits (Westerman et al., 2002). The Tg2576 mice in Ohno et.al.'s study have significant impairments in social recognition, as measured by a social recognition index, or percentage of time spent investigating a mouse that they had been previously exposed to prior to a 3-hour separation. In contrast to wild-type animals, BACE1-/- and BACE1-/-Tg2576+ mice displayed recall of the previous social encounter, with a reduction in time spent investigating the familiar mouse. In Y-maze alternation, Tg2576 mice had lesser spontaneous arm alternation compared to

wild-type animals, while BACE1-/- mice had alternation levels that was intermediate between the two. In contrast, BACE1-/-Tg2576+ mice had wild-type-like levels of Y-maze alternation. In counts of arms entered, BACE-/- mice had a phenotype like that of wild-type animals, while both Tg2576 and BACE-/-Tg2576+ mice had a significantly higher number of arm entries than wild-type control animals.

Examination of cholinergic function was assessed by measuring post-burst afterhyperpolarization (AHP) in the CA1 region of the hippocampus. Administration of the pharmacological agent carbachol (CCh) acts to inhibit the slower component of the AHP without affecting the overall peak of neuronal activity, and in response to depolarization in the presence of CCh, Tg2576+ brain slices show lesser neuronal excitability compared to all other genotypes. BACE1-/- and BACE-/-Tg2576+ hippocampal response to CCh was like that of wild-type mice, suggesting normal capacity for neuronal excitability.

Within the parameters of these experiments, it appears that removal of BACE1 in the presence of hAPP overexpression ameliorates certain cognitive and physiological deficits associated with Aβ, although BACE1 KO itself confers an aberrant phenotype in one aspect of Y-maze exploration. Both Y-maze alternation and social recognition tasks rely on some aspect of normal hippocampal function, but their relationship to clinical AD impairments is unclear. However the AHP data from Ohno et al. (2004) suggests that the hippocampal cholinergic function that underlies learning and memory processes itself is restored in BACE-/-Tg2576+ compared to Tg2576+ mice. The functional improvements described by Ohno et.al. are based in non-aversive, internally motivated tasks, and conceptually the processes that underlie these tasks exist on a continuum of cognitive function with aversive spatial memory tasks like the water maze. It is possible that certain types of cognitive processes are differentially sensitive to the presence of functioning BACE1 enzyme, or even that BACE1 and its substrates may play an entirely different role in various cognitive processes. Finally, it is interesting to note that genetic BACE1 removal in the presence of hAPP overexpression in Tg2576 mice

ameliorates histological pathology and reduces particular types of cognitive deficits. In addition, experimental data indicates the converse is also true, as BACE1/hAPP transgenic mice have accelerated histological pathology, again emphasizing the importance of maintaining critical levels of biochemical entities involved in cognitive function.

Given the broad expression of BACE throughout the body, with higher levels in the pancreas and brain, it is somewhat surprising to find no significant abnormalities or mechanistic toxicity in BACE knockout mice had been reported in the published literature. Concerns about possible mechanistic toxicity due to actions on an unknown substrate of BACE similar to the case of γ–secretase and Notch were allayed with this data, although the full learning and memory profiles of these BACE-null animals are yet unknown. While previous genetic knockout mouse model work defined the need for APP and other amyloid processing pathway entities like Aβ and the presenilins for normal function in behavioral memory tasks (and even fundamental postnatal development), the removal of BACE may cause lesser phenotypes. It is possible that the products of an intact α–secretase Aβ pathway and intact BACE2 will be sufficient to allow normal cognitive performance, alternatively, Aβ could be requisite for the synaptic plasticity that underlies highly regulated learning and memory processes.

1.8 Evaluation of behavioral and spatial memory phenotypes of BACE KO x PDAPP mice: using a genetically modified animal model to approximate the risks and benefits of therapeutic BACE inhibition

Evaluation of the cognitive status of the BACE knockout animals using standard aversive spatial memory tasks like the MWM would answer many of these questions regarding deleterious effects of BACE removal on synaptic plasticity. A series of behavioral studies that approximate the effects of BACE reduction in various genetic paradigms would shed light on the function and necessity of various products of APP metabolism in the processes underlying learning and memory.

A reasonable initial proof-of-principle experiment would entail behavioral and histological testing of BACE knockout mice on a PDAPP background at various ages to catalogue the range of possible effects of BACE removal in AD. While these analyses would be informative about theoretical mechanistic toxicity from complete removal of BACE, an absolute genetic knockout of BACE on an APP overexpressing background is not directly analogous to the situation that would arise from therapeutic BACE inhibition. Any putative BACE inhibitor would likely be a small molecule drug that acts to bind the excess BACE enzyme that exists in the neurons of AD patients.

This dissertation will feature the behavioral and histological analyses of BACE homozygous and hemizygous mice on a PDAPP background. Indeed, a much more relevant therapeutic model would involve a *reduction* of BACE on an human mutant APP overexpressing background. This can be accomplished with conditional mutants of BACE, or alternatively with a mouse line that has a partial deletion of the BACE gene. Heterozygous BACE knockout mice overexpressing hAPP mutations would more closely approximate partial BACE inhibition than even conditional mutants of BACE, as some level of BACE activity would be retained throughout any potential BACE inhibition via small molecule drug. Given what has been discussed about the effects of removal of APP, overexpression of Aβ and the potential role for the APP processing pathway in regulating neuronal activity, here are some of the potential outcomes from behavioral analysis of these BACE KO x PDAPP lines:

- Deletion of BACE on a background overexpressing Aβ could rescue the mouse cognitive deficits associated with the PDAPP transgene.
- Deletion of BACE and subsequent loss of β-CTF and Aβ could worsen the phenotype if these metabolites and/or some other substrate of BACE are required for normal learning and memory as well as neuronal activity regulation in mice.
- Deletion of BACE could produce an intermediate phenotype that improves/worsens the cognitive phenotype of PDAPP mice, dependent on

the dosage of the gene (e.g. partial vs. complete BACE gene deletion).

- Deletion of BACE could have no effect the PDAPP mouse phenotype.

Thus, the behavioral and histological studies with both BACE homozygous and hemizygous mice crossed to the PDAPP line can provide valuable cautionary or validative information for the therapeutic BACE inhibition rationale for AD. Finally, these BACE KO x PDAPP mouse studies would also examine the role of APP, BACE and Aβ in normal and pathological learning and memory processes.

1.9 Summary

Alzheimer's Disease is the major debilitating and ultimately terminal neurological affliction of the elderly. As the world's population ages, AD will become a public health crisis of global proportions if no disease-modifying prophylactic or treatment is developed. In the past two decades there have been numerous critical discoveries about the evolution of AD pathology, and the amyloid-beta peptide has emerged as a major culprit in the pathogenesis of this disease. Various experimental strategies to reduce or otherwise modify the levels of Aβ are currently being developed, ranging from administration of Aβ vaccines, Aβ antibodies, anti-inflammatory agents, to inhibitors of the secretase enzymes that generate mature amyloidogenic Aβ. Therapeutic inhibition of the recently identified BACE enzyme is an appealing method of Aβ control, as it is solely responsible for β-site cleavage of amyloidogenic Aβ, BACE activity is highly elevated in AD brains, inhibitor design is aided by the known BACE crystal structure, and BACE-null animals are viable and fertile.

To help determine the true benefits and possible deleterious effects on synaptic plasticity and function of therapeutic BACE enzyme reduction, detailed behavioral analyses of BACE-modified mice on various genetic backgrounds must be initiated. Spatial memory testing of homozygous and heterozygous BACE knockout mice on hAPP mutant and BACE2 knockout backgrounds would provide significant information about the therapeutic and toxic effects of BACE reduction in AD, and

generate information on the requirements of the various APP metabolites and BACE substrates in learning and memory. Even before it was identified, the BACE activity of APP was the focal point of intense research efforts in the field of AD. This dissertation will focus on the behavioral and histological analyses of BACE homozygous and hemizygous mice on a PDAPP background, in hopes of answering some of the questions regarding the value and theoretical risks of AD therapeutic BACE inhibition, and the role of the amyloid-processing pathway in normal and perturbed memory function.

Chapter 2 Materials and Methods

2.1 General practices

2.1.1 Animal use

Examination of the phenotypic characteristics of genetically modified mice and tissue from such mice was the experimental basis for this dissertation. All mice used in these experiments have been cared for and studied in a manner in accordance with general Institutional Animal Care and Use Committee policies, as well as with the specific animal research protocols in place at Elan Pharmaceuticals, South San Francisco, CA, USA.

2.1.2 Animal care

Mice were raised offsite by a protocol-approved vendor (Charles River Laboratories or Taconic Farms) and transported by air and truck to Elan Pharmaceuticals in South San Francisco (Elan SSF). After shipment the mice were transferred to their permanent caging within the Research Animal Facility (RAF), and given *ad libitum* access to food and water. The maximum number of adult animals per cage was 4, and every effort was made to cage multiple animals of the same gender together, to reduce socialization anxieties. Animals were maintained in ventilated rooms with 24 complete air changes per hour, with a microisolator cover on each cage to reduce spread of airbourne diseases. All animals were housed under a 12 hour light/dark cycle, and all experimentation was conducted during the hours of light between 0700 to 1900. Animals are allowed to acclimatize for one week following shipment.

2.1.3 Animal handling

After acclimatization, mice were handled and examined prior to testing. Handling included 2-4 sessions of gentle restraint by tail, transfer between cage to table surface and transfer to the experimenter's hip in a manner akin to what would be required during later behavioral testing. Upon initial handling animals were examined for any injuries or abnormalities that would exclude them from further study, including: wounds and other open skin lesions, abnormal or damaged limbs, tumors, obvious blindness, pregnancy, pathological circle-pacing or spinning, hemiplegia and seizure.

If at any time during later behavioral testing a mouse displayed any of the aforementioned exclusionary characteristics it was removed from further study. All efforts were made to ensure a clean and quiet experimental environment for the research animals in these studies.

2.1.4 Preparation and collection of animal tissues

After *in vivo* experimentation was completed, animals were euthanized and their tissues collected for further analysis. Mice were placed in an acrylic box and euthanized by carbon dioxide asphyxiation until breathing stopped. Blood was collected by cardiac puncture, prior to transcardiac perfusion. Mice were abdominally bisected to expose the heart in preparation for 2 minutes of transcardiac perfusion by 0.9% saline solution. Brain tissue was dissected with one whole hemibrain drop-fixed in 4% paraformaldehyde (stored at 4˚C for 48h) and the other hemibrain further dissected to collect cortical and hippocampal regions for ELISA measurements (~0.100 and 0.025g wet weight respectively, stored at –80˚C).

2.2 sensorimotor behavioral testing

2.2.1 Activity monitoring

Locomotor activity was measured as part of the basic phenotypic characterization of the BACE KO mouse lines. Previous work with the PDAPP mouse had shown behavioral abnormalities related to motor function and possibly anxiety, which could be affected by the alteration of the BACE gene (Dodart et al., 1999; Gerlai et al., 2002).

Spontaneous motor activity data was quantitatively captured using the Versamax activity monitoring system (Accuscan Instruments, Columbus, Ohio, USA)(Figure 2.1). This system features 4 separate 50cm x 50cm acrylic activity chambers which can be partitioned to produce 8 activity arenas from which mouse activity can be reliably detected (Figure 2.2). The activity chambers are framed by an array of infrared beam emitters and receivers spaced 2cm apart on the x, y, and z axes. The Versamax program, which controls the monitoring session via a Dell Optiplex computer, records all movements that break the line of the infrared beams.

Once the activity data was collected the resultant .VMX files could be filtered for specific types of motor activity using the Versamap program. Using Versamap, various zones could be created within the 25cm x 25cm mouse activity arena to measure the duration and quantity of generalized movements (e.g. total distance moved, time spent in movement, and number of rests in movement), spatially constrained movements (e.g. number of entries into an adjacent sector, Figure 2.2) and specific movements (e.g. rearing and stereotypic movements). Filtered data from the Versamap program were compiled into a format that can be read by the spreadsheet software program Excel (Microsoft Corporation, Redmond, WA, USA). Any number of mapping filters could be applied to the .VMX data so this represents a very detailed source of spontaneous locomotor data (Dow-Edwards, 1998).

In most experiments, activity detection time was 15min per each primary and secondary session, of which the primary session is performed to allow animals to habituate to a less explorative state. In Study 011B, only one detection session was recorded due to mechanical error. All chambers are cleaned with unscented cloths (Clorox Wipes, Oakland, CA, USA) prior to entry by any animal to prevent motor behaviors based in social examination of scents.

A subset of the motor activities measured within the Versamax system was analysed to generate open field-like test information. The open field is a test in which a rodent is exposed to novel arena and assessed for the quantity, quality and duration of movements within the open central area of the novel environment. Anxious animals will move notably less within the central area of the space, while normal animals will increasingly enter this area. Distance traveled, time spent in area, and number of vertical movements in the central open field (25% of total area) were calculated (Figure 2.2)

Figure 2.1 Activity monitor chambers (top) and an individual mouse activity arenas (bottom). Animals are placed in each chamber and all movements are recorded via infrared beam breaks.

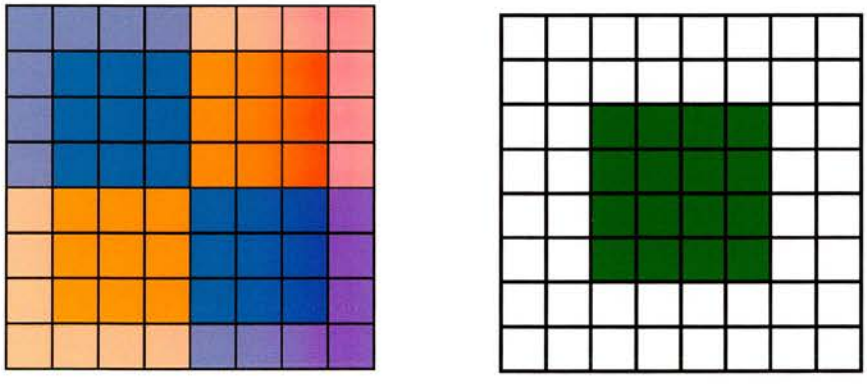


Figure 2.2 Activity chamber map schematics for rearings (pink), stereotypy (purple) sector crossings (movement from blue to red or red to blue area, left) and open field maps (green area indicates central area, right)

2.2.2 Grip strength/positional sense

There is evidence in the literature that links the metabolism of Aβ and the expression of BACE and BACE2 to a rare muscular condition called Inclusion Body Myositis (IBM) (Askanas V, 1992; Vattemi et al., 2001; Vattemi et al., 2003). Patients with IBM have myopathies that feature congophilic Aβ intracellular aggregations which vacuolate muscle cells. BACE and BACE2 colocalize to areas of dying muscle fibers in IBM and accumulate at the postsynaptic site of the neuromuscular junction. To

determine whether experimental animals lacking in the products of the BACE gene have muscular phenotypes, forelimb grip strength tests were conducted.

The mice were weighed immediately before testing and the body mass used to calculate a body strength ratio. Using a grip strength apparatus (San Diego Instruments, San Diego, CA, USA) with a digital force transducer readout, mice were brought in proximity to the gripping plate (Figure 2.3). Once a grip was taken with the forelimbs, each mouse was gently pulled away from the transducer along the plane of the apparatus until the grip was broken (Meyer et al., 1979). Maximum force per three successive trials was used in creating an average grip strength for that day. After three successive days, the grip force data was taken to calculate the grip strength ratio:

Average maximal grip force (g)/mouse body mass (g) = Grip Strength Ratio

After performing 2 studies using the digital force transducer the device malfunctioned, and a manual test was done instead to similarly assess the limb muscle tone (Roberds et.al. 2001). In this positional sense/tone test, individual mice were constrained to a small surface area and nudged at the shoulder in an effort to upend the animal. Mice were scored for their ability to remain upright using their limbs, with 0 for normal ability to resist upending, 1 for staggering to remain upright, 2 for losing footing in 2 or more limbs, and 3 for immediate loss of upright position.

Figure 2.3 Grip strength apparatus. Mice are allowed to grip the square foil plate, which is attached to the digital force transducer at the right to provide a measure of limb strength.

2.2.3 Rotorod

In continuing with the motor phenotype characterization of the BACE KO mouse lines, Rotorod testing was also performed to provide information on ambulatory movement. The measurement of motoric coordination in rodents is commonly done using a mechanized rotorod apparatus, which causes minimal distress to animals when applied properly with a sensible protocol (Forster and Lal, 1999). The apparatus in these experiments is an EZRod system (Accuscan Instruments, Columbus, Ohio, USA), consisting of 6 test chambers, which have 44.5cm x 14cm x 51cm dimensions (Figure 2.4). A Dell Optiplex computer running the EZRod and EZDiag programs was used to control the EZRod.

Figure 2.4 Rotorod motor activity chambers. Mice are placed on the rod in each chamber, suspended above an electrified grid floor. Latency to

fall from each motorized rod is recorded, as a measure of motor coordination.

At the height of 35cm, there is a mechanized plastic rod 70mm in diameter that is turned by a driver motor located behind the chamber. Below the rod is an electrifiable grid that delivers the footshock to mice that fall from the rod. The rod can be programmed to rotate at specific speeds over time, giving information on the motor capacity of animals at various rotational speeds. The metal grid below the rotation chambers is electrified with a 0.8mA current, which is noxious but not painful to touch once the mice have fallen, serving to prevent the mice from prematurely leaving the rod without respect to their motor capabilities.

The test animals are placed on the motorized rod (40mm or 70mm in diameter) in an enclosed chamber. An individual trial begins with the rotation of the rod and ends when the rodent falls from the rod or completes the trial without falling for the duration of the trial. This latency to fall is the primary measure for rotorod testing. The animals are then returned to their home cages to recoup before their next trial in 10 minutes.

There are three stages to the rotorod testing performed at Elan to assess motor coordination and capacity, comprising a 3-day test regime:

- Day 1 - Animals are acclimated to the test chambers, in which they are placed on an immobile rod for 30s for 4 trials with 10 minutes intertrial breaks.
- Day 2 - Animals are placed on a rod that rotates at constantly at 10rpm for 4 trials of 90s duration with 10 minutes intertrial breaks.
- Day 3 - Animals are subjected to 4-7 trials of 150s maximum duration with rotation increasing steadily from 0-40rpm with 10 minutes intertrial breaks.
- Primary measurements are made from calculating average latencies over trials from both constant and accelerating speed tests.

2.3 Spatial Memory Testing

AD patients suffer from declarative memory dysfunction, particularly episodic memory that is attributed to neuropathology in the hippocampus as well as cortical

regions of the brain. Concomitantly, behavioral analysis of transgenic models of AD also focuses on cognitive deficits based in the hippocampus and cortex. While there are many behavioral tasks that are based on a component of hippocampal function, the Morris water maze has become the gold standard task for detecting spatial memory impairments in transgenic mouse models of AD. The task entails placing a test rodent into a pool with a platform of some kind that the animal must locate and land to escape the aversive task of swimming. When released into the pool over a series of trials, rodents improve their navigational performance by engaging spatial learning and memory abilities. The original water maze spatial learning and memory studies were conducted in rats, and were extended to show that the ability to solve watermaze tasks is highly dependent on the function of the hippocampus, and because of these early publications, the task is sometimes referred to as the Morris Water Maze (MWM) (Morris, 1984; Schenk F. and Morris, 1985).

At the present time every major transgenic model of AD has been assessed in some version of the MWM (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997; Holcomb et al., 1998; Chen et al., 2000; Chishti et al., 2001). Initial reports of the behavioral deficits in the PDAPP mouse utilized a protocol like the classic rat experiments, in which the mice were tested for their ability to solve a single task (Morris, 1984; Justice and Motter, 1997). While the PDAPP mice did display a spatial learning deficit, it was not age-related as it was apparent in even young mice and, surprisingly at the time, did not reflect accumulating amyloid plaque burden. Chen et al. (2000), revisited this issue by publishing a report in 2000 in which PDAPP mice were tested with a modified MWM protocol for the ability to learn a series of spatial locations (Learning capacity) as well as to complete tasks to a certain performance level (testing to criterion). Using this MWM study design Chen et.al., 2000 were able to demonstrate a cognitive spatial learning and memory deficit that is related to age and accumulating amyloid burdens. Much of the watermaze experimentation for the BACE KO mouse lines described here is based specifically on the protocol developed by Chen et al. (2000).

2.3.1 The Water Maze Apparatus

The watermaze is a system encompassing the tank in which the animal swims, a CCD video camera, and a computer that contains the Watermaze software (Actimetrics, Evanston, IL, USA) that collects the image data and manages the running of the study. At Elan, the watermaze tank is an adapted circular livestock trough 1.52m in diameter, 61cm high that sits on an 183cm x 183cm wooden platform 30cm high. When filled, the Watermaze tank contains about 1800L of water to which 300mL of white flat latex paint (Home Depot, Colma, CA, USA) is added to make the water opaque enough to occlude a submerged platform. Each escape platform is made of acrylic with a circular 18cm landing surface covered in a cross-hatched polyfoam shelf liner (Rubbermaid, Atlanta, GA, USA) to facilitate landing and is submerged 1cm below the surface of the water.

The camera, videoboard, imaging analysis program and Watermaze software are all from Actimetrics as part of their complete Watermaze system. Images of the swimming mice are collected via the CCD camera mounted centrally over the pool. Ceiling rods hang from the ceiling, suspending the curtains that can be drawn around the tank to exclude extramaze cues. 500W Lights are mounted at various places along the walls at 90cm from the floor to illuminate the maze for video tracking. To aid in tracking of light-colored animals, a black non-toxic marker was applied to the shoulder areas of the mice. The room in which the animals are tested is rectangular with separate tank, computer and the cage rack areas. Water temperature during testing is typically $23\pm2°$. When released into the pool, mice are positioned with their heads facing the wall of the tank to prevent bird's eye viewing of the pool as they are placed. Upon landing the platform, mice are given 30s to explore and fix the location spatially using visual cues. Mice are removed from the platform using a immobilized paint roller with a foam cover (Home Depot, Colma, CA, USA) and returned their home cage for a 10 minute rest interval. During this time, animals are warmed using a space heater (Costco, Mountain View, CA, USA).

2.3.2 Visual Cued Navigation (VCN)

After sufficient pre-study handling, mice are tested for the ability to navigate to an escape or landing platform marked by a visible intramaze cue, a darkened cylinder standing 25cm from the center of the platform (Figure 2.5). Extramaze cues are

occluded by the drawing the curtains around the watermaze. Animals are released from random sites and given 90s to navigate to the visibly cued platform independently and given 30s on the platform after landing. If after 90s have elapsed and any mouse has not independently landed on the platform, it is guided manually to the landing platform, allowed to land, and also given 30s on the platform.

VCN exposes the animals to swimming and allows them to associate landing the platform with being returned to their home cages. VCN is conducted for 4 trials/day, over 3 days with the platform in the center of the maze on Days 1 and 2 (Figure 2.6). On Day 3 a new platform location is utilized to ensure the animals are swimming to the visible cue and not a specific location. Animals not averaging Day 3 swim times of less than 20s are removed from the study. Typically these animals are not simply slower swimmers, as even aged PDAPP mice are capable of performing to this basic criterion.

Figure 2.5 Watermaze in VCN mode, with curtains hiding extramaze spatial cues, and a visible object marking the submerged platform site.

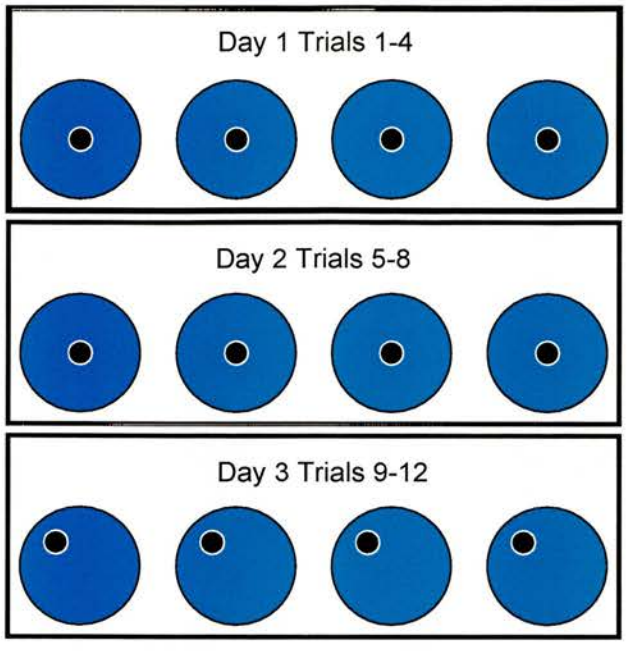


Figure 2.6 Watermaze in VCN mode, schematic of visible platform locations (black circle) within the pool over days 1-3. Note the change in platform location on day 3, for trials 9-12.

Instead, these animals have characteristics that would confound the interpretation of swim performance as a function of spatial memory: neophobic animals that avoid the visibly cued platform, "floaters" who are uninclined to swim in order to find the platform, and other mice that have obvious visual impairment. Typically very few mice are excluded from study for these reasons, between 5-10%.

2.3.3 Serial Spatial Navigation from Memory (SNM)

Mice that have been judged fit to swim from previous VCN testing proceed to SNM, in which their performance depends on the formation and use of strategies based in memory to successfully locate the now hidden platform. The occluding curtains are removed to reveal several extramaze features within the watermaze room that can be used to help form a stable representation of space outside the pool. These features include one curtain wall that is left extended, light stands, a pair of purple gloves attached to the wall, the hose and piping system, as well as a large vertical shelf system (Figure 2.7). All efforts is made to keep the extramaze environment stably

positioned not just during any given test day, but throughout a single experiment and indeed throughout all experiments across age and colonies tested.

In the SNM paradigm, animals were trained on a series of spatial locations into finding each one to a predetermined criterion of performance (<21s over 3 successive trials). The mice were exposed to a series of platform locations over 10 days, with a maximum of 8 90s trials per day separated by a 10 minute intertrial period (Figure 2.8). Mice are released from 4 different release sites (at the base of the NW-SE, SW-NE diagonals crossing the pool) along the edge of the tank, in 1-4, 1-4 order. The number of locations the animals will experience depends how quickly they reach the specific performance level at the previous platform location.

To reduce spatial location bias, half of the animals tested in one experiment are tested on platform locations in order 1-10, while the other group experiences the platforms in order 10-1. In order to gain sufficient information about the spatial

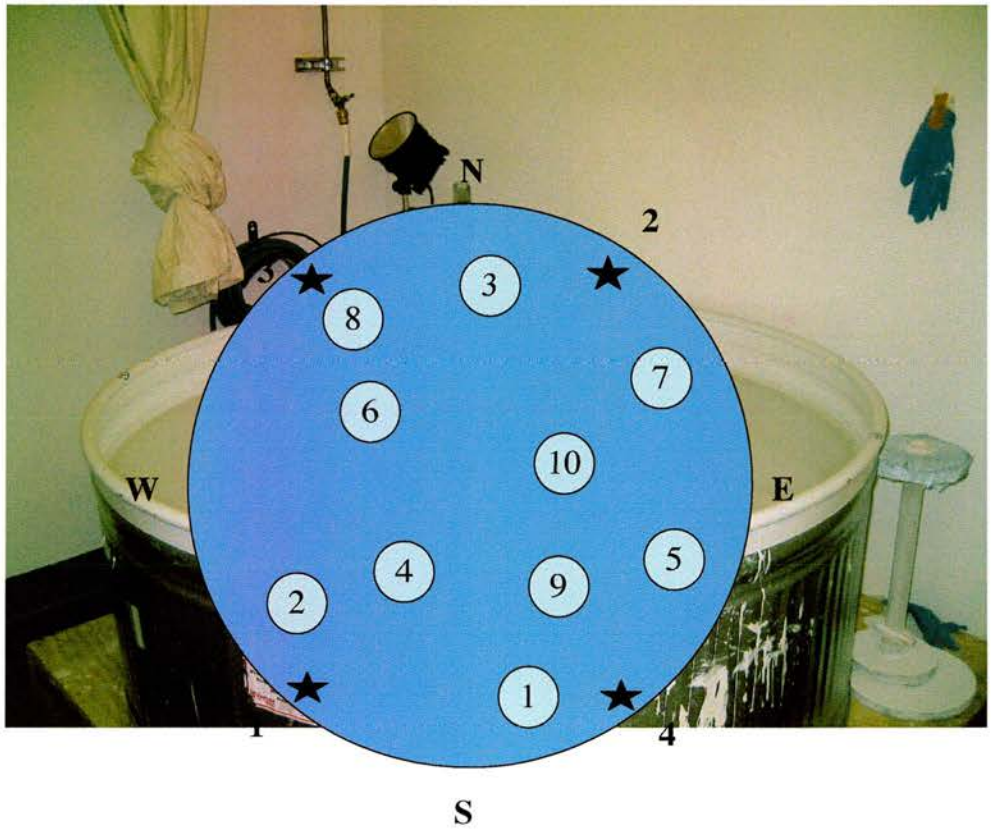


Figure 2.7 Water maze in SNM mode. The submerged platform is not visible, but several extramaze cues are available to form a spatial map.

Figure 2.8 Map of SNM platform locations (numbered) and release sites (starred, in order of release).

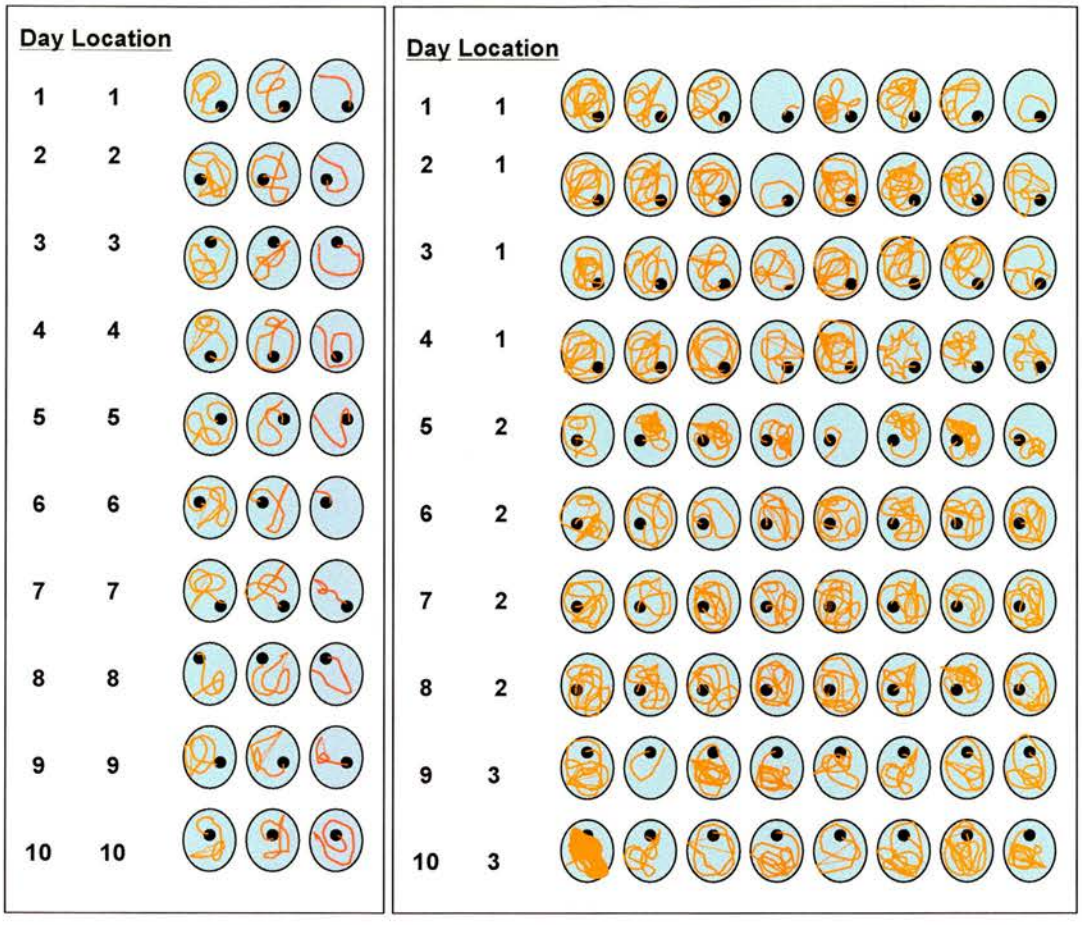


Figure 2.9 Example swim performance paths (in red) for mice with unimpaired (left panel) and impaired (right panel) spatial learning by day and platform. Impaired animals require several more trials to learn any given location.

on the following day. Using these parameters an animal with the highest possible spatial performance levels will solve the full set of 10 spatial location tasks in the minimum of 30 total trials (3 per location) (Figure 2.9). Similarly poorest performance level would be to be tested on only 3 platform locations over the maximum 80 trials overall (with exposure to the second and third locations only possible via the automatic platform change rule after 32 unsuccessful trials).

Thus the number of platforms learned provides a measure of 'learning capacity,' while the mean number of trials to reach the learning criterion (TTC) for any one location is a measure of acquisition rate, especially in the case of the first platform location. Performance on the first platform location alone is analogous to the classic version of the watermaze in which animals are trained on a single task.

2.4 Seizure Experiments

During the initial watermaze experiments to characterize the homozygous BACE KO mice on a PDAPP mouse line, some animals were observed to have spontaneous clonic seizures (alternation of muscle contraction and relaxation) prior to, during and after swim trials. Mice observed to have seizures often were subsequently noted to have extreme muscle weakness, no forelimb grip responses and in some cases were hemiplegic to either the right or left side of the body. After each cohort of water maze experiments was completed and the study blind was removed, it was apparent that spontaneous seizures were occurring in mice lacking BACE. This suggested that the absolute deficiency of the BACE gene product had some mechanistic involvement in this seizure activity, and accordingly, formal seizure testing was done to investigate this possibility.

2.4.1 Induction of Seizures

Induction of seizures was achieved by administration of the compound pentylenetetrazole (PTZ, from Sigma Chemicals, St. Louis, MO, USA). PTZ is a commonly used seizure-inducing drug that acts via the GABAA/benzodiazepine receptor complex, possibly by blocking Cl- influx (Vitek et al., 1965; Zhang et al., 1989). By employing intraperitoneal (i.p.) injections of PTZ at varying doses in rodents, seizures of a range of strengths, types and durations can be initiated. A typical experiment involves removal of an animal from its home cage to a larger observation area. Animals are then dosed i.p. with PTZ at 25 or 60mg/kg made with 5-10ml/kg dose volume in 0.9% saline solution and observed for seizure profile for 30 minutes. The observation arena used is an acrylic cylinder 30cm in diameter, 45cm high placed on a bench liner sheet (Figure 2.10). The cylinders are cleaned with Clorox cloth wipes between uses.

Figure 2.10 Seizure observation arenas. Animals were placed in clear plexiglass cylinders for seizure activity scoring.

The low (25 mg/kg) and high (60 mg/kg) doses were chosen for their published ability to reliably elicit mild clonic seizures and severe tonic-clonic seizures respectively in the vast majority of treated animals. One unavoidable aspect of experimental tonic-clonic seizure induction, whether by chemical agent or direct electrocorneal kindling, is that it is lethal to the majority of animals . Indeed, any treatment that causes 95% of controls animals to display tonic-clonic seizures will typically also be near the 50% lethality range for the treatment, such that half of the experimental animals will not survive the induction. Given these dismaying losses, every effort was made to reduce the discomfort of the animals and to reduce the numbers of animals used. For example, animals were all euthanized immediately after seizure testing, and in the third seizure experiment (Study 011C), the N of animals used per treatment group was reduced from N~10-15 to N~8, as prior analysis of Studies 011A and 011B showed that this number was sufficient to produce statistically meaningful results. Details of the ethical concerns and scientific concerns considered in performing this study are addressed in the study protocol presented to the Elan Institutional Animal Care and Use Committee (IACUC), which is included in the appendix of this dissertation (Ch. 10.2).

2.4.2 Seizure observations

The PTZ seizure profile includes observations of the time to onset, the severity and description of seizure, number of seizures and duration of seizure activity as well as time to death and group percentage of lethality. Seizure activity is generally divided into partial clonic, general clonic and tonic seizures. Partial clonic seizures are observed when animals exhibit localized single head or limb twitches that are brief, lasting no more than the time it takes to perform the activity. General clonic seizures are characterized by a combination of smaller clonic activities that may occur over a wider area of the body, e.g. tremor of the forebody, haunches, which may have duration of several seconds to minutes. Tonic seizures are the most severe type of seizures induced by PTZ and is often lethal. Tonic seizure activity is defined by the extension of muscles, with head, limbs, torso and tail elongated in an extreme caudal direction.

The capacity to seize in response to exposure to seizure-inducing compounds in rodents is a function of the individual animal's resistance to seizure. Generally, previous spontaneous seizure activity predisposes experimental animals towards having greater seizure activity in response to PTZ (Kosobud and Crabbe, 1990; Kosobud et al., 1992; Ferraro et al., 1999). In experiments where 25mg/kg of PTZ was used, the objective of the study was to examine the resistance to clonic-type seizures by BACE and/or PDAPP genotype. Similarly the 60mg/kg doses of PTZ were used to examine resistance to tonic seizures by genotype. Seizure types and their common abbreviations are described below:

Clonic type: alternative contraction and relaxation of the voluntary muscles

C = Clonic - co-coordinated, unsymmetrical convulsion occurring while natural, purposeful like movements are also present, e.g. head or tail twitches.
Ch = Champing - clonus of the jaws only
Cs = Clonic symmetrical - repetitive symmetrical jerks or twitches of the limbs, can include whole-body tremors that do not interfere with locomotion.
Rn = Running excitement - often preceded by partial or symmetric clonus; a severe clonic convulsion that features rapid and jerky walking or running.

Tonic type: persistent contraction and spasm of a set of voluntary muscles.

PE = Paw extension - sustained extension of hindlimbs, usually preceded by tonic flexion **(Tf). Tf** is used if linear tonic flexion occurs without extension; **Tf** is uncommon.
Op = Opisthotonus - head, body and limbs are rigidly extended and arched backwards.
Em = Emprosthonus - opposite of **Op** i.e. extended forward.
EPE = Extreme paw extension – Tonic flexation with underlying tremor that involves the whole body, directed towards the caudal aspect; typically a pre-lethal observation.

Mixed Clonic/Tonic type

Pop = Popcorn - seizure where animal repeatedly "pops" into the air; this seizure is a mixture between clonic and tonic types, as the rapid alternation between the two in the hindlimbs propel the animals into unrestrained leaps around the arena.
Rr = Rock and roll - animal is prostrate on its back and rocks from side to side in a seeming effort to right itself, occasionally rolling over (overshooting) and continuing to rock again; often due to asymmetrical tonic activity.
Su = Sitting up - sits upright on hindlimbs during the seizure
Pr = Praying - sitting up seizure in which forelimbs are held together or crossed in manner resembling prayer.

2.4.3 Seizure scoring

Experiments using the PTZ model of seizure induction frequently use seizure scores to grade the severity of seizure activity in test animals (Matsumoto, 1990; Ferraro et al., 1999). In collecting the seizure observations, it is possible to distinguish between the types of seizure activity and allocate them to partial clonic (PC), general clonic (GC) or tonic (T) seizure categories. By recording the latencies in minutes to onset of the three seizure classes, a formula can be applied that generates a composite seizure severity score that differentially weights seizures by severity:

$$\text{Seizure score} = 0.2/(\text{onset PC}) + 0.3/(\text{onset GC}) + 0.5/(\text{onset T})$$

2.5 Histological Experiments

One of the classical pathological features of AD is the presence of Aβ deposits in the brains of patients. While amyloid plaques in the cortex, hippocampus and entorhinal cortex are often used post-mortem to confirm the clinical diagnosis of AD, there is a much controversy regarding the relationship between levels of brain amyloid burdens and cognitive status (Terry et al., 1991; Nagy et al., 1995; Gomez-Isla et al., 1996;

Naslund et al., 2000; Bussiere et al., 2002, see also Ch.1, p.8 of this dissertation). With respect to the PDAPP transgenic mouse model of AD, there is some relationship between amyloid burdens in the cortex and behavioral deficits and progressive synaptic loss is observed, however unlike in the AD there is no global neuronal loss present in this model (Irizarry et al., 1997; Chen et al., 1998; Chen et al., 2000).

Among the various synaptic proteins that are emerging as biomarkers for synaptic function, Calbindin levels have been directly correlated in a transgenic mouse model that overexpresses mutant human APP to spatial memory performance in the water maze (Lally et al., 1997; Eriksson et al., 1998; Jiang et al., 2003; Palop et al., 2003; Shetty, 2004). Calbindin (CB) is a calcium-binding protein expressed throughout the brain, and is associated with homeostasis and protection from apoptosis in neurons as well as developmental and pathologically-induced neurogenesis in the granule cell layer of the hippocampus (Lally et al., 1997; Eriksson et al., 1998; Jiang et al., 2003; Shetty, 2004). In these studies, CB is measured and correlated to behavior in all animals where possible. Also, while it is not the objective of this work to examine the levels of APP and amyloid in the transgenic BACE KO mice, this type of histology is used to confirm the genotypic identities of animals tested.

2.5.1 Calbindin Histology

After submersion in 4% paraformaldehyde, hemibrain tissues are placed in 0.05% Sodium Azide (NaN) in phosphate buffered saline pH 7.4 (PBS, Gibco, Carlsbad, CA, USA) solution until further processing. Brain tissues are cut coronally to 40um thickness on a Leica vibratome (Wetzlar, Germany) and stored free-floating in 48-well Costar plates (Corning, NY, USA) in a 10% glycerol PBS solution. Quantitative CB immunostaining is conducted with one section per brain that contains hippocampal structures, particularly the outer molecular layer of the dentate gyrus.

Sections are collected into a 96-well Costar plate filled with PBS using a fine camelhair paintbrush. All incubation and wash steps are done with gentle agitation applied by a Nutator or an orbital shaker (Becton-Dickinson, Franklin Lakes, NJ, USA). Tissues are washed 2x for 10 minutes. Tissues are quenched with a 3%H_2O_2, 0.05% Triton X-100 (Fisher Scientific, Hampton, NH, USA) in PBS solution for 20

minutes. After 2 more 10 minute rinses, a blocking step follows with 10% normal horse serum (NHS) (Vector Labs, Burlingame, CA, USA) for 1h. Following a single 10 minute wash, sections are incubated overnight at 4°C in 3mg/mL mouse anti-CB antibody (Sigma Chemicals, St. Louis, MO, USA) in PBS solution. The following morning, sections are washed 2x for 10 minutes in PBS and incubated for 1h in the dark with a 1:75 horse anti-mouse fluorescein-isothicyanate secondary antibody (Vector Labs, Burlingame, CA, USA) and again washed 2x for 10 minutes in PBS afterwards. Sections are mounted onto Fisher charged superfrosted slides with Vectashield from Vector Labs.

CB-stained sections are imaged using a Biorad/Zeiss 5000 laser confocal microscope system operating from a Dell Optiplex computing platform. Images are collected using the fluorescein filter on the Zeiss microscope at 10% laser power. Images are collected at 60x magnification at the level of the dorsal granule cells of the outer molecular layer of the dentate gyrus in the hippocampus. Quantitative analysis of the images was performed using the NIH Image 1.63 program available for free download at the http://rsb.info.nih.gov/nih-image/download.html webpage. CB density is presented as a value normalized to the levels of CB from 6 different brains from 18mo non-transgenic littermates from the PDAPP line concurrently stained in each staining run.

2.5.2 APP/Aβ Histology

Staining for hAPP and hAβ was performed using the monoclonal mouse antibodies 8E5 and 3D6, respectively. 8E5 is an antibody raised to the 444-592 amino acid residue stretch of the hAPP protein that is used to stain dystrophic neurites, while 3D6 is a synthetic antibody for the N-terminus 1-5 amino acid stretch of hAβ capable of binding diffuse and compact deposited amyloid. Prestaining, processing, and anatomical regions of interest for tissues immunoreacted with 8E5 and 3D6 is the same as with CB staining. Again all incubation and wash steps were done with gentle agitations on a nutator device.

All sections are washed 2x for 10 minutes in PBS with the same quenching step as with CB. After another set of 2 10 minute washes in PBS, sections are incubated overnight in 3ml/mL of biotinylated 3D6 (1:433 concentration) or 8E5 (1:233 concentration) in a 1% NHS in PBS solution at 4°C. On the following morning after 2 10 minute washes in PBS, sections are incubated in a secondary antibody complex solution, A/B Vector Elite kit from Vector Labs at 1:75 in PBS for 1h. After 2 10 minute washes in PBS, sections are reacted in a 0.5 mg/mL diamino benzidine solution in 100mM Tris-HCl buffer activated with 1:2400 H_2O_2 for no more than 10 minutes. Sections are then mounted on superfrosted charged slides and air dried overnight. Slides are cleaned with a 10 minute dip in Propar (Anatech, Denver NC, USA) and coverslipped in Permount (Fisher Scientific) mounting solution. Images of the 8E5 and 3D6-immunoreacted sections are collected using an Axiocam microscope system from Zeiss. Under the light phase 4x photomicrographs are taken of the hippocampal and cortical regions of the tissues. As these images are used solely for the qualitative confirmation of genotype by visible hAPP and hAb expression, no further analysis was performed with these images.

2.6 Statistical Analyses

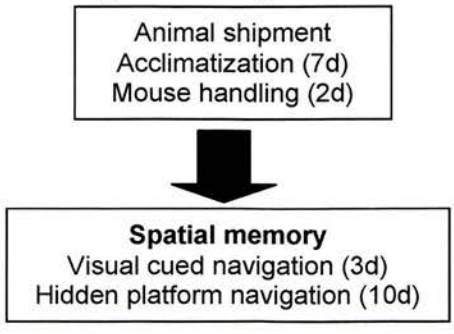
Analyses were performed using Prism 4 from Graphpad Software (San Diego, CA USA) and Matlab7 (The Mathworks, Natick, CA, USA). A MANOVA-based analysis of factors sufficient to handle wide differences in group values and N was done to determine the importance of genotype, age, gender, and color factors on measure results prior to other tests. Following the factorial MANOVA all further analyses were based in genotype, age and Genotype*age. For some of the watermaze studies, especially where there are multiple age- and genotype-related variables imposed on an experimental design with a by-day variable, a simple ANOVA (analysis of variance) was insufficient to analyse the data. ANOVAs would make assumptions about experimental parameter simplicity or sphericity that are untrue in this case. Instead a Two-Way ANOVA is employed when measures involved trials over multiple days (e.g. VCN speed, latencies). In other cases, like learning capacity, which is presented as a single number column per age/genotype. a simple ANOVA with the Cochran-Mansel-Heusen test was used to determine between-group differences. Other tests

were used as necessary and described in text (e.g. Seizure Lethality was measured using Fisher's exact Chi-squared test).

Correlational analyses were conducted between behavioral measures, CB levels and pharmacological seizure data are conducted using Spearman's Correlation test. This type of correlation analysis produces an R-value that describes the amount of variability in one measure that can be described by values in another. These value relationships are generated by a log ranking method, which is preferable to comparison of actual values due to the extreme differences in sources of variability between the methodologies producing the data. Arbitrary numerical assignments were given to data with text values to allow the numeric correlational analyses: within color albino=0, agouti=1, black=2, while with gender male=1, female=2. In Study 001 the following genotype assignments were made: Control=1, PDAPP=2, BACE KO=3, and BACE KO; PDAPP=4. Finally in Study 006 genotype assignments were as follows: PDAPP=1, BACE pKO; PDAPP=2.

2.7 Experimental Overview

This dissertation work revolves around three major experiments: the spatial memory characterization of mice with homozygous and hemizygous deletions of the BACE gene crossed to a heterozygous PDAPP mouse line, and the profiling of these mice for sensorimotor phenotypes and susceptibility to pharmacologically-induced seizures. The terminology homo- and hemizygous refers specifically to genetic knockout of either both (homo) or one (hemi) BACE gene allele, while heterozygous refers to mice carrying one copy of the PDAPP transgene. These experiments will be referred to as **Study 001** (BACE homozygous KO x PDAPP lines), **Study 006** (BACE hemizygous KO x PDAPP lines) and **Study 011** (PTZ seizure experiments on mice from both Studies 001 and 006), with an experimental roadmap presented below.



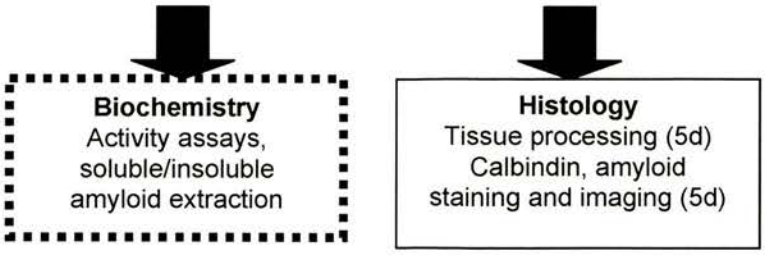


Chart 2.1 Flowchart for Studies 001, 006. This schema represents the workflow for each individual cohort, with a total of 18 cohorts. Red boxes indicate research was conducted outside the scope of this thesis by other collaborators on the Elan/Pharmacia BACE project team. Brains collected from each mouse undergoing spatial memory testing were partitioned to one hemi-brain each for the purposes of biochemical/histological analysis.

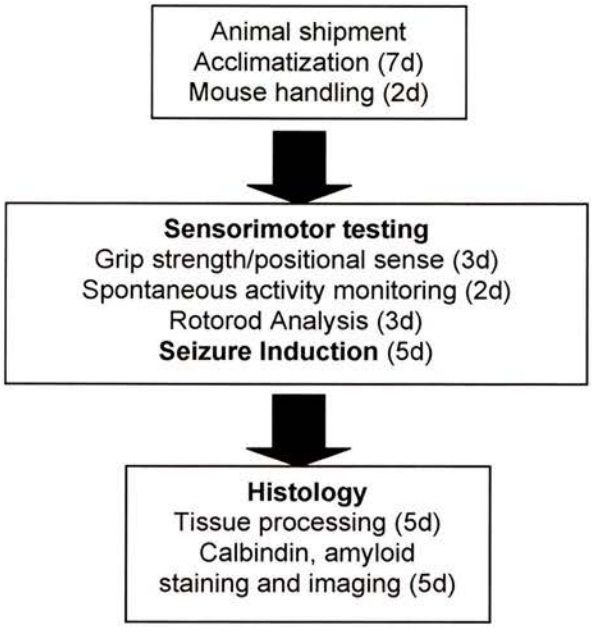


Chart 2.2 Flowchart for Studies 011A-C. This schema represents the workflow for each study, which was similar to Study 001 in Chart 2.1 above.

2.7.1 Study 001, 006 Animals

The Study 001 and 006 mice have been generated from a triple-strained background comprising the C57Bl6, DBA/2J, and Swiss-Webster mouse lines. At the time the various BACE KO x PDAPP lines were generated, the PDAPP breeding stock was

12-14 generations from the F1 progeny of the original Line 109 mice first described in Games et al. (1995). As a point of reference, the published work of Chen et al. (2000) describing the spatial memory phenotypes of PDAPP mice involved animals from generations 6-10, while currently PDAPP mice are 22-25 generations from F1. All study cohorts utilize both male and female mice, with albino as well as agouti- and black-coated pigmented animals. Best efforts were made to balance each cohort by genotype, gender and color.

BACE KO mice were created by an exon deletion process (Roberds et al., 2001). A lambda KOS vector containing the BACE gene from 129 mice was used to create the exon 1 deletion of the BACE1 gene. A 165 base pair deletion was introduced into the vector BACE1 exon1 sequence, and replaced with an expression cassette containing a neomycin-resistance gene and an HSV-thymidine kinase gene to select for specific homologous recombination. The vector was electroporated into 129/ SvEvBrd ES cells and selected positively for neomycin resistance with G4189 (Gibco) and negatively for thymidine kinase with gancylcovir (Roche Bioscience) resistance. Upon successful BACE1 gene targeting, the embryonic stem cells were injected into C57/Bl6 blastocysts. These chimeric offspring were bred to C57/Bl6 mice to produce BACE1 knockout heterozygotes. Heterozygous BACE1 KO mice were subsequently bred together to produce progeny homozygous for the BACE1 knockout allele (1/2 would be BACE1 KO heterozygotes, 1/4 would be wild-type, 1/4 would be BACE1 KO homozygotes.

Mice BACE1 knockout heterozygotes were bred to PDAPP transgene homozygous mice. The resulting progeny for that breeding are the Study 006 mice, with animals that are heterozygous for the PDAPP transgene with both or one BACE1 genes. The subset of the Study 006 mice that were heterozygous for both BACE1 and the PDAPP transgene were then bred to mice of the same genotype to produce the Study 001 mice (Figure 2.11).

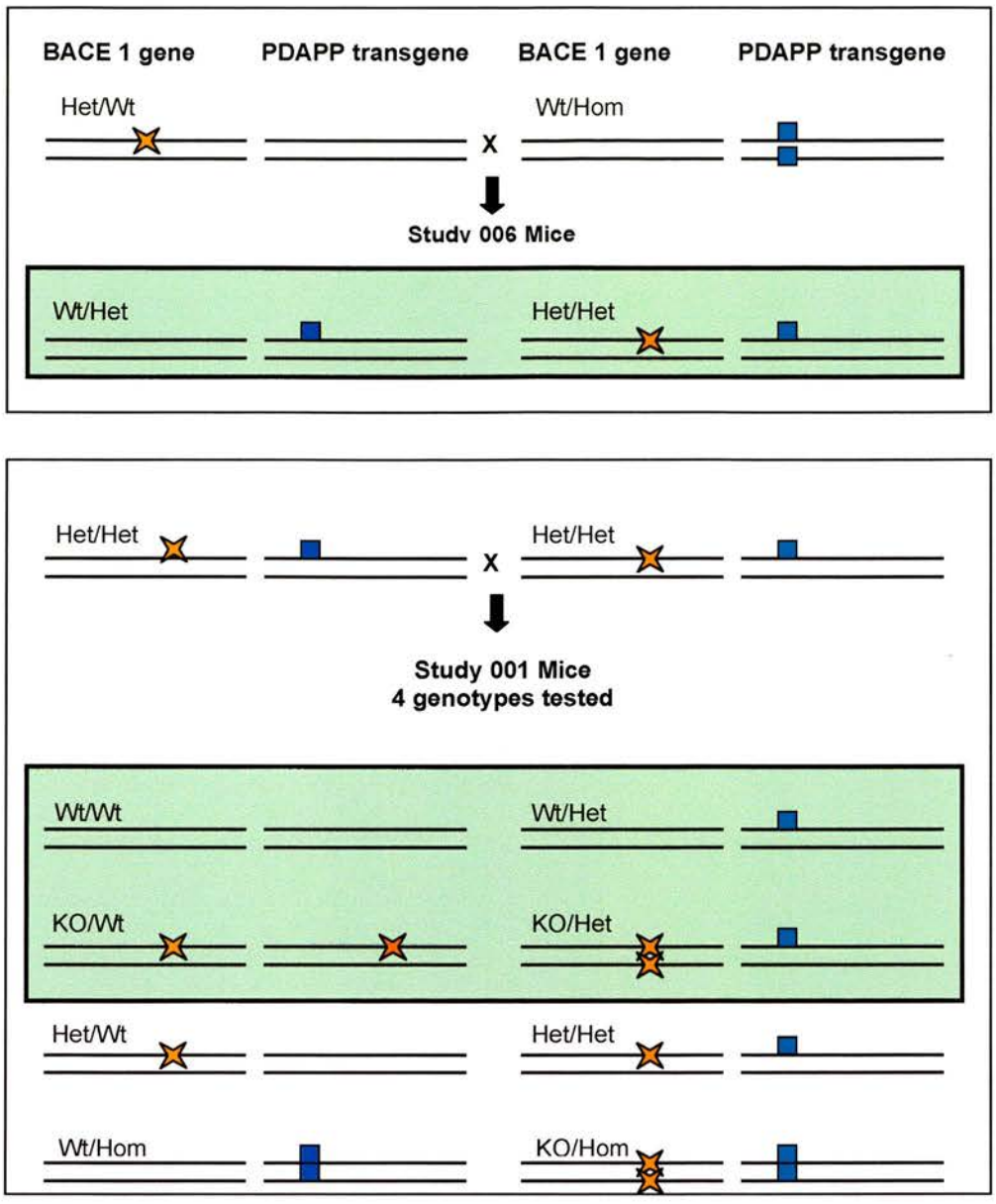


Figure 2.11 Genetic Lineage of BACEKO mice in Studies 001 and 006. Upper panel: A mouse with one copy of BACE1 is bred to a mouse with 2 copies of the PDAPP transgene, the resulting Study 006 progeny are all PDAPP heterozygotes, 50% with one copy of BACE1 (Het/Het), 50% with 2 copies of BACE1 (Wt/Het). Lower panel: PDAPP heterozygotes with one copy of BACE1 are bred, producing an assortment of genotypes. Of the 8 possible genotypes, 4 were assessed in Study 001; a Control animal with both copies of BACE1 and no PDAPP transgene (Wt/Wt), PDAPP heterozygous animals (Wt/Het), BACE1 KO animals with no copies of the gene (KO/Wt), and BACE1 KO; PDAPP animals with no BACE1 genes and one copy of the PDAPP transgene (KO/Het). Black lines represent the BACE1 gene and PDAPP transgene, red crosses indicate a BACE1 gene deletion, while blue boxes represent copies of the PDAPP transgene. Green boxes indicate genotypes tested in Studies 001 and 006.

2.7.2 Study 001 Experimental Design

Study 001 was designed to examine the spatial memory phenotypes of BACE homozygous KO x PDAPP mice of various genotypes across ages that would give a wide representation of amyloid plaque burdens (young pre-plaque mice, middle-aged mice with moderate plaque depositions, and old mice with heavy amyloid deposits). The animals tested in the watermaze by age, genotype and gender are described in the following tables; the majority of mice tested in the watermaze also were analysed for hippocampal levels of CB and a selection of mice were qualitatively genotyped for APP/Aβ expression.

2.7.3 Study 006 Experimental Design

The objectives of Study 006 were similar to that of Study 001, with the major difference stemming from the difference in the dosage of the BACE knockout allele. Study 006 compared head-to-head the spatial memory performances of animals with a hemizygous deletion of BACE on a heterozygous PDAPP transgene background to mice with intact BACE genes on a heterozygous PDAPP background. As a result of the breeding scheme (Figure 2.11 upper panel) there were only two genotypes produced in Study 006 animals, which were also tested at young, middle and old ages to understand the spatial memory phenotypes in a context that would normally produce high levels of pathogenic amyloid. Mice in Study 006 were assessed for spatial memory profiles in the watermaze, tested for CB immunoreactivity in the hippocampus and a select number of tissues were analysed to ascertain genotype with APP/Aβ histology.

2.7.4 Study 011 Experimental Design

Using the same lines of animals from studies 001 and 006, Study 011 was conducted to develop a larger set of behavioral phenotype data including tests to determine the relative propensity to seizures in the BACE KO and PDAPP mouse lines. Three separate experiments were conducted within Study 011, with differing ages and also different doses of the seizure-inducing agent, PTZ. In Study 011A 18mo old mice of the same lines as the Study 001 (BACE homozygous KO colony) were examined for

body mass, grip strength, spontaneous locomotor activity, motor coordination, and response to administration of 60mg/kg of i.p. PTZ. In Study 011B, 5mo old mice from the same lines as Study 006 (BACE hemizygous KO colony) were also analysed for the same functional measures as with Study 011A. In Study 011C 18mo old mice from the Study 006 lines were profiled with the same measures as the previous studied, and further tested on response to both 25 and 60mg/kg of PTZ. Study 011 tissues were tested for CB immunoreactivity in the hippocampus with a select number of tissues immunostained to ascertain genotype with APP/Aβ histology.

The research data and conclusions (Ch. 3-8) that follows is confidential as of October 21st, 2005, although an attempt to publish the majority of the data will be made in late Fall 2005. Publication of data from this thesis will be done as a stand-alone document. However, the manuscript generated from this work will include references to related work done by Elan researchers involving biochemical and detailed histological analyses of the BACE1 KO x PDAPP mouse model that will be published around the same time.

Dione T. Kobayashi

Ch.3 Study 001: Spatial memory characterization and histological analysis of homozygous BACE KO x PDAPP mice

In the opening chapter of this dissertation, information about the biology of Alzheimer's Disease and the rationale for in vivo examination of BACE KO x PDAPP mice was presented. These BACE KO x PDAPP mice are fully or partially deficient in the β-secretase gene, while transgenically overexpressing a human mutant form of the Amyloid Precursor Protein driven by the PDGF promoter (PDAPP transgene). In effect, they represent a gross genetic model of therapeutic BACE inhibition.

This chapter focuses on the experimental data gathered from these Study 001 mice, which have a homozygous BACE gene knockout on a transgenic PDAPP background. Behavioral and histological data are derived from analysis using a modified serial spatial reversal water maze paradigm, histological analysis of Calbindin (CB) levels in the hippocampus, confirmation of APP-overexpressing genotypes with APP and Aβ staining, and correlational analyses of behavioral and CB histological measures are also presented. The genotypic identities of Study 001 animals are described in section 2.7 in the previous chapter, while the details of the animals tested are presented below in Tables 3.0a.

	Non-Tg (Wt)	PDAPP -/+ (Het)
BACE +/+	**Control**	**PDAPP**
BACE -/- (KO)	**BACE KO**	**BACE KO; PDAPP**

Table 3.0a Study 001 Table of Genotypes

Due to the variability inherent in spatial memory testing of PDAPP-based mice of both genders and on triple-strained background (C57Bl6, DBA/2J, Swiss-Webster), it was essential to test N~20 mice to obtain sufficiently powered statistical analysis. As the effects of β-secretase gene ablation may vary as a function of age, animals in Study 001 were tested at young (3mo), middle (13mo) and old ages (18mo). In addition, the constraints of breeding a colony of mice with 4 genotypes of interest required an effort to balance animals by coat colors (albino, black and agouti) as well as gender. The numbers of Study 001 animals separated by genotype, coat color and gender groupings are presented in Table 3.0b with N_{total}=251 mice.

		Female				Male				ALL			
Age (mo)	Genotype	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL
3	BACE KO; PDAPP	2	2	4	8	2	0	6	8	4	2	10	16
	BACE KO	2	5	4	11	4	0	7	11	6	5	11	22
	PDAPP	1	2	8	11	1	5	5	11	2	7	13	22
	Control	3	1	7	11	3	1	5	9	6	2	12	20
	ALL	8	10	23	41	10	6	23	39	18	16	46	80
13	BACE KO; PDAPP	2	4	5	11	1	0	8	9	3	4	13	20
	BACE KO	2	2	7	11	2	2	5	9	4	4	12	20
	PDAPP	3	2	6	11	3	2	7	12	6	4	13	23
	Control	1	4	4	9	3	1	8	12	4	5	12	21
	ALL	8	12	22	42	9	5	28	42	17	17	50	84
18	BACE KO; PDAPP	1	2	8	11	2	2	4	8	3	4	12	19
	BACE KO	1	3	6	10	2	4	3	9	3	7	9	19
	PDAPP	8	3	2	13	2	3	10	15	10	6	12	28
	Control	4	3	3	10	0	1	10	11	4	4	13	21
	ALL	14	11	19	44	6	10	27	43	20	21	46	87
ALL	BACE KO; PDAPP	5	8	17	30	5	2	18	25	10	10	35	55
	BACE KO	5	10	17	32	8	6	15	29	13	16	32	61
	PDAPP	12	7	16	35	6	10	22	38	18	17	38	73
	Control	8	8	14	30	6	3	23	32	14	11	37	62
	ALL	30	33	64	127	25	21	78	124	55	54	142	251

Table 3.0b Study 001 mice by genotype, age, gender and color.

Details of the statistical analysis of Study 001 must be mentioned here. While best efforts were taken to balance the genotypes and gender of animals studied, it was not possible to also balance by color or pigmentation. A cross-categorization analysis (MANOVA) similar to general linear modeling to determine the contribution of each factor to results was performed across animals and measures (Table 3.0c). The major finding was that the most important factor in determining data outcomes was genotype, followed by age. Although gender was an insignificant factor in all measures, color was a significant factor in several measures (TTC1, Average TTC, Swim speed and Swim latencies). Further analysis revealed that when the disparity in number of Albino versus Black and Agouti animals was built into the model, color became an insignificant factor that was dependent on genotype and age (overall p=0.62). Thus all statistics further will be described by genotype, age and genotype*age.

Outcome	Gender	Color	Age	Genotype
VCN Latency	0.48	<0.0001	<0.0001	0.078
VCN Speed	0.06	0.035	<0.0001	0.0013
TTC1	0.29	0.022	0.0015	0.038
TTC2	0.87	0.22	0.16	<0.0001
TTC3	0.66	0.34	0.065	<0.0001
Average TTC1-3	0.47	0.036	0.2	<0.0001
Platforms Learned	0.74	0.069	0.42	<0.0001
Calbindin	0.042	0.46	0.0022	<0.0001

Table 3.0c Statistical summary of factor significance in Study 001.

3.1 Visible platform testing

Prior to spatial memory testing in the hidden platform serial locations task, Study 001 mice were assessed in visual cued navigation (VCN). In this first phase of water maze testing, lasting 3 days, 4 trials/day, the mice learned to swim to a visible platform as described in Ch 2, p.9-11. VCN is a general associative task that also provides insight into the sensorimotor function of the test mice. The primary measures of performance are latency to escape which should show a decline across trials, and swim speed, which should remain relatively static.

Qualitatively, all lines of the Study 001 mice were equally able to perform the rudiments of the swim task, although with progressive age more animals were removed from the study due to their inability to complete the VCN testing in a satisfactory enough manner to progress to hidden platform testing. If by the third day of VCN any animals were not escaping to the visible platform in <20s, they were removed from further study as they could not be expected to meet the criterion for serial learning in the hidden platform testing. Study 001 mice swam at mean speeds ranging from 22-30cm/s, with a typical swim path sequence beginning with the mice swimming at the perimeter of the pool. In later trials mice steadily learned to climb onto the visible platform and treat it as an escape route from the aversive task of swimming and their increasingly efficient swim path reflect this learning.

Figure 3.1.1 shows the steady decline in escape latency across days at all ages (Tables 3.1.1a-d). The greatest differences between genotypes were visible on the Day 1 of testing, varying from 21.4-26.4s in 3mo animals, 34-54.8s in 13mo animals, and 42.3-56.9s in 18mo animals. In Study 001 mice there was a strong effect of age on performance (Age: $F=13.88$, $p<0.0001$). At 3mo, there were no genotypic differences in VCN swim latency. By 13mo, BACE KO mice had longer swim latencies than Control mice (BACE KO $p<0.01$). At 18mo as BACE KO swam for shorter trials than all mice carrying the PDAPP transgene (BACE KO; PDAPP $p<0.01$, PDAPP $p<0.01$).

Most of the Study 001 groups' VCN swim latencies were longer on the first testing day as a function of age, except for the PDAPP group, suggesting that this was an age-related influence upon search patterns rather than sensorimotor function as swim speeds were equivalent with age. Although mean latency values were higher with age, there was no indication that any of the transgenic mice completing VCN testing on day 3 then had any significant difference from the Control mice. All genotype groups were able to navigate to the visible platform with a mean escape latency of 10s, with no genotypic deficiency at any age, by day 3 of VCN.

BACE KO; PDAPP mice had the slowest average swim speeds of mice at 3 and 18mo of age as seen in Figure 3.1.2 and Tables 3.1.2a-d. In addition, all of the genotypes experienced age-related declines in swim speed (Table 3.1.2c). However, even these aged BACE KO; PDAPP mice were competent to perform within the strictures of a trials to criterion-based serial learning paradigm as their average swim latencies by Day 3 of VCN testing were <20s, making their inclusion in the experiment reasonable. In addition it appears that the BACE KO mice may have a genotypic deficiency in learning-based performance of the VCN, as their swim speeds are similar to that of Control mice, but still swam for longer periods.

Study 001 albino mice had longer swim latencies than other pigmented mice, but did not have slower swim speeds compared to other mice. This difference was only apparent on Day 3 of testing (Table 3.1.1e), still well within the <20s criterion set in hidden platform testing. Visual inspection of trials in the Watermaze program showed that albino mice were tracked with less precision than pigmented animals, which led to artifactually extended trials for ~1-4s, as trials automatically ended when animal are tracked "on platform" for >1s. This color-based swim speed difference is not treated as a meaningful difference in this measure. Later serial spatial testing with a hidden platform featured better tracking of albino mice than in VCN as there was greater tonal contrast between mice and the platform area.

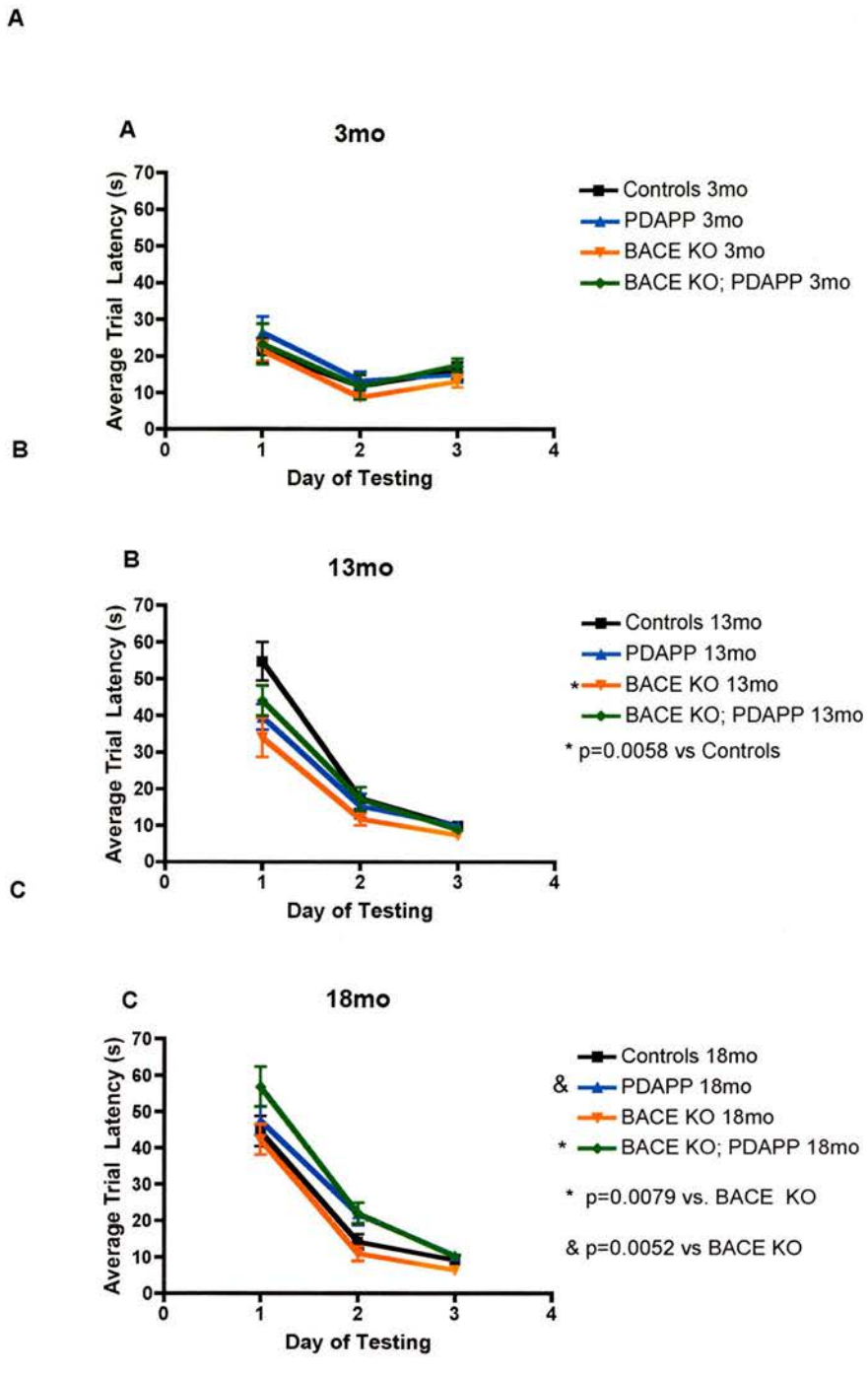


Figure 3.1.1 Visual cued navigation swim latencies of Study 001 mice by age and genotype. A: Latencies for 3mo mice do not differ by genotype. B: At 13mo BACE KO mice had longer swim latencies than Control mice. C: Again at 18mo BACE KO mice require the longest trials overall, swimming for longer time than PDAPP or BACE KO;PDAPP mice. These data suggest a BACE KO specific deficit in associative memory or swimming efficiency.

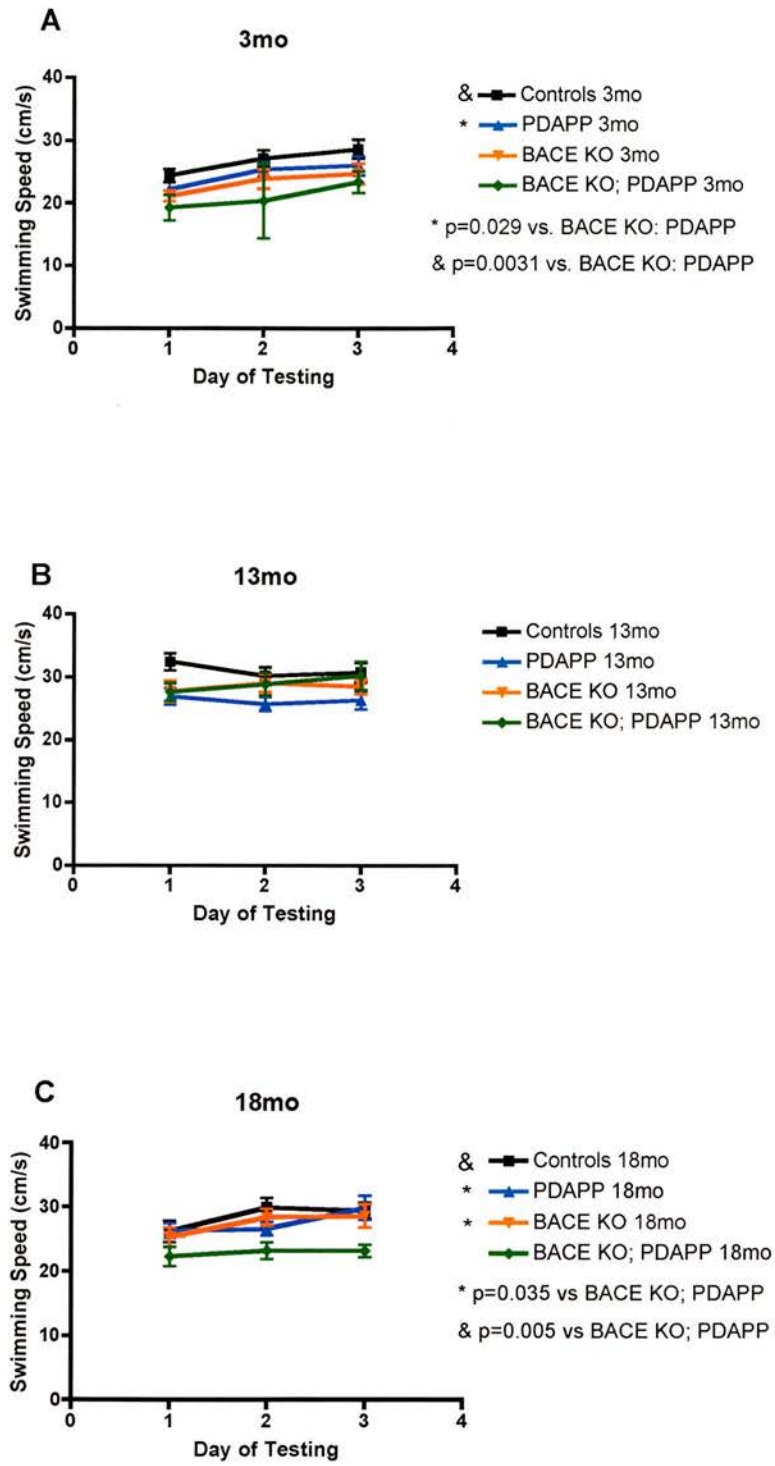


Figure 3.1.2 Visual cued navigation swim speeds of Study 001 mice by age and genotype. A: At 3mo BACE KO; PDAPP mice swim slower than Control and PDAPP mice. B: There were no genotypic differences in swim speed at 13mo. C: 18mo old BACE KO; PDAPP mice swim significantly slower than Control and PDAPP mice although their swim latencies (Figure 3.1.1C) are indistinguishable at this age.

Age (Mo)	Genotype	3 months N	3 months Mean	3 months Std	13 months N	13 months Mean	13 months Std	18 months N	18 months Mean	18 months Std	All N	All Mean	All Std
3	BACE KO; PDAPP	16	23.21	22.39	20	43.98	18.51	19	56.87	23.78	55	42.39	25.19
	BACE KO	22	21.36	13.16	20	33.95	23.95	19	42.25	18.22	61	31.99	20.44
	PDAPP	22	26.4	20.3	23	39.51	16.77	28	47.53	21.12	73	38.64	21.21
	Control	20	21.32	15.39	21	54.75	24.22	21	44.59	19.08	62	40.53	24.12
	ALL	80	23.11	17.68	84	43.06	22	87	47.71	20.97	251	38.31	22.87
13	BACE KO; PDAPP	16	11.55	13.57	20	17.09	14.41	19	22.04	12.96	55	17.19	14.07
	BACE KO	22	8.67	4.6	20	11.71	7.7	19	10.85	8.47	61	10.46	7.01
	PDAPP	22	13.22	11.53	23	15.25	15.91	28	21.81	16.79	73	17.16	15.36
	Control	20	11.69	13.88	21	17.34	13.74	21	14.04	9.3	62	14.4	12.48
	ALL	80	11.34	11.18	84	15.37	13.36	87	17.59	13.49	251	14.85	12.96
18	BACE KO; PDAPP	16	17.45	7.02	20	8.79	5.9	19	10.13	4.42	55	11.77	6.8
	BACE KO	22	13.06	8.07	20	7.29	5.58	19	6.31	3.25	61	9.07	6.72
	PDAPP	22	14.9	10.38	23	9.83	6.27	28	10.29	5.71	73	11.54	7.79
	Control	20	15.91	10.74	21	9.7	5.26	21	9.09	6.3	62	11.5	8.22
	ALL	80	15.16	9.25	84	8.84	5.76	87	9.1	5.31	251	10.98	7.48
ALL	BACE KO; PDAPP	16	17.4	16.05	20	23.39	20.44	19	29.68	25.34	55	23.78	21.62
	BACE KO	22	14.46	10.53	20	17.65	18.77	19	19.8	19.38	61	17.17	16.72
	PDAPP	22	18.18	15.67	23	21.53	18.83	28	26.54	22.19	73	22.44	19.59
	Control	20	16.53	13.83	21	27.26	25.55	21	22.57	20.21	62	22.14	20.9

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.6	6	0.053
Age	13.88	2	<0.0001
Genotype	11.73	3	0.078

	p-value				
Comparison	CE KO; PDA	BACE KO	PDAPP	Controls	ALL
3 vs 13 months	<0.0001	0.0015	0.0015	<0.0001	<0.0001
3 vs 18 months	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
13 vs 18 months	0.34	0.11	0.052	0.77	0.37

	p-value			
Comparison	3 mo	13 mo	18 mo	ALL
BACE KO; PDAPP VS BACE KO	0.21	0.13	0.0079	0.0076
BACE KO; PDAPP VS PDAPP	0.094	0.49	0.82	0.89
BACE KO; PDAPP VS CONTROL	0.36	0.68	0.21	0.76
BACE KO VS PDAPP	0.74	0.062	0.0052	0.016
BACE KO VS CONTROL	0.9	0.0058	0.5	0.058
PDAPP VS CONTROL	0.79	0.38	0.2	0.83

	Day								
	1			2			3		
Color	N	Mean	(Std)	N	Mean	(Std)	N	Mean	(Std)
Albino	142	38.66	(24.55)	142	16.25	(13.42)	142	13.38	(7.48)
Agouti	55	38.19	(19.58)	55	13.10	(10.51)	55	8.24	(7.20)
Black	54	37.52	(21.73)	54	12.97	(13.76)	54	7.44	(5.14)

Tables 3.1.1a-e Descriptive, MANOVA and pairwise ANOVA statistics for VCN swim latencies in Study 001 mice by age and genotype.

Age (Mo)	Genotype	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
3	BACE KO; PDAPP	16	19.28	3.95	20	27.59	6.27	19	22.31	6.58	55	23.35	6.67
	BACE KO	22	21.16	4.13	20	27.69	7.57	19	25.36	6.65	61	24.61	6.72
	PDAPP	22	22.22	3.52	23	26.9	6.29	28	26.32	6.11	73	25.27	5.82
	Control	20	24.38	4.78	21	32.37	6.15	21	26.25	7.66	62	27.72	7.11
	ALL	80	21.76	4.41	84	28.64	6.82	87	25.06	6.8	251	25.24	6.7
13	BACE KO; PDAPP	16	20.39	23.86	20	28.82	7.96	19	23.24	5.63	55	24.36	6.99
	BACE KO	22	23.9	7.06	20	29.03	6.32	19	28.48	5.44	61	27.01	6.68
	PDAPP	22	25.4	5.85	23	25.69	5.28	28	26.66	5.59	73	25.98	5.52
	Control	20	27.1	6.1	21	30.14	6.14	21	29.95	6.86	62	29.1	6.43
	ALL	80	24.2	6.29	84	28.34	6.54	87	27.11	6.27	251	26.65	6.55
18	BACE KO; PDAPP	16	23.41	6.8	20	30.18	10.07	19	23.23	4.1	55	25.81	8.08
	BACE KO	22	24.74	7.71	20	28.53	5.45	19	28.66	7.85	61	27.2	7.22
	PDAPP	22	26.02	7.25	23	26.27	6.83	28	29.91	9.84	73	27.59	8.32
	Control	20	28.6	6.85	21	30.71	7.16	21	29.44	5.82	62	29.6	6.58
	ALL	80	25.79	7.3	84	28.85	7.61	87	28.07	7.84	251	27.6	7.67
ALL	BACE KO; PDAPP	16	21.03	5.25	20	28.87	8.18	19	22.93	5.45	55	24.51	7.3
	BACE KO	22	23.27	6.57	20	28.42	6.42	19	27.5	6.77	61	26.27	6.94
	PDAPP	22	24.55	5.9	23	26.29	6.09	28	27.63	7.51	73	28.8	6.72
	Control	20	26.69	6.13	21	31.07	6.47	21	28.55	6.91	62	26.52	7.05

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.1	6	0.35
Genotype*Gender	0.38	9	0.95
Gender*Age	1.24	6	0.28
Gender	3.51	1	0.035
Age	8.27	2	<0.0001
Genotype	3.07	3	0.0013

	p-value				
Comparison	CE KO; PDA	BACE KO	PDAPP	Controls	ALL
3 vs 13 months	<0.0001	0.0056	0.04	0.0016	<0.0001
3 vs 18 months	0.18	0.045	0.1	0.49	0.003
13 vs 18 months	0.018	0.62	0.34	0.0043	0.0018

	p-value			
Comparison	3 mo	13 mo	18 mo	ALL
BACE KO; PDAPP VS BACE KO	0.23	0.98	0.035	0.036
BACE KO; PDAPP VS PDAPP	0.029	0.42	0.035	0.1
BACE KO; PDAPP VS CONTROL	0.0031	0.13	0.005	<0.0001
BACE KO VS PDAPP	0.74	0.29	0.25	0.38
BACE KO VS CONTROL	0.15	0.11	0.95	0.062
PDAPP VS CONTROL	0.58	0.045	0.14	0.039

Tables 3.1.2a-e Descriptive, MANOVA and pairwise ANOVA statistics for VCN swim latencies in Study 001 mice by age and genotype.

Serial spatial reversal learning

Following the 3 days of VCN, Study 001 mice were tested in cross-sectional age groups on a series of spatial locations for measures of learning acquisition rates and memory capacity. These direct measures can be further analysed to provide interpretations about changes in learning and memory by genotype with age. As described in Ch.2 p.10-15, the specified learning criterion for each individual mouse was to locate the hidden escape platform in <21s in three consecutive trials. Upon reaching this criterion on a given day, mice were tested on a new spatial location on the following day. In Chen et al.'s (2000) study using an MWM protocol, trials to criterion (TTC) was measured in a distinct experiment in which all animals were tested until they learned 5 spatial locations, after which the animals were tested for learning capacity over a period of 10 days in which it was possible for each mouse to experience up to 10 separate platform locations. In addition, Chen et al. (2000) validated their MWM protocol in both cross-sectional and longitudinal groups of PDAPP and Control animals at various ages. In this experiment however, TTC and platform learning capacity measures were combined into a single cross-sectional experiment where over 10 days mice of young (3mo), middle (13mo) and old (18) ages were tested on a maximum of 10 platform locations and minimum of 3 locations.

3.2 Acquisitional and serial learning deficits

The first spatial memory measure that is generated by Study 001 MWM testing is the mean number of trials required for an animal to reach the specific learning criterion on platform location one. Analysis of number of trials to criterion for location one alone is akin to the classical non-serial reference memory version of the watermaze in which only one spatial task is solved. Deficits in learning this task are thus the earliest opportunity to observe impairments in spatial memory .

By taking the average of TTC on this first platform location across ages so that the major variable is genotype, the only significantly different group was the BACE KO; PDAPP line, which required 11 trials to learn location one compared to 7.5

trials in Control mice as seen in Figure 3.2.1A ($F=2.86$, df 3/239, $p<0.05$). By examining performances to criterion on location one, it was possible to detect impairments in 3 mo PDAPP and BACE KO; PDAPP mice relative to Control mice with a post-hoc analysis (Table 3.2.1a-d). Mice carrying the PDAPP transgene at 3mo required an average of 7.23 and 11.19 trials to learn what Control mice acquired in 5.25 trials ($p<0.05$, $p<0.025$). By 18mo all groups have performance levels that are indistinguishable from each other. At all ages it is clear that both in Study 001 PDAPP and BACE KO; PDAPP mice were impaired in single-task spatial learning relative to Control mouse lines. BACE KO mice were statistically indistinguishable from Controls in this task whether compared by age group or with performances averaged over ages, suggesting that they were unimpaired in initial spatial learning.

By expanding this single-platform learning analysis to platform locations 2 and 3, it is possible to ascertain the status of the first two memory reversals. Learning successive tasks requires not only the ability to learn a new location but also to 'rewrite' the memory of the prior location. Deficits in learning platforms 2 and 3 by genotype revealed a genotypic impairment in spatial memory reversal that was static with age, but amplified with respect to successive locations (Figure 3.2.2). Control mice learned the locations of platforms in about 4.05-5.40 trials, while PDAPP mice required 6.82-10.39 trials, BACE KO mice needed 4.70-7.0 trials, and BACE KO; PDAPP mice had to swim 7.3-12.21 trials to reach the same learning criterion on location 2 (by genotype $F=8.75$ df 3/239, $p<0.0001$). Differences in TTC on Location 3 were even wider, as the genotypic deficits of the PDAPP, BACE KO and especially the BACE KO; PDAPP mouse lines expanded (by genotype $F=21.35$, df 3/238, $p<0.001$). Tables 3.2.1a-d and 3.2.2a-h summarizes the statistics for both the initial spatial learning deficits on Location 1 as well as the reversal deficits on Locations 2 and 3 by genotype and age.

At all ages, the BACE KO; PDAPP mice required the greatest number of trials to reach the same performance criterion on the first spatial location as other mice in this study and were also most impaired in reversal-based learning. This general

pattern is evident when average trials to criterion for locations 1-3 are assessed across ages and at individual ages (Figure 3.2.3, Table 3.2.3a-d). Averaged across all ages, BACE KO; PDAPP mice have generalized learning and memory deficits that appear to be additives of PDAPP and BACE KO deficits alone as they require overall about 12 trials per location relative to the 8.1-9.45 trials for PDAPP and BACE KO mice, and 5.8 trials for Control animals (Figure 3.2.3a, Table 3.2.3a). When broken into age groups, PDAPP and BACE KO mice have a progressive spatial memory impairment that is present by 3mo relative to Controls, both averaging 7.2-10.2 trials per location compared to 5.4-6.4 trials in Controls.

This increased BACE KO; PDAPP deficit finding was unexpected, as the concept of therapeutic inhibition of BACE for AD as well as the Amyloid Hypothesis would lead one to expect BACE KO would abolish or at least reduce the cognitive deficits associated with amyloid overexpression in PDAPP mice. Thus far the severe impaired acquisitional learning phenotype of BACE KO; PDAPP mice instead argues that BACE activity, whether via APP cleavage to Aβ or by action on some other substrate, is a requirement for intact spatial memory processes.

	3 months			13 months			18 months			All		
Genotype	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	16	11.19	8.78	20	10.95	7.13	19	11	5.85	55	11.04	7.12
BACE KO	22	6.32	3.81	20	8.55	4.59	19	9.42	7.11	61	8.02	5.36
PDAPP	22	7.23	4.82	23	12.61	8.54	28	10.21	7.91	73	10.07	7.55
Control	20	5.25	2.69	21	7.43	3.85	21	9.86	7.93	62	7.55	5.6
ALL	80	7.28	5.55	84	9.95	6.61	87	10.13	7.23	251	9.16	6.63

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	0.68	6	0.67
Age	6.68	2	0.0015
Genotype	2.86	3	0.038

			p-value		
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.71	0.12	0.015	0.11	0.0036
3 vs 18 months	0.54	0.13	0.16	0.009	0.0026
13 vs 18 months	0.79	0.98	0.24	0.31	0.92

			p-value	
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.05	0.37	0.35	0.021
BACE KO; PDAPP VS PDAPP	0.15	0.67	0.34	0.24
BACE KO; PDAPP VS CONTROL	0.013	0.14	0.47	0.0063
BACE KO VS PDAPP	0.58	0.18	0.77	0.2
BACE KO VS CONTROL	0.51	0.57	0.65	0.66
PDAPP VS CONTROL	0.24	0.05	0.85	0.083

Tables 3.2.1a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 001 mice on TTC1 performance by age and genotype.

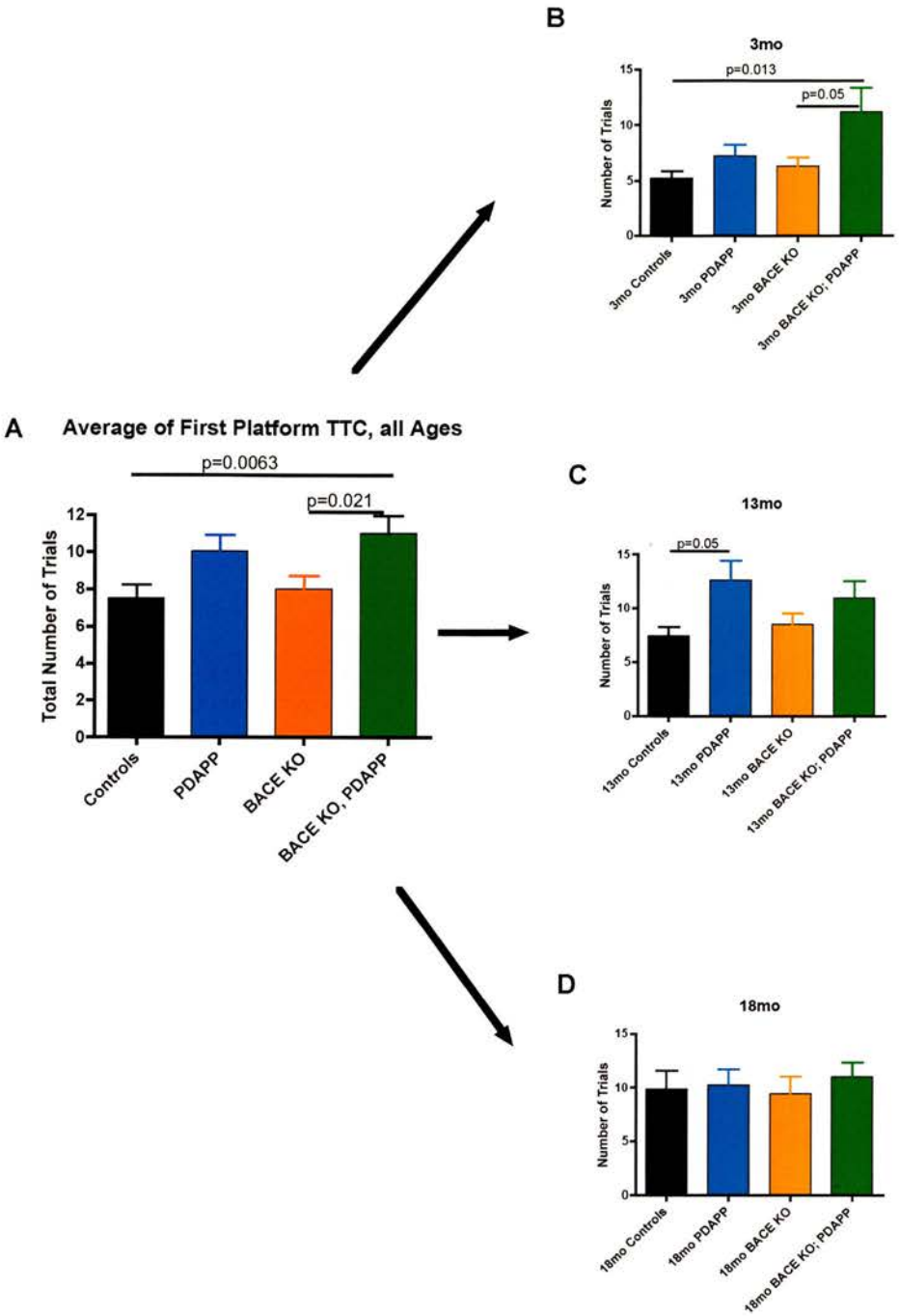


Figure 3.2.1 Deficits in initial spatial memory acquisition in Study 001 mice by age and genotype. A: Average of first platform location trials to criterion across all ages, by genotype. By this measure BACE KO; PDAPP mice required more trials to learn this task than BACE KO and Control mice. B: At 3mo BACE KO; PDAPP mice show a non-age-related deficit in the ability to learn location 1 compared to BACE KO and Control mice. C: By 13mo, PDAPP mice are deficient in TTC1 relative to Control mice. D: There are no differences in initial spatial learning between genotypes by 18mo.

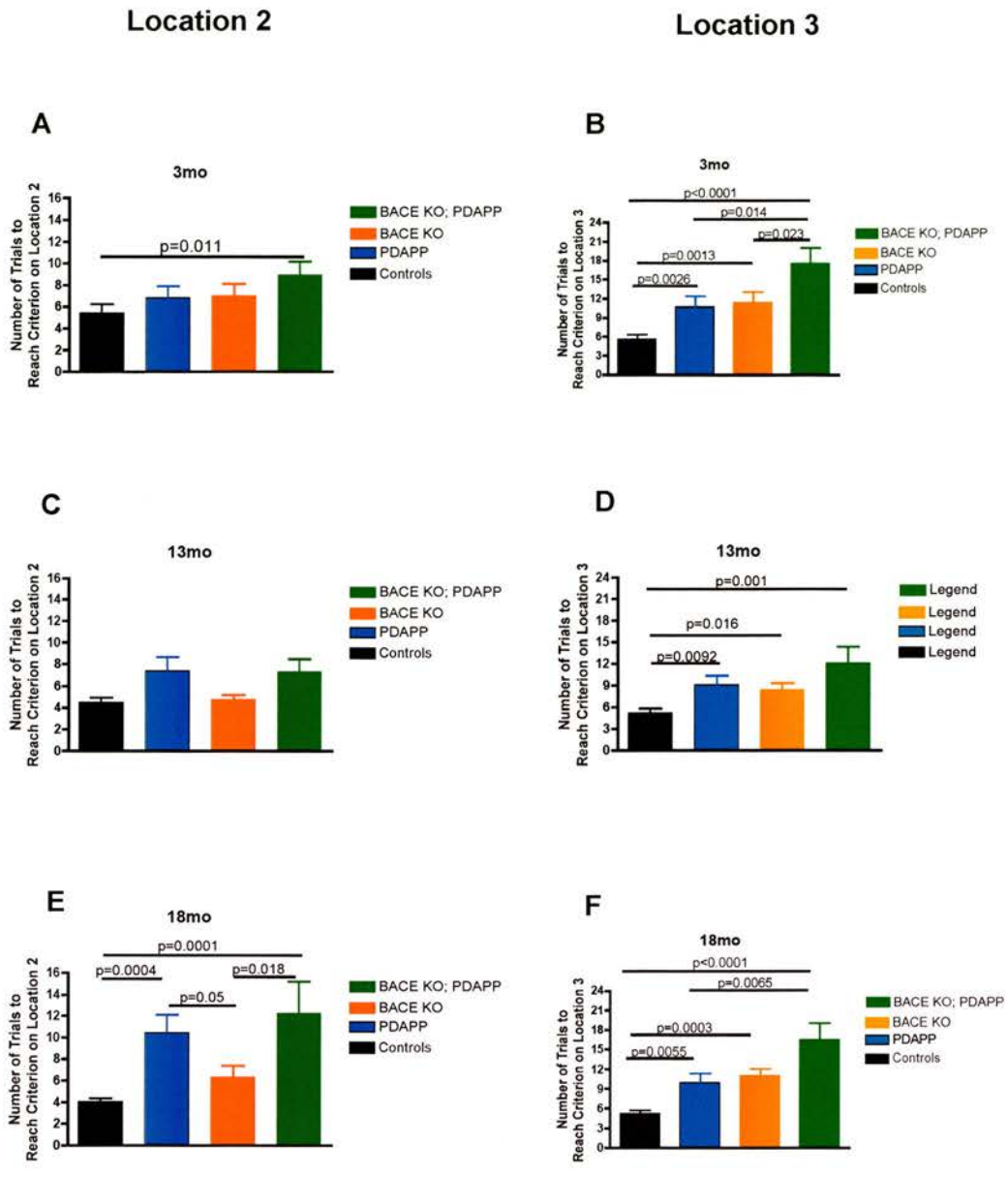


Figure 3.2.2 Deficits in spatial reversal memory on locations 2 and 3 in Study 001 mice by age and genotype. A,C: At 3 the BACE KO; PDAPP mice have perseverative deficits relative to Control mice on Location 2, with no differences at 13mo. E: All mice carrying the PDAPP transgene are impaired on second platform learning compared to BACE KO and Control mice. BACE KO mice have no perseverative deficits at any age tested. B: On location 3, both PDAPP and BACE KO mice have learning deficits compared to Controls, while BACE KO; PDAPP mice have additive PDAPP and BACE KO-derived impairments. D: 13mo old BACE KO; PDAPP, BACE KO and PDAPP mice have decreased ability to learn location 3 compared to Control mice. F: At 18mo BACE KO; PDAPP, BACE KO and PDAPP mice display location 3 learning deficits relative to Control mice, and the BACE KO; PDAPP mouse deficits again appear to be synergistic to BACE KO and PDAPP impairments.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	16	8.94	4.89	20	7.3	5.31	19	12.21	13.1	55	9.47	8.86
BACE KO	22	7	5.33	20	4.7	2.11	19	6.32	4.64	61	6.03	4.33
PDAPP	22	6.82	5.2	23	7.39	6.04	28	10.39	9	73	8.37	7.22
Control	20	5.4	3.75	21	4.48	1.89	21	4.05	1.24	62	4.63	2.52
ALL	80	6.94	4.91	84	6	4.47	87	8.37	8.75	251	7.12	6.44

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.23	6	0.29
Age	1.86	2	0.16
Genotype	8.75	3	<0.0001

	p-value				
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.19	0.19	0.84	0.68	0.14
3 vs 18 months	0.75	0.66	0.11	0.41	0.78
13 vs 18 months	0.085	0.4	0.16	0.68	0.078

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.11	0.13	0.018	0.0017
BACE KO; PDAPP VS PDAPP	0.098	0.9	0.51	0.15
BACE KO; PDAPP VS CONTROL	0.011	0.082	0.0001	<0.0001
BACE KO VS PDAPP	0.94	0.015	0.05	0.057
BACE KO VS CONTROL	0.27	0.84	0.15	0.12
PDAPP VS CONTROL	0.31	0.094	0.0004	0.0004

Tables 3.2.2a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 001 mice on TTC2 performance by age and genotype.

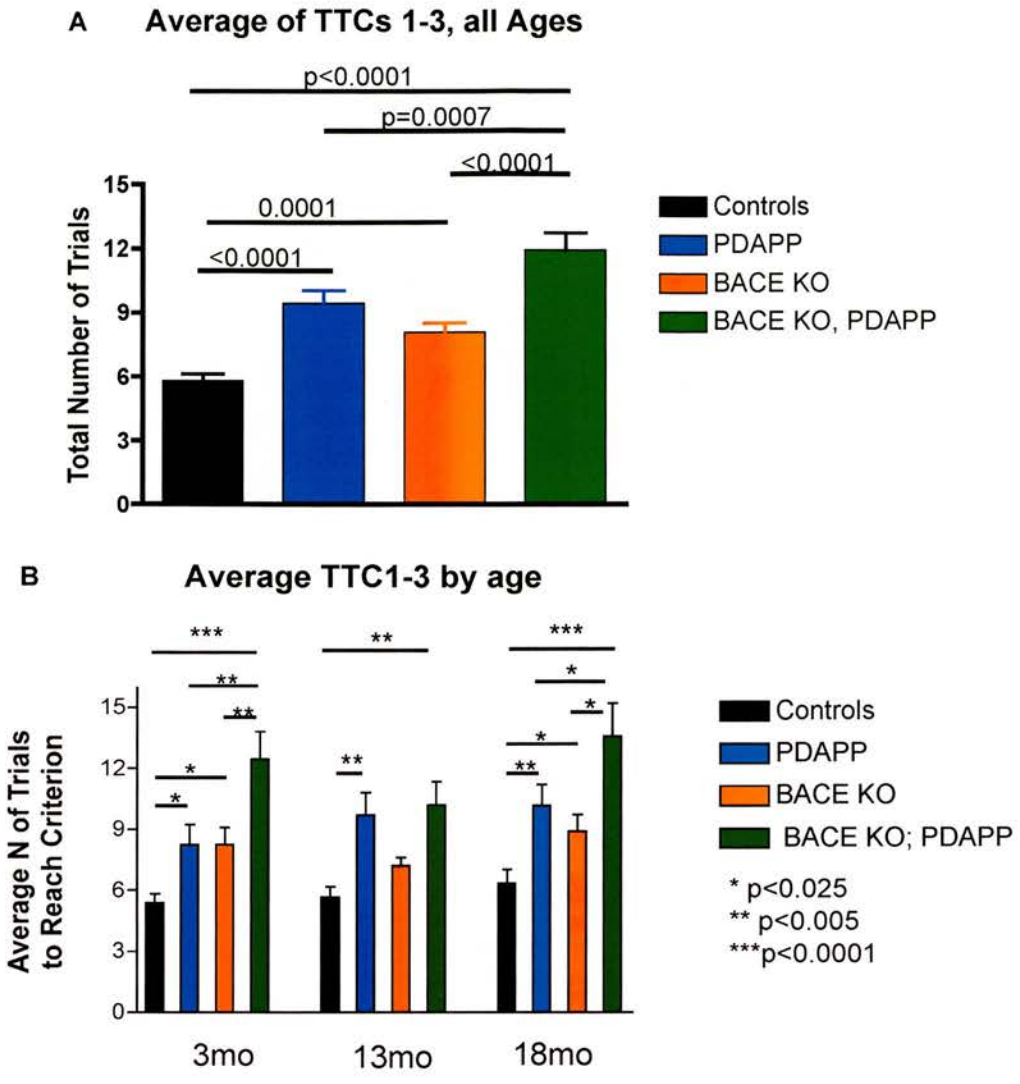
Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	16	17.5	10	20	12.1	9.98	18	16.56	10.7	54	15.19	10.3
BACE KO	22	11.41	7.96	20	8.35	4.21	19	10.95	4.88	61	10.26	6.07
PDAPP	22	10.68	8.1	23	9.13	5.65	28	9.96	7.38	73	9.92	7.05
Control	20	5.55	3.36	21	5.14	2.83	21	5.19	2.52	62	5.29	2.88
ALL	80	10.96	8.5	84	8.65	6.58	86	10.4	7.85	250	9.99	7.7

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	0.58	6	0.75
Age	2.76	2	0.065
Genotype	21.35	3	<0.0001

	p-value				
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.019	0.3	0.46	0.81	0.026
3 vs 18 months	0.69	0.63	0.59	0.95	0.8
13 vs 18 months	0.045	0.14	0.8	0.86	0.045

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.023	0.38	0.16	0.0086
BACE KO; PDAPP VS PDAPP	0.014	0.42	0.0065	0.0006
BACE KO; PDAPP VS CONTROL	<0.0001	0.001	<0.0001	<0.0001
BACE KO VS PDAPP	0.83	0.92	0.22	0.4
BACE KO VS CONTROL	0.0013	0.016	0.0003	<0.0001
PDAPP VS CONTROL	0.0026	0.0092	0.0055	<0.0001

Tables 3.2.2e-h Descriptive, MANOVA, and pairwise ANOVA statistics for Study 001 mice on TTC3 performance by age and genotype.



3.2.3 Deficits in spatial memory average TTC Location 1-3 in Study 001 mice by age and genotype. A: Grouped across ages, PDAPP and BACE KO animals show impaired spatial learning and memory relative to Controls; BACE KO; PDAPP mice have spatial memory deficits in excess of the PDAPP and BACE KO mice, suggesting an additive deficit. B: Separated by ages, it is apparent that BACE KO; PDAPP mice have the poorest learning memory and profile at any given age. PDAPP mice develop deficits in spatial criterion-based learning by 3mo, which are still present at 18mo, while BACE KO mice have milder deficit that is significantly different at 3 and 18mo compared to Control mice, but not at 13mo.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	16	12.48	5.33	20	10.22	5.16	19	13.58	7.08	55	12.02	5.99
BACE KO	22	8.24	4.08	20	7.2	1.8	19	8.89	3.71	61	8.1	3.39
PDAPP	22	8.24	4.67	23	9.71	5.26	28	10.19	5.55	73	9.45	5.2
Control	20	5.4	1.98	21	5.68	2.2	21	6.37	3.05	62	5.82	2.46
ALL	80	8.39	4.74	84	8.2	4.28	87	9.72	5.61	251	8.79	4.95

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	0.8	6	0.57
Age	1.6	2	0.2
Genotype	22.96	3	<0.0001

	p-value				
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.1	0.69	0.26	0.73	0.71
3 vs 18 months	0.73	0.45	0.17	0.32	0.094
13 vs 18 months	0.038	0.26	0.86	0.5	0.038

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.003	0.086	0.0089	<0.0001
BACE KO; PDAPP VS PDAPP	0.0025	0.72	0.015	0.0007
BACE KO; PDAPP VS CONTROL	<0.0001	0.0004	<0.0001	<0.0001
BACE KO VS PDAPP	0.93	0.16	0.67	0.31
BACE KO VS CONTROL	0.01	0.068	0.023	0.0001
PDAPP VS CONTROL	0.013	0.001	0.0035	<0.0001

Tables 3.2.3a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 001 mice on averaged TTC1-3 performance by age and genotype.

3.3 Learning capacity (Number of platforms learned)

Beyond the initial three platform locations learned, Study 001 mice tested in this serial spatial learning task may learn up to 10 locations depending on their ability to sufficiently demonstrate their knowledge of test locations on any one day. The measure that corresponds to the number of total platform locations learned within this criterion-based context is called learning capacity (LC), although because of the use of a learning criterion, this is not strictly a measure of capacity.

Figure 3.3.1 and Table 3.3.1a-d shows the LC measure results from Study 001 mice across age and genotype. Control mice at all ages have the highest LC levels, learning between 8.3-8.95 out of a possible 10 locations for each age tested. BACE KO mice had LC performances that were intermediate between Controls and transgenic groups, as they were statistically distinct from both at most ages (Table 3.3.1a). At all ages PDAPP mice performed more poorly than Controls in LC, learning between 6.9-7.5 locations over time (genotype: $F=15.59$, df 3/241, $p<0.0001$). In turn, PDAPP mice performed significantly better than BACE KO; PDAPP mice at 3 and 18mo. Again, BACE KO; PDAPP mice consistently had the poorest memory measure profile, with LC numbers ranging from 5.8-6 platforms learned. At nearly every age BACE KO; PDAPP mice performed significantly worse than other genotype groups, with the sole exception of 13mo PDAPP mice. There was no significant effect of an LC deficit due to age within any genotype group ($F=0.88$).

These data argue that both the PDAPP and BACE KO genotypes conferred a spatial learning capacity deficiency in comparison to non-transgenic Control mice. At all

	3 months			13 months			18 months			All		
Genotype	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	16	5.94	1.57	20	6.45	1.85	19	5.84	1.98	55	6.09	1.81
BACE KO	22	7.77	1.88	20	7.55	1.36	19	6.95	1.72	61	7.44	1.68
PDAPP	22	7.45	1.9	23	6.91	2.23	28	7.25	1.92	73	7.21	2
Control	20	8.95	1.28	21	8.86	1.46	21	8.33	1.71	62	8.71	1.5
ALL	80	7.61	19.5	84	7.44	1.97	87	7.14	2	251	7.39	1.97

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	0.77	6	0.6
Age	2.71	2	0.069
Genotype	15.59	3	<0.0001

	p-value				
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.39	0.68	0.31	0.87	0.76
3 vs 18 months	0.87	0.14	0.69	0.27	0.12
13 vs 18 months	0.29	0.29	0.5	0.34	0.2

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.0018	0.05	0.056	<0.0001
BACE KO; PDAPP VS PDAPP	0.0098	0.39	0.0081	0.0005
BACE KO; PDAPP VS CONTROL	<0.0001	<0.0001	<0.0001	<0.0001
BACE KO VS PDAPP	0.55	0.24	0.57	0.48
BACE KO VS CONTROL	0.033	0.019	0.014	<0.0001
PDAPP VS CONTROL	0.0068	0.0003	0.035	<0.0001

ages the genotype with the poorest performance was the BACE KO; PDAPP group, while the deficits of the PDAPP and BACE KO were similar in scale, implicating an additive effect of deficits from the presence of the PDAPP transgene and the homozygous deletion BACE KO. This pattern reproduced the implication from TTC measures that BACE gene deletion and the PDAPP transgene independently contribute to the BACE KO; PDAPP spatial memory phenotype.

Tables 3.3.1a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 001 mice on learning capacity by age and genotype.

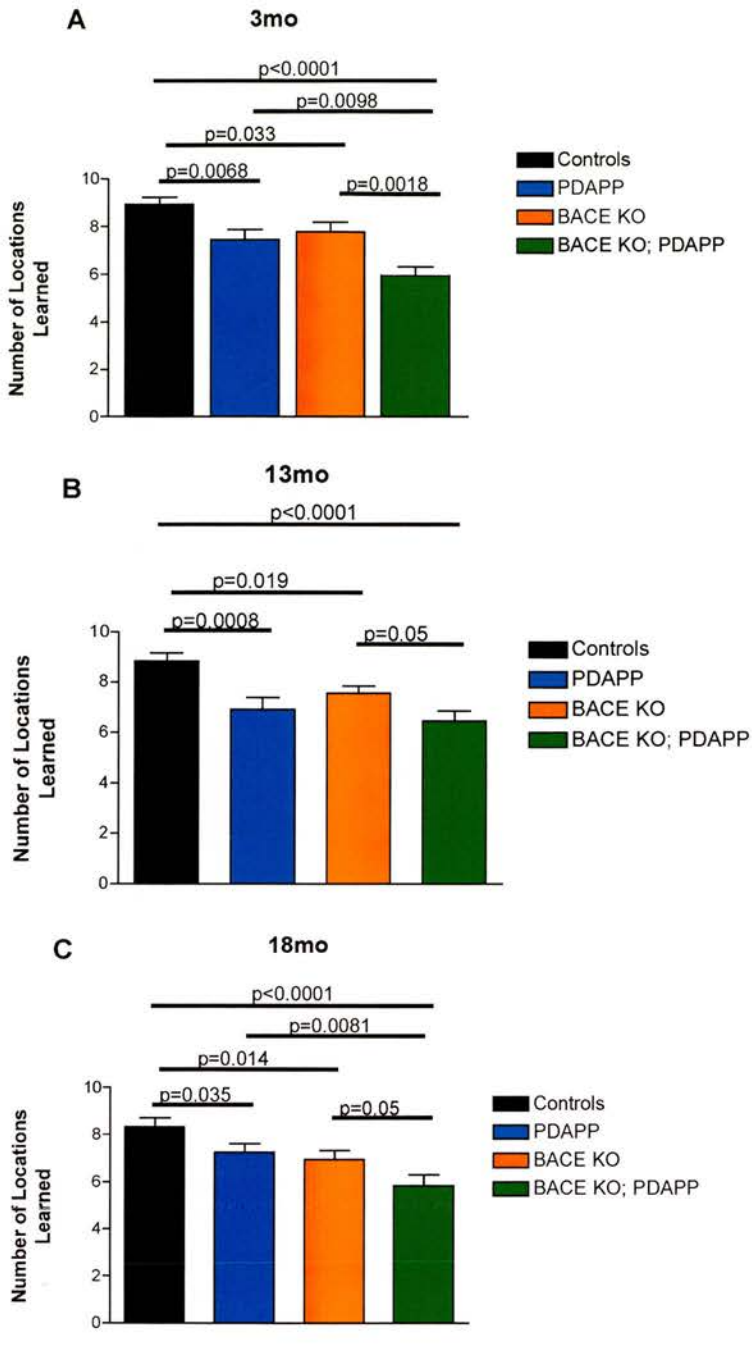


Figure 3.3.1 Deficits in spatial memory capacity in Study 001 mice by age and genotype. A: At 3mo of age, PDAPP and BACE KO mice learn fewer locations than Control animals, although BACE KO; PDAPP learn significantly less than all groups. B: As at 3mo, PDAPP and BACE KO mice have impairments relative to Control animals, but the BACE KO mice perform better than BACE KO; PDAPP mice. C: BACE KO; PDAPP mice again have the worst spatial memory capacity profiles relative to BACE KO, PDAPP and Control mice, while BACE KO and PDAPP mice are also deficient relative to Controls.

Non-Memory Phenotypes

3.4 Seizures and other observations

In the course of handling and testing the Study 001 mice, a number of spontaneous seizures were observed. The seizures were clonic-tonic type, in which mice would run around their cages, with intermittent whole-body tremors and asymmetric paw waving. Post-seizure mice would be extremely weak, with little to no grip strength, hemiplegia and would have to be removed from study (Figure 3.4, Table 3.4A). Animals were also removed from study for other reasons, including excessive anxiety, spinning, floating and blindness, with greater percentage of removals linked to the PDAPP transgene. It is notable to mention that the total numbers per genotype group between Tables 3.4A and 3.4B are not equal. This was due to the fact that the percentage of animals removed from study reflected the current number of animals in the study at that point, which may have been reduced by prior removals. Analysis of the seizure observations by genotype revealed a pattern related to the BACE KO phenotype (Table 3.4B). It is also possible that animals observed to have hemiplegia and weakness and inability to swim may have had unobserved seizures. In light of these observations, it may be that homozygous BACE gene deletion predisposes Study 001 mice to have seizure activity, a hypothesis explored extensively in Study 011.

3.5 Death and survival rates

Animals observed to have seizures often died within 24h of the seizure. These in-house deaths rates motivated an analysis of the death rates of Study 001 mice at the remaining throughout life at the vendor site, thus not exposed to the stress of shipment and behavioral testing. Using a survival analysis method, the mortality ages of 50% of the various Study 001 genotypes groups was calculated with information regarding the breeding colony kept at the vendor site (Table 3.5, Figure 3.5). Disparities between numbers of animals between groups do not affect analysis, which is based on rate of death within each group only.

It appears that the degree of genetic modification (e.g. ablation of one or both BACE1 genes, carrying one or two PDAPP transgenes) was related to the life expectancy of the Study 001 mice, although the BACE KO genotype (2 degrees of modification) lives longer on average than the PDAPP mice (one degree of modification). Although mice carrying one copy of the PDAPP transgene (PDAPP 1x, always heterozygous for transgene unless otherwise noted) reached 50% group mortality in the same 6 month timeframe as the animals carrying 2 PDAPP transgenes (PDAPP 2x), PDAPP 1x mice survived at a much higher rate that PDAPP 2x mice after 8 months (Figure 3.5A). Combinations of the BACE KO and PDAPP transgene appear to be additive in that they had shorter lifespans than either of the component genotypes. In-house deaths are highest in animals with BACE KO, with a similar pattern of deaths at the vendor site.

Finally, it has been noted that even the non-transgenic Control mice have relatively short lifespans, which is about 15mo on average (Figure 3.5A). This is not unexpected, given their DBA/2J background strain heritage, as this line has been noted to have unusually short lifespans, varying between 15-20 months (Goodrick, 1975).

Age (Mo)	Control	PDAPP	BACE KO	BACE KO; PDAPP
3	16.6% (N=4 of 24)	8.3% (N= 2 of 24)	8.3% (N= 2 of 24)	35.7% (N=10 of 28)
13	12.5% (N=3 of 24)	36.1% (N= 13 of 36)	23.2% (N= 6 of 26)	31.1% (N=9 of 29)
18	8.7% (N=2 of 23)	31.7% (N= 13 of 41)	24% (N= 6 of 24)	47.2% (N= 17 of 36)

Table 3.4A Study 001 mice removed from study by age and genotype.

Age (Mo)	Control	PDAPP	BACE KO	BACE KO; PDAPP
3	0%	0%	4.2% (N= 1 of 23)	17.9% (N=4 of 21)
13	0%	0%	8.0% (N= 2 of 22)	12.0% (N=3 of 23)
18	0%	0%	4.0% (N= 1 of 20)	14.0% (N= 3 of 22)

Table 3.4B Spontaneous seizures in Study 001 mice by age and genotype.

	N	Age of 50% Group Mortality (mo)	Degrees of Genetic Modification	PDAPP Transgenes	Deleted Alleles
Control	48	14	0	0	0
BACE KO	49	8	2	0	2
PDAPP 1x	143	6	1	1	0
PDAPP 2x	52	6	2	2	0
BACE KO; PDAPP 1x	100	4	3	1	2
BACE KO; PDAPP 2x	82	2.75	4	2	2

Table 3.5 50% Mortality ages of Study 001 mice, 1x indicates one copy of PDAPP transgene (heterozygous), 2x indicates two copies of PDAPP transgene (homozygous).

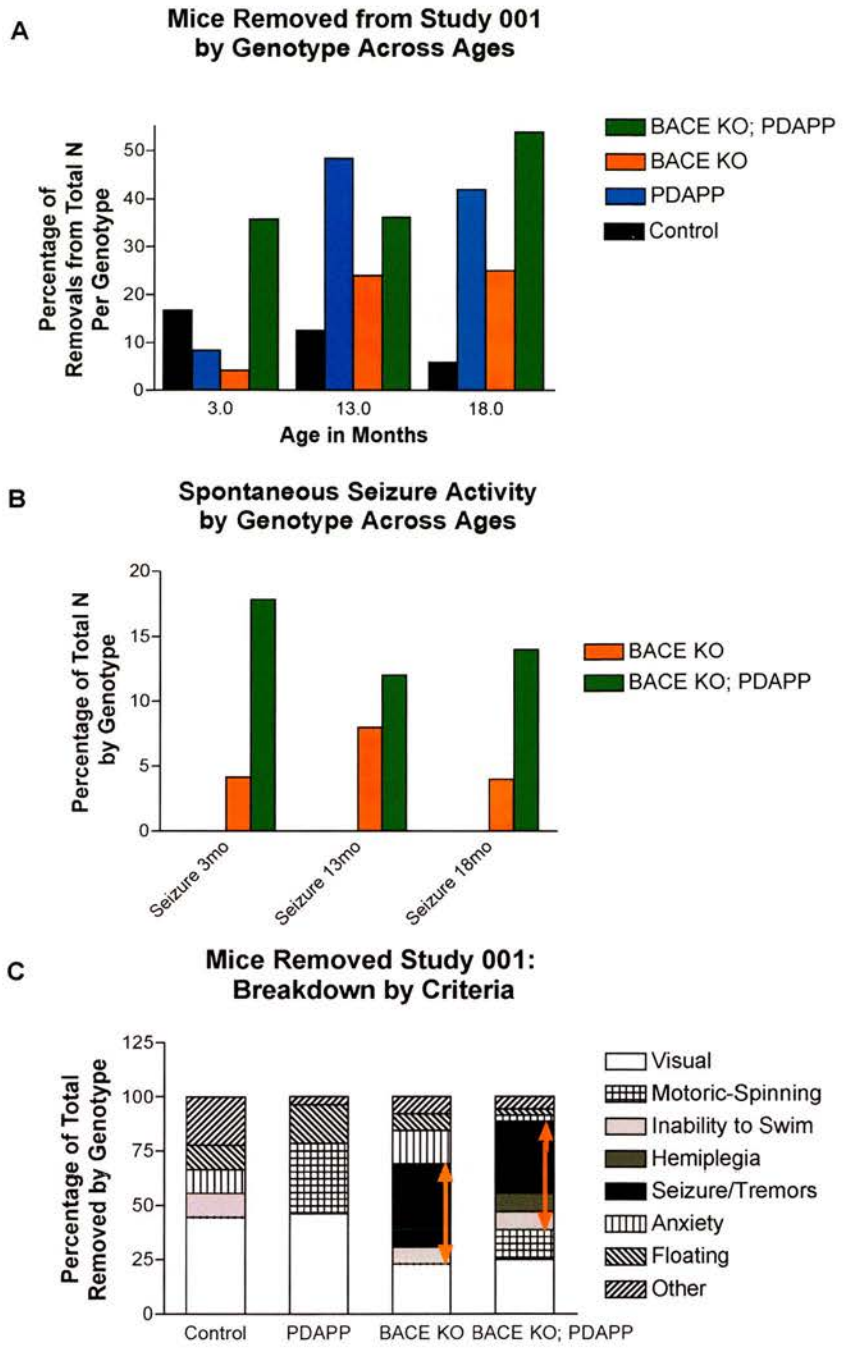


Figure 3.4 Seizures and other observations in Study 001 mice. A; Percentage of mice removed by genotype/age, note the preponderance of PDAPP transgenic mouse removals relative to BACE KO and Control mice. B: Spontaneous seizures are observed in mice with BACE gene deletions, with more seizure activity in BACE KO; PDAPP mice. C: Study removal reasons by genotype. Red arrows indicate removals due to seizure or possible seizure-related activity, like hemiplegia or acute inability to swim, which were prevalent in mice lacking BACE.

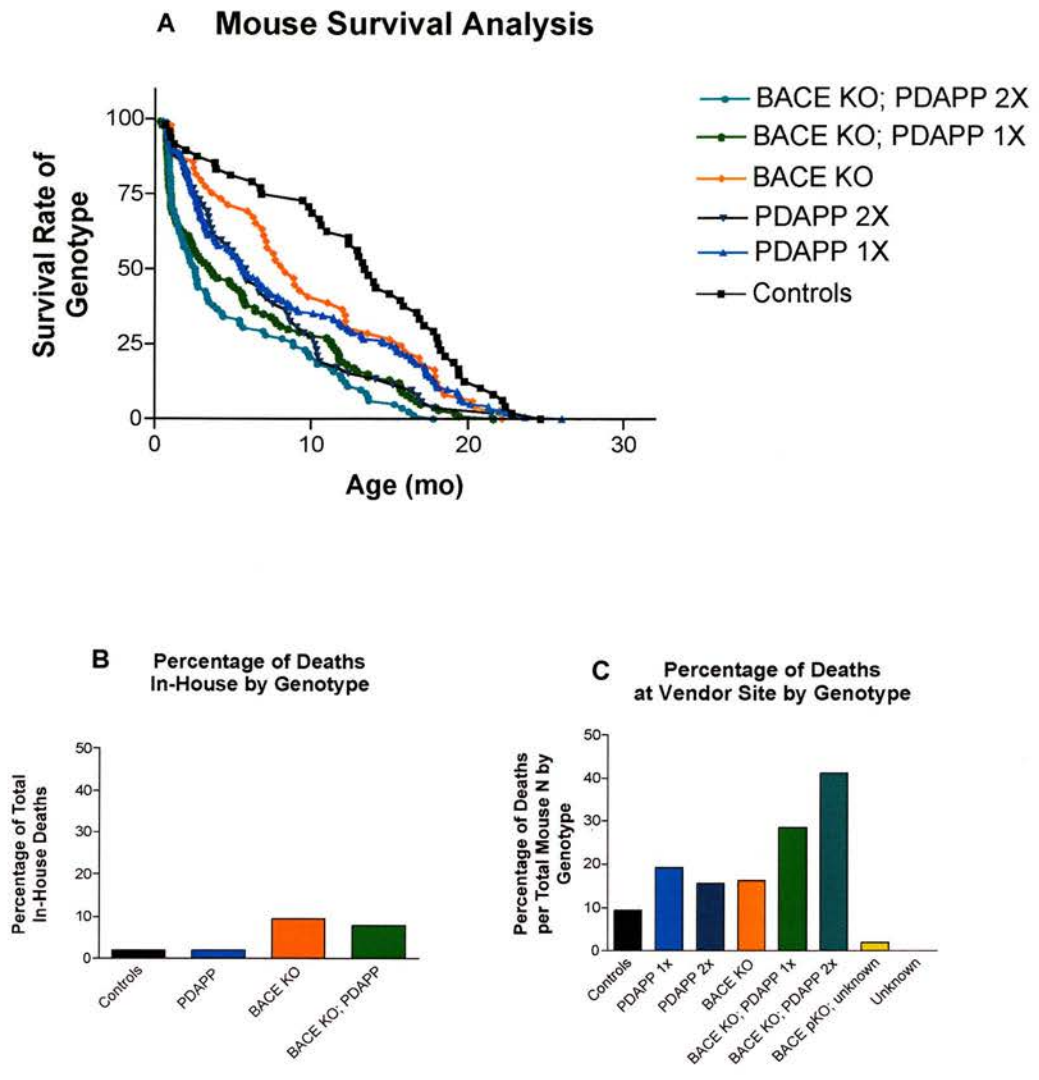


Figure 3.5 Death and survival rates in Study 001 mice. A: Survival analysis by genotypes. A: Survival analysis of Study 001 mice by genotype, housed at the vendor site. The degree of genetic manipulation apparently has a deleterious effect on mouse lifespan, as mice with 3-4 gene modifications have the shortest lives (BACE KO, PDAPP 2X, BACE KO; PDAPP 1X). The PDAPP transgene also negatively impacts lifespan as mice with one or two transgene copies live shorter lives than BACE KO or Control mice. B: Spontaneous death rates by genotype after shipment to Elan, animals with BACE KO appear to die at higher levels than PDAPP or Control mice. C: Spontaneous deaths at the vendor site appear to generally follow a pattern of greater deaths in mice with more genetic modifications, similar to Figure 3.5A.

Calbindin and Amyloid Histology

3.6 Calbindin histology in the hippocampal Outer Molecular Layer

Calbindin (CB) is a Ca^{++}-binding protein ubiquitously expressed in cerebral neurons often used as a surrogate marker for neurogenesis related to seizures, which were observed in Study 001 mice (Figures 3.6.1-3.6.2). Previous experiments by Palop et al. (2003) showed that behavioral performance in the MWM correlated positively with levels of CB in the outer molecular layer of the hippocampus of J20 hAPP transgenic mice. CB measurements were made in the BACE x PDAPP mice of Study 001 to determine whether CB is a marker for cognitive function in the PDAPP mouse lies as well.

CB immunostaining was performed with an anti-CB antibody from Sigma Chemicals at 3ug/mL concentrations with a fluorescent secondary antibody. Fluorescence images were collected with a laser confocal microscope, and imaged using the NIH Image 1.63 program. Mean intensity measurements in the neurite region of the outer molecular layer were taken and normalized against the average intensity levels of a group of 18mo non-transgenic mice. Non-specific immunoreactive artifacts like blood vessels were not included in the CB mean intensity measurements.

At 3mo in Study 001 mice CB levels were decreased in all animals carrying the PDAPP transgene relative to either the Control or BACE KO mice, as seen in Figure 3.6.1a and Table 3.6a-d ($F=5.07$, df 3/52610, $p<0.005$). At 13mo there are no significant differences in CB immunoreactivity in any measure by genotype groups. By 18mo, there was an unexpected phenotypic shift in CB levels, as all mice with homozygous BACE gene deletion had higher mean CB intensity levels than PDAPP mice (BACE KO; PDAPP $p=0.025$, BACE KO $p<0.0005$). Indeed, BACE KO mice had higher CB levels than Control mice (BACE KO $p<0.05$). This pattern of increased CB immunoreactivity coupled with the behavioral epileptic activity suggested a link between the CB histology and the seizures observed in BACE KO mice (section 3.4). This CB-seizure relationship would be explored more deeply in

study 011A to better determine whether genotype plays a role in the apparent seizure activity of BACE KO mice.

	3 months			13 months			18 months			All		
Genotype	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE KO; PDAPP	6	64.21	13.7	12	71.24	15.7	19	104.6	32.7	37	87.24	31.1
BACE KO	8	100.2	32.7	10	81.36	27.9	16	124.4	45.8	34	106	41.9
PDAPP	18	66.03	20.4	16	81.45	17.1	22	82.48	25.8	55	77.09	22.8
Control	10	103	51.2	13	94.38	50.9	19	95.76	30.5	42	97.11	41.9
ALL	41	81.43	36	51	82.38	31.2	76	100.2	36.2	168	90.19	35.7

Source Factor(s)	F statistic	DF	p-value
Genotype*Age		6	
Age	6.35	2	0.0022
Genotype	5.07	3	0.0022

	p-value				
Comparison	**BACE KO; PDAPP**	**BACE KO**	**PDAPP**	**Controls**	ALL
3 vs 13 months	0.55	0.22	0.05	0.58	0.85
3 vs 18 months	0.0052	0.18	0.05	0.96	0.0025
13 vs 18 months	0.0067	0.0045	0.84	0.55	0.0017

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE KO; PDAPP VS BACE KO	0.028	0.46	0.19	0.012
BACE KO; PDAPP VS PDAPP	1	0.32	0.025	0.63
BACE KO; PDAPP VS CONTROL	0.038	0.17	0.42	0.063
BACE KO VS PDAPP	0.0059	0.87	0.0005	0.0009
BACE KO VS CONTROL	0.81	0.58	0.037	0.41
PDAPP VS CONTROL	0.0074	0.65	0.16	0.0079

Table 3.6a-d Descriptive, MANOVA and pairwise ANOVA statistics for hippocampal Calbindin levels in Study 001 mice by age and genotype.

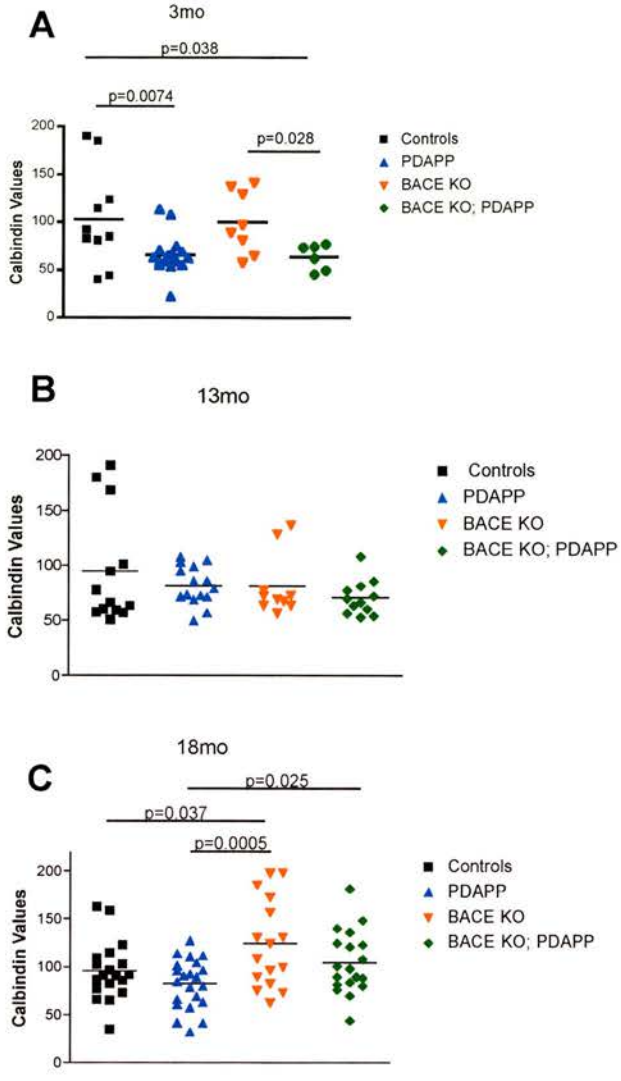


Figure 3.6.1 Calbindin immunoreactivity in the hippocampal outer molecular layer in Study 001 mice. A: At 3mo animals carrying the PDAPP transgene (PDAPP and BACE KO; PDAPP mice) have significant depletions of Calbindin (CB) relative to Control mice. BACE KO mice have CB levels similar to Controls, and significantly higher levels than BACE KO; PDAPP mice. B: At 13mo there are no significant differences in CB level by genotype. C: In contrast the PDAPP-specific CB depletion of 3mo animals, 18mo Study 001 mice feature a significant BACE KO-specific enrichment of CB relative to PDAPP mice. In addition, BACE KO mice have higher hippocampal CB levels relative to Control mice, suggesting an age-related shift in CB phenotypes in BACE-deficient animals.

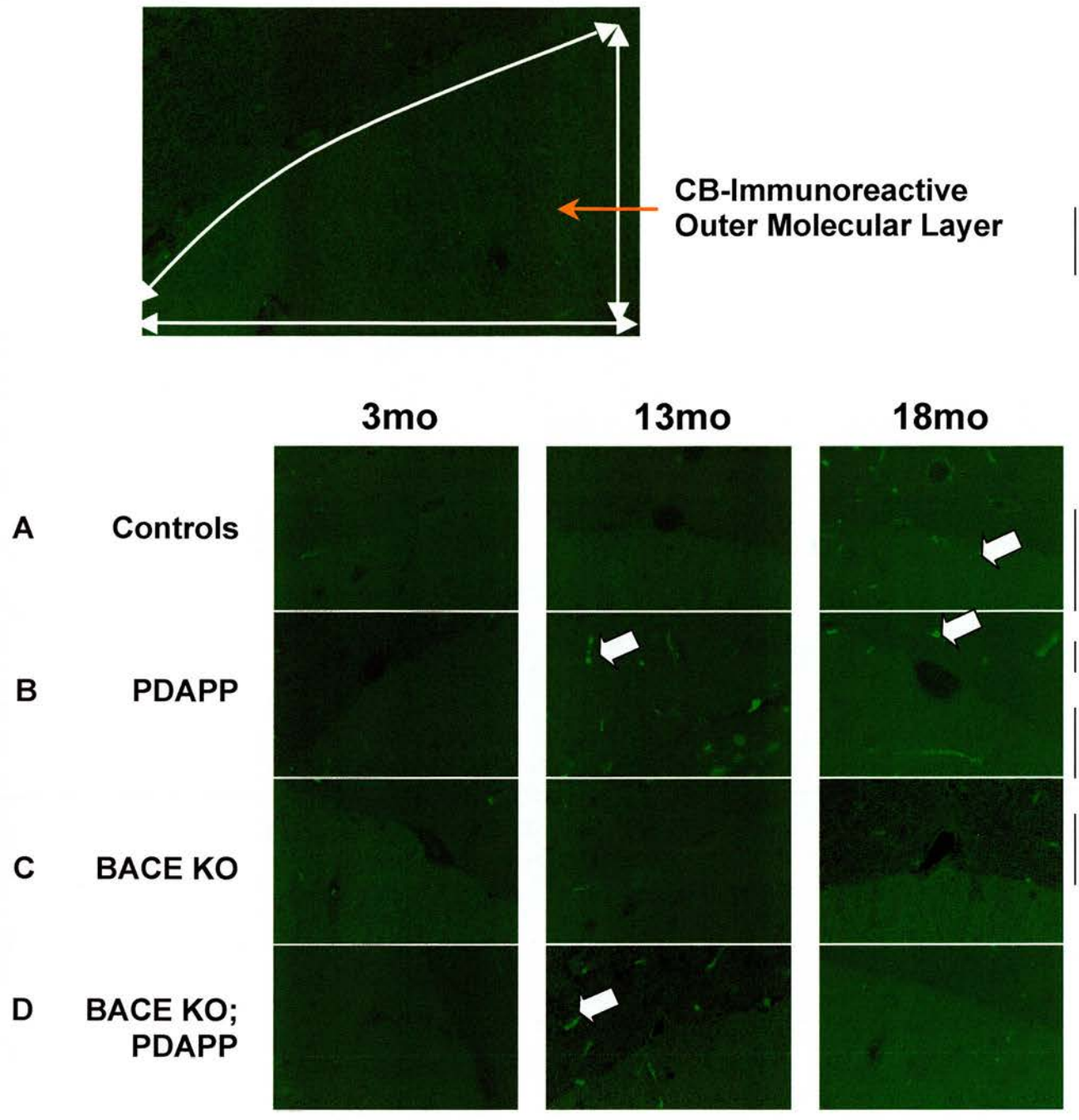


Figure 3.6.2 Calbindin images from the hippocampal outer molecular layer in Study 001 mice. Images with CB immunoreactivity levels close to group averages are presented, and the immunoreactivity of blood vessels (white arrows) is artifactual. 3mo: PDAPP and BACE KO; PDAPP mice have depletions of CB relative to Control and BACE KO mice. 13mo: There is no distinction between CB levels from any genotype. 18mo: BACE KO and BACE KO; PDAPP mice have visibly higher levels of CB compared to Control and PDAPP mice, suggesting an age-related shift from PDAPP-specific CB depletion in young animals to a BACE-specific CB enrichment in aged mice.

3.7 hAmyloid Precursor Protein immunoreactivity and Aβ processing

Expression of hAPP in the PDAPP and BACE KO; PDAPP animals was confirmed by antibody staining with 8E5, a monoclonal antibody raised to the 444-592 amino acid residue stretch of the hAPP protein. The same staining was used to confirm the lack of hAPP expression in Control and BACE KO (Figure 3.7.1). As this was a qualitative confirmation of genotypes it is difficult to draw further conclusions, although it is clear which animals transgenically overexpressed hAPP with the 8E5 staining and which individuals did not (Figure 3.7.1 rows a and c vs. rows b and d).

Confirmation of hAβ processing was conducted via antibody staining with 3D6, a synthetic antibody for the 1-5 amino acid stretch of the N-terminus of the hAβ peptide (Figure 3.7.2). Using this antibody amyloid deposits are visible in the hippocampus and cortex of PDAPP mice at 13 and 18mo of age, while none is visible in the Control and BACE KO mice that do not carry the APP transgene. Homozygous knockout of the BACE gene on the background of a PDAPP transgene in BACE KO; PDAPP animals effectively abolishes processing of APP to hAβ, as seen in Row D of the 13 and 18mo BACE KO; PDAPP panels of Figure 3.7.2.

ELISA hAβ1-4x data was not collected from these Study 001 animals as their brain homogenates were depleted in separate biochemical fractionation and extraction experiments to confirm lack of BACE enzyme activity.

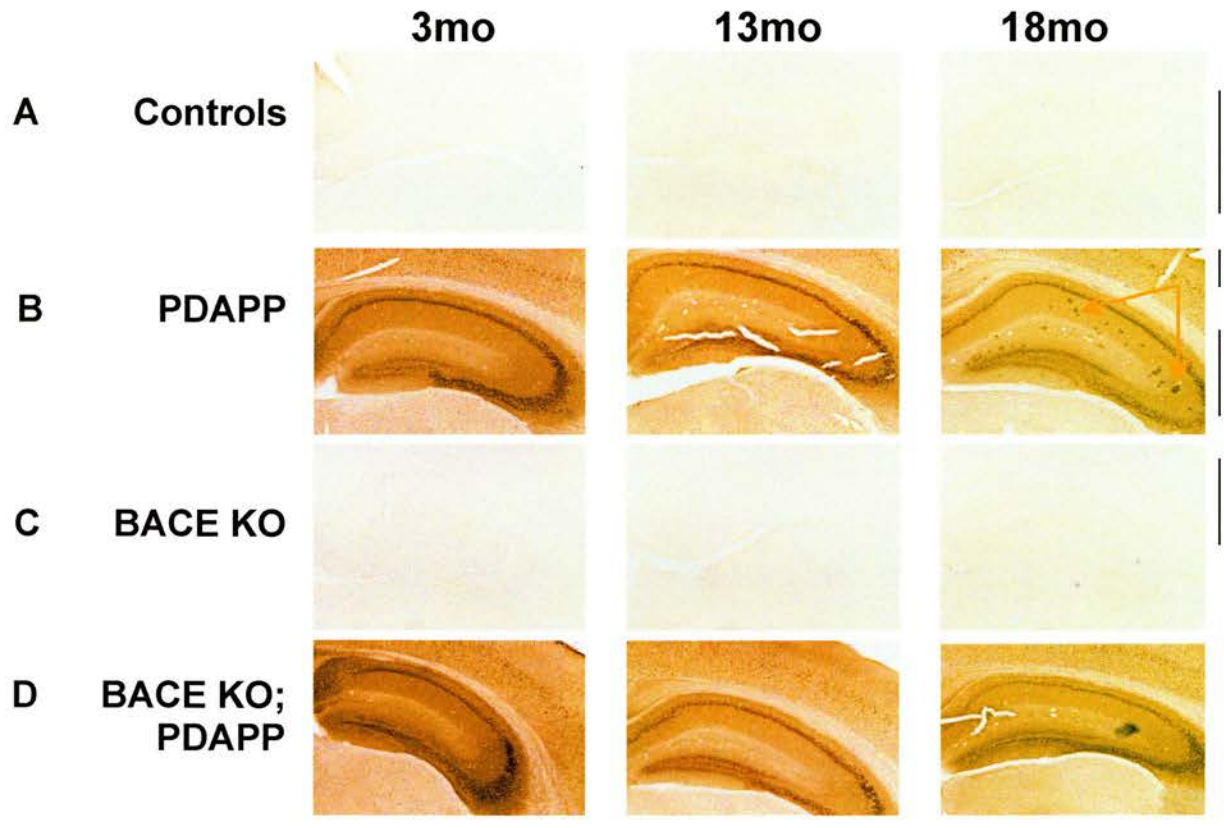


Figure 3.7.1 APP brain immunoreactivity in Study 001 mice. 3mo, 13mo: hAPP is detected by the antibody 8E5 in PDAPP and BACE KO; PDAPP mice. 18mo: hAPP is present in PDAPP and BACE KO; PDAPP mouse brains, however, the dystrophic neurites present (plaque-like accumulations within the hippocampus, delineated by red arrows) in 18mo PDAPP mice are abolished in 18mo BACE KO; PDAPP mice.

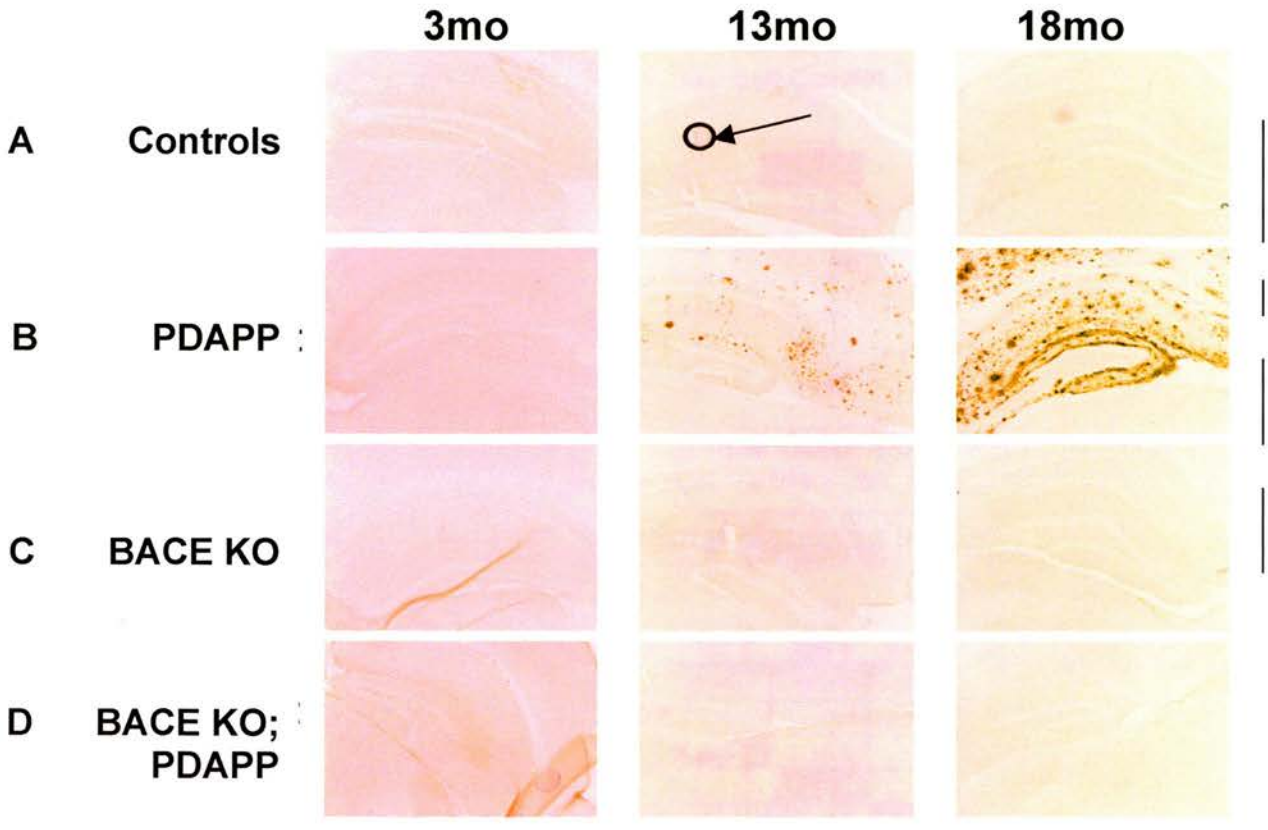


Figure 3.7.2 BACE KO abolishes Aβ processing in Study 001 mice. 3mo: No Aβ depositions detected by the 3D6 antibody are present in young mice, as this neuropathological feature does not manifest until at least 6-7mo in PDAPP mice. 13mo: Plaque-like deposits of Aβ are present in 13mo animals, while BACE KO; PDAPP brains are devoid of any Aβ. The black arrow in the 13mo Controls panel points to an air bubble. 18mo: The cortex and hippocampus of PDAPP animals have heavy Aβ burdens, while BACE KO; PDAPP brains remain free of such deposits, suggesting that homozygous BACE gene deletion alone is sufficient to abolish all Aβ processing.

3.8 Correlation analyses of behavioral and histological data

Correlation Analysis Cell Key

R-Values Colorimetrics | P-Values Colorimetrics | Self-Correlation | Insignificant p- or Non-correlative r-value,

Column Abbreviations

Plats = Number of Platforms Learned
TTC N = Trials to Criterion, Location N
Ave1-3 = Average TTC Locations 1-3
CB = Calbindin Intensity in the Hippocampal Outer Molecular Layer

Example Correlation Table, Behavioral Measures Interrelationships

R-values from correlation analysis of each measure

	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3
#Platforms		-0.2208	-0.4811	-0.677	-0.7621
TTC 1	0.0476				0.3994
TTC 2	P<0.0001			0.3305	0.6643
TTC 3	P<0.0001		0.0026		0.8075
ave1-3	P<0.0001	0.0002	P<0.0001	P<0.0001	

p-values from correlation analysis of each measure

Figure 3.8.1a Example correlation tables. A: Correlation table of relationships between various behavioral measures, with R-values presented in upper diagonal section and p-values presented in lower diagonal section. Corresponding p-values and R-values are found in the same coordinate distance from the black diagonals separating the two types of values, with R-values at x_r,y_r coordinates, and p-values at y_p,x_p coordinates where $x_r=y_p$ and $y_r=x_p$

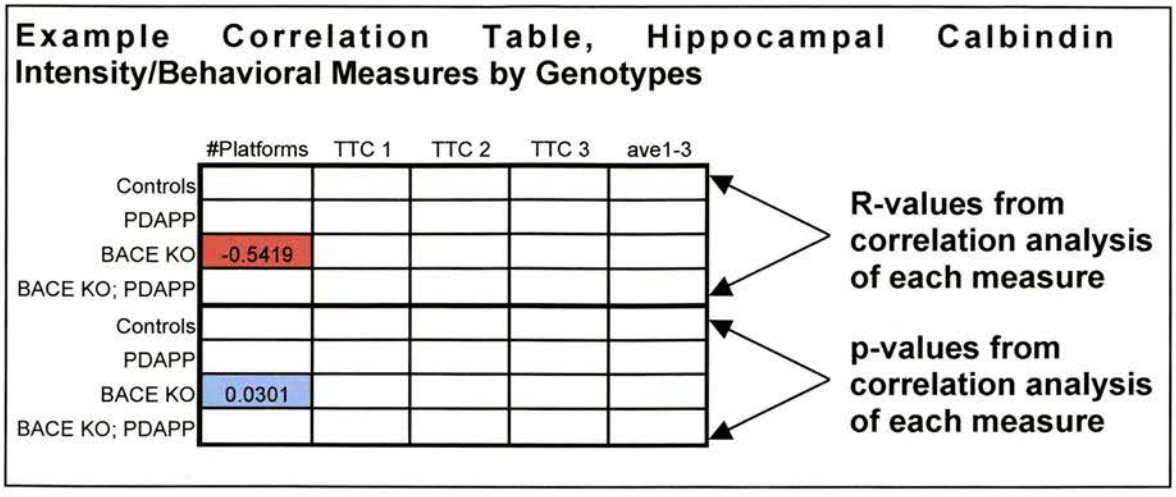


Figure 3.8.1B Example correlation tables B: Correlation table of calbindin values to behavioral performance measures, with individual genotypes presented. R-values are in upper sections and p-values are in lower section.

One method of determining whether behavioral and histological markers such as CB are functionally related is to perform correlation analyses between them. Correlation tests compare the distribution of values from one set of measures to the value set for another, and generate statistical coefficients that describe how much variance in one set is due to changes in values on the other set (the R-value). Data from Study 001 mice were tested using the Spearman's Correlation test, which does an evaluation of value sets based on a log-ranking methodology. These correlation results were checked for reliability by creating R- and p-value tables in which the same behavioral and CB measures formed the rows and columns. Reliable and reflexive values generated from one row*column set should equal the values from the same column*row set (e.g. TTC1 vs. TTC3 == TTC3 vs. TTC1). Upon analysis, certain variables were found to fail the reflexivity test, largely because they based on comparison of derived values with wide variability and were not presented (e.g. averages of several trials over several animals like latencies for day one of VCN). Two correlation tables are presented for each age group tested in Study 001, with the R- and p-values from the reflexive behavioral measures for all groups combined as the first table (Tables 3.8.1-3.8.3), and by-genotype comparisons of CB to behavioral measures for the second table (Tables 3.8.4-3.8.6).

Analysis of the relationships between the various behavioral measures in all Study 001 mice reveals a relatively stable pattern with age of connections between specific measures (Tables 3.8.1-3.8.3). By examining both the statistical p-value and correlation coefficient R generated by a non-parametric Spearman's correlation test, it possible to make three important interpretations about the functional directionality of correlations, the grouping of similar behavioral measure sets and the grouping of dissimilar behavioral measure sets. In addition, by including the Genotype, Gender and Color factors one can also see the relationships that were implied in more traditional statistical analyses presented in Sections 3.2-3.3, and 3.6. Overall the major factor related to spatial memory performance is genotype, which has high degrees of association Platforms Learned (memory capacity), TTC2, TTC3 and Average TTC1-3 (serial memory and learning) at all ages. Surprisingly there were data associations as well with aged animals by color and gender in TTC1, TTC2, Average TTC1-3 and Calbindin Intensity (CB). These findings were in part reflected in the more traditional ANOVA analyses, but were lesser factors in those analyses compared to genotype.

With respect to directionality of measures, the best example was that of platform locations learned and all other measures, which were based in number of trials (see Correlation Key above). While an animal that had intact spatial memory learned a higher number of spatial locations, if their acquisitional rate was also unimpaired the measures like TTCs, Ave1-3 will be low as animals needed fewer trials to learn any specific location to the performance criterion. At all ages there was a negative R-value correlation between Plats and all other measures, satisfying this intuitive condition.

Behavioral tasks with a high degree of statistically significant positive R-values like Platforms learned and TTC1-3 at 3mo can be theoretically grouped together as a similar set of measures. The high degree of correlation in this particular task set suggests that the processes that are the basis for these measures are also highly related if not the same. The patterns of related measures did change between age

groups, suggesting that the if the pattern of related values was due to relatedness of underlying functional processes, then these were age-related changes. At 13mo TTC2 and TTC3 were no longer correlated highly, as many mice were deficient in learning platform location 3. This lack of correlation is another statistical representation of the limits of learning and memory in the Study 001 mice. By 18mo the TTC3 and TTC1 and TTC2 measures were all highly correlated, and overall average learning rate was correlated to number of platforms learned ($R=-0.24$, $P<0.025$) indicative of an even greater age-related decline in memory performances as animals are able to learn fewer locations. This pattern of changing relatedness of similar sets of values may be due to utilization of different memory processes as more tasks are presented to the animals.

Finally, TTC1 and TTC3 can be considered unrelated sets of measures as they shared no strong statistical relationships in Study 001 mice until 18mo (18mo: $R=0.235$, $p<0.05$). One interpretation of this finding is that the processes that generate the TTC1 and TTC3 performance data rely on distinct spatial learning and memory capabilities to acquire these locations, as TTC1 is analogous to single location acquisition rate while the serial learning of TTC3 requires elements of flexibility in spatial learning and/or capacity as well as acquisition. By 18mo, many more animals rely on components of long-term memory processes to learn even platform location one to the specified criterion (because they need >8 trials, >1 testing day to learn this location). This age-related change may make the fundamental process of learning location one more akin to that of learning on later platforms when serial learning capacity impairments also force animals to use long-term memory processes and potentially overnight memory consolidation to solve their tasks.

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		-0.034	0.015	-0.468	0.184	0.271	0.475	0.524	-0.127
Gender	0.767		0.060	0.043	0.096	-0.068	-0.047	-0.008	-0.083
Color	0.895	0.599		-0.077	0.005	0.037	0.104	0.078	0.011
#Platforms	P<0.0001	0.705	0.499		-0.248	-0.533	-0.705	-0.779	0.193
TTC 1	0.102	0.399	0.964	0.027		0.137	0.107	0.440	-0.406
TTC 2	0.015	0.552	0.745	P<0.0001	0.227		0.377	0.691	0.011
TTC 3	P<0.0001	0.677	0.358	P<0.0001	0.346	0.001		0.813	-0.050
ave1-3	P<0.0001	0.943	0.494	P<0.0001	P<0.0001	P<0.0001	P<0.0001		-0.181
CBInt	0.4197	0.9481	0.2443	0.8339	0.1383	0.4983	0.2111	0.6025	

Table 3.8.1 Correlation of behavioral measures, R- and P-values of all 3mo Study 001 mice. Spatial memory capacity (#Platforms) is highly correlated to learning each of 2 spatial locations (TTC2, TTC3). The ability to learn locations 2 and 3 (TTC2, TTC3) are also highly correlated, suggesting a relationship between the ability to rewrite memory once (TTC2) or more times (TTC3). Genotype appears to have a major association with spatial memory performance values.

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		0.097	-0.055	-0.397	0.085	0.136	0.301	0.335	-0.121
Gender	0.378		0.179	-0.065	0.192	0.054	0.051	0.187	-0.072
Color	0.622	0.104		-0.046	-0.198	-0.067	-0.092	-0.204	-0.285
#Platforms	0.000	0.559	0.676		-0.055	-0.050	-0.022	-0.072	0.041
TTC 1	0.440	0.081	0.071	0.620		0.294	0.099	0.730	0.024
TTC 2	0.216	0.623	0.547	0.651	0.007		0.150	0.545	0.251
TTC 3	0.005	0.645	0.403	0.840	0.369	0.172		0.608	0.236
ave1-3	0.002	0.088	0.062	0.515	P<0.0001	P<0.0001	P<0.0001		0.200
CBInt	0.3979	0.6142	0.0426	0.7753	0.8676	0.0759	0.0955	0.1597	

Table 3.8.2 Correlation of behavioral measures, R- and P-values of all 13mo Study 001 mice. As in 3mo mice, spatial memory capacity (#Platforms) in 13mo animals is highly correlated to learning on spatial location 3 (TTC3). At 13mo the ability to learn locations 1 and 2 (TTC1, TTC2) are now highly correlated, suggesting a relationship between initial memory acquisition (TTC1) and the ability to rewrite memory once (TTC2). Again, Genotype appears to be a significant factor in spatial memory performance, although Color is related to CB Intensity.

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		0.079	0.016	-0.421	0.063	0.253	0.515	0.419	0.182
Gender	0.466		0.150	-0.069	-0.119	-0.076	-0.017	-0.034	-0.303
Color	0.880	0.167		0.048	-0.297	-0.260	-0.064	-0.280	-0.153
#Platforms	P<0.0001	0.526	0.658		-0.202	-0.068	-0.150	-0.241	0.042
TTC 1	0.563	0.272	0.005	0.061		0.088	0.235	0.623	-0.057
TTC 2	0.018	0.486	0.015	0.533	0.417		0.268	0.542	0.126
TTC 3	P<0.0001	0.874	0.557	0.168	0.029	0.013		0.775	0.116
ave1-3	P<0.0001	0.755	0.009	0.024	P<0.0001	P<0.0001	P<0.0001		0.110
CBInt	0.0731	0.0166	0.2617	0.9618	0.7276	0.3511	0.3064	0.2799	

Table 3.8.3 Correlation of behavioral measures, R- and P-values of all 18mo Study 001 mice. As in 3 and 13mo mice, spatial memory capacity (#Platforms) in 18mo animals is highly correlated to learning 2 spatial locations (TTC2, TTC3). At 18mo the ability to learn locations 1 and 2 (TTC1, TTC2) are correlated to learning location 3, suggesting a wider relationship with age between initial and subsequent spatial learning. At this age there is stronger relationship between Color and spatial memory performance than seen at younger ages, and Gender appears to be associated with higher Calbindin Intensity.

In analyzing patterns of correlation between CB histology and various cognitive spatial measures in animals by genotype, it is apparent that with age, the functional relationships are changing and there is little overall relationship between CB levels and behavioral measures (Tables 3.10.4 - 3.10.6). At 3mo there were no significant correlations between CB and any other measure (Table 3.10.4). The actual directionality of the few meaningful CB-behavioral R-values in Study 001 mice was surprising considering the Palop et.al. (2002) report that claimed CB as a surrogate marker for spatial memory function, in which greater CB levels were associated with better learning performance in both transgenic and non-transgenic hAPP mice. However, in Study 001, only mice at 13mo were higher CB values associated by R-values with higher numbers of spatial location learned only in the PDAPP mouse (R=0.549, P=0.028, with no extension of this pattern to Control animals. Other CB relationships with other measures were found, as at 13mo albino Control mice had higher CB levels (R=-0.660, P=0.014) and at 18mo CB was higher in male Control mice (R=-0.567, P=0.009). Overall these data and their lack of repeating patterns across age or genotypes imply that CB had little value as a robust predictor of spatial memory function (Tables 3.8.4-3.8.6).

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3
BACE KO; PDAPP	0.157	0.086	-0.559	0.416	0.389	0.265	0.556
BACE KO	-0.088	0.473	-0.323	-0.149	0.156	0.302	0.235
PDAPP	-0.164	0.302	0.041	-0.069	-0.037	0.031	0.045
Control	0.064	0.412	-0.205	-0.002	0.064	0.379	0.341
BACE KO; PDAPP	0.627	0.792	0.059	0.178	0.211	0.405	0.060
BACE KO	0.747	0.064	0.223	0.582	0.563	0.255	0.381
PDAPP	0.503	0.208	0.867	0.779	0.881	0.901	0.855
Control	0.820	0.127	0.464	0.995	0.822	0.164	0.213

Table 3.8.4 Correlation of hippocampal calbindin intensity to behavioral measures, R- and P-values by genotype in 3mo Study 001 mice. CB levels in BACE KO mice do not correlate to other measures in Study 001 mice of this age.

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3
BACE KO; PDAPP	-0.171	-0.269	-0.345	-0.392	0.074	0.526	0.109
BACE KO	-0.174	-0.174	-0.149	-0.213	0.139	0.290	-0.024
PDAPP	-0.260	-0.058	0.549	0.111	0.076	0.349	0.227
Control	0.178	-0.660	0.057	0.389	0.282	0.048	0.365
BACE KO; PDAPP	0.594	0.398	0.272	0.207	0.818	0.079	0.737
BACE KO	0.631	0.631	0.681	0.555	0.703	0.416	0.947
PDAPP	0.332	0.832	0.028	0.683	0.781	0.186	0.398
Control	0.560	0.014	0.854	0.189	0.351	0.877	0.221

Table 3.8.5 Correlation of hippocampal calbindin intensity to behavioral measures, R- and P-values by genotype in 13mo Study 001 mice. At this age PDAPP mice had a positive correlation with higher CB levels and the ability to learn more spatial locations. In Control animals there was no comparable pattern, although albino Control mice had higher CB levels.

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3
BACE KO; PDAPP	-0.234	-0.139	0.194	0.073	0.174	-0.096
BACE KO	-0.380	-0.102	0.135	-0.240	-0.038	-0.063
PDAPP	-0.222	0.007	0.091	0.028	0.391	0.196
Control	-0.567	-0.418	-0.122	0.101	-0.049	0.025
BACE KO; PDAPP	0.336	0.570	0.426	0.766	0.475	0.704
BACE KO	0.147	0.709	0.619	0.372	0.888	0.817
PDAPP	0.321	0.974	0.687	0.900	0.072	0.382
Control	0.009	0.067	0.608	0.673	0.837	0.917

Table 3.8.6 Correlation of hippocampal calbindin intensity to behavioral measures, R- and P-values by genotype in 18mo Study 001 mice. At this age the only statistically significant correlation was that higher CB levels occur in male Control mice. Overall CB does not appear to be a robust biomarker for spatial memory in Study 001 mice.

Ch.4 Study 006: Spatial memory characterization and histological analysis of hemizygous BACE pKO x PDAPP mice

In the previous chapter of this dissertation, the details of spatial memory and histological characterization of homozygous BACE KO x PDAPP mice were presented. While the original working hypothesis was that deletion of the BACE gene would ameliorate both the cognitive and histological pathologies in PDAPP mice, the data Study 001 did not support this hypothesis. Complete knockout of murine BACE genes on a PDAPP background prevented plaque-like deposition of amyloid in the mouse brain, but spatial memory performance in the modified MWM in these mice was poorer than that of PDAPP mice. At the same time, there was a mild spatial learning capacity impairment associated with the BACE KO genotype on a wild-type background -- suggesting that BACE activity is required for normal spatial memory processes.

Following on these findings, it was of interest to perform the same assessments on animals with a partial BACE gene deletion, to determine whether the deleterious phenotypes of BACE KO x PDAPP mice are due to the absolute lack of BACE in development. In addition, a partial deletion of the BACE gene may confer cognitive rescue of the PDAPP phenotype. Study 006 focuses on the MWM spatial learning and memory characterization and histological analysis on mice with one functioning allele of the BACE gene on a PDAPP background, relative to PDAPP mice.

The genotypic identities and creation of Study 006 animals are described in section 2.7, while the details of the animals tested are presented below in Tables 4.0a-c. Due to the variability inherent in spatial memory testing of PDAPP-based mice of both genders and on a triple-strained background (C57Bl6, DBA/2J, Swiss-Webster), N~20 mice were tested to sufficiently power statistical analysis. As the effects of b-secretase gene ablation may vary as a function of age, animals in Study 001 were tested at young (3mo), middle (13mo) and old ages (18mo). In addition, the constraints of breeding a colony with two genotypes of interest required examination by coat colors (albino, black and agouti) and gender, with a preponderance of agouti animals produced. Study 006 animals are presented in Table 4.0b (N_{total}=143 mice).

	PDAPP +/+
BACE -/+	PDAPP BACE pKO; PDAPP

Table 4.0a Study 006 table of genotypes.

		Female				Male				ALL			
		Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL
Age (mo)	Genotype												
3	BACE pKO; PDAPP	9	3	2	14	10	1	1	12	19	4	3	26
	PDAPP	12	0	3	15	10	0	5	15	22	0	8	30
	ALL	21	3	5	29	20	1	6	27	41	4	11	56
13	BACE pKO; PDAPP	10	0	1	11	10	0	1	11	20	0	2	22
	PDAPP	3	0	3	6	9	0	4	13	12	0	7	19
	ALL	13	0	4	17	19	0	5	24	32	0	9	41
18	BACE pKO; PDAPP	8	0	3	11	4	3	5	12	12	3	8	23
	PDAPP	10	1	2	13	8	0	2	10	18	1	4	23
	ALL	18	1	5	24	12	3	7	22	30	4	12	46
ALL	BACE pKO; PDAPP	27	3	6	36	24	4	7	35	51	7	13	71
	PDAPP	25	1	8	34	27	0	11	38	52	1	19	72
	ALL	52	4	14	70	51	4	18	73	103	8	32	143

Table 4.0b Study 006 mice.

Statistical analysis of the contribution of genotype, age gender and color on the behavioral and histological measures in Study 006 mice were performed. Overall the major factors impacting measure outcomes were age and genotype, with some contribution from color and gender. In many instances there was also a significant age*genotype interaction, suggesting these were indeed the most importance factors affecting performance. Further analysis revealed that when the disparity in number of Albino versus Black and Agouti animals was built into the model, color became an insignificant factor (overall p=0.06). Furthermore when the unequal numbers of colors among genotypes was analysed, color was found to not be the driving factor in the significance of color (p=0.17 by Cochran-Mantel-Haenszel test). Thus all statistics further will be described only by genotype, age and genotype*age.

Outcome	Gender	Color	Age	Genotype	Interactions
VCN Latency	0.22	<0.0001	0.035	0.82	None
VCN Speed	0.21	0.13	<0.0001	0.7	Genotype*Age (p=0.0057)
TTC1	0.086	0.0064	0.05	0.78	None
TTC2	0.67	0.19	0.0087	0.82	Genotype*Age (p=0.0057)
TTC3	0.3	0.9	0.0043	0.0034	None
Average TTC1-3	0.088	0.14	<0.0001	0.086	Genotype*Age (p<0.0001)
Platforms Learned	0.1	0.72	<0.0001	<0.0001	Genotype*Age (p<0.0001)
Calbindin	0.19	0.37	<0.0001	<0.0001	Genotype*Age (p<0.0001)

Table 4.0c Statistical summary of factor significance in Study 006.

Visual Cued Navigation

4.1 Visible Platform Testing

Prior to spatial memory testing in the hidden platform serial locations task, Study 006 mice were assessed in visual cued navigation (VCN). VCN is a procedural learning task that also provides insight into the sensorimotor function of the test mice with trial latency and swim speed measurements (Figure 4.1.1-4.1.2). Animals are tested for 4 trials/day for 3 days in a pool with a visible platform.

Over successive trials the mice learned to climb the platform to escape from the task of swimming. The mice became more efficient at the task over the 3 days of VCN testing, with shorter trial latencies each day (Figure 4.1.1). Among all the mice tested at 3, 13, and 18mo, the swimming performances of PDAPP and BACE pKO; PDAPP mice featured a strong effect of age, such that older animals of both genotypes swam slower and had longer swim trials (Tables 4.1.1a-d-4.1.2a-d). At 13mo BACE pKO; PDAPP mice located the visible platform faster than PDAPP mice ($F=2.3$, $p<0.05$). There were no genotypic differences in Study 006 swim speeds overall.

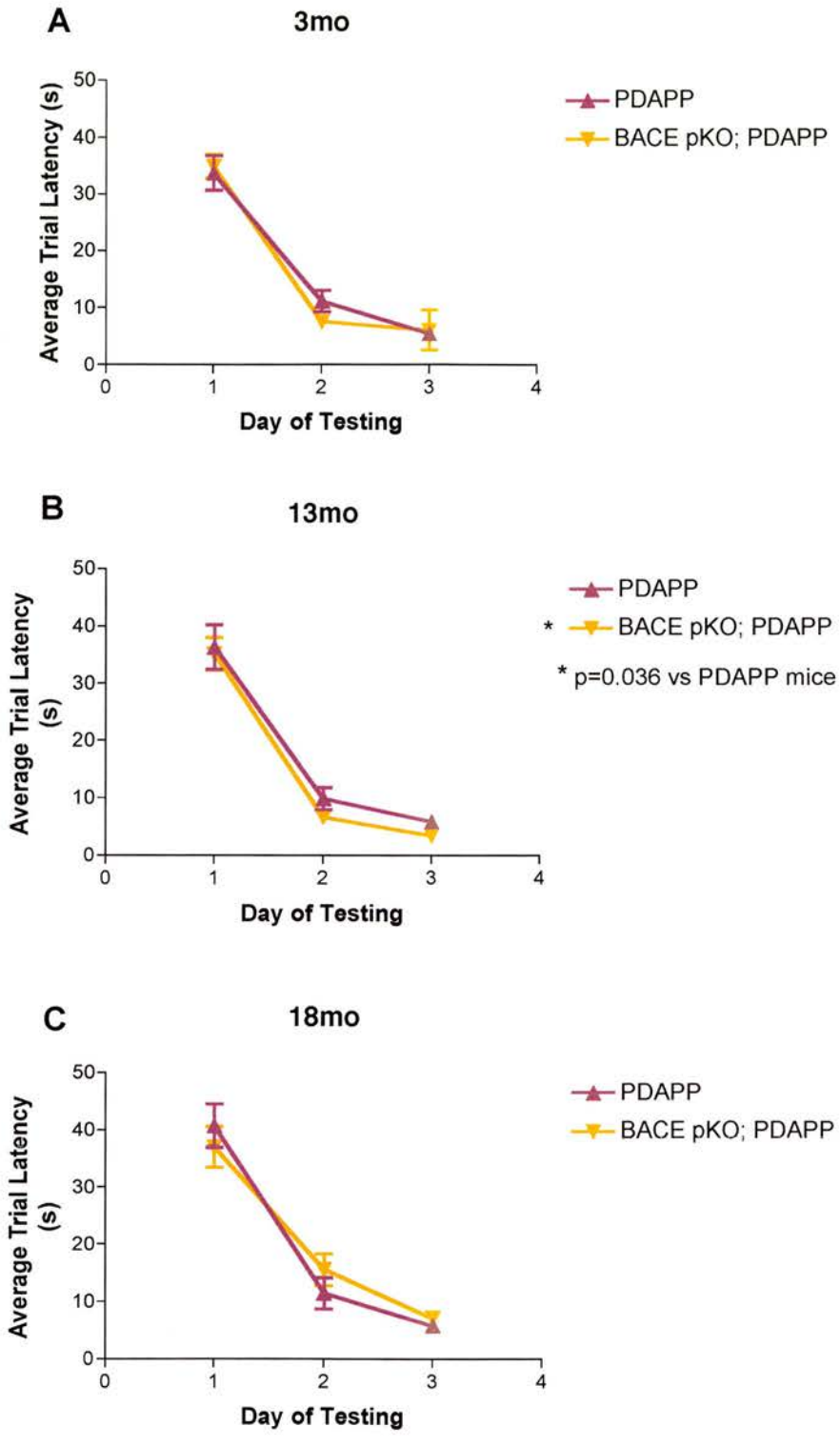


Figure 4.1.1 Visual cued navigation swim trial latencies in Study 006 mice by age and genotype. A-C: At 3 and 18mo VCN trial latencies between PDAPP and BACE pKO; PDAPP mice are similar, while at 13mo BACE pKO; PDAPP mice have shorter trials than PDAPP mice.

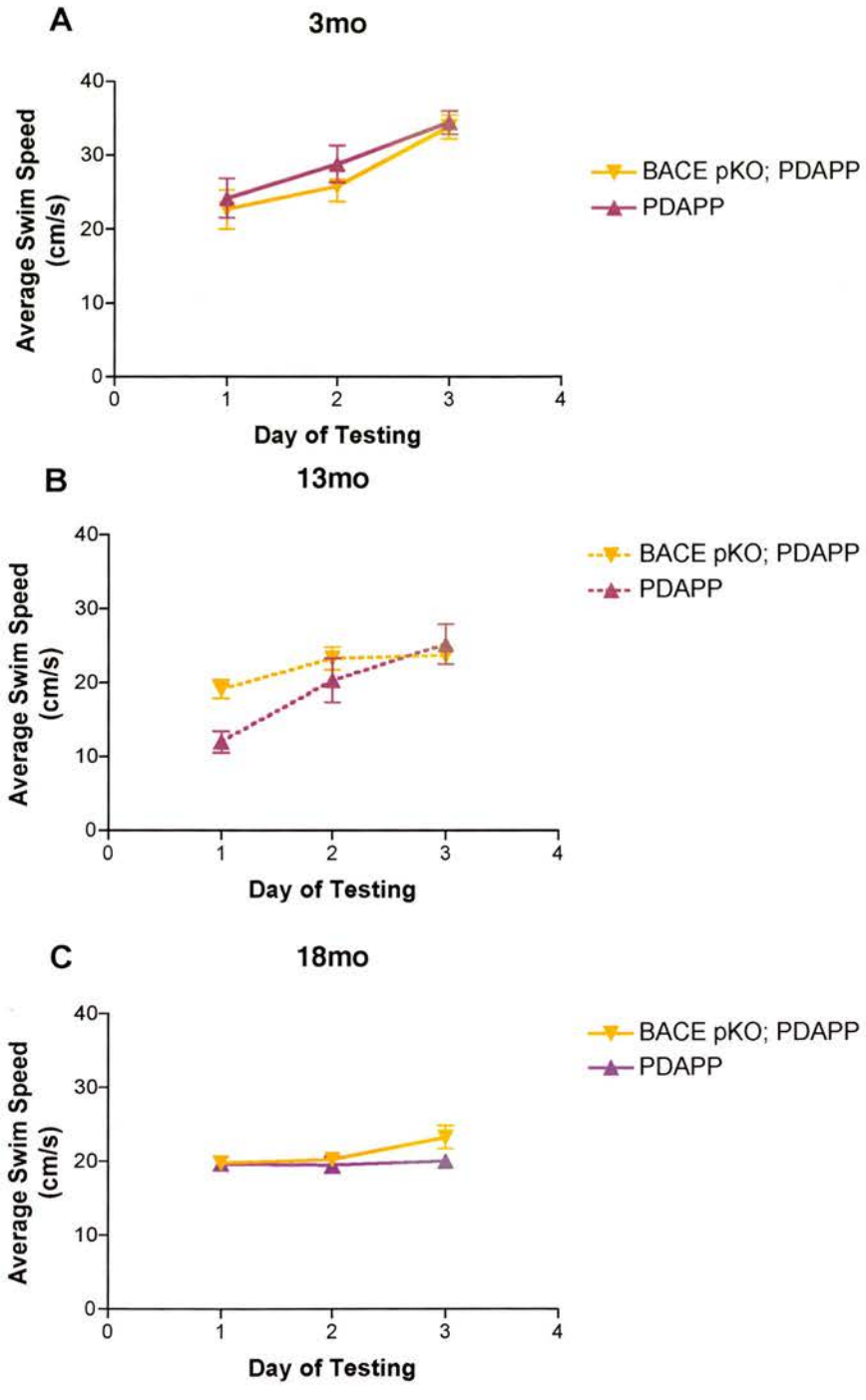


Figure 4.1.2 Visual cued navigation swim trial speeds in Study 006 mice by age and genotype. A,C: Swimming speed in 3 and 18mo animals are equivocal. B: At 13mo, PDAPP mice swim slower than BACE pKO; PDAPP mice on Day 1, but their performance becomes indistinguishable by Day 2 and 3, suggesting that subsequent criterion-based hidden platform testing is not affected by differences in swim speed.

Day	Genotype	3 months			13 months			18 months			All		
		N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
1	BACE pKO; PDAPP	26	34.96	10.56	22	35.24	13.42	23	37.03	17.03	71	35.72	13.63
	PDAPP	30	33.77	16.65	19	36.38	16.87	23	40.73	18.01	72	36.44	17.03
	ALL	56	34.32	14.05	41	35.76	14.93	46	38.71	17.36	143	36.07	15.37
2	BACE pKO; PDAPP	26	7.61	5.71	22	6.69	4.38	23	15.56	13.32	71	9.9	9.43
	PDAPP	30	11.24	10.48	19	9.92	8.29	23	11.48	13.14	72	10.94	10.62
	ALL	56	9.55	8.72	41	8.19	6.61	46	13.72	13.24	143	10.41	10.01
3	BACE pKO; PDAPP	26	6.12	3.56	22	3.5	1.82	23	7.14	5.31	71	5.64	4.08
	PDAPP	30	5.43	2.1	19	5.92	3.22	23	5.81	3.74	72	5.68	2.93
	ALL	56	5.76	2.88	41	4.62	2.81	46	6.54	4.66	143	5.66	3.55
ALL	BACE pKO; PDAPP	26	16.23	15.13	22	15.14	16.5	23	19.91	17.92	71	17.09	16.54
	PDAPP	30	16.94	16.74	19	17.4	17.4	23	19.34	20.06	72	17.74	17.85
	ALL	56	16.61	15.97	41	16.19	16.89	46	19.65	18.84	143	17.41	17.18

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.98	2	0.069
Age	2.3	2	0.035
Genotype	0.3	1	0.82

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.0053	0.84	0.12
3 vs 18 months	0.027	0.51	0.17
13 vs 18 months	0.0009	0.91	0.07

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.34	0.036	0.25	0.79

Tables 4.1.1a-d Descriptive, MANOVA and pairwise ANOVA statistics for VCN swim latencies in Study 006 mice by age and genotype

Day	Genotype	3 months N	3 months Mean	3 months Std	13 months N	13 months Mean	13 months Std	18 months N	18 months Mean	18 months Std	All N	All Mean	All Std
1	BACE pKO; PDAPP	26	22.73	13.44	22	19.11	5.93	23	19.61	3.63	71	20.6	9.05
	PDAPP	30	24.22	14.42	19	12.01	6.25	23	19.77	3.8	72	19.56	11.41
	ALL	56	23.53	13.87	41	15.82	5.99	46	19.68	3.66	143	20.09	10.25
2	BACE pKO; PDAPP	26	25.81	10.67	22	23.29	6.99	23	19.49	5.31	71	22.98	8.43
	PDAPP	30	28.82	13.75	19	20.31	13.06	23	20.25	4.09	72	24.05	12.26
	ALL	56	27.42	12.4	41	21.91	10.23	46	19.84	4.76	143	23.5	10.46
3	BACE pKO; PDAPP	26	33.81	8.25	22	23.69	4.2	23	19.99	3.69	71	26.2	8.37
	PDAPP	30	34.38	8.67	19	25.21	11.8	23	23.23	7.6	72	28.7	10.57
	ALL	56	34.12	8.4	41	24.4	8.51	46	21.46	5.94	143	27.42	9.56
ALL	BACE pKO; PDAPP	26	27.45	11.82	22	22.03	6.1	23	19.7	4.22	71	23.26	8.88
	PDAPP	30	29.14	13.09	19	19.13	11.93	23	21.08	5.56	72	24.11	11.98
	ALL	56	28.35	12.51	41	20.71	9.34	46	20.33	4.9	143	23.67	10.51

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.19	6	0.31
Age	9.45	2	<0.0001
Genotype	0.47	1	0.7

Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.0045	0.0007	<0.0001
3 vs 18 months	<0.0001	0.0007	<0.0001
13 vs 18 months	0.3	0.081	0.079

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.99	0.092	0.7	0.38

Tables 4.1.2a-d Descriptive, MANOVA and pairwise ANOVA statistics for VCN swim speeds in Study 006 mice by age and genotype

Serial Spatial Navigation from Memory

Study 006 mice were tested in a water maze task across cross-sectional age groups for measures of learning and memory acquisition and capacity. As in Study 001, these measures were based on a specified criterion at each spatial location, requiring the animals to swim to the hidden platform in 3 successive trials in <21s. Measures of learning rates and the ability to modify acquired spatial memory through serial platform locations (reversal) provides information about patterns and changes in learning and memory by genotype with age.

4.2 Acquisitional and Serial Learning Deficits

Analysis of number of trials to criterion for the first platform location is akin to the classical version of the watermaze in which only one spatial task is presented (TTC1). Deficits in learning this task are thus the earliest observable impairments in rodent spatial memory as shown in Figure 4.2.1. Overall, the mice took approximately 7 test trials to learn this first problem (Figure 4.2.1). The ANOVA analysis shows no difference as a function of genotype ($F<1$), but there was an overall slowing in the rate of learning as a function of age ($F=2.89$, df 2/136, $p<0.05$). While the age*genotype interactions were not significant, 13mo BACE pKO; PDAPP mice did learn location one in fewer trials than PDAPP mice (Table 4.2a-d $p=0.05$). However, there was a tendency for PDAPP mice to learn location 1 faster than BACE pKO; PDAPP mice by 18mo ($p=0.06$).

Analysis of performance on TTC2 and TTC3 provides a measure of spatial memory reversal, as animals must have the capacity to learn new locations and 'overwrite' the residual memory of the previous spatial location. As seen in Tables 4.2e-h and 4.2i-l, both genotypes exhibit decline in serial TTC2 and TTC3 learning. Study 006 mice have genotypic differences at various ages, with BACE pKO; PDAPP mice performing better than PDAPP mice at 13mo, with a reversal of this pattern by 18mo on TTC2 and TTC3. Indeed in TTC2 there is a significant age*genotype interaction, which approaches significance on TTC3 (TTC2 $F=5.37$, 2/136, $p<0.01$; TTC3 $F=3.0$ df 2/136, $p=0.053$).

Another way of displaying these differences is by taking the average of TTC over locations 1-3 (Figure 4.2.3, Table 4.2m-p). The same significant patterns that were seen with analysis of individual locations were reproduced analysis of average TTC of locations 1-3 (age*genotype: $F=14.28$, df 2/1291, $p<0.0001$). This spatial memory improvement relative to PDAPP mice at 13mo in a reversal-like task on TTC2 suggested that the partial deletion of the BACE gene on a PDAPP background confers some rescue of the deleterious spatial memory phenotype of hAPP mice. However by 18mo, this BACE pKO; PDAPP genotype becomes a cognitive liability in both TTC2 and TTC3. This may indicate a shift in the relative function of BACE gene products with age, such that decreased BACE may further impair memory in mice with transgenic APP overexpression.

The other major utility of this analysis was that it was easier to visualize an age-related decline in spatial memory acquisition rates by genotype. In particular, PDAPP mice reach the nadir of their performance levels at 13mo, while BACE pKO; PDAPP mice have steady age-related declines that continue from 13-18mo and from 3-18mo overall (Figure 4.3.2b-c). This difference reflects, in part, the apparent "rescue" of the PDAPP associated decline in performance that occurs by the end of the first year of life by the partial BACE knock-out. If this is due to a relative decline in Aβ levels at 13mo, it suggests that this cannot be sustained at older ages, by which time, the transgenic overproduction of Aβ can no longer be compensated for by the partial inactivation of BACE. Alternatively it could be that BACE pKO; PDAPP mice become susceptible to a separate deleterious phenotype due to their reduced BACE levels. It must be noted that by 18mo the PDAPP mice appear to have very little spatial memory decline, as performance at this age is significantly better than at 13mo. As these PDAPP mice have overall fewer deficits in this colony than others reported (Chen et al. 2000), it may be that longitudinal analysis between ages is not appropriate without prior confirmation that crosse-sectional analyses will yield the same results.

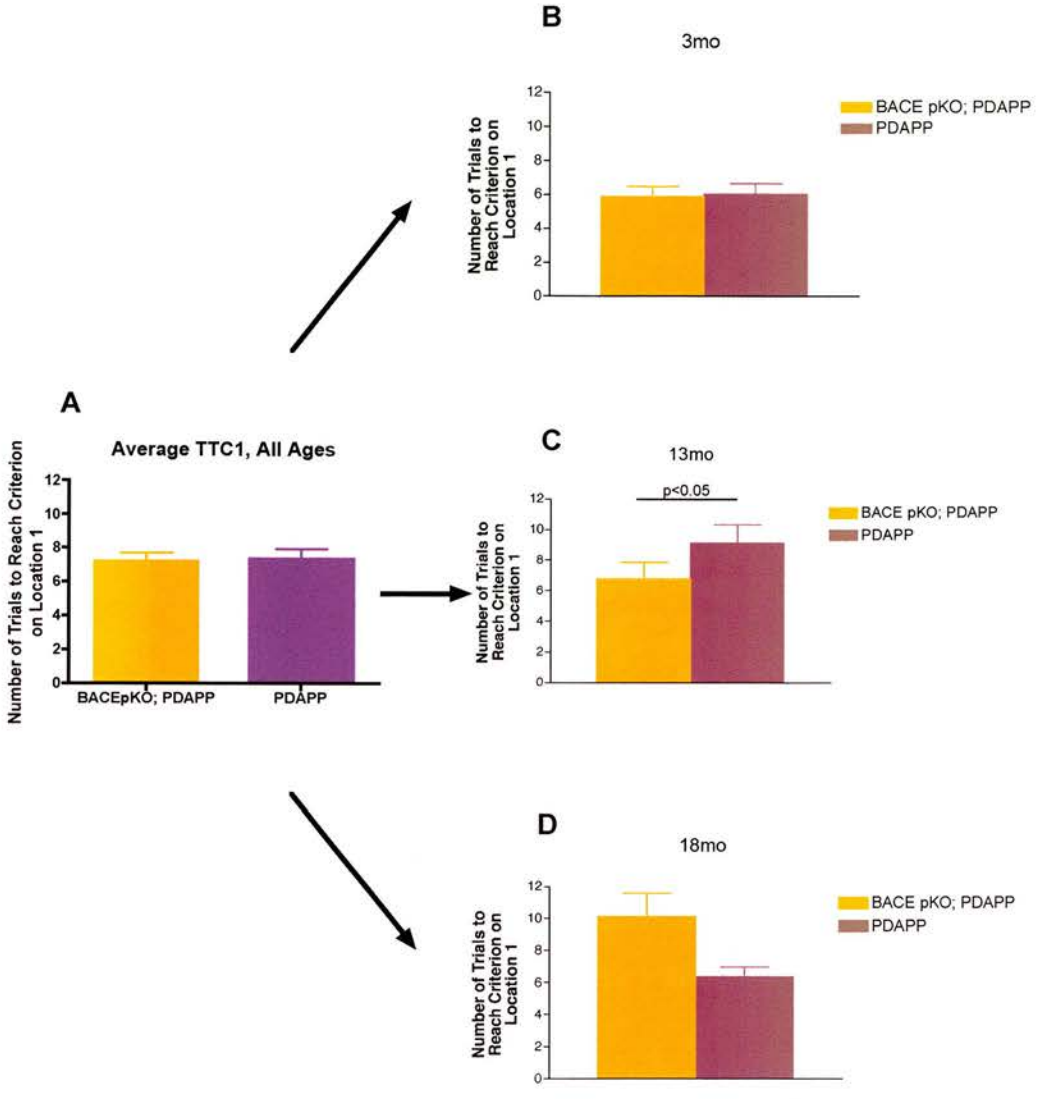


Figure 4.2.1 Deficits in initial spatial memory acquisition in Study 006 by age and genotype. A: PDAPP and BACE pKO; PDAPP require on average the same number of trials to learn location 1 when grouped across all ages. B: At 3mo, Study 006 animals both require about 6 trials to learn location 1 to criterion. C: By 13mo, BACE pKO; PDAPP mice learn location 1 in fewer trials than PDAPP mice. D: The difference in PDAPP and BACE pKO; PDAPP mouse performance at 18mo on location 1 approaches statistical significance (p=0.06), such that PDAPP mice appear to learn location 1 faster than BACE pKO; PDAPP mice.

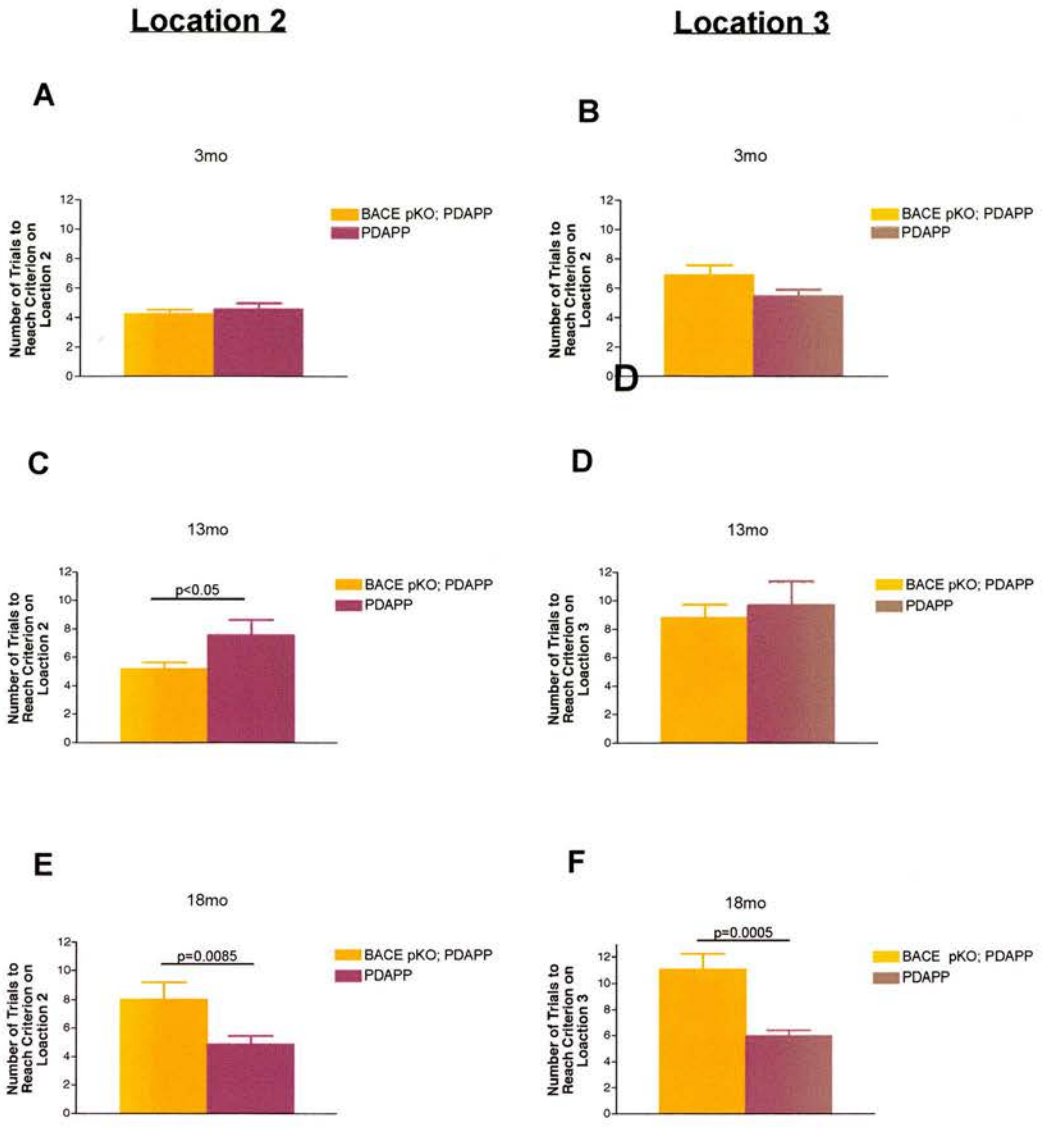


Figure 4.2.2 Deficits in serial spatial reversal memory on locations 2 and 3 in Study 006 mice by age and genotype. A,B: At 3mo Study 006 mice have equivalent performance in a test of initial (location 2) and serial (location 3) spatial reversal. C: BACE pKO; PDAPP mice at 13mo have improved ability to learn a new spatial location compared to PDAPP mice. D: Performance on location 3 by 13mo Study 006 mice is equivocal. E,F: At 18mo BACE pKO; PDAPP display significant impairments in learning locations 2 and 3 compared to PDAPP mice.

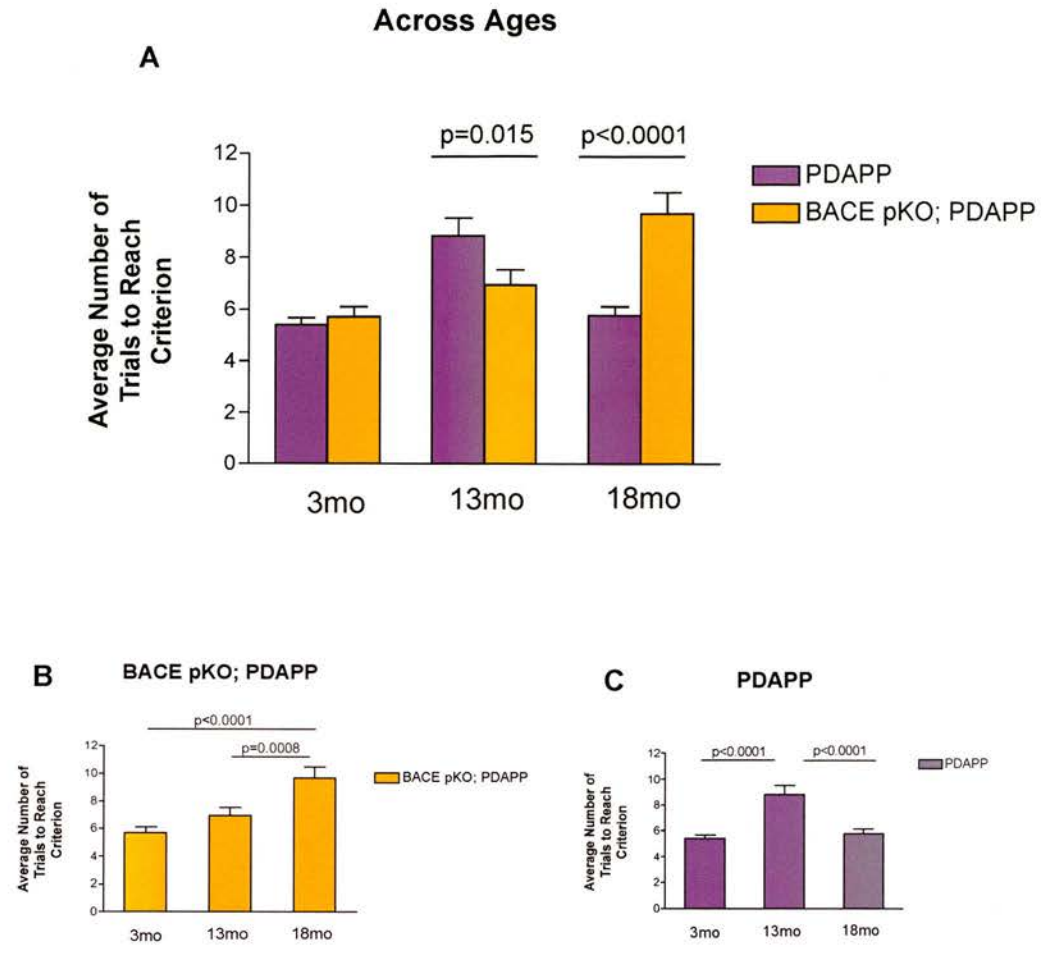


Figure 4.2.3 Deficit in memory acquisition average trials to criterion in Study 006 mice by age and genotype. A: At 13mo BACE pKO; PDAPP mice require on average fewer trials to learn each spatial location to criterion compared to PDAPP mice, but by 18mo PDAPP mouse performance is superior to that of BACE pKO; PDAPP mice. B: BACE pKO; PDAPP mice show a progressive age-related deficit in memory acquisition and serial learning between 13 and 18mo, and 3 and 18mo groups. C: PDAPP mice show a non-progressive memory deficit between 3mo and older animals, with the worst overall performance in average trials to criterion at 13mo, not 18mo of age.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	26	5.88	3.02	22	6.77	5.14	23	10.13	7.11	71	7.54	5.52
PDAPP	30	6.03	3.3	19	9.16	5.19	23	6.39	2.71	72	6.97	3.91
ALL	56	5.96	3.14	41	7.88	5.24	46	8.26	5.65	143	7.25	4.77

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	1.49	2	0.23
Age	2.89	2	0.05
Genotype	0.08	1	0.78

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.7	0.019	0.05
3 vs 18 months	0.0095	0.51	0.02
13 vs 18 months	0.033	0.098	0.77

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.97	0.05	0.06	0.93

Tables 4.2.1a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 006 mice on TTC1 performance by age and genotype.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	26	4.31	1.46	22	5.18	2.42	23	8.04	5.79	71	5.79	3.96
PDAPP	30	4.62	2.26	19	7.58	4.71	23	4.91	2.75	72	5.51	3.42
ALL	56	4.47	1.91	41	6.29	3.81	46	6.48	4.75	143	5.65	3.69

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	5.37	2	0.0057
Age	4.92	2	0.0087
Genotype	0.05	1	0.82

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.3	0.0059	0.0072
3 vs 18 months	0.0012	0.76	0.011
13 vs 18 months	0.032	0.019	0.83

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.78	0.05	0.0085	0.82

Tables 4.2.2a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 006 mice on TTC2 performance by age and genotype.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	26	6.92	3.51	22	8.82	4.36	23	11.09	5.62	71	8.83	4.78
PDAPP	30	5.5	2.37	19	9.74	7.28	23	6	2.24	72	6.78	4.53
ALL	36	6.16	3.01	41	9.24	5.83	45	8.49	4.92	143	7.79	4.75

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	3	2	0.053
Age	5.68	2	0.0043
Genotype	8.86	1	0.0034

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.095	0.0062	0.002
3 vs 18 months	0.0027	0.45	0.0069
13 vs 18 months	0.19	0.054	0.64

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.15	0.85	0.0005	0.0036

Tables 4.2.2e-h Descriptive, MANOVA and pairwise ANOVA statistics for Study 006 mice on TTC3 performance by age and genotype.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	26	5.71	1.94	22	6.92	2.78	23	9.7	3.92	71	7.38	3.37
PDAPP	30	5.39	1.56	19	8.82	2.94	23	5.77	1.62	72	6.42	2.48
ALL	56	5.54	1.74	41	7.8	2.98	46	7.74	3.57	143	6.9	2.98

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	14.28	2	<0.0001
Age	15.19	2	<0.0001
Genotype	2.98	1	0.086

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.063	<0.0001	<0.0001
3 vs 18 months	<0.0001	0.44	<0.0001
13 vs 18 months	0.0008	<0.0001	0.59

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.58	0.015	<0.0001	0.086

Tables 4.2.3a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 006 mice on averaged TTC1-3 performance by age and genotype.

4.3 Learning Capacity (Number of platforms learned)

Over the course of the Study 006 experiment, mice were exposed to a series of platform locations. The number of spatial locations or platforms learned serves as a measure representative of the learning and memory capacity of these animals. The PDAPP and BACE pKO; PDAPP genotypes are indistinguishable with respect to their learning capacity (LC) in the watermaze until they reach 18mo of age, when BACE pKO; PDAPP mice have significantly less memory capacities than PDAPP mice ($F=17.94$, df 1/138, $p<0.0001$, Table 4.3a-d, Figure 4.3.1). In addition there is a significant age-related decline in LC measures within genotypes across ages from learning an average of 9 locations at 3mo to about 7 locations by 13m-18mo ($F=32.97$, df 2/138, $p<0.0001$, Table 4.3.1). Overall there is a strong interaction of age*genotype that impacts spatial memory capacity in Study 001 mice ($F=27.17$ df 2/138, $p<0.0001$). The data implies that partial loss of the BACE gene confers a severely deleterious memory capacity impairment in mice that overexpress transgenic mutant APP.

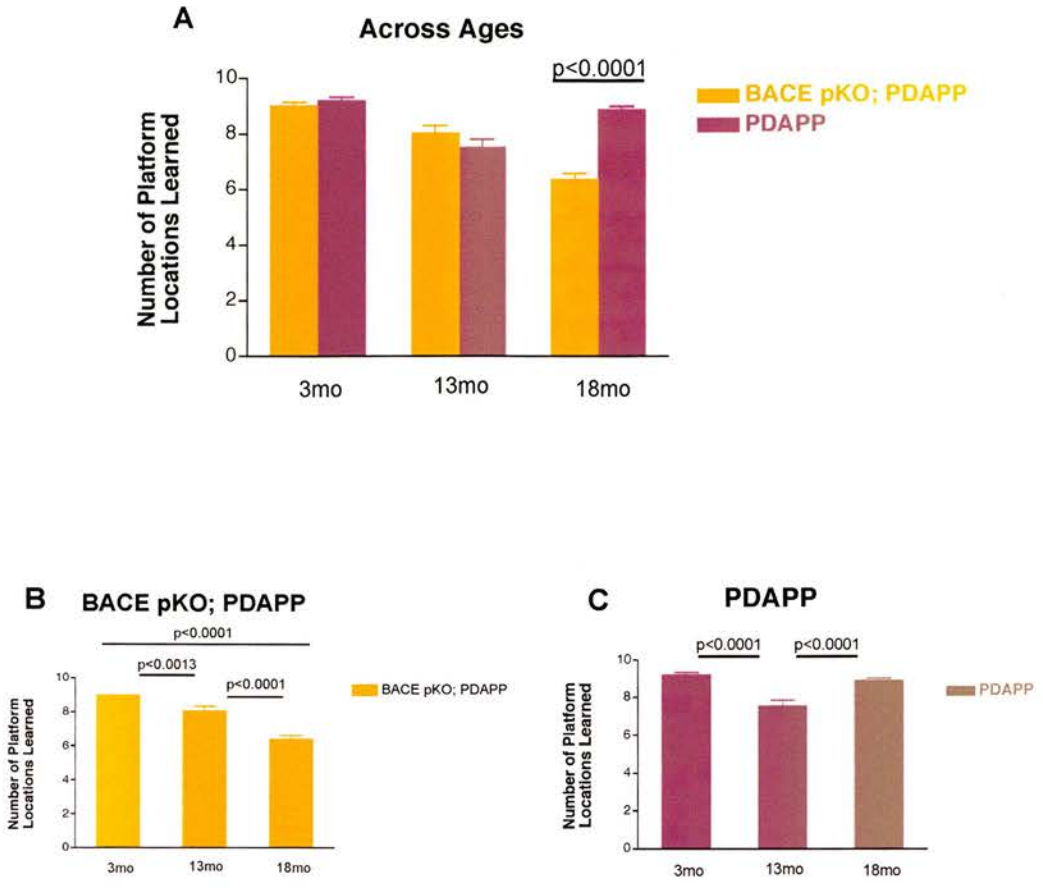


Figure 4.3.1 Deficits in spatial memory capacity in Study 006 mice by age and genotype. A: Across ages BACE pKO; PDAPP mice are significantly worse in their spatial memory capacity in aged mice. B: BACE pKO; PDAPP mice display an age-related decline in their total spatial memory capacity, as performance mice aged between 3 and 13mo, 13 and 18mo, as well as 3 and 18mo are significantly different. C: PDAPP mice show a non-progressive deficit in serial spatial memory capacity, with the poorest spatial memory capacity at 13mo.

Genotype	3 months			13 months			18 months			All		
	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	26	9	0.85	22	8.05	1.33	23	6.39	1.08	71	7.86	1.53
PDAPP	30	9.2	0.76	19	7.53	1.31	23	8.87	0.69	72	8.65	1.14
ALL	56	9.11	0.8	41	7.8	1.33	46	7.63	1.54	143	8.26	1.4

Source Factor(s)	F statistic	DF	p-value
Genotype*Age	27.17	2	<0.0001
Age	32.97	2	<0.0001
Genotype	17.94	1	<0.0001

Comparison	p-value		
	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.0013	<0.0001	<0.0001
3 vs 18 months	<0.0001	0.24	<0.0001
13 vs 18 months	<0.0001	<0.0001	0.47

Comparison	p-value			
	3 mo	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP **VS** PDAPP	0.46	0.1	<0.0001	<0.0001

Tables 4.3.1a-d Descriptive, MANOVA and pairwise ANOVA statistics for Study 006 mice on learning capacity by age and genotype.

Non-Memory Phenotypes

4.4 Seizures and Other Observations

In comparison to Study 001 mice with homozygous deletions of the BACE gene, Study 006 mice displayed a lesser seizure phenotype with only about 3% of animals having observed seizures compared to 17% of BACE KO; PDAPP animals in Study 001. (Figure 3.4B, 4.4B). Indeed, Study 006 mice had fewer deleterious phenotypes altogether, and those that remained, including seizures and circling behavior or spinning, could be attributed to the presence of the PDAPP transgene as the hemizygous BACE gene deletion appeared to confer no additional abnormal phenotype itself.

Animals were also removed from study for other reasons, including excessive anxiety, spinning, floating and blindness, with nearly equivalent numbers of animals removed by genotype across ages (PDAPP: 18.1% of original N=72, BACE pKO; PDAPP: 16.2% of original N=74). Different removal reasons ("Other") that were unrelated to deleterious phenotypes included shipment of incorrectly aged animals, body wounds, or removal of animals prior to behavioral testing for biochemical analysis (Figure 4.4C). Observation of Study 006 animals suggests that the hemizygous removal of the BACE gene did not predispose mice to spontaneous seizure activity, although this possibility is explored more extensively in Study 011.

4.5 Death and Survival Rates

Study 006 mice have much lower percentages of deaths in-house than Study 001 mice, suggesting that these mice are more capable of responding to the stresses of travel, new caging environments, and behavioral testing (Figures 3.5, 4.5). In addition, although BACE pKO; PDAPP mice were on the whole more genetically altered than PDAPP mice, the partial deletion of the BACE gene on a PDAPP background appeared to promote longer lifespans as these mice lived on average to 10.5mo while PDAPP mice lived on average to 8.5mo when vendor inventories were analysed (PDAPP N=117, BACEpKO; PDAPP N=167, Table 4.5). This somewhat surprising data suggest that partial deletion of BACE on a PDAPP

background provides a meaningful rescue of the early death phenotype associated with PDAPP animals, possibly by a beneficial reduction of Aβ levels.

Age (Mo)	PDAPP	BACE KO; PDAPP
3	26.1% (N=6 of 23)	21% (N=7 of 24)
13	24% (N=6 of 25)	8.3% (N=2 of 24)
18	4.2% (N=1 of 24)	12% (N= 3 of 26)

Table 4.4A Study 006 Mice Removed from Experiment by Age and Genotype

Age (Mo)	PDAPP	BACEpKO; PDAPP
3	0% (N= 0 of 23)	4.2% (N=1 of 24)
13	0% (N= 0 of 25)	0% (N=0 of 24)
18	4.2% (N= 1 of 24)	4% (N= 1 of 26)

Table 4.4.B Spontaneous Seizures in Study 006 Mice by Age and Genotype

	Age of 50% Group Mortality (mo)	Degrees of Genetic Modification	PDAPP Transgenes	Deleted Alleles
PDAPP	8.5	1	1	0
BACE pKO; PDAPP	10.5	2	1	1

Table 4.5 50% Mortality Ages of Study 006 Mice

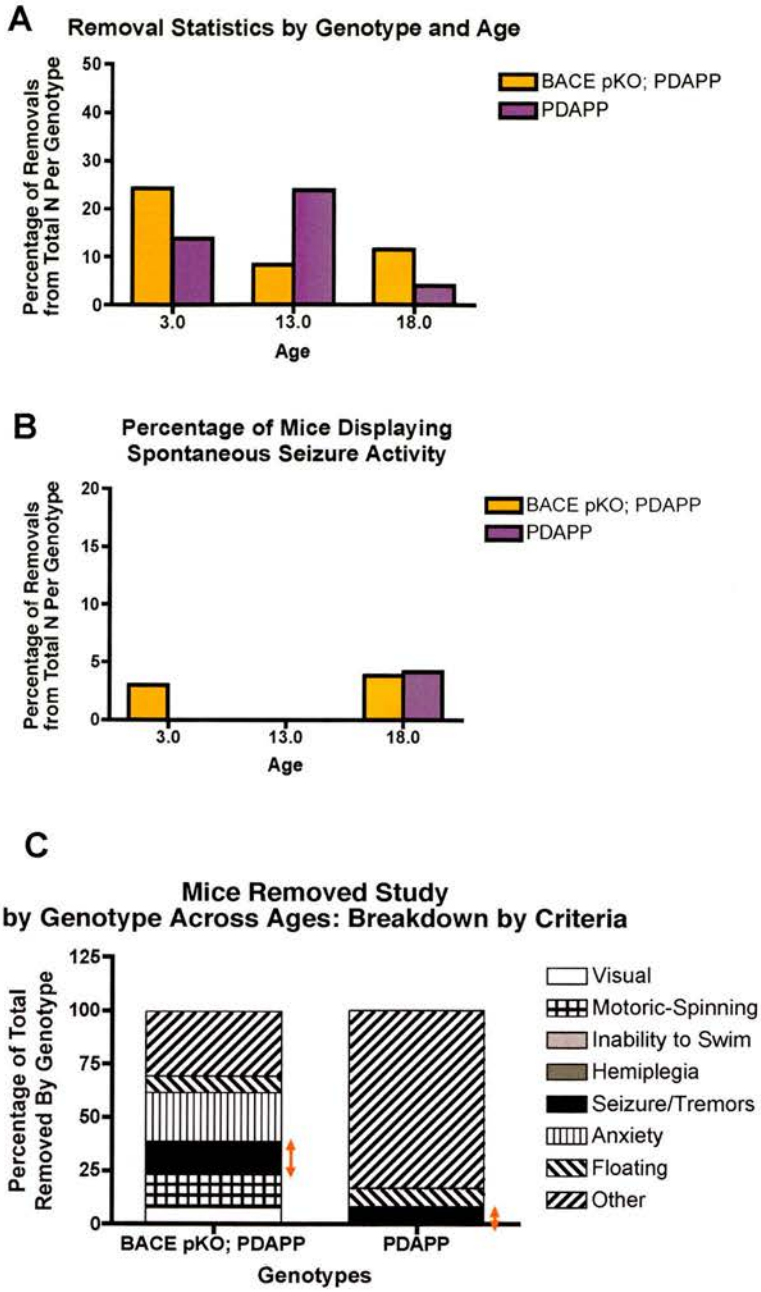


Figure 4.4. Seizures and other observations in Study 006 mice. A: The percentage of mice removed from study various from 5-25% when broken down by PDAPP and BACE pKO; PDAPP mice across ages. B: A small number of mice display spontaneous seizure activity, with about 3% in 3mo BACEpKO; PDAPP mice and about 4% each for 18mo PDAPP and BACE pKO; PDAPP mice. Unlike the Study 001 mice, seizure activity in Study 006 mice is not limited to animals with BACE gene deletions. C: Breakdown of removal reasons for PDAPP and BACE pKO: PDAPP mice. BACE pKO; PDAPP mice were removed for more reasons than the PDAPP mice, including anxiety, spinning and inability to swim. Red arrows indicate removals related to seizure activity.

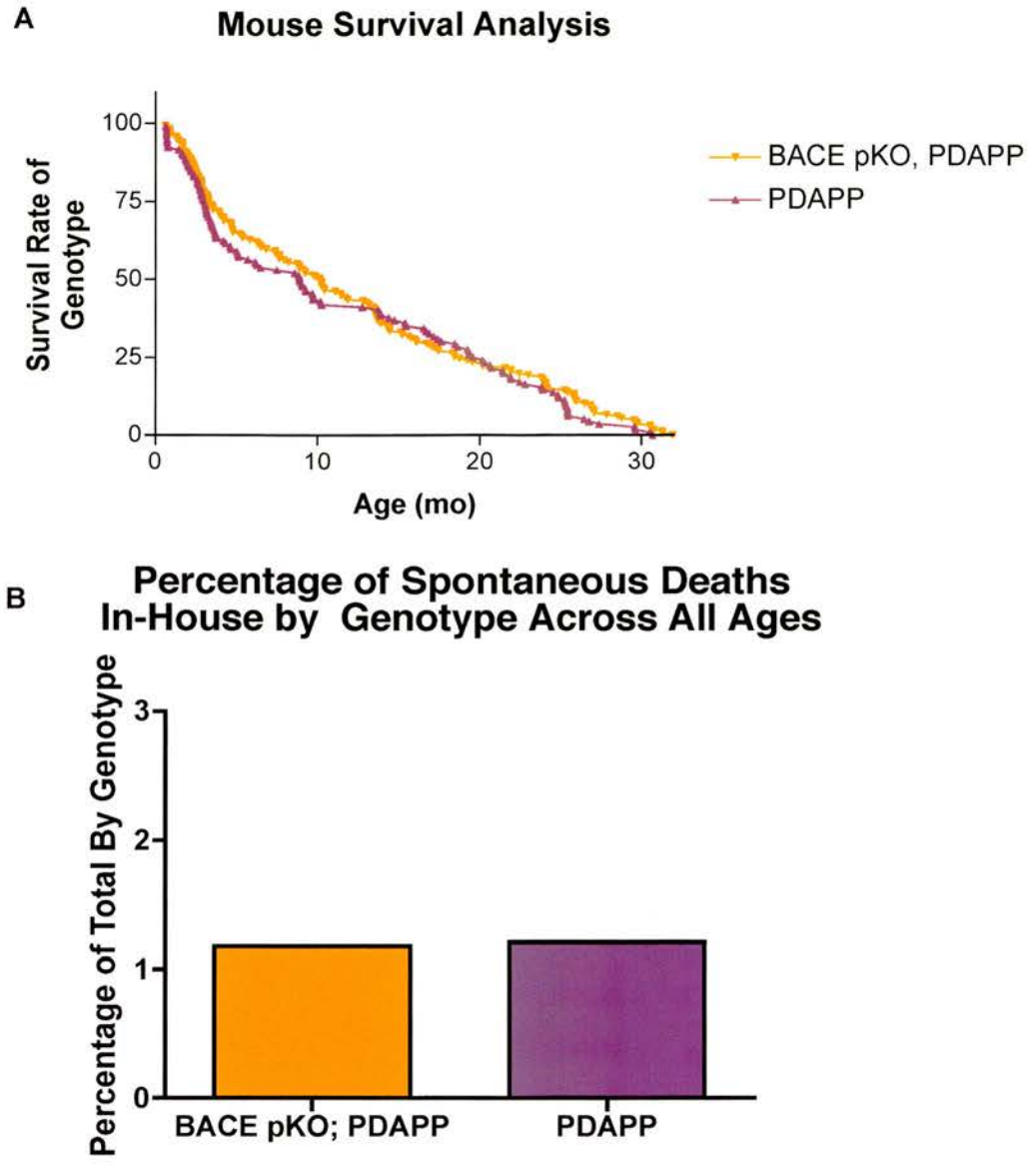


Figure 4.5 Death and survival rates in Study 006 mice. A: BACE pKO; PDAPP mice live on average longer than PDAPP mice, with average lifespans of 10.5mo and 8.5mo (Table 4.5), and oldest ages at 32.5mo and 30.5mo respectively. B: A similar number of BACE pKO; PDAPP and PDAPP mice died after shipment to Elan prior to testing. This 1% death rate is much lower than the Study 001 death rates which ranged from 3% in Control and PDAPP mice to 10% in mice with BACE gene deletions.

Calbindin and Amyloid Histology

4.6 Calbindin Histology in the Hippocampal Outer Molecular Layer

Calbindin (CB) is a Ca^{++}-binding protein ubiquitously expressed in cerebral neurons, which has been linked to spatial memory performance in transgenic hAPP mice (Palop et.al.; 2003). CB has also often been used as a surrogate marker for neurogenesis related to seizures in rodents, which were originally observed at a high rate in Study 001 mice. By using an antibody to CB and probing it with a fluorescent secondary antibody, measurements of the average intensity of hippocampal CB in 006 mice were made with the intention of relating spatial memory performance to hippocampal CB immunoreactivity.

While there was far less spontaneous seizure activity in Study 006 mice, there was also a dynamic range of CB intensity patterns in the hippocampus with age. At 3mo, there was tendency towards BACE pKO; PDAPP mice having higher CB levels than PDAPP mice ($p=0.078$). However, by 18mo PDAPP mice had the greater CB intensities compared to BACE pKO; PDAPP mice ($p=0.0008$, Figure 4.6.1C). This pattern of CB immunoreactivity mirrors the behavioral findings in which the BACE pKO; PDAPP mice display a significant decline in spatial memory relative to PDAPP mice. Overall this pattern translated to a significant age*genotype interaction in Study 006 mice ($F=7.0$ df 6/138, p.0014).

It is possible that if CB is functionally related to memory status, the BACE hemi-deletion may protect against the loss of CB in the hippocampus at early ages as was also observed in Study 001 PDAPP mice, but by 18mo some other process is taking place (Tables 4.6a-d). Alternatively, it is possible that 18mo PDAPP mice were experiencing greater unobserved seizure activity, which could contribute to the elevation of CB levels. Again, the hypothesis of hemizygous BACE deletions protecting against seizure and, with it, changes in CB levels will be explored in greater detail in Study 011c.

	3 months			13 months			18 months			All		
Genotype	N	Mean	Std	N	Mean	Std	N	Mean	Std	N	Mean	Std
BACE pKO; PDAPP	22	59.23	9.91	13	45.21	9.83	21	59.18	19.1	56	55.96	15.1
PDAPP	25	52.82	12.2	15	49.04	9.35	19	81.71	30.5	59	59.63	19.2
ALL	47	55.82	11.5	28	47.26	9.6	40	67.62	21.7	115	57.84	17.3

Genotype*Age	7	2	0.0014
Age	15.43	2	<0.0001
Genotype	2.55	1	0.11

	p-value		
Comparison	BACE pKO; PDAPP	PDAPP	ALL
3 vs 13 months	0.0018	0.45	0.0052
3 vs 18 months	0.69	<0.0001	0.0021
13 vs 18 months	0.0058	<0.0001	<0.0001

	p-value			
Comparison	**3 mo**	**13 mo**	**18 mo**	**ALL**
BACE pKO; PDAPP VS PDAPP	0.078	0.36	0.0008	0.11

Table 4.6a-d Descriptive, MANOVA and pairwise ANOVA statistics for hippocampal Calbindin levels in Study 006 mice by age and genotype.

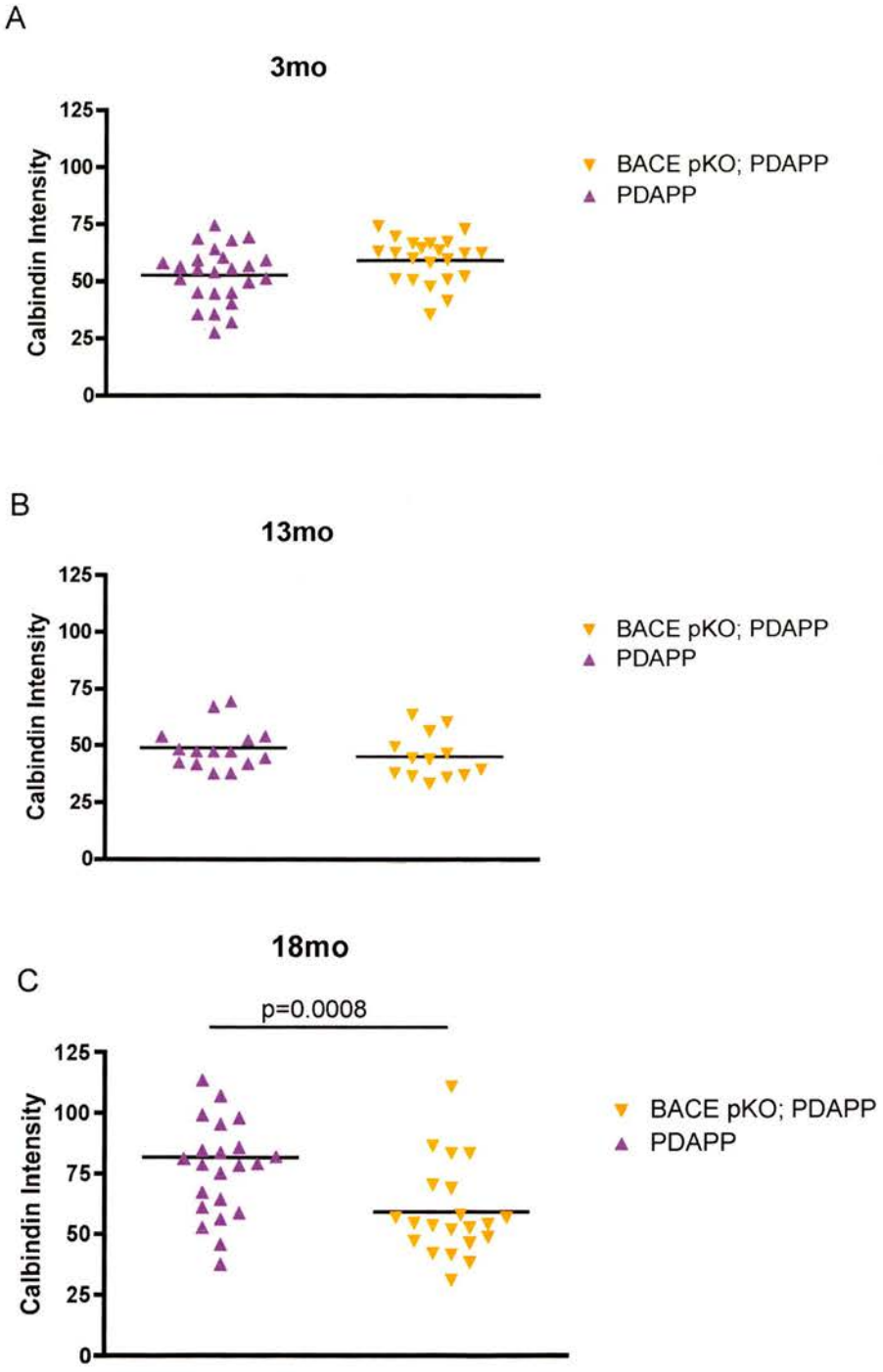


Figure 4.6.1 Calbindin intensity in the hippocampal outer molecular layer of Study 006 mice. A: Although there is a trend towards BACE pKO; PDAPP mice having higher Calbindin (CB) levels than PDAPP mice, they are statistically equivocal at 3mo. B: At 13mo BACE pKO; PDAPP and PDAPP mice have indistinguishable CB levels. C: By 18mo, there is a significant deficit in CB levels in BACE pKO; PDAPP mice relative to PDAPP mice, mirroring the behavioral deficits seen in these animals.

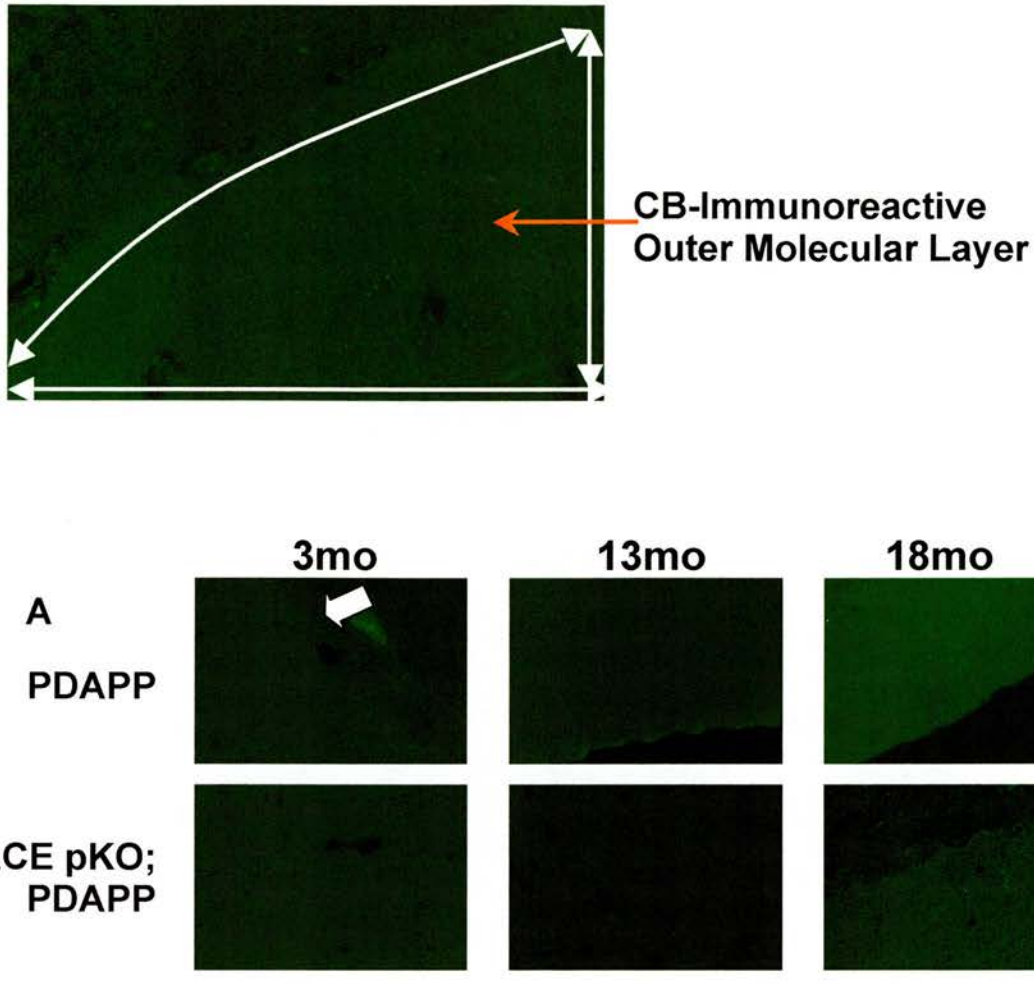


Figure 4.6.2 Calbindin images from the hippocampal outer molecular layer in Study 006 mice. Images with CB immunoreactivity levels close to group averages are presented, and the immunoreactivity of blood vessels (white arrow) is artifactual (3mo PDAPP). 3mo: PDAPP and BACE pKO; PDAPP mice have equivalent CB levels. 13mo: There is no distinction between CB levels from any genotype. 18mo: BACE KO; PDAPP mice have visibly lower levels of CB compared to PDAPP mice, suggesting an age-related decline in CB immunoreactivity in mice with partial BACE gene deletion.

4.7 hAmyloid Precursor Protein Immunoreactivity and Aβ processing

Expression of hAPP in the PDAPP and BACE pKO; PDAPP animals was confirmed by antibody staining with 8E5. hAPP expression was evident in cortical and hippocampal tissues of all Study 006 mice (Figure 4.7.1a-b). Confirmation of hAβ processing was conducted via antibody staining with 3D6 (Figure 4.7.2). Using this antibody, amyloid deposits were visible in the hippocampus and cortex of PDAPP mice at 13 and 18mo of age, with attenuated amyloid deposition in BACE pKO; PDAPP brain tissues at the same ages. Deletion of one murine BACE allele was sufficient to reduce Aβ metabolism, especially at the 13mo age (Figure 4.7.2a-b middle panels). This finding was intriguing as this was the age in which BACE pKO; PDAPP mice had superior spatial memory performance in acquisitional measures (Figures 4.2.2c, 4.2.3a). By 18mo the accumulation of amyloid in BACE pKO; PDAPP mice still appeared to be less than that of PDAPP mice but comparable in regions of the hippocampus to that of 13mo PDAPP mice (Figures 4.7.2a middle panel, Figure 4.7.2b right panel). This observation suggests that hemizygous BACE gene deletion partially rescues PDAPP mice from amyloid neuropathology, as it seems that accumulations are delayed in their development. In PDAPP mice there was little spatial memory decrement between the ages of 13-18mo, so it was perhaps not surprising that there is no distinction between 18mo performance in PDAPP and BACE pKO; PDAPP mice. By 13mo BACE pKO; PDAPP mice may have also reached a level of saturation in histological pathology/spatial memory performance.

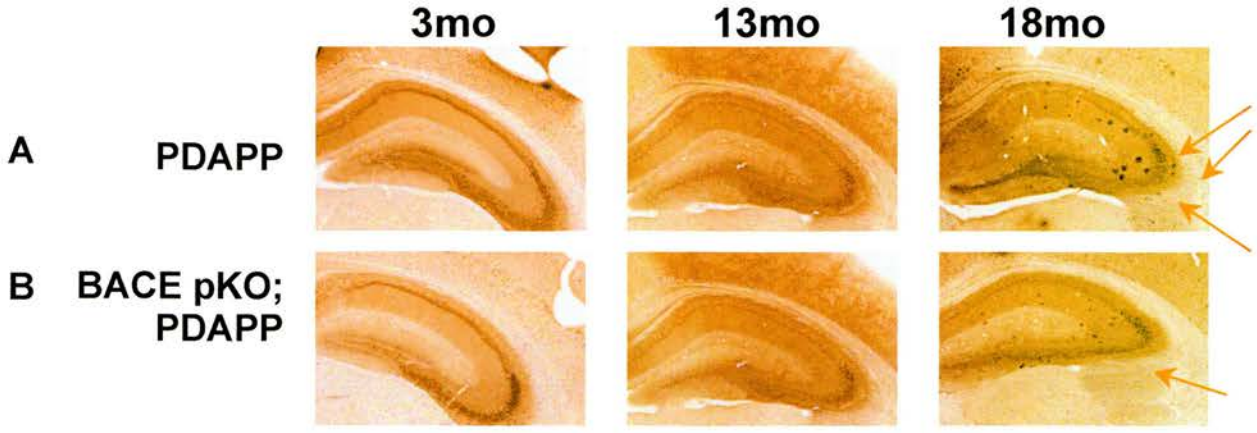


Figure 4.7.1 APP brain immunoreactivity in Study 006 mice. 3mo, 13mo: hAPP is detected by the antibody 8E5 in PDAPP and BACE pKO; PDAPP mice. 18mo: hAPP is present in PDAPP and BACE KO; PDAPP mouse brains, however, the extent of neuritic dystrophy pathology present (plaque-like accumulations within the hippocampus, delineated by red arrows) in 18mo PDAPP mice appears to be greater than that of 18mo BACE KO; PDAPP mice.

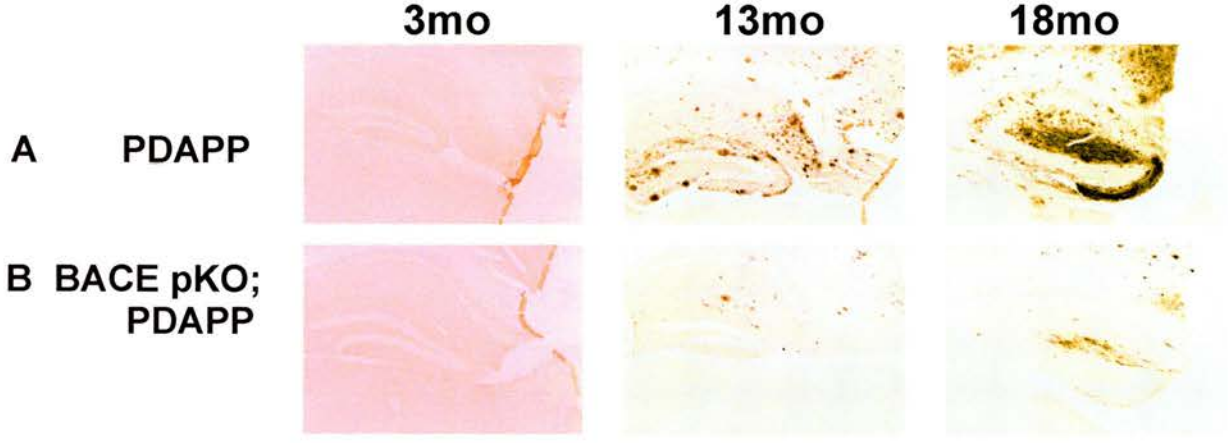


Figure 4.7.2 Partial BACE gene deletion ameliorates cerebral Aβ deposition in Study 006 mice. 3mo: No Aβ depositions detected by the 3D6 antibody are present in young mice, as this neuropathological feature does not manifest until at least 6-7mo in PDAPP mice. 13mo: Plaque-like deposits of Aβ are present in 13mo animals, while BACE KO; PDAPP brains appear to have attenuated levels amyloid burdens. 18mo: The cortex and hippocampus of PDAPP animals have heavy Aβ burdens, while BACE KO; PDAPP brains have fewer deposits than even 13mo PDAPP mice, suggesting that hemizygous BACE gene deletion is sufficient to diminish and/or delay Aβ deposition throughout life.

4.8 Correlation Analyses of Behavioral and Histological Data

Correlation Analysis Cell Key

R-Values Colorimetrics	P-Values Colorimetrics	Self-Correlation	Insignificant p- or Non-correlative r-value,
0.3<R<1 / -1<R<-0.3	p<0.05		

Column Abbreviations

Plats = Number of Platforms Learned
TTC N = Trials to Criterion, Location N
Ave1-3 = Average TTC Locations 1-3
CB = Calbindin Intensity in the Hippocampal Outer Molecular Layer

Example Correlation Table, Behavioral Measures Interrelationships

R-values from correlation analysis of each measure

	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3
#Platforms		-0.2208	-0.4811	-0.677	-0.7621
TTC 1	0.0476				0.3994
TTC 2	P<0.0001			0.3305	0.6643
TTC 3	P<0.0001		0.0026		0.8075
ave1-3	P<0.0001	0.0002	P<0.0001	P<0.0001	

p-values from correlation analysis of each measure

Figure 4.8.1a Example correlation tables. A: Correlation table of relationships between various behavioral measures, with R-values presented in upper diagonal section and p-values presented in lower diagonal section. Corresponding p-values and R-values are found in the same coordinate distance from the black diagonals separating the two types of values, with R-values at x_r,y_r coordinates, and p-values at y_p,x_p coordinates where $x_r=y_p$ and $y_r=x_p$

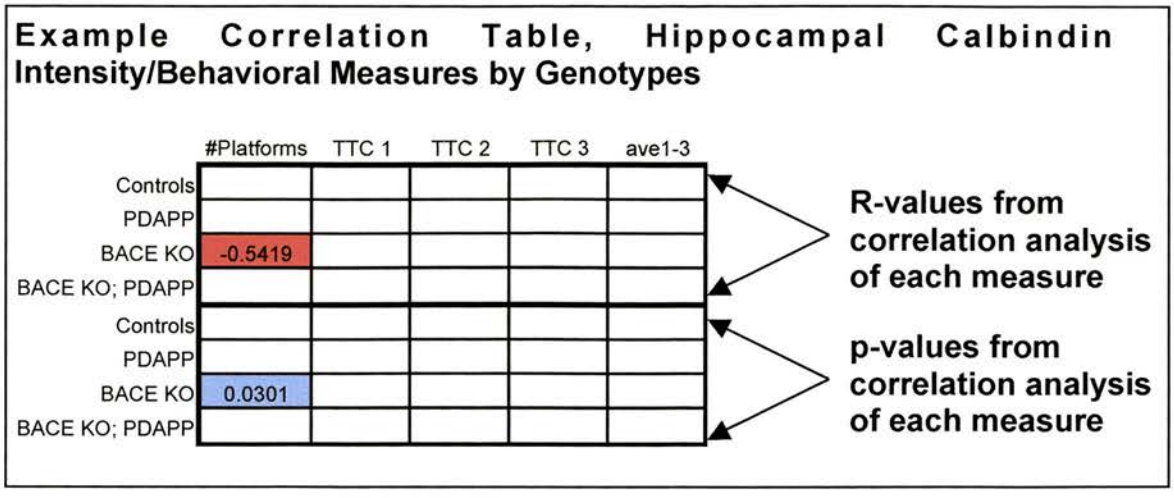


Figure 4.8.1B Example correlation tables. B: Correlation table of calbindin values to behavioral performance measures, with individual genotypes presented. R-values are in upper sections and p-values are in lower section.

The patterns of intra-behavioral measures in Study 006 were reminiscent to that of Study 001, as once again, numbers of platforms learned at all ages was inversely related to trial-based measures like TTC, and there were certain measures that were linked and also unrelated at specific ages. Study 006 correlation patterns for behavioral measures differ in that all the TTC measures were unrelated to each other from the earliest age tested (3mo) and remained distinct at 13mo and 18mo as well (Tables 4.8.1-4.8.3). However the TTC1 and TTC3 measures were inversely correlated to LC at 3mo and expanded to include all TTC measures to LC by 13 and 18mo.

At all ages TTC2 appeared to be functionally disconnected in value patterns compared to all other measures. While TTC1 performance is measure of spatial memory acquisition, learning the TTC2 task requires the ability to "unlearn" a previous task to successfully solve a new task. The lack of correlation between TTC1 and TTC2 performance values suggests that they may be fundamentally different processes.

Finally, the fact that many of the general features of the behavioral correlations patterns between 001 and its progenitor colony 006 are similar even though these are

not directly comparable colonies suggests that these behavioral measure relationships were genuine. These patterns of relationship implicate separate anatomical and/or synaptic processes, a concept which could be further explored with electrophysiological studies.

Correlational analysis conducted between genotype, color and gender factors and behavioral and Calbindin (CB) measures revealed several patterns of relationships. At 3mo, Genotype is related to Color (R=0.295, P=0.0275), which is the correlational affirmation of the earlier data that shows that the majority of BACE pKO; PDAPP mice are pigmented (Table 4.0a). However, both gender and color show associations to behavioral performance in 3mo Study 006 mice, such that female mice appear to learn more platform locations (R=0.287, P=0.032), and dark-coated mice learn location 1 faster (R=-0.371, P=0.0049). In addition female mice and dark-coated mice had higher CB intensities. By 13mo, there only by-factor correlation in Study 006 was that dark-coated mice now had lower levels of CB. By 18mo, the influence of genotype was stronger, indicating that overall BACE pKO; PDAPP mice had lesser memory capacity (#Platforms), took longer to learn location 2 (TTC2), and had lower CB levels, all of which were presented directly in Sections 4.2-4.3, and 4.6.

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		0.038	0.295	-0.119	-0.007	-0.006	0.196	0.072	0.277
Gender	0.7788		0.111	0.287	-0.043	0.173	-0.107	-0.093	0.442
Color	0.0275	0.4140		-0.047	-0.371	-0.112	0.095	-0.252	0.319
#Platforms	0.3809	0.0320	0.7319		-0.287	-0.115	-0.432	-0.547	0.123
TTC 1	0.9607	0.7551	0.0049	0.0320		0.033	0.039	0.593	-0.082
TTC 2	0.9647	0.2054	0.4152	0.4040	0.8122		0.131	0.467	-0.198
TTC 3	0.1472	0.4306	0.4855	0.0009	0.7777	0.3413		0.664	-0.063
ave1-3	0.5969	0.4948	0.0609	P<0.0001	P<0.0001	0.0003	P<0.0001		-0.193
CBInt	0.0598	0.0018	0.0288	0.4097	0.5849	0.1878	0.6719	0.1929	

Table 4.8.1 Correlation of behavioral measures, R- and P-values of all 3mo Study 006 mice. Spatial memory capacity (#Platforms) is highly correlated to initial and subsequent spatial locations (TTC1, TTC3). Higher Calbindin intensity is correlated to gender (female) and color, (agouti, black). The correlational analysis also implies that there are more agouti and black BACE pKO; PDAPP mice (Table 4.0b).

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		0.179	0.184	-0.110	-0.107	-0.088	0.105	-0.121	0.073
Gender	0.1564		0.027	0.021	-0.177	-0.159	-0.018	-0.163	0.126
Color	0.1457	0.8307		0.154	-0.087	-0.210	0.035	-0.186	-0.360
#Platforms	0.3866	0.8698	0.2245		-0.379	-0.296	-0.526	-0.734	-0.218
TTC 1	0.3992	0.1616	0.4923	0.0020		-0.015	0.181	0.405	0.056
TTC 2	0.4911	0.2091	0.0952	0.0174	0.9070		-0.058	0.671	-0.073
TTC 3	0.4126	0.8858	0.7856	P<0.0001	0.1547	0.6510		0.612	-0.001
ave1-3	0.3392	0.1995	0.1411	P<0.0001	P<0.0001	0.0009	P<0.0001		0.008
CBInt	0.6230	0.3942	0.0119	0.1375	0.7039	0.6210	0.9935	0.9585	

Table 4.8.2 Correlation of behavioral measures, R- and P-values of all 13mo Study 006 mice. As in 3mo mice, spatial memory capacity (#Platforms) in 13mo animals is highly correlated to learning spatial locations 1 and 3, but at 13mo memory capacity is also correlated to location 2 (TTC2). Higher Calbindin intensity is correlated to animal color (agouti, black).

	Genotype	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	ave1-3	CBInt
Genotype		0.000	-0.125	-0.838	0.241	0.317	0.472	0.623	-0.425
Gender	1.0000		0.059	-0.044	-0.102	-0.080	-0.050	-0.167	-0.335
Color	0.4097	0.6948		0.011	0.092	-0.184	0.037	0.006	-0.245
#Platforms	P<0.0001	0.7739	0.9411		-0.326	-0.404	-0.585	-0.753	0.337
TTC 1	0.1073	0.4989	0.5412	0.0273		0.146	0.120	0.629	0.084
TTC 2	0.0321	0.5988	0.2216	0.0054	0.3333		0.263	0.646	-0.112
TTC 3	0.4126	0.8858	0.7856	P<0.0001	0.1547	0.6510		0.606	-0.232
ave1-3	P<0.0001	0.2661	0.9684	P<0.0001	P<0.0001	P<0.0001	P<0.0001		-0.207
CBInt	0.0056	0.0324	0.1233	0.0312	0.6009	0.4846	0.1494	0.1946	

Table 4.8.3 Correlation of behavioral measures, R- and P-values of all 18mo Study 006 mice. Spatial memory capacity (#Platforms) in 18mo animals is highly correlated to learning each of 3 spatial locations (TTC1, TTC2, TTC3). Genotype is also correlated to spatial memory and CB intensity, as BACE pKO; PDAPP mice learn fewer platform locations, need more trials to learn location 2, and have lower CB levels. Female Study 006 mice have overall higher CB levels.

The histological and behavioral correlations in Study 006 are similar to that of Study 001, as in both CB does not appear to be a rigorous predictor or biomarker of spatial memory status (Tables 4.8.4-4.8.6). CB content in hippocampal neurons was absolutely non-predictive of spatial memory function at 3mo in Study 006 mice, although female BACE pKO; PDAPP mice had higher CB levels (R=0.604, P=0.003, Table 4.8.4). By 13mo Study 006 mice had no significant correlations between CB and any other measure (Table 4.8.5). At 18mo CB was also not correlated to spatial memory performance, although male PDAPP and albino BACE pKO. PDAPP mice had higher CB levels (table 4.8.6). These Study 006 findings

argue against the proposal that CB levels are a metric for spatial memory performance in mice in opposition to the transgenic hAPP mouse data from Palop et.al. (2003).

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	AVG TTC 1-3
PDAPP							
BACE pKO, PDAPP	0.604						
PDAPP							
BACE pKO, PDAPP	0.003						

Table 4.8.4 Correlation of hippocampal calbindin intensity to behavioral measures, R- and P-values by genotype in 3mo Study 006 mice. At 3mo there is only apparent CB immunoreactivity relationship in Study 006 is to animal gender, such that female BACE pKO; PDAPP mice have higher CB levels.

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	AVG TTC 1-3
PDAPP	0.314	0.367	0.012	-0.476	-0.375	0.013	-0.390
BACE pKO, PDAPP	0.124	-0.232	-0.338	0.126	-0.192	0.254	0.166
PDAPP	0.275	0.197	0.969	0.085	0.187	0.964	0.168
BACE pKO, PDAPP	0.687	0.447	0.259	0.681	0.529	0.402	0.587

Table 4.8.5 Correlation of hippocampal calbindin intensity to behavioral measures, R- and P-values by genotype in 13mo Study 006 mice. At 13mo there are no significant correlations between Calbindin and any other measure.

	Gender	Color	#Platforms	TTC 1	TTC 2	TTC 3	AVG TTC 1-3
PDAPP	-0.567						
BACE pKO, PDAPP		-0.742					
PDAPP	0.009						
BACE pKO, PDAPP		0.000					

Table 4.8.6 Correlation of hippocampal calbindin (CB) intensity to behavioral measures, R- and P-values by genotype in 18mo Study 006 mice. Pigmented BACE pKO; PDAPP mice have lesser hippocampal CB immunoreactivity, while albino animals tend to have greater CB immunoreactivity. Aged Male PDAPP mice have higher levels of CB than females. Overall it does not appear that CB is a biomarker of memory function in Study 006 mice.

Ch.5 Study 011A: General Sensorimotor Behavioral Phenotyping and Response to Seizure Induction of 18mo Homozygous BACE KO x PDAPP Mice

The purpose of Study 011A was to explore the genotypic basis for the spontaneous seizure phenotype observed in Study 001 mice with homozygous deletions of the BACE gene. While BACE KO and BACE KO; PDAPP mice had mild to severe spatial memory phenotypes in the water maze, some part of this impairment may be linked to irregular regulation of neuronal activity that itself is the basis for seizures.

In this study homozygous BACE KO x PDAPP mice were screened by genotype for their responses to chemically induced tonic seizures with the drug pentylenetetrazole (PTZ). PTZ is a seizure-inducing drug that acts via the GABAA/benzodiazepine receptor complex, possibly by blocking Cl- influx (Vitek et al., 1965; Yu et al., 1986). PTZ is commonly used in mice as a model of epileptiform activity, and has well-documented dose-response effects (Engstrom and Woodbury, 1988; Martin et al., 1988; Kosobud et al., 1992; Ferraro et al., 1999). Using this chemically-induced seizure model, aged Study 001 mice were administered PTZ in an effort to determine if BACE is normally involved in regulation of neuronal activity in epiletogenic regions of the brain like the hippocampus. In addition, mice in this Study 011A were also tested on a number of sensorimotor tasks (including forelimb grip strength, spontaneous locomotor activity monitoring, and rotorod motor coordination) to provide a broad general behavioral profile of homozygous BACE KO mice (Table 5.0).

Experimentation was limited to the number of homozygous BACE KO x PDAPP mice available, and it served the purpose of the study hypothesis to utilize aged animals to better determine whether they had differential seizure-induction profiles based on lifetime spontaneous activity. However, there were very few BACE KO animals alive at this age, and all were female mice, so this must be considered in examining the results of Study 011A. Around 18mo of age the survival rates of BACE KO mice dropped off sharply, which may indicate that there is an age-related

genotypic effect of BACE KO on survival (Ch.3 Figure 3.5a). Total N for this study was 45 mice.

	Female				Male				ALL			
	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL
Genotype												
BACE KO; PDAPP	2	0	5	7	3	0	2	5	5	0	7	12
BACE KO	1	0	2	3	0	0	0	0	1	0	2	3
PDAPP	2	2	2	6	4	0	5	9	6	2	7	15
Control	3	0	3	6	3	2	4	9	6	2	7	15
ALL	8	2	12	22	10	2	11	23	18	4	23	45

Table 5.0a Study 011A mice, all aged 18mo.

The overall findings from Study 011A are summarized below (Table 5.0b). One-way ANOVA tests were conducted on all measures except for grip strength and the rotorod, which had serial timepoints and was subject to MANOVA testing, and the Lethality measure which was analysed with Fisher's exact Chi-squared test. Due to the scarcity of animals across all gender and genotype groups, statistical analysis of performance by color was not possible, nor was any analysis of interactions between gender and genotype. However, descriptive statistics are presented for all factors. Although Study 011A mice had equivalent sensorimotor task responses on the majority of responses, it appears that BACE gene ablation results in an anxiety phenotype (which may have influenced the rotorod performance) and these animals also have less resistance to kindling severe seizures. Only one measure featured gender-based statistical differences (constant speed rotorod).

Measure	P-Values Gender	Genotype
Masses	0.21	0.028
Grip Strength	0.23	0.16
Crossings	0.44	0.61
Distance	0.43	0.2
Rests	0.64	0.38
Movement Time	0.45	0.28
Vertical Actvity	0.59	0.12
Stereotypy	0.72	0.37
Open Field Distance	0.62	0.2
Open Field Time	0.55	0.0023
Open Field Vertical Activity	0.83	0.24
Rotorod Constant Speed	0.0002	0.0097
Rotorod Accelerating	0.29	P<0.0001
PC Latency	0.58	0.15
GC Latency	0.14	0.63
TC Latency	0.26	N/A
PC Score	0.8	0.028
GC Score	0.2	0.094
TC Score	0.63	N/A
Seizure Score	0.31	0.45
Percentage Lethality	1	0.57
Death Latency	0.71	0.19
Calbindin Intensity	0.7	0.4

Table 5.0b Statistical summary of factor significance in Study 011A.

Mass and Muscular Function

5.1 Body mass and grip strength

In the immediate aftermath of spontaneous seizure activity in Study 001 mice, animals were observed to have very weak forelimb grip strength. This lack of limb strength is one of the common sequelae of severe clonic-tonic seizures in mice, in which limbs are rigidly extended during convulsions, as previously described in section 2.3.2 of the Materials and Methods chapter. Thus, it was hypothesized that animals that experience spontaneous seizure throughout life would have weaker forelimb grip strength when tested quantitatively for this characteristic.

Using an apparatus that digitally records transduced force, Study 011A mice were assessed for their forelimb grip strength as a function of their individual body mass. Animals were brought in proximity to a foil screen attached to a mass-displacement measuring device and allowed to grasp the surface with their forelimbs. The mice were then gently pulled laterally by the tail until their grip on the screen was broken. Mice were tested on three successive days, with three trials per day. The expected normal result would be for an animal to have relatively stable grip strength performances over the testing period, with no detectable reduction or increases in average grip strength. The effects of relative body mass or size differences between animals are minimized using a unitless grip strength ratio. For example, a mouse with a mass of 50g that displaces 100g with its forelimbs, or one of 25g that displaces 50g, each will have the same grip strength ratio of 2. A typical grip strength ratio for aged mice will vary between 2.0-3.0, with higher ratios of 2.5-3.5 in younger animals.

Grip strength ratio = (mass displaced by grip) / (body mass of mouse)

Study 011A mice were separated by gender and compared by genotype for body mass. Analysis of body mass revealed no statistical differences between the female mice of any genotypes as all had average masses between 34-45g. However, male PDAPP and BACE KO; PDAPP mice weighed significantly less than Control

males, with average group body masses at about 35g and 45g respectively (genotype: F=3.343, df 2/22, $p<0.05$; Table 5.1a-c). This finding was somewhat surprising given the largely anecdotal belief held by experimenters working with PDAPP-based mice that they are predisposed to obesity. Unfortunately, there were no male BACE KO mice available for body mass analyses.

Grip strength was also determined for Study 011A mice, using a ratio of maximal masses displaced prior to breaking of forelimb screen grip to individual body mass. As with the body mass comparisons, female Study 011A mice by genotype had statistically equivocal performances with grip strength ratios of about 2.5-3.0. Male mice carrying the PDAPP transgene all had significantly stronger grip strengths than Control mice, at ratios of 2.5-2.7 versus 2.0-2.2 respectively (Table 5.1d-f).

Factor	N	Mean (g)	STD
Color			
Agouti	18	36.24	5.91
Black	4	38.23	6.93
Albino	23	38.57	6.91
Gender			
Female	22	35.7	5.73
Male	23	39.43	6.74
Genotype			
BACE KO; PDAPP	12	38.21	5.57
BACE KO	3	34.01	3.52
PDAPP	15	39.5	11.32
Control	15	34.35	5.4
All	45	36.1	5.94

Source Factor(s)	F statistic	DF	p-value
Gender	1.579	1	0.21
Genotype	3.343	3	0.028

Comparison	P-Values
BACE KO; PDAPP VS PDAPP	0.27
BACE KO; PDAPP VS CONTROL	0.019
PDAPP VS CONTROL	0.034

Table 5.1a-c Descriptive and One-Way ANOVA statistics for body masses in Study 011A mice by gender and genotype

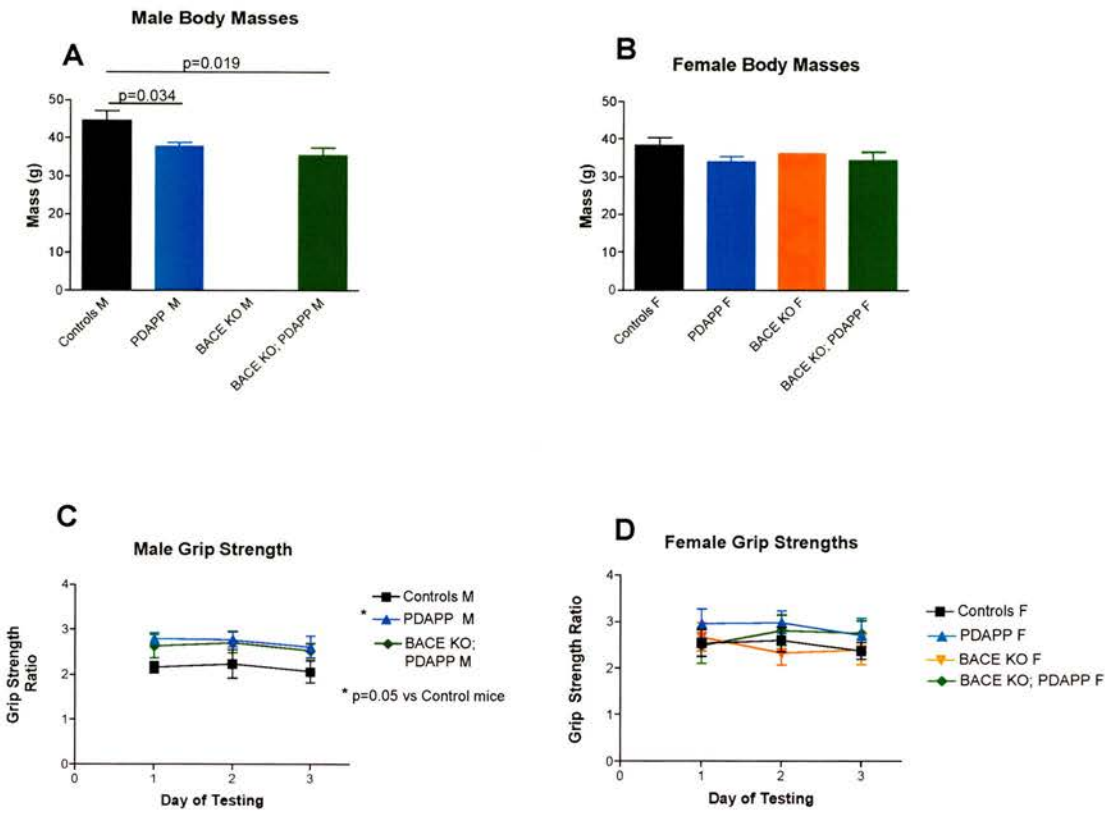


Figure 5.1 Body mass and grip strength of 18mo Study 011A mice by gender and genotype. A: Male PDAPP and BACE KO; PDAPP mice weighed significantly less than Control male mice; there were no male BACE KO mice in this experiment. B: There were no significant differences in body weights between 18mo female mice in Study 011A. C: Male mice with the PDAPP transgene have stronger forelimb grips than Control males. D: There were no significant differences in grip strength among female mice of the various Study 011A genotypes.

Factor	N	Trials					
		1		2		3	
Color		Mean	Std	Mean	Std	Mean	Std
Agouti	18	2.71	0.7	2.84	0.62	2.74	0.66
Black	4	2.92	0.57	2.93	0.73	2.63	0.61
Albino	23	2.48	0.75	2.48	0.75	2.34	0.66
Gender							
Female	22	2.39	0.69	2.5	0.66	2.42	0.61
Male	23	2.79	0.62	2.83	0.73	2.63	0.71
Genotype							
BACE KO; PDAPP	12	2.58	0.82	2.79	0.7	2.68	0.54
BACE KO	3	2.46	0.72	2.25	0.81	2.63	0.88
PDAPP	15	2.93	0.6	2.93	0.59	2.64	0.81
Control	15	2.29	0.53	2.39	0.74	2.27	0.56
All	45	2.59	0.68	2.67	0.71	2.53	0.67

Source Factor(s)	F	DF	p-value
Gender	1.51	3	0.23
Genotype	1.61	6	0.16

Comparison	p-Values
BACE KO; PDAPP VS PDAPP	0.5
BACE KO; PDAPP VS CONTROL	0.29
PDAPP VS CONTROL	0.05

Tables 5.1d-f Descriptive and One-Way ANOVA statistics for grip strengths in Study 011A mice by gender and genotype

Motoric Phenotypes

5.2 Spontaneous locomotor activity monitoring

Measurement of spontaneous locomotor activity is one of the most basic assessments made in phenotyping a transgenic mouse line as it provides information on the fundamental motor activity levels that are often the basis for other behavioral tasks. General activity levels can be recorded objectively by automated systems to produce a wide array of locomotor data, which inform the experimenter on aspects of locomotion, exploration, and anxiety states in test animals. In this section homozygous BACE KO x PDAPP mice were tested in an automated spontaneous locomotor monitoring system for measures of total distance moved, total time spent in motion, number of sectors crossed, number of total rests in movement, vertical activity (a measure related to motivation to explore and lack of anxiety) and repetitive or stereotypic movements (often associated with seizure propensity) as described in section 2.2.1. In addition open field exploration measurements were also made, to gain information about any underlying anxiety phenotypes in the BACE KO x PDAPP mice.

Examination of spontaneous motor phenotypes in Study 011 mice revealed no significant differences in horizontal activity profiles between genotypes except for a greater level of vertical activity in BACE KO; PDAPP mice compared to PDAPP animals ($p=0.05$, Figure 5.2.2b). Interestingly, PDAPP mice tended towards traveling shorter distances and spending less time in motion than Control mice (Tables 5.2.1a-c; Figure 5.2.1a-b). PDAPP mice have a noted anxiety phenotype, which these measures seem to support if anxiety indeed prevented the mice from further exploration (Gerlai et al., 2002). Alternatively, these PDAPP mice (which had no motor impairment in later locomotor testing on the rotorod), could simply be incurious with respect to novelty, a feature that has been noted in AD patients (Daffner et al., 1999). At the same time these findings suggest that the lower body masses described in PDAPP mice relative to Control mice in Figure 5.1a are not likely due to leaner body profiles due to overexploratory hyperactivity.

The increased vertical activity finding in which BACE KO; PDAPP mice reared more than PDAPP mice is in disagreement with the overall timid phenotype of 6-7w old BACE1 KO mice described by Harrison et.al. in 2003. This may be due to differences in the measures used or the age of the mice tested. One alternative hypothesis is that excess vertical activity of BACE KO; PDAPP is related to their propensity to spontaneous seizures, not anxiety per se.

To better examine the possibility of an anxiety phenotype in BACE KO animals, an open field-like analysis was performed. Exploration of an unfamiliar open area is commonly used to assess anxiety levels in rodents, as animals with higher anxiety levels will spend greater time exploring the periphery of an arena, spending less time, traveling shorter distances and rearing less in open central areas (Delbarre et al., 1970; Britton and Britton, 1981). The Accuscan activity monitoring system was modified to extract open field-like activity in a 12.5x12.5cm square centered within the larger 25x25cm arena. Over the same two 15min exploration sessions, mouse activity as recorded for distance traveled, time spent within, and number of rearings in the open field area.

BACE KO; PDAPP mice spent significantly less time in the open field area than both Control and PDAPP mice during monitoring sessions, which suggests a strong anxiety phenotype in these mice (Table 5.2.3a-c, Figure 5.2.3b, $F= 7.16$, df 2/68, $p<0.005$). Open field distance and vertical activity was not significantly different between any of the genotypes, although there was a trend towards greater activity in BACE KO mice compared to other genotypes in these as well as the open field time measure. The PDAPP mouse pattern of reduced exploration is repeated in the open field anxiety analysis, although they spend similar amounts of time in the open region compared to Control mice. As the PDAPP mice appear to ambulate less, and do not spend time in spatially distinct areas relative to Control mice, it is possible that the altered pattern of exploration present in PDAPP mice is not based in anxiety, but perhaps reduced motivation to explore. In this the BACE KO; PDAPP mice appear to be distinct from PDAPP mice, as they appear to have a more

classical anxiety phenotype, in which they travel shorter distances and also spend less time in the central open field area compared to Control mice.

The unusual pattern of higher activity levels but significantly less time spent in the open field area in BACE KO mice could be explained by an anxiety phenotype superimposed on a hyperactive phenotype. This concept is supported by the pattern of overall greater activity in BACE KO mice in distance traveled and time spent in motion, albeit with high variability, although this interpretation is still not completely aligned with the findings of non-exploratory BACE KO mice by Harrison et al. (2003).

		Distance (cm)		Time (s)		Motion Rests	
Factor	**N**	Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	18	379.97	300.57	47.14	37.66	80.28	33.42
Black	4	266.6	122.12	35.93	18.35	60.75	17.42
Albino	23	423.61	235.28	53.83	30.5	81.13	22.87
Gender							
Female	22	443.88	297.82	55.75	37.7	80.91	30.19
Male	23	342.77	203.3	43.65	26.44	77.13	24.84
Genotype							
BACE KO; PDAPP	12	415.99	231.17	52.94	33.03	76.17	26.66
BACE KO	3	669.37	423.43	79.33	51.04	83	29.31
PDAPP	15	281.62	251.26	36.82	34.21	72.87	32.25
Control	15	428.31	209.82	53.65	23.59	86.53	22.71
All	45	392.2	256.15	49.56	32.64	78.98	27.33

Source Factor(s)		F	DF	p-value
Distance	Gender	0.63	1	0.43
	Genotype	1.69	2	0.2
Time	Gender	0.59	1	0.45
	Genotype	1.31	2	0.28
Rests	Gender	0.22	1	0.64
	Genotype	1	2	0.38

	P-Values		
Comparison	**Distance**	**Time**	**Rests**
BACE KO; PDAPP VS PDAPP	0.18	0.23	0.82
BACE KO; PDAPP VS CONTROL	0.8	0.86	0.3
PDAPP VS CONTROL	0.092	0.14	0.19

Tables 5.2.1a-c Descriptive and One-Way ANOVA statistics for distance, time and motion rests activity monitoring measures in Study 011A mice by gender and genotype.

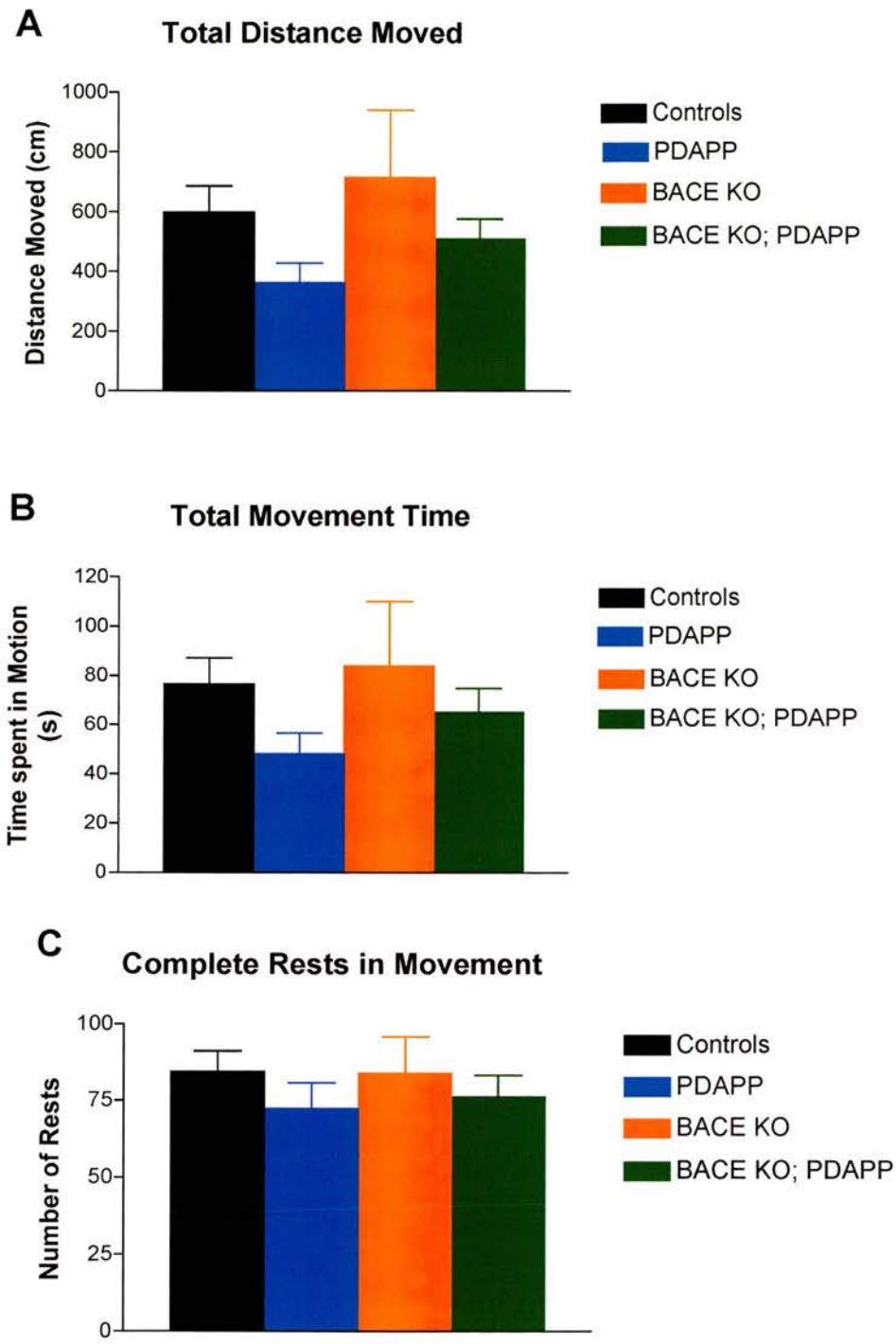


Figure 5.2.1 Spontaneous locomotor activity monitoring in 18mo Study 011A mice. A: Across genotypes there are no significant differences in their total horizontal exploration distances. B: As in horizontal distance explored, there are no differences in total time spent in motion for Study 011A mice. C: There was no genotypic differentiation in the number of pauses in movement in Study 011A mice.

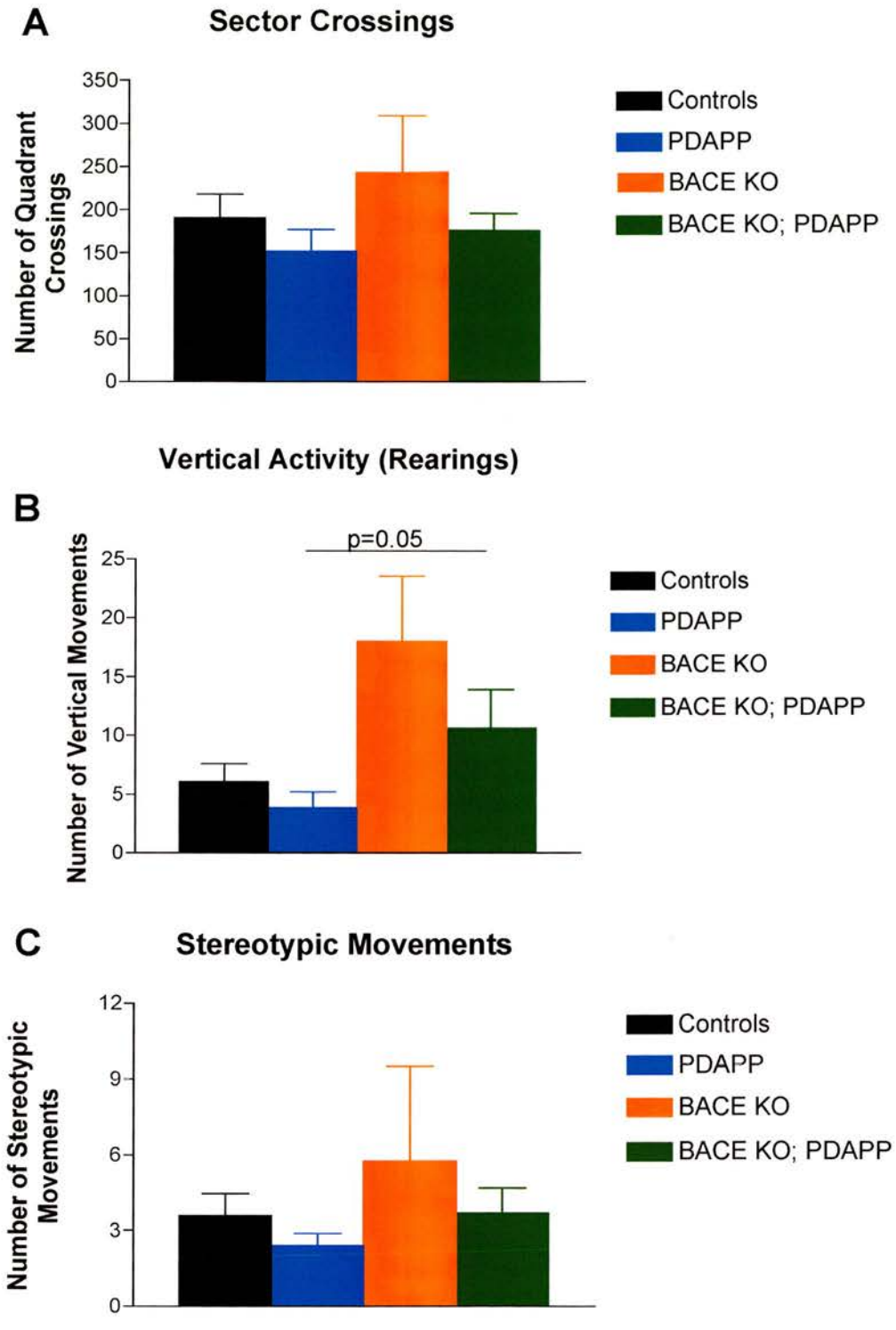


Figure 5.2.2 Spontaneous locomotor activity monitoring in Study 011A mice. A: There was no genotypic differentiation in the number of quadrants crossed in Study 011A mice. B: BACE KO; PDAPP mice reared significantly more than PDAPP mice. C: Study 011A mice by genotype were indistinguishable in their levels of stereotypic or repetitive movements.

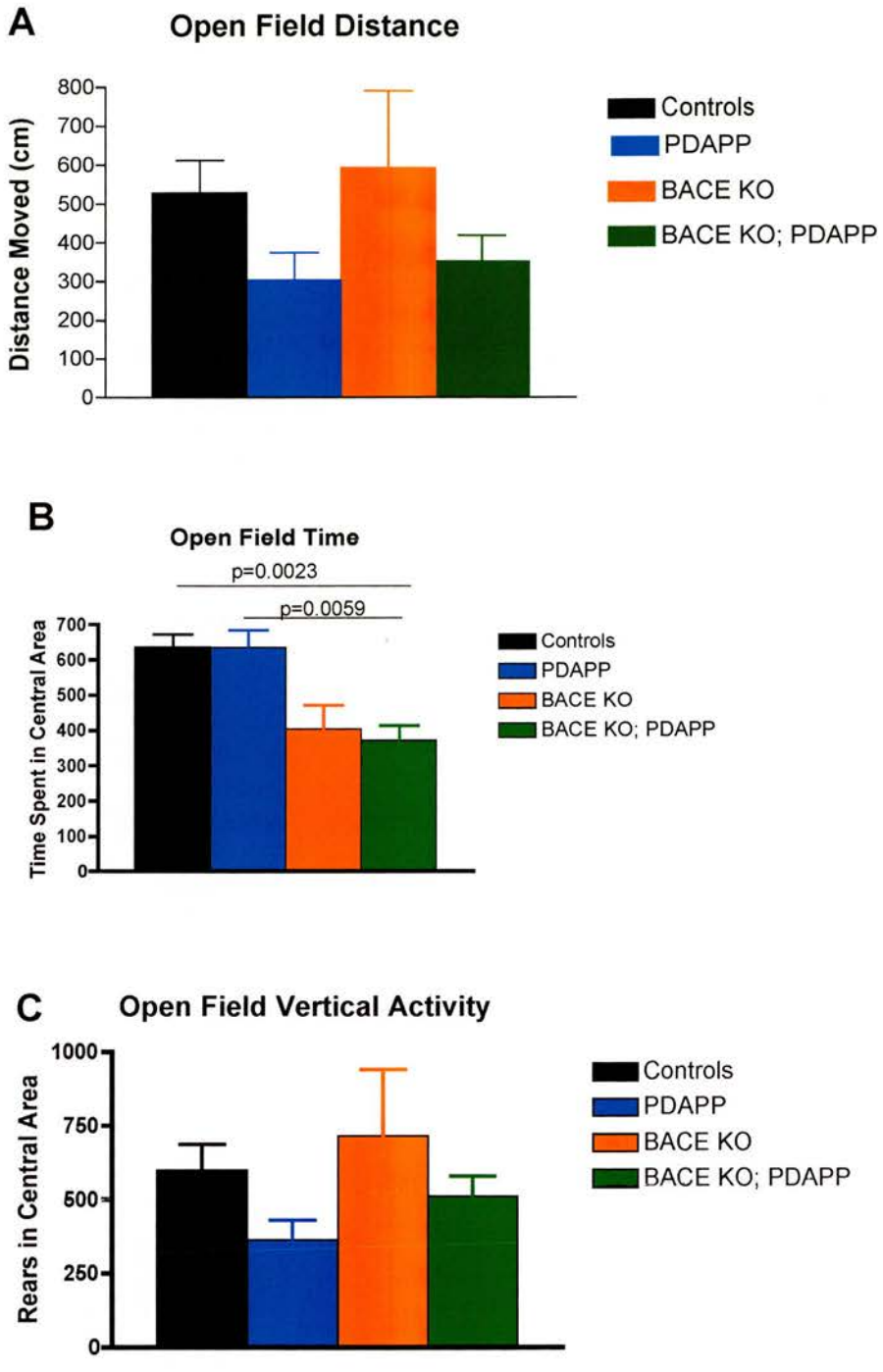


Figure 5.2.3 Open field activity in Study 011A mice. Open field exploration in rodents is associated with anxiety status, as anxious animals avoid open central areas in novel environments, and bold animals explore all areas to a high degree. A: Study 011A mice do no differ in the distances traveled in open fields. B: BACE KO; PDAPP mice spent less time than Control or PDAPP animals in the open field region of the arena, suggesting an anxiety phenotype. C: There was no difference in open field vertical rearing activity in Study 011A mice.

Factor	N	Crossings		Rears		Stereotypy	
		Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	18	177.72	106.68	9.28	11.49	3.17	2.81
Black	4	121.75	27.79	3	2.94	2.25	2.06
Albino	23	193.74	97.37	7.3	7.51	4	4.26
Gender							
Female	22	197.77	111.5	9.73	11.48	3.64	4.26
Male	23	164.83	82.32	5.78	5.66	3.39	2.86
Genotype							
BACE KO; PDAPP	12	175.25	72.93	11.25	12.22	3.75	3.77
BACE KO	3	265	160.69	20.33	11.93	6.67	8.96
PDAPP	15	157.87	109.93	4.8	5.48	2.4	1.96
Control	15	191.73	89.32	5.27	5.71	3.8	3.17
All	45	180.93	97.97	7.71	9.11	3.51	3.57

Source Factor(s)		F	DF	p-value
Crossings	Gender	0.6	1	0.44
	Genotype	0.5	2	0.61
Rears	Gender	0.29	1	0.59
	Genotype	2.27	2	0.12
Stereotypy	Gender	0.13	1	0.72
	Genotype	1.03	2	0.37

	P-Values		
Comparison	Crossings	Rears	Stereotypy
BACE KO; PDAPP VS PDAPP	0.72	0.05	0.24
BACE KO; PDAPP VS CONTROL	0.58	0.079	0.99
PDAPP VS CONTROL	0.33	0.88	0.21

Table 5.2.2a-c Descriptive and One-Way ANOVA statistics for crossings, rears and stereotypic activity monitoring measures in Study 011A mice by gender and genotype.

Factor	N	OF Distance (cm)		OF Time (s)		OF Rears	
		Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	18	407.89	302.42	536.57	210.56	2631.67	1358.43
Black	4	291.95	91.62	647.4	150.96	2460.75	405.25
Albino	23	432.88	314.96	560.28	179.61	2914.43	1009.8
Gender							
Female	22	430.89	338.91	520.07	173.28	2684.59	1084.57
Male	23	390.72	252.25	595.33	199.77	2834.09	1178.33
Genotype							
BACE KO; PDAPP	12	390.03	246.95	409.13	128.61	2334.58	878.74
BACE KO	3	429.57	386.23	448.8	126.39	2776	1213.97
PDAPP	15	320.53	323.6	605.87	201.4	2753	1277.99
Control	15	512.61	281.7	652.69	151.15	3107.13	1108.8
All	45	410.36	295.04	558.54	189.03	2761	1123.1

Source Factor(s)		F	DF	p-value
OF Distance	Gender	0.25	1	0.62
	Genotype	1.69	2	0.2
OF Time	Gender	0.36	1	0.55
	Genotype	7.16	2	0.0023
OF Rears	Gender	0.05	1	0.83
	Genotype	1.47	2	0.24

	P-Values		
Comparison	OF Distance	OF Time	OF Rears
BACE KO; PDAPP VS PDAPP	0.6	0.0059	0.37
BACE KO; PDAPP VS CONTROL	0.26	0.0008	0.094
PDAPP VS CONTROL	0.079	0.45	0.4

Table 5.2.3a-c Descriptive and One-Way ANOVA statistics for crossings, rears and stereotypic activity monitoring measures in Study 011A mice by gender and genotype.

5.3 Rotorod motor coordination

In addition to assessing the homozygous BACE x PDAPP mice for spontaneous locomotor activity, it was also necessary to examine motor coordination in an involuntary paradigm. Motoric coordination in rodents is typically tested using a rotorod apparatus. While the details of the apparatus may vary, the basic rotorod involves placing a mouse on a circular rod that is driven by a motor, which can turn at a constant or accelerating rate of speed. Time to fall is the primary measure for motor coordination on the rotorod, and increases in these fall latencies (indicative of procedural motor learning) are expected over a series of trials. In this study mice were tested for 4 trials in a static rotorod paradigm (10 rpm constant speed) and 7 trials on the following day in an accelerating rotorod paradigm (0-40 rpm) as described in section 2.2.3 of this dissertation.

Testing for motor coordination with the rotorod revealed significant motor phenotypes in mice carrying the PDAPP transgene as well as BACE KO mice (Figure 5.3.1a-b). The overall ANOVA analysis showed a highly significant difference between groups ($F=3.39$, df 3/86, $p=0.0097$). These results show that Control mice in constant velocity paradigms were able to stay on the rotorod for 2-4s, while PDAPP mice had superior motor performance compared to other genotypes, with fall latencies of 3-4s. In contrast animals with BACE gene deletions had constant rotorod falling latencies that were between 1.5-2.5s with little overall improvement over a series of trials.

In the accelerating rotorod paradigm, mice experience a more challenging task that requires more attention to perform in each trial and typically rodents respond with greater falling latencies. In Study 011A, the accelerating rotorod profile of Controls and PDAPP mice showed the typical pattern of motor improvement over the 7 trials of testing. The performance of PDAPP and Control mice was indistinguishable, improving in fall latencies over 7 trials from 10-23s. As there is an element of procedural motor learning in rotorod testing, it appears that PDAPP mice were not deficient in this kind of learning, just as they were similarly unimpaired in cued

navigation of the water maze over 12 trials. However there was no such overall improvement in the performances of either BACE KO or BACE KO; PDAPP mice, which averaged between 9-13s in 7 trials at 0-40 rpm (genotype: $F=11.7$, df 14/308, $p<0.0001$; Figure 5.3.1b).

The presence of the PDAPP transgene did not appear to ameliorate the poor motor coordination phenotype conferred by the deletion of the BACE gene, suggesting this may be a dominant functional phenotype of BACE KO animals. BACE KO animals were unimpaired in grip strengths and measures of visually cued swimming ability in the water maze, but the rotorod task is more motorically complex than simple grip tests and requires a different type of motivation to perform than the water maze (aversion to shock / falling versus aversion to swimming). The poor motor coordination phenotype of BACE KO mice may thus be based not just in neuromuscular impairment, but perhaps also altered motivational states. These speculations have some empirical basis, as BACE has been implicated in the maintenance of muscular fibres at the motor endplate in human, and BACE KO mice have also been found to have alterations in the dopaminergic and serotonergic neurochemistry (Vattemi et al., 2001; Harrison et al., 2003; Vattemi et al., 2003).

In addition, gender was a highly significant factor in performance on the constant speed rotorod (Figure 5.3.2a, Table 5.3c). The impact of this finding on the genotype-based analysis is unclear, but it may be based in excess falling fear in female mice ($F=14.1$, df 1/86 $p=0.0002$). This gender-based difference did not extend to performances on the accelerating rotorod, while the genotypic impact on rotorod performance did -- implying that genotype is more broadly important in determining rotorod motor coordination in Study 011A mice (Figure 5.3.2b).

Factor	N	Trials							
		1		2		3		4	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	18	2.6	2.96	3.27	5.54	3.17	3.25	3.43	3.52
Black	4	3.88	3.29	8.44	6.95	4.24	2.41	2.43	1.5
Albino	23	1.62	1.59	3.06	3.2	2.26	2.65	2.54	2.33
Gender									
Female	22	1.56	1.46	2.09	1.32	1.74	1.35	2.4	1.98
Male	23	2.84	2.98	5.09	6.24	3.81	3.58	3.34	3.38
Genotype									
BACE KO; PDAPP	12	1.19	0.64	1.84	0.74	1.42	0.43	2.88	2.42
BACE KO	3	1.17	1.57	1.45	0.29	1.22	0.31	1.49	0.73
PDAPP	15	3.22	3.19	5.2	6.64	4.11	3.56	3.75	4.04
Control	15	2.23	2.33	3.91	4.46	2.9	3.08	2.29	1.4
All	45	2.21	2.43	3.62	4.75	2.8	2.9	2.86	2.76

Source Factor(s)	F	DF	p-value
Gender	14.1	1	0.0002
Genotype	3.39	3	0.0097

Comparison	P-Values
BACE KO; PDAPP VS PDAPP	0.034
BACE KO; PDAPP VS CONTROL	0.1
PDAPP VS CONTROL	0.16
Male VS Female	0.011

Tables 5.3a-c Descriptive and One-Way ANOVA statistics for performance on the constant speed rotorod in Study 011A mice by gender and genotype.

Factor	N	Trials													
		1		2		3		4		5		6		7	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	18	9.19	5.98	13.52	9.59	16.76	9.83	20	10.1	18.37	11.16	17.31	9.43	2.03	11.72
Black	4	16.76	1.96	9.67	3.68	12.94	7.43	18.39	5.76	17.02	6.8	19.38	7.75	19.15	1.73
Albino	23	10.89	5.86	14.06	8.48	14.21	11.3	16.01	10.7	14.48	8.94	17.05	11.4	16.06	12.63
Gender															
Female	22	10.3	6.42	11.27	6.65	13.63	11.1	16.65	10.6	13.44	8.52	15.53	9.55	16.75	10.51
Male	23	11.13	5.61	15.55	9.81	16.54	9.57	18.93	9.74	18.97	10.23	19.1	10.68	19.25	12.88
Genotype															
BACE KO; PDAPP	12	9.3	5.92	10.51	5.2	12.83	11.7	14.7	8.01	10.14	4.15	13.45	7.21	12.3	8.16
BACE KO	3	8.84	1.68	9.29	5.36	7.99	2.85	9.14	3.43	12.79	7.93	8.87	4.54	8.6	4.97
PDAPP	15	13	7.51	18.1	10.7	20.37	9.81	20.69	10.8	20.4	11.97	21.36	11.93	22.71	13.93
Control	15	10.27	4.79	12	7.53	13.12	9.07	19.16	10.9	17.72	8.76	18.18	9.69	19.8	10.37
All	45	10.72	5.97	13.45	8.6	15.12	10.4	17.81	10.1	16.26	9.74	17.36	10.19	18.03	11.72

Source Factor(s)	F	DF	p-value
Gender	1.3	7	0.29
Genotype	11.7	14	P<0.0001

Comparison	P-Values
BACE KO; PDAPP VS PDAPP	0.032
BACE KO; PDAPP VS CONTROL	0.17
PDAPP VS CONTROL	0.061

Tables 5.3d-f Descriptive and MANOVA statistics for performance on the accelerating rotorod in Study 011A mice by gender and genotype.

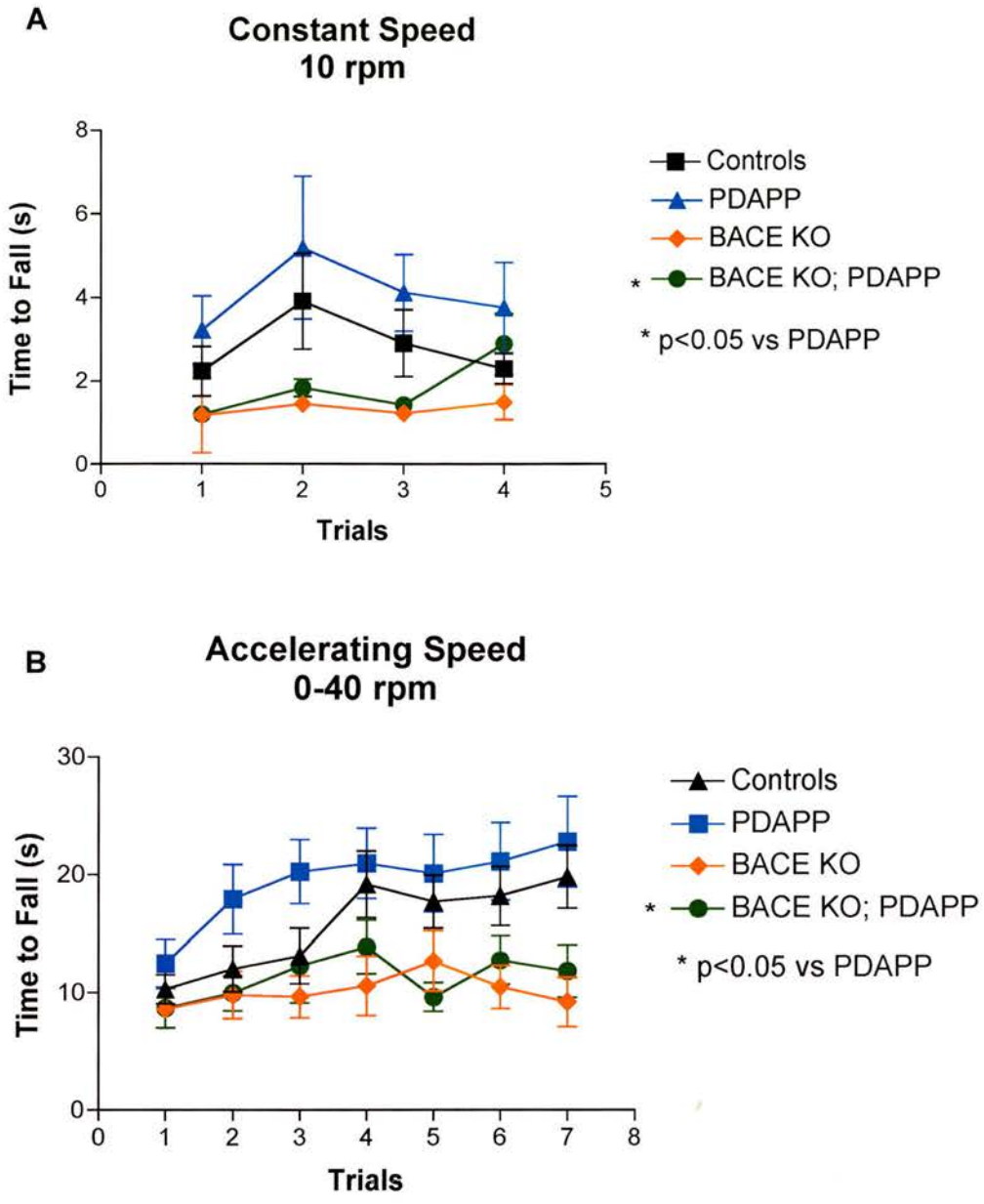


Figure 5.3.1 Motoric coordination on the rotorod in Study 011A mice by genotype. A: When placed on a rod turning at a constant slow speed, BACE KO; PDAPP mice fall significantly faster than PDAPP mice on all trials, and do not improve in their falling latencies. B: BACE KO; PDAPP mice fall faster from an accelerating rotorod than PDAPP mice. These mice are able to improve on their rotorod performance over trials, suggesting a BACE KO-specific motor coordination impairment.

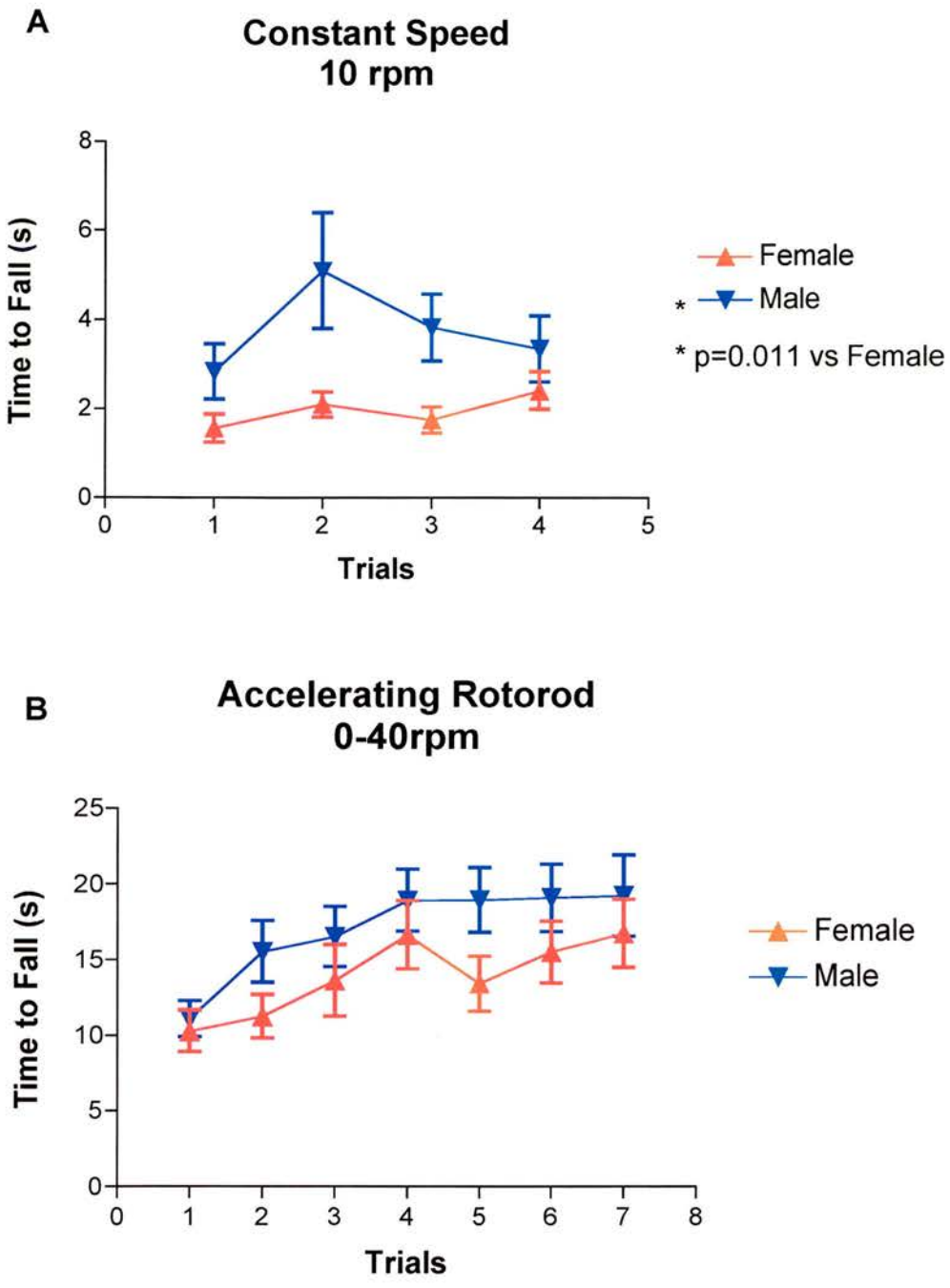


Figure 5.3.1 Motoric coordination on the rotorod in Study 011A mice by gender. A: When placed on a rod turning at a constant slow speed, female Study 011A mice fall significantly sooner than male mice on most trials, with little improvement in their falling latencies. B: In an accelerating rotorod paradigm male and female Study 011A mice have equivalent performances over 7 trials, both improving over time.

Seizure Phenotypes

5.4 PTZ-Induced seizures

Animals with homozygous BACE gene deletions in Study 001 were observed to have severe spontaneous seizures, implicating BACE or one or more of its substrates as a necessary regulator of neuronal activity, which can give rise to epileptiform activity when unchecked. Given the random nature of observations of spontaneous seizures it was decided to examine the deliberate induction of seizures, in a controlled experimental setting. To accomplish this, the animals were administered the agent PTZ, commonly used to induce seizure activity in mice, as described in section 2.3 of this dissertation (no spontaneous seizure activity was observed prior to seizure induction). Indeed, many anti-seizure medications are pre-clinically tested for efficacy in preventing or reducing PTZ-induced seizures in rodents (Zhang et al., 1989; Ferraro et al., 1999).

Conceptually, it is possible to determine the prior seizure activity levels of homozygous BACE KO x PDAPP mice given PTZ as the mice will have divergent levels of resistance to seizure with different doses of intraperitoneal PTZ. For example, mice that have experienced spontaneous tonic seizures throughout their lives generally display significantly shorter latencies to severe seizures and even death compared to seizure-naïve animals upon treatment of 60mg/kg of PTZ. Conversely and counter intuitively, animals with a history of severe seizures will be resistant to developing signs of mild clonic seizure, while seizure-inexperienced animals will have shorter latencies to these kinds of seizures. In addition, seizure scores (based on a formula that weights seizure type latency depending on seizure severity) can also be used to determine whether the overall seizure profile of BACE KO x PDAPP mice diverges with genotype.

Seizure severity order: Partial Clonic (PC) < General Clonic (GC) < Tonic-Clonic (TC)

$$\text{Seizure score} = 0.2/(\text{onset PC}) + 0.3/(\text{onset GC}) + 0.5/(\text{onset TC})$$

Unfortunately, only two BACE KO animals survived to this point and ANOVA analyses require more than 2 values per group, so these animals were not included in

any PTZ-induction analysis. Testing of Study 011A animals in the PTZ-seizure induction paradigm overall revealed that the presence of the PDAPP transgene and complete deletion of the BACE gene predisposes such mice to earlier and more severe seizure activity (Table 5.4; Figures 5.4.1, 5.4.2). The first seizures seen after PTZ treatment are of the partial clonic (PC) type, and onset to first sign of this mildest form of seizure is generally faster in seizure-naïve animals. In keeping with this idea, Control animals had partial clonic seizure onset at about 50s after PTZ induction, while all other PDAPP and BACE KO mice had partial clonic seizures at 70-80s (Figure 5.4.1a). Subsequently, Control mouse composite seizure scores were largely based on the rapid onset of PC convulsions, as opposed to more severe types of seizures (Figure 5.4.1d, 5.4.2a). PDAPP mice had significantly smaller PC seizure scores (indicative of longer latencies to mild clonic seizures) than Controls (Figure 5.4.2a, $F=3.92$, df 2/44, $p=0.028$). Similarly, the BACE KO genotype mice also had a tendency towards having smaller PC scores, implying that these mice have experienced seizures at some point in their lives (Figure 5.4.1a, 5.4.2a,c).

To obtain a high rate of animals with severe tonic-clonic seizures, a dose of PTZ (60mg/kg) must be given, resulting in many animal deaths (see section 2.3). In Study 011A there was 40% lethality in Control mice and 70% in all other genotypes (Figure 5.4.1b, Table 5.4.1d). Examination of the mice that died showed that compared to Controls, BACE KO; PDAPP mice tended to have average times to death that were significantly shorter, by about half at 12 min compared to 22 min. (Figure 5.4.1c). Death latencies also tended to be shorter for PDAPP and BACE KO mice, which died on average about 15 min after PTZ administration.

Composite seizure scores did not differ between any statistically analyzable genotypic groups in Study 011A mice had as all animals had average scores between 0.38-0.45, but the breakdown of these composite scores into their PC, general clonus (GC) and severe tonic-clonic seizures (TC) components were more informative (Composite Seizure Scores $F<1$; Figure 5.4.1d, Table 5.4.1b). As mentioned above, Control animals were not resistant to the development of PC convulsions and had high PC scores of about 0.3, while animals carrying the

PDAPP transgene and/or homozygous BACE gene deletions were slower to kindle these mild PC seizures and had scores ranging from 0.18-0.2 (Figure 5.4.2a). There were no genotypic differences between groups in the onset of seizures (PC Scores), BACE KO; PDAPP mice had higher GC scores than PDAPP mice, implying a divergence in their resistance to developing more severe types of convulsions (p=0.034, Figure 5.4.2b, Table 5.4.2a-c).

However, with respect to the most severe kind of seizure activity, BACE KO; PDAPP mice have significantly higher tonic seizure scores of about 0.25 compared to either Controls or even PDAPP mice which also have a tendency towards higher tonic scores than Controls mice at scores of 0.10 and 0.15 respectively (F=5.25, df 3/44, p=0.048; Figure 5.4.2c). This last finding suggests that combining the BACE gene deletion with the PDAPP transgene in mice had an additive effect on the PDAPP propensity to severe seizure activity. Taken together this data implicates some entity within the APP metabolic processing pathway as having an important role in the fundamental regulation of neuronal activity, whether in development or in the adult maintenance of brain function.

	Seizure Latency (min)			Death Latency			Seizure Score		
Factor	**N**	Mean	Std	**N**	Mean	Std	**N**	Mean	Std
Color									
Agouti	18	1.54	2.14	11	6.48	5.94	18	0.43	0.22
Black	4	1.28	0.52	2	18.18	12.55	4	0.35	0.03
Albino	23	1.11	0.67	14	7.26	7.29	23	0.45	0.2
Gender									
Female	22	1.23	0.68	13	8.76	7.48	22	0.4	0.21
Male	23	1.36	1.92	14	6.81	7.56	23	0.46	0.2
Genotype									
BACE KO; PDAPP	12	1.93	2.66	8	3.68	3.25	12	0.48	0.29
BACE KO	3	1.15	0.24	2	7.3	0.85	3	0.33	0.04
PDAPP	15	1.28	0.43	10	7.26	7.44	15	0.39	0.15
Control	15	0.83	0.38	7	13.24	9.32	15	0.45	0.19
All	45	1.3	1.44	27	7.75	7.44	45	0.43	0.2

	Source Factor(s)	F	DF	p-value
Seizure Latency	Gender	0.3	1	0.58
	Genotype	2.02	2	0.15
Death Latency	Gender	N/A	1	0.71
	Genotype	N/A	2	0.19
Seizure Score	Gender	1.06	1	0.31
	Genotype	0.83	2	0.45

	P-Values	
Comparison	**Seizure Onset**	**Seizure Score**
BACE KO; PDAPP VS PDAPP	0.23	0.21
BACE KO; PDAPP VS CONTROL	0.05	0.55
PDAPP VS CONTROL	0.4	0.48

Table 5.4.1a-c Descriptive and One–Way ANOVA statistics for seizure responses and lethality in Study 011A mice by gender and genotype.

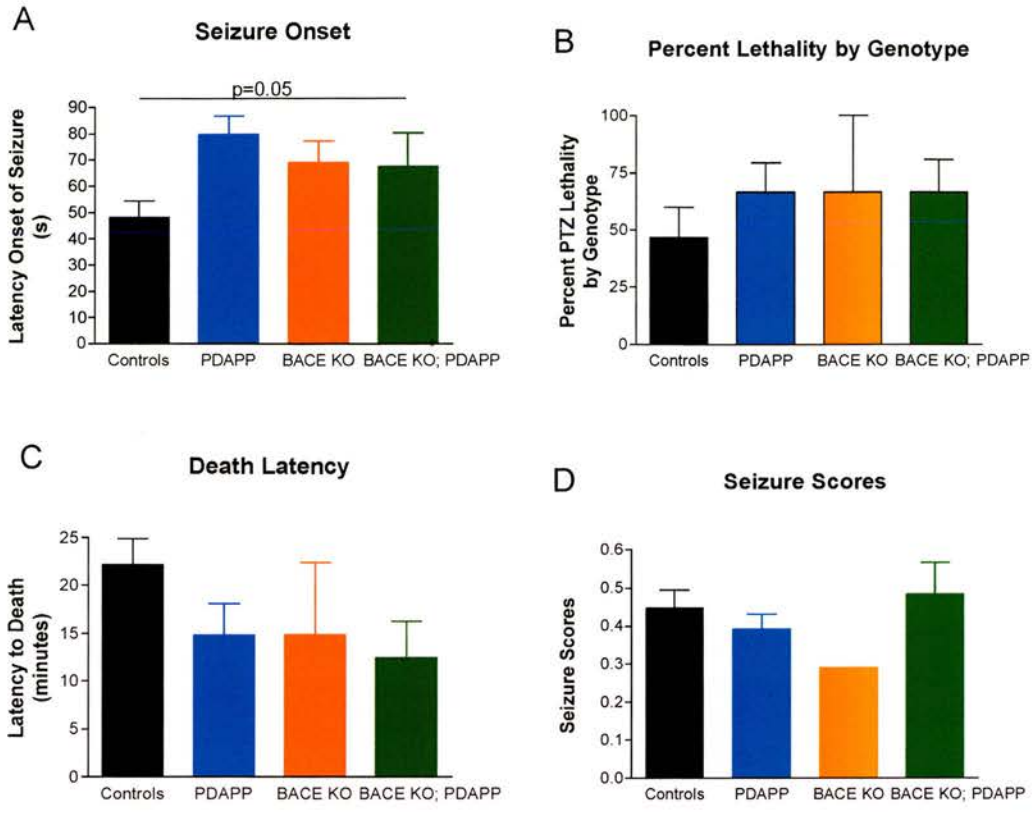


Figure 5.4.1 PTZ-induced seizure activity in Study 011A mice. Mice were given 60 mg/kg of a seizure-inducing agent to kindle mild to severe seizures. A: Onset to initial seizure observation following treatment was significantly shorter in BACE KO; PDAPP mice compared to Control animals. There was a trend towards animals with PDAPP transgenes or BACE gene deletion having later mild seizure onset, suggesting tolerance to mild seizures. B: More PDAPP, BACE KO and BACE KO; PDAPP mice (~70%) die in response to PTZ treatment than Control mice (`40%). C: PDAPP, BACE KO, and BACE KO; PDAPP mice have a tendency to die sooner than Control mice following seizure induction, although this is not a significant effect. D: Composite seizure scores consisting of mild (partial clonic), moderate (general clonic) and severe (tonic) seizures are equivocal between Study 011A mouse genotypes.

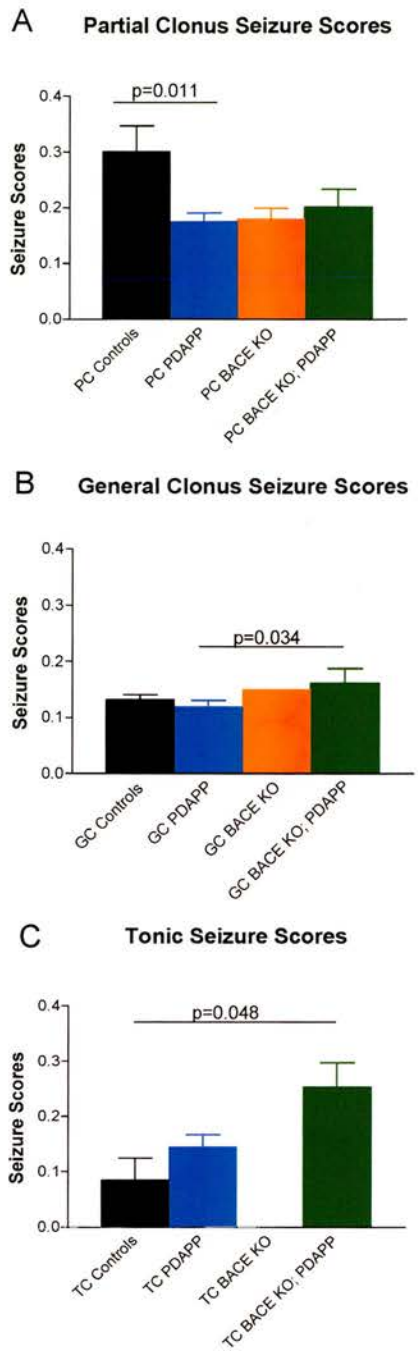


Figure 5.4.2 PTZ-induced component seizure scores in Study 011A mice. While composite seizure scores based on latency between Study 011A mice was similar, contributions from each type of seizure was also analysed. A: Control mice develop mild seizures faster than PDAPP mice, with a trend towards the same compared to BACE KO and BACE KO; PDAPP mice. B: BACE KO; PDAPP mice develop general clonic convulsions more rapidly than PDAPP animals. C: BACE KO; PDAPP mice have a faster onset to severe seizure activity than Control mice, suggesting lesser resistance to kindling major seizures.

Genotype	Female		Male		All	
	Number of Mice	n (%) that died	Number of Mice	n (%) that died	Number of Mice	n (%) that died
BACE KO; PDAPP	7	5 (71.4)	5	3 (60.0)	12	8 (66.7)
PDAPP	6	3 (50.0)	9	7 (77.8)	15	10 (66.7)
Control	6	3 (50.0)	9	4 (44.4)	15	7 (46.7)
All	19	11 (57.9)	23	14 (60.9)	42	25 (59.5)

	Source Factor(s)	P-Values
Lethality	Gender	1
	Genotype	0.57

Tables 5.4.1d Descriptive and Fisher's Chi-squared test statistics for seizure component scores in Study 011A mice by gender and genotype.

		Partial Clonus Score			General Clonus Score			Tonic-Clonic Score	
Factor	N	Mean	Std	N	Mean	Std	N	Mean	Std
Color									
Agouti	18	0.21	0.12	15	0.15	0.05	10	0.16	0.11
Black	4	0.18	0.07	4	0.11	0.01	2	0.13	0.05
Albino	23	0.24	0.15	22	0.14	0.06	10	0.18	0.12
Gender									
Female	22	0.21	0.13	20	0.13	0.06	9	0.16	0.12
Male	23	0.23	0.14	21	0.14	0.05	13	0.18	0.1
Genotype									
BACE KO; PDAPP	12	0.2	0.11	10	0.16	0.08	7	0.25	0.12
BACE KO	3	0.18	0.04	3	0.15	0	0	NA	NA
PDAPP	15	0.17	0.06	14	0.12	0.04	11	0.14	0.07
Control	15	0.3	0.18	14	0.13	0.03	4	0.09	0.08
All	45	0.22	0.13	41	0.14	0.05	22	0.17	0.11

	Source Factor(s)	F	DF	p-value
PC Score	Gender	0.07	1	0.8
	Genotype	3.92	2	0.028
GC Score	Gender	1.73	1	0.2
	Genotype	2.53	2	0.094
TC Score	Gender	0.23	1	0.63
	Genotype	5.25	2	0.015

	P-Values		
Comparison	PC Score	GC Score	TC Score
BACE KO; PDAPP VS PDAPP	0.57	0.034	0.065
ACE KO; PDAPP VS CONTROL	0.06	0.1	0.048
PDAPP VS CONTROL	0.011	0.54	0.19

Tables 5.4.2a-c Descriptive and One–Way ANOVA statistics for seizure component scores in Study 011A mice by gender and genotype.

Calbindin and Amyloid Histology

5.5 Calbindin histology in the hippocampal outer molecular layer

Previous reports regarding a positive correlation between spatial memory performance and hippocampal Calbindin (CB) levels by Palop et al. (2003) motivated a similar attempt to reproduce these findings in the BACE KO x PDAPP mice. In addition, increases in CB have been found to be associated with seizure-induced neurogenesis and resistance to post-seizure neuronal toxicity (Yang et al., 1997; Gary et al., 2000; Lee et al., 2002; Jiang et al., 2003). In 18mo Study 001 BACE KO mice had higher levels of hippocampal CB intensity than PDAPP mice as described in section 3.6. These differences in Study 001 were based on an analysis of mouse brains that may have had spontaneous seizure activity, and it was of interest to determine the genotypic changes in CB in response to chemically induced seizures with PTZ treatments.

Using a monoclonal antibody to CB, hippocampal tissues from PTZ-treated Study 011A mice were analysed for CB intensity levels in the hippocampal outer molecular layer. This quantitation of CB intensity revealed no significant differences between any genotypes ($F<1$, Figure 5.5a, Tables 5.5a-c). It is possible that all that the indistinguishable results are based in unequal levels of CB intensities prior to PTZ administration that differentially increased to a post-seizure equivocal level. Previous results from the 18mo Study 001 animals (Figure 3.6.1c) argue against this explanation, as do the reports from other authors claiming that seizure activity alters CB levels in the brain. One perhaps salient feature is that each genotype group had a specific profile of seizure activity, and also time to death. Given these facts, each animal would have upregulated CB levels dependent on the amount of time to death and tissue processing. Thus another explanation for the lack of distinction between CB levels between Study 011A genotypes is that by-genotype changes simply exist as a function of time since seizure induction, and PTZ treated animals reach equivalent levels of CB over different timescales.

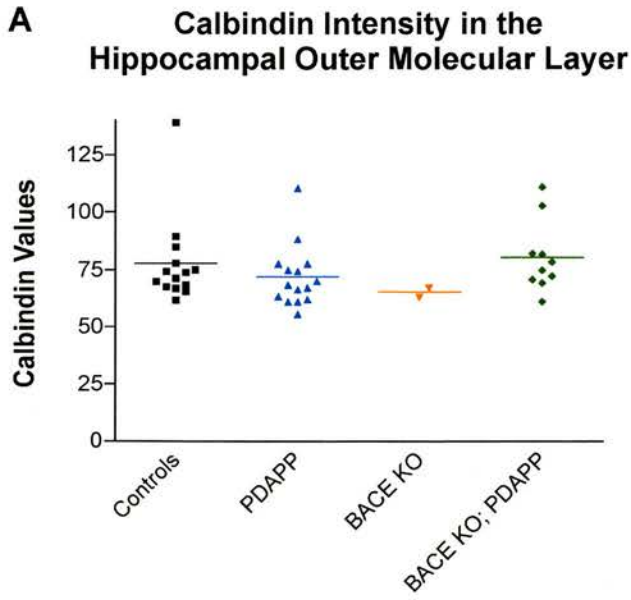
Factor	N	Mean	STD
Color			
Agouti	16	74.96	20.89
Black	4	74.77	11.88
Albino	21	75.97	12.78
Gender			
Female	19	74.22	16.21
Male	22	76.53	16.13
Genotype			
BACE KO; PDAPP	10	80.24	15.42
BACE KO	2	65.11	2.86
PDAPP	15	71.77	13.61
Control	14	77.48	19.22
All	41	75.46	16.01

Source Factor(s)	F statistic	DF	p-value
Gender	0.15	1	0.7
Genotype	0.93	2	0.4

Comparison	P-Values
BACE KO; PDAPP VS PDAPP	0.2
ACE KO; PDAPP VS CONTROL	0.67
PDAPP VS CONTROL	0.37

Tables 5.5a-c Descriptive and One–Way ANOVA statistics for Calbindin immunoreactivity in Study 011A mice by gender and genotype.

APP antibody staining of Study 011A animals to confirm their hAPP genotypes revealed one mistyped animal, which was removed from analysis (described as a Control animal by the vendor, the animal in fact did express hAPP). All other mice were of expected genotypes (images not shown).



B Calbindin Images Hippocampal Outer Molecular Layer

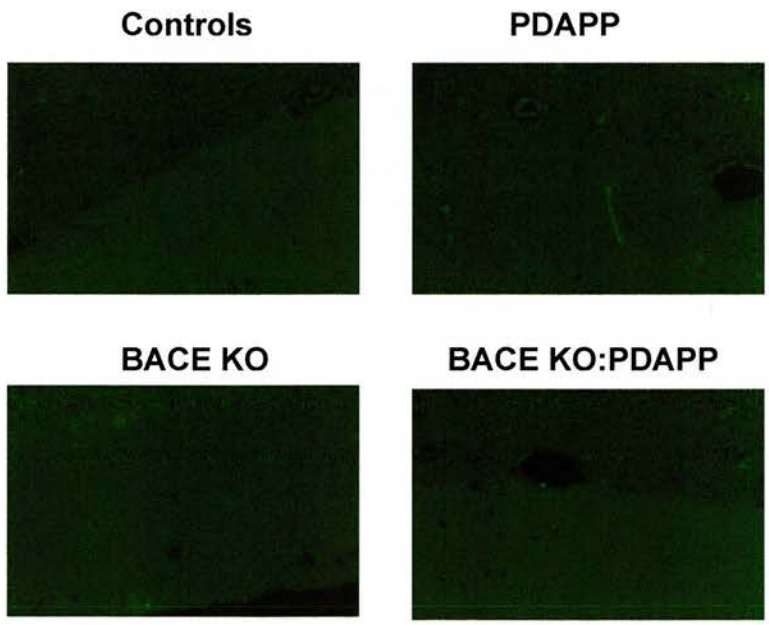


Figure 5.5 Calbindin (CB) immunoreactivity and images in the hippocampal outer molecular layer of Study 011A mice. A: Aged Study 011A mice had equivocal levels of CB following acute and largely lethal treatment with PTZ. B: Images of the CB immunoreactivity by genotype are shown, with sections close to the genotype group average Cb intensity value.

5.6 Correlation analyses of sensorimotor, seizure, and histological data

Correlation analysis cell key

R-Values Colorimetrics P-Values Colorimetrics Self-Correlation

Column Abbreviations

PC Lat =Latency to Partial Clonus
GC Lat= Latency to General Clonus
TC Lat = Latency to Tonic Seizure
Score = Composite seizure score
DeathT = Latency to death
PCscore= Partial clonus component of seizure score
GCscore= General clonus component of seizure score
Tcscore = Tonic seizure component of seizure score
CB Int = Calbindin Intensity in the Hippocampal Outer Molecular Layer
GS1 = Grip strength day 1
GS2 = Grip strength day 2
GS3 = Grip strength day 3
2RR1 = Constant speed rotorod trial 1
2RR4 = Constant speed rotorod trial 4
3RR1 = Accelerating speed rotorod trial 1
3RR4 = Accelerating speed rotorod trial 4
3RR7 = Accelerating speed rotorod trial 7
SectX1 = Sector crossings, session 1
Rest1 = Complete rests in motion, session 1
Dist1 = Total distance traveled, session 1
Time1 = Total time spent in motion, session 1
Rear1 = Vertical activity counts, session 1
Stereo1 = Number of stereotypic movements, session 1
Open Dist1 = Total open field distance traveled, session 1
Open Dist2 = Total open field distance traveled, session 2
Open Time1 = Total time spent in motion in open field, session 1
Open Time2 = Total time spent in motion in open field, session 2
Open Vert1 = Vertical activity counts, session 1
Open Vert2 = Vertical activity counts, session 2

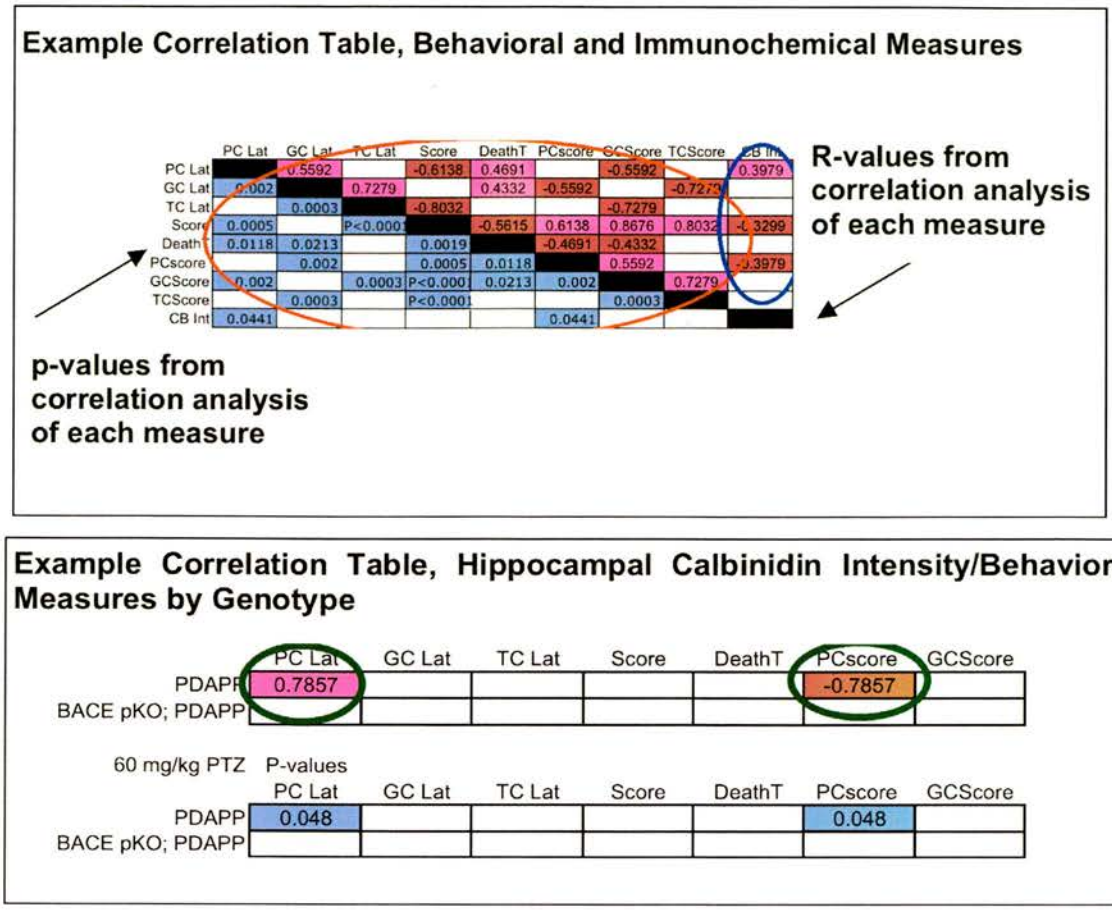


Figure 5.6.1a-b Example correlation tables. A: Correlation table of relationships between various behavioral measures, with R-values presented in upper diagonal section and p-values presented in lower diagonal section. Corresponding p-values and R-values are found in the same coordinate distance from the black diagonals separating the two types of values, with R-values at x_r,y_r coordinates, and p-values at y_p,x_p coordinates where $x_r=y_p$ and $y_r=x_p$ Values contained within red circle indicate significant intrameasure correlations, e.g. clonic to tonic seizure latency. Values contained within blue circles indicate significant intermeasure correlations, e.g. clonic seizure latency to Calbindin intensity. B: Correlation table of various behavioral measures and Calbindin intensity separated by genotype. R-value tables are above while P-values are below. Values in green circles indicate significant behavioral/Calbindin correlations.

Functional analysis of diverse behavioral and histological measures allows for the discovery of mathematical relationships that may be based in similar neuronal activational processes. Every behavioral response is inherently based in some complex network of neuroanatomical connections and correlational analyses makes

it possible to make predictions regarding functional relatedness of various inter-methodology measures.

In conducting Spearman's correlation analyses of Study 011A animals' behavioral and histological measures, several statistically meaningful relationships were discovered between measures that formed two broad patterns of correlations. The first pattern of correlation featured measurements within groups were on average interchangeable in their prediction of function within the larger feature group, forming a group of reliable and consistent correlates (seizure activity, motor coordination, etc.). These intratask correlates are exemplified by grip strength data, as performance on day 1 was predictive of performance on day 2. The second pattern of correlation centered on numerical relationships from intermeasure datasets that were on the surface unrelated, like anxiety and seizure propensity. These novel findings also include some measures that were predictive of PTZ-induced seizure activity, and suggest that there is indeed some underlying functional connection based in anatomy between these phenotypes (Table 5.6). For the overall analysis presented in Table 5.6 N=45. For the CB/sensorimotor phenotype analysis presented in Table 5.7, N=41, as the BACE KO group had too few values to conduct the analysis and this group was excluded from the correlation (Controls N=15, PDAPP N=14, BACE KO; PDAPP N=12).

Intrameasure relationships

There were five main sets of intrameasure relationships seen in Table 5.6:

- General Clonic (GC)/Tonic Seizures)TC)
- Grip Strengths
- Constant and Accelerating Rotorod
- Horizontal locomotor activity (including open field measures)
- Vertical/Stereotypic locomotor activity (including open field measures)

Table 5.6 Correlation of pharmacologic, behavioral and histological measures, with resulting R- and p-values of Study 011A mice. Table is located in pocket at back cover of document.

This table of correlation values underlines several intrameasure and intermeasure relationships.

Intrameasure values are highly correlated within each set, as performance on one measure is likely to be functionally related to performance on a similar measure. These intrameasure correlations are circled in red, and are closest to the table diagonal separating R- and p-values. Thus many measures within PTZ-seizure induction, grip strength, rotorod, general and open field activity monitoring have high degrees of correlations within each set.

Intermeasure relationships occur between different task measures and are circled in blue, typically distant from the R-/p-value diagonal. Correlations between these metrics suggest that although the tasks vary methodologically, the underlying functional bases for their performance are similar, and even predictive of one another. In particular, seizure response appears to be correlated to CB intensity, rotorod fall latency, as well as vertical activity or rearing. In addition grip strength is related to the tendency to have fewer repetitive movements, and CB intensity is correlated to spending less time in open field areas.

GC and TC latencies and scores were positively correlated ($R=0.65$, $p=0.0009$) to each other and also in latency to death (latency/scores: $R=0.596$, $p<0.0001$; death latency: $R=0.885$, $p=<0.0001$), but not related to PC (partial clonus) measures. This suggested that the processes underlying onset to PC vs. GC/TC were distinct in Study 011A mice. Grips strength measures from day-to-day were highly related due to their expected stability of values between days ($R>0.70$, $p<0.001$). Rotorod performance in both the constant and accelerating rod paradigms were also strongly correlated between days, such that performance on the static rod appeared to be predictive of performance on the accelerating rod ($R>0.40$, $p<0.008$). Interestingly, the automated locomotor activity monitoring produced two separate groups of correlated measures, the horizontal activity set (which included measures of distance traveled, time spent in movement, sector crossings, and rests; for all horizontal activity measures: $R>0.798$, $p<0.001$) and the vertical/stereotypic measure set ($R=0.329$, $p<0.05$).

By definition, the open field measurements should be highly related to the general activity monitoring measures, as they are simply taken from a spatially constrained subarea of the whole activity monitoring arena. OF dist1 and OF Vert1 and OF Vert2 values were overall correlated with all horizontal activity measures (dist $R>0.82$, $p<0.001$, Vert1/2 $R>0.46$, $p<0.01$). Total rearing activity was also correlated to OF dist1 but inversely related to OF time1 and OF time2, suggesting that mice that had high levels of vertical activity did not spend much time in open areas, but still traversed the central area, at a higher speed (OF dist1 $R>0.32$, $p<0.05$, OF time1/2 $R<-0.46$, $p<0.01$). This finding suggests that there is an anxiety phenotype involved as animals who are averse to spending time an open field would do so by not spending time there and by moving rapidly through the area. Oddly, there was no apparent correlation between overall rearing activity and open field rearing activity, suggesting that the majority of rearing did not occur within the circumscribed open field area.

Intermeasure relationships

Interset correlations were also significant between a number of measures, suggesting similarities in underlying physiological processes. Most notable of these relationships are those that are related to seizure activity, to the point where they can be predictive of PTZ-induced seizure resistance and susceptibility. Resistance to severe seizures, represented by high TC latencies, was positively correlated to better performance on the static and accelerating rotorod ($R>0.33$, $p<0.025$). In contrast, lower TC latencies and thus greater susceptibility to seizures were associated with greater levels of vertical activity ($R=-0.522$, $p<0.025$). Patterns of correlation with the open field behaviors were also interesting, as higher seizure scores were associated with longer times spent as well as more rearings in the center of the arena, suggesting that animals with less anxiety were resistant to seizure ($R>0.32$, $p<0.03$).

Finally, although there were no genotypic differences in CB levels when analysed as a single measure (Section 5.5), there was a significant correlation between CB intensity in the hippocampus and total seizure scores, providing a mathematical linkage between a neuroanatomical measure with a gross set of behavior observations ($R=-0.317$, $p<0.05$; Table 5.7). This finding argues that use of correlational analyses can define functional phenotypes that are difficult to discern by examining any individual. The direction of this relationship was in fact positive, so that increased CB intensity was associated overall higher seizure activity scores. In addition, CB intensity was correlated with lesser time, distance and vertical activity within the open center of the monitoring arena, suggesting a link between the levels of hippocampal Ca^{++} and anxiety ($R<-0.37$, $p<0.025$). These findings support the previous information from Study 001 and Study 006, in which greater CB immunoreactivity was related to poorer spatial memory performance. Thus not only was CB implicated as a measure of spatial memory dysfunction, but it also was linked to reduced neuronal activity regulation with greater propensity to seizure activity and anxiety phenotypes.

Analysis of CB measures by genotype revealed significant correlations with certain open field activity measures (Table 5.7). Controls animals had an inverse correlation between CB levels and OF vertical activity, in that greater CB intensities were seen in mice with lesser open field rearings ($R=-0.582$, $p<0.05$). PDAPP mice also had inverse relationships between CB intensity and OF time and distance ($R<-0.625$, $p<0.025$). These findings argue that CB levels do not correspond to generally normal behavior, as even in non-transgenic mice high levels relate to increased deleterious behaviors like anxiety in open field and increased propensity to seize. CB in Control mice also had a tendency towards correlation with PC Lat/Scores (PC Lat/Scores: $R=|0.508|$, $p=0.064$). If this was a real trend then this strengthens the concept of CB as a marker for neuronal dysfunction, as the Controls mice have no genetic modifications and no underlying neuropathology they represent a more default state of relationships between CB and seizure response.

Table 5.7 Correlation of Calbindin (CB) to all other measures, R- and P-values of Study 011A mice. Table is located in pocket at back cover of document.

Specific assessment of CB correlations to behavioral and pharmacological metrics was done by genotype to discern wider patterns of predictive functional relationships. The only significant correlations with CB were in open field exploration during the second activity monitoring session. High levels of CB in Control mice were related to lesser vertical activity in the open field, while PDAPP mice moved less and spent less time in open field areas. While Control and PDAPP mice typically differ in many other respects, the cross-genotype pattern of open field avoidance in mice with high CB suggests that anxiety behaviors may be influenced by neuronal Ca^{++} homeostasis.

Ch.6 Study 011B: General Behavioral Phenotyping and Response to Seizure Induction of 5mo Hemizygous BACE pKO x PDAPP Mice

Study 011B was designed to examine the resistance to induction of severe seizures in animals with a partial deletion of the BACE gene, and to broadly profile other sensorimotor phenotypes in these mice. In Study 011A PDAPP mice were found to have tendencies for lowered resistance to tonic seizure kindling, supernormal performance in a motor coordination task, and what could be interpreted either as an anxiolytic or incurious phenotype. Other findings from this prior study revealed a deficit in motor coordination in mice with homozygous BACE KO; PDAPP animals. Also in Study 011A, homozygous BACE KO; PDAPP mice had the least resistance to developing tonic seizures, suggesting that the loss of BACE on a PDAPP background amplified the seizure-prone phenotype of PDAPP mice.

The BACE partial knockout (pKO); PDAPP mice in Study 011B were compared directly to littermate PDAPP mice, analysed for body masses, grip strength, spontaneous locomotor activity, motor coordination and response to chemically-induced seizures. One critical objective of Study 011B was to determine if the BACE pKO; PDAPP rescue of spatial memory impairments, relative to the PDAPP mice, described in the water maze experiments of Study 006 would be extended to the amelioration of propensity to seize and other PDAPP-related sensorimotor phenotypes. In addition, younger animals were used in Study 011B (5mo) compared to Study 011A (18mo) in an effort to assess the affect of age on sensorimotor and seizure resistance phenotypes in BACE KO mice (Table 6.0a). Total N for this study was 28 mice.

	Female				Male				ALL			
	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL
Genotype												
BACE pKO; PDAPP	2	1	0	3	2	2	5	9	4	3	5	12
PDAPP	4	4	3	11	5	0	0	5	9	4	3	16
ALL	6	5	3	14	7	2	5	14	13	7	8	28

Table 6.0a Study 011B mice, all aged 5mo.

The overall performance of Study 011B mice is summarized below (Table 6.0b). One-way ANOVA tests were conducted on all measures except for grip strength and

the rotorod, which had serial timepoints and was subject to MANOVA testing, and the Lethality measure which was analysed with Fisher's exact Chi-squared test. Due to the scarcity of animals across all gender and genotype groups, statistical analysis of performance by color was not possible, nor was any analysis of interactions between gender and genotype. However, descriptive statistics are presented for all factors. Although Study 011A mice had equivalent sensorimotor task responses on the majority of responses, it appears that BACE gene ablation impacts resistance to kindling moderate to severe seizures (GC Latency/Score). However, gender was also a significant factor in resistance to developing moderate seizures, with male mice having more susceptibility to seizures.

	P-Values	
Measure	**Gender**	**Genotype**
Masses	0.21	0.028
Grip Strength	0.55	0.37
Crossings	0.15	0.43
Distance	0.25	0.4
Rests	0.73	0.8
Movement Time	0.19	0.4
Vertical Actvity	0.27	0.66
Stereotypy	0.97	0.34
Open Field Distance	0.27	0.43
Open Field Time	0.54	0.3
Open Field Vertical Activity	0.92	0.1
Rotorod Constant Speed	0.24	0.86
Rotorod Accelerating	0.73	0.45
PC Latency	0.072	0.077
GC Latency	0.0032	0.0044
TC Latency	0.67	0.39
PC Score	0.1	0.083
GC Score	0.025	0.01
TC Score	0.15	0.19
Seizure Score	0.018	0.066
Percentage Lethality	1	0.57
Death Latency	0.71	0.19
Calbindin Intensity	0.7	0.4

Table 6.0b Statistical summary of factor significance in Study 011B.

Mass and Muscular Function

6.1 Body mass and grip strength

BACE pKO; PDAPP and PDAPP mice in Study 011B mice were separated by gender and compared for differences in body mass and forelimb grip strength relative to body mass (Figure 6.1). Grip strength was an important measurement in phenotyping the BACE pKO mice, as feeble forelimb grips are associated with previous seizure activity, and observations of spontaneous seizures in Study 001 and 006 mice motivated these seizure induction experiments. Individual mice were assessed for grip strength using a grip strength apparatus with a digital readout for transduced force. Mice were allowed to grasp a foil screen attached to the force transducer and gently pulled laterally until their grip on the foil was broken. The expected normal result would be for an animal to have static grip strength performances over the three-day testing period, with no significant fluctuation in average grip strength.

Grip strengths were presented as ratios of force required to break their grips over individual body mass, measured in 3 successive trials each for 3 days as described in section 2.2.2. Using a unitless grip strength ratio, effects of relative body mass or size differences between animals are minimized, since grip strength is presented as a function of body mass.

Grip strength ratio = (mass displaced by grip on screen) / (body mass of mouse)

Analysis of body mass revealed no statistical differences between genders by genotype as males and females of both genotypes had average body masses of 37-42g and 27-33g respectively, although male mice did weigh more than female mice ($F<1$, Table 6.1a-b). Examination of grip strength ratios between the two genotypes in this study also were equivocal, so that with respect to both body mass and forelimb muscle tone and grip PDAPP and BACE pKO; PDAPP mice are equals, with Male grip strengths ranging from 3.0-4.0, and Female grip strengths ranging from 3.5-4.5 (Gender $F<1$; Genotype $F<1.1$; Table 6.1d-ef). One comment that can

be made is that all of these mice have relatively high grip strengths compared to the expected values for wild-type mice of this age, which typically varies between 2.5-3.5. While there were no littermate non-transgenic control mice to compare to PDAPP and BACE pKO; PDAPP mice in Study 011B, this follows the trend set in Study 011A, in which male mice carrying the PDAPP transgene had greater average grip strengths than male Control mice (Ch. 5, Figure 5.1c). These data would suggest that the PDAPP transgene confers supranormal muscular strength to mouse carriers, which is in apparent contradiction to the hypothesis that animals

Factor	N	Mean (g)	STD
Color			
Agouti	13	36.24	5.91
Black	7	38.23	6.93
Albino	8	38.57	6.91
Gender			
Female	14	31.57	3.55
Male	14	39.64	4.34
Genotype			
BACE pKO; PDAPP	12	38.1	6.56
PDAPP	16	33.73	4.17
ALL	28	35.6	5.66

Source Factor(s)	F	DF	p-value
Gender	9.478	1	P<0.0001
Genotype	0.96	1	0.11

experiencing spontaneous seizures would have weaker grip strengths.

Table 6.1a-b Descriptive and One-Way ANOVA statistics for body masses in Study 011B mice by gender and genotype.

Factor	N	Trials					
		1		2		3	
Color		Mean	Std	Mean	Std	Mean	Std
Agouti	13	3.78	0.69	3.82	0.67	3.78	0.7
Black	7	3.49	0.35	3.23	0.39	3.78	0.65
Albino	8	3.45	0.77	3.65	0.78	4.13	1.84
Gender							
Female	14	3.73	0.74	3.76	0.77	4.18	0.56
Male	14	3.47	0.57	3.5	0.58	3.63	1.58
Genotype							
BACE pKO; PDAPP	12	3.39	0.54	3.64	0.71	3.75	1.74
PDAPP	16	3.76	0.72	3.62	0.68	4.03	0.58
ALL	28	3.6	0.66	3.63	0.68	3.91	1.2

Source Factor(s)	F	DF	p-value
Gender	0.72	3	0.55
Genotype	1.09	3	0.37

Table 6.1c-d Descriptive and One-Way ANOVA statistics for grip strengths in Study 011B mice by gender and genotype.

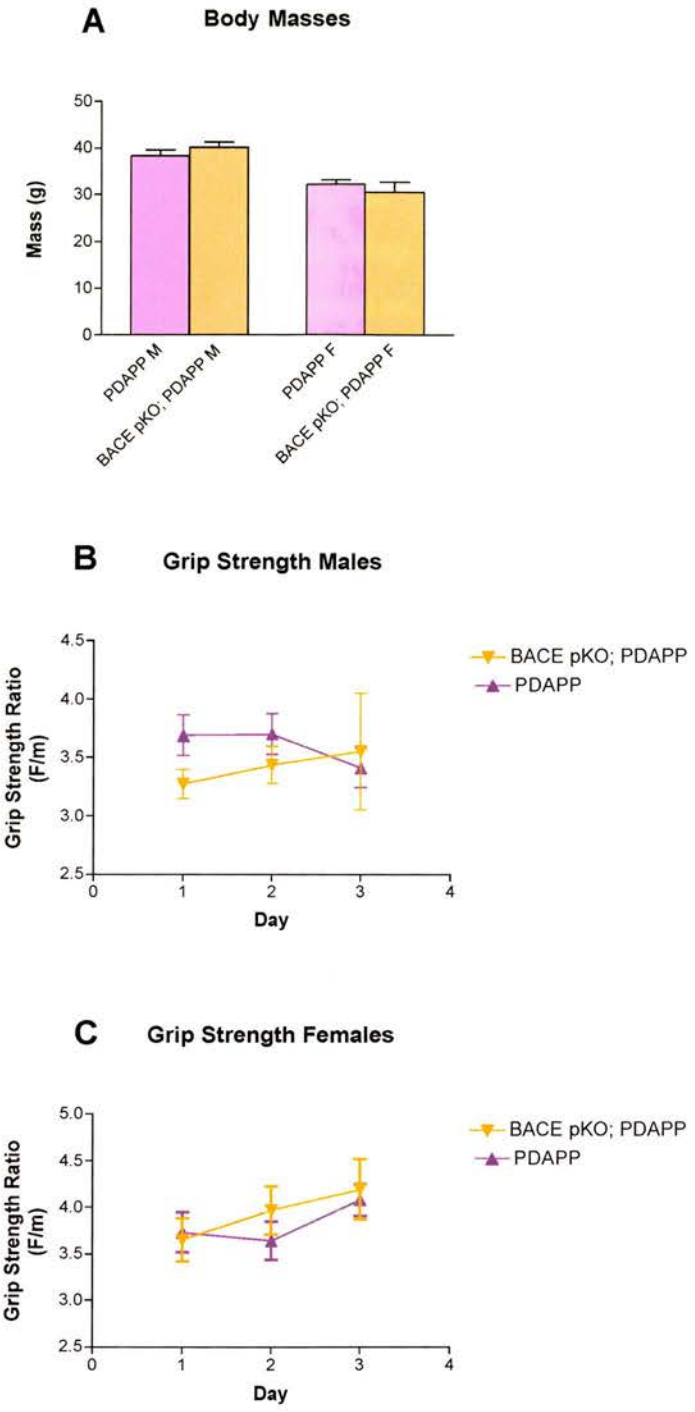


Figure 6.1 Body mass and grip strength of 5mo Study 011B mice by gender and genotype. A: Body weights between male and female mice of BACE pKO; PDAPP and PDAPP genotypes are indistinguishable within gender groups. B: Male Study 011B mice do not differ significantly in measures of their forelimb grip strength when grouped by genotype. C: Female Study 011B mice are also equivocal in grip strength by genotype.

Motoric Phenotypes

6.2 Spontaneous locomotor activity monitoring

Basic initial behavioral phenotyping of transgenic mice almost invariably includes some aspect of spontaneous locomotor activity monitoring, which can inform the researcher about effects of genetic manipulation on exploration and anxiety phenotypes. The mice in Study 011B were assessed for horizontal activity including total distance traveled, total time of movement, crossings into specified test areas, and stoppages in movement. Study 011B mice were also tested for vertical activity in the form of rearing, and repetitive motions (stereotypy), which includes circling, grooming or even seizures. Finally, these mice were also tested for specific anxiety phenotypes using an open field activity measures. The details of the system utilized to collect these data are described in section 2.2.1.

Comparison of horizontal and vertical spontaneous locomotor activity as well as repetitive movement between BACE pKO; PDAPP and PDAPP mice resulted in no significant differences between groups (Figure 6.2.1-6.2.2). There was a tendency for BACE pKO; PDAPP mice to move shorter distances over less time on the horizontal plane in a more restricted set of sector areas. Overall however, this can be attributed to the far greater variability in activity levels in PDAPP in these activity measures (Tables 6.2.1a-b, 6.2.2a-b; Figure 6.2.1-6.2.2). If the reduced activity in BACE pKO; PDAPP mice is not simply due to trivial vagaries of statistical variation, then another possible interpretation is that partial deletion of the BACE gene confers some kind of hypoexploratory, incurious or possibly an anxious phenotype, in excess of the overall lesser exploration seen in PDAPP mice of Study 011A. Harrison et.al. in 2003 reported that their homozygous BACE knockout mice had a non-exploratory phenotype, and the BACE pKO; PDAPP may be more analogous to these animals, although BACE KO mice in Study 011A were not found to be hypoexploratory (possibly due to a very small N=3, with all female animals in this group).

Open field testing in which mice were monitored for movements in the central region of the arena also revealed no significant differences between PDAPP and BACE pKO; PDAPP mice (Table 6.2.3a-b, Figure 6.2.3). As in the other general motor activity measures, there was a non-significant trend towards lowered activity in BACE pKO; PDAPP mice in the open field. Taken together these data argue that although there are intimations otherwise, partial removal of the BACE gene product confers no statistically meaningful change in spontaneous motor or anxiety behavior of PDAPP animals.

	Total Distance (cm)			Total Time (s)			Complete Rests in Motion		
Factor	**N**	Mean	Std	**N**	Mean	Std	**N**	Mean	Std
Color									
Agouti	12	1171.33	2041.17	12	116.28	157.47	12	118.5	43.95
Black	6	2471.05	3751.19	6	207.48	236.31	6	135.5	59.94
Albino	10	692.88	439.24	10	90.94	57.62	10	134.6	52.57
Gender									
Female	14	1970.36	2985.68	14	179.81	204.08	14	130.43	52.37
Male	14	587.57	240.75	14	73.74	33.9	14	125.36	48.13
Genotype									
BACE pKO; PDAPP	12	576.08	236.12	12	75.66	32.89	12	129.17	48.78
PDAPP	16	1806.13	2817.04	16	165.11	194.65	16	126.94	51.47
ALL	28	1278.96	2194.47	28	126.78	153.37	28	127.89	49.42

Source	Factor(s)	F	DF	p-value
Distance	Gender	1.39	1	0.25
	Genotype	0.73	1	0.4
Time	Gender	1.83	1	0.19
	Genotype	0.72	1	0.4
Rests	Gender	0.12	1	0.73
	Genotype	0.06	1	0.8

Tables 6.2.1a-b Descriptive and One-Way ANOVA statistics for distance, time and motion rests activity monitoring measures in Study 011B mice by gender and genotype.

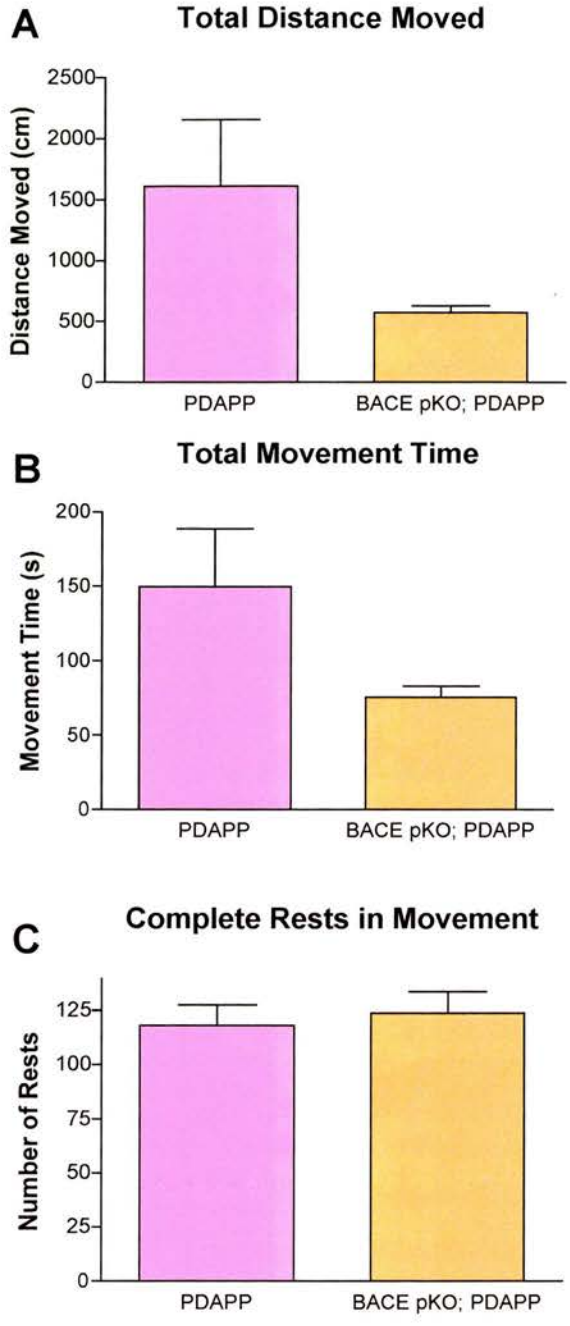


Figure 6.2.1 Spontaneous locomotor activity monitoring in 5mo Study 011B mice. A: BACE pKO; PDAPP and PDAPP mice do not significantly differ in total exploration distance, although PDAPP mice have a tendency towards greater activity levels. B: PDAPP mice spend a non-significantly greater amount of time in movement compared to BACE pKO; PDAPP mice, suggesting that partial BACE gene deletion confers a less exploratory phenotype. C: Study 011B mice are equivocal in the number of stoppages in movement.

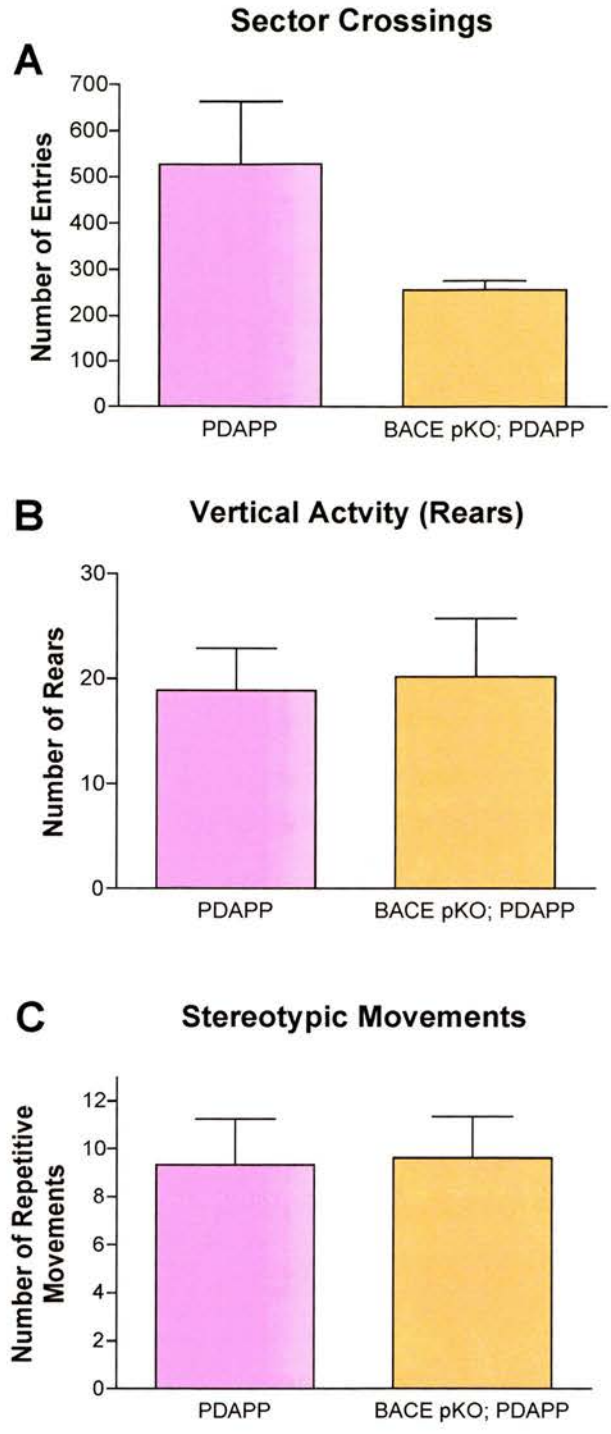


Figure 6.2.2 Spontaneous locomotor activity monitoring in 5mo Study 011B mice. A: PDAPP mice have a tendency to cross more arena quadrants than BACE pKO: PDAPP, although this is non-significant difference. B: There is no difference in the level of vertical exploration as measures by rearings between BACE pKO; PDAPP and PDAPP mice. C: Study 011B mice are indistinguishable in their number of stereotypic or repetitive movements during the observation period.

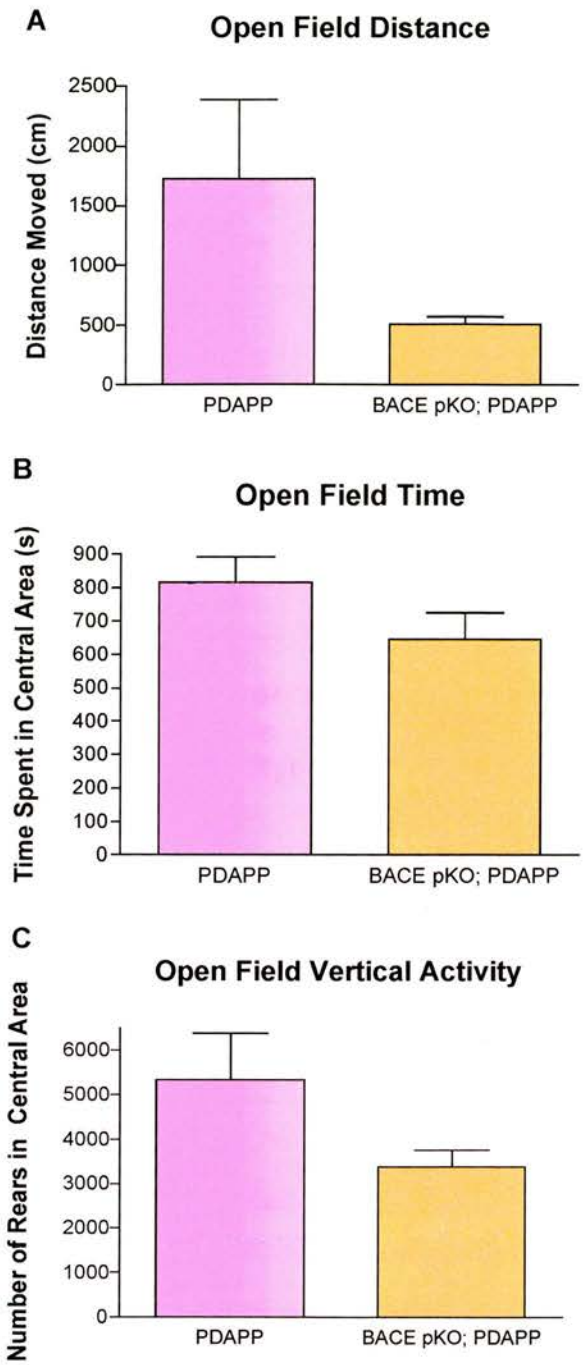


Figure 6.2.3 Open field behavior of 5mo Study 011B mice. Open field exploration in mice is associated with anxiety status, as anxious animals avoid open central areas in novel environments, and bold animals exploring all areas to a high degree. A: PDAPP mice tend to traverse greater distances in an open field area. Study 011B mice do not differ in the amount of time they spend in an open field area. C: PDAPP mice tend to rear more in the open field area than BACE pKO;PDAPP mice, suggesting these mice are anxious in novel environs.

		Sector Crossings			Vertical Activity (Rears)			Stereotypic Movements	
Factor	**N**	Mean	Std	**N**	Mean	Std	**N**	Mean	Std
Color									
Agouti	12	397.5	496.63	12	10.42	12.01	12	6.25	6.88
Black	6	770.67	1010.8	6	40.5	33.32	6	15.83	11.72
Albino	10	304.2	160.29	10	22.9	16.82	10	12.2	7.54
Gender									
Female	14	653.79	763.11	14	15.21	16.91	14	9.57	10.55
Male	14	234.5	93.51	14	27.43	25.69	14	11.29	7.16
Genotype									
BACE pKO; PDAPP	12	252.25	94.41	12	26.33	27.72	12	12.58	7.37
PDAPP	16	588.06	732.27	16	17.56	17.05	16	8.81	9.8
ALL	28	444.14	574.6	28	21.32	22.23	28	10.43	8.89

Source Factor(s)		F	DF	p-value
Crossings	Gender	2.18	1	0.15
	Genotype	0.65	1	0.43
Rears	Gender	1.26	1	0.27
	Genotype	0.2	1	0.66
Stereotypy	Gender	0	1	0.97
	Genotype	0.95	1	0.34

Table 6.2.2a-b Descriptive and One-Way ANOVA statistics for crossings, rears and stereotypic activity monitoring measures in Study 011B mice by gender and genotype.

		Open Field Distance			Open Field Time (s)			Open Field Vertical Activity (Rears)	
Factor	**N**	Mean	Std	**N**	Mean	Std	**N**	Mean	Std
Color									
Agouti	12	1305.23	2552.85	12	863.61	350.62	12	4647.67	3527.15
Black	6	2814.27	5030.69	6	862.58	446.9	6	2955.17	2150.39
Albino	10	624.35	441.8	10	612.89	294.16	10	3317	1661.87
Gender									
Female	14	2248.76	3884.53	14	855.3	411.26	14	4369.36	3398.47
Male	14	522.09	286.57	14	692.39	297.18	14	3250.14	1779.1
Genotype									
BACE pKO; PDAPP	12	513.52	263.14	12	658.09	317.53	12	4402.58	3756.68
PDAPP	16	2039.35	3664.02	16	860.66	377.9	16	3365.13	1579.91
ALL	28	1385.42	2842.16	28	773.85	361.71	28	3809.75	2722.07

Source Factor(s)		F	DF	p-value
OF Distance	Gender	1.29	1	0.27
	Genotype	0.65	1	0.43
OF Time	Gender	0.39	1	0.54
	Genotype	1.14	1	0.3
OF Rears	Gender	3.07	1	0.092
	Genotype	2.87	1	0.1

Tables 6.2.3a-b Descriptive and One-Way ANOVA statistics for crossings, rears and stereotypic activity monitoring measures in Study 011B mice by gender and genotype.

6.3 Rotorod motor coordination

While the Study 011B mice were statistically indistinguishable in spontaneous locomotion measures, examination of involuntary movement and motor coordination was also necessary to fully characterize motor activity levels in these animals. The rotorod is a mechanized rod that can rotate at constant or accelerating speeds, and animals placed on this rod are tested for their latency to fall. Typically mice placed on the rotorod improve their performance and increase their falling latencies over a number of trials, in a protocol described in section 2.2.3.

On the rotorod, PDAPP and BACE pKO; PDAPP mice in Study 011B have motor coordination phenotypes that are also indistinguishable (Tables 6.3a-d, Figure 6.3a-b). Mice of both genotypes not only stay on the rotating rod for similar amounts of time in static and accelerating paradigms, but also have a similar pattern of improvement of falling latencies over time. This results of this forced locomotion task affects the interpretation of the prior spontaneous locomotion data in which BACE pKO; PDAPP mice had a tendency towards hypoactivity. As BACE pKO; PDAPP mice do not apparently have any basic locomotor deficiencies when challenged on the rotorod, their decreased horizontal activity patterns as more likely a result of changes in exploratory motivation or anxiety. In addition, mice with homozygous BACE KO in Study 011A had poor performances on the rotorod that was statistically worse than PDAPP mice. That the BACE pKO; PDAPP and PDAPP mice in this study had indistinguishable rotorod performances suggests that unlike homozygous deletions, partial deletion of the BACE gene does not confer a deleterious motor coordination phenotype.

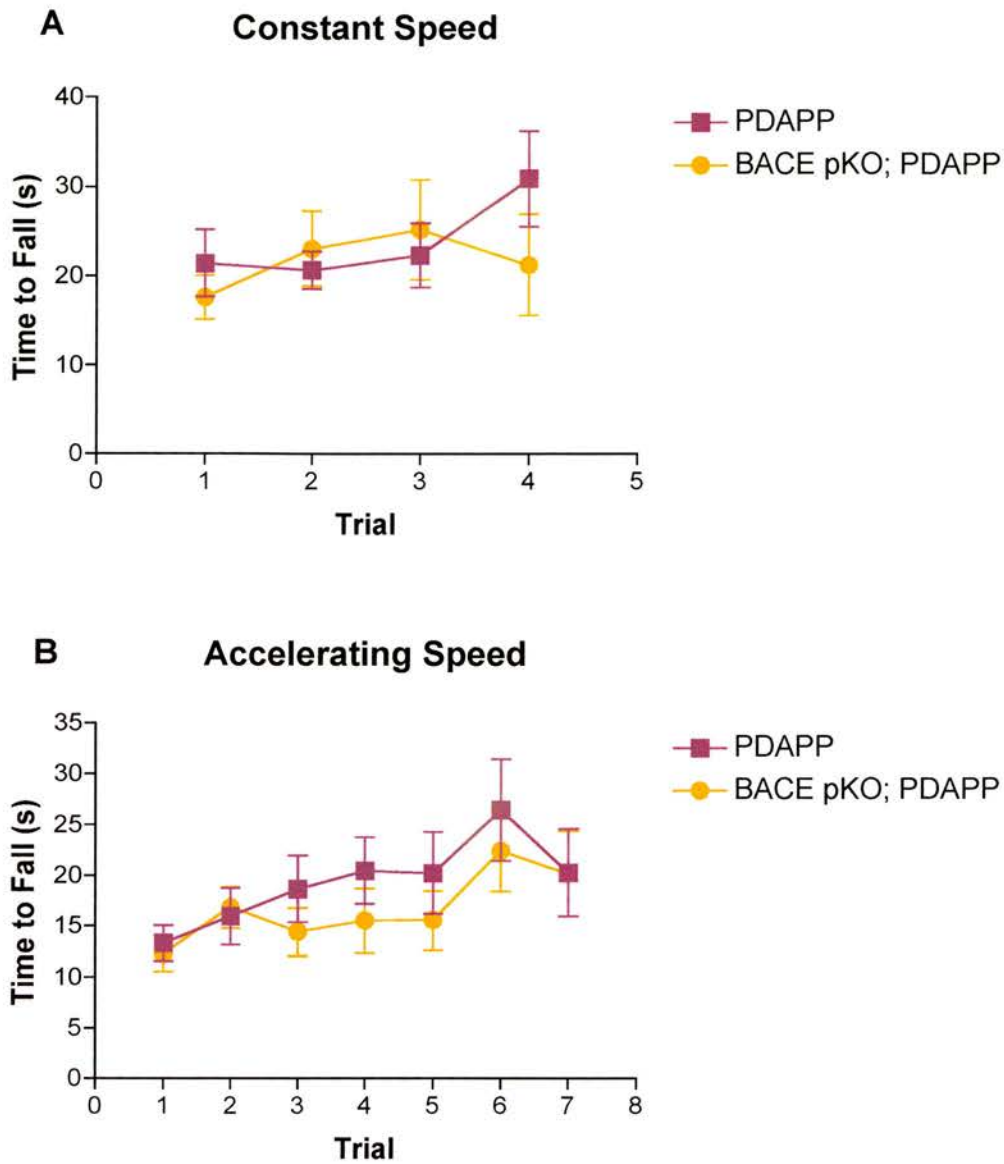


Figure 6.3 Motoric coordination on the rotorod in Study 011B mice. A: When placed on a rotorod turning at a constant slow speed of 10rpm, Study 011B mice perform equivalently by genotype over 4 trials. B: BACE pKO; PDAPP and PDAPP mice are equivalent in their ability to stay of a rotorod accelerating from 0-40rpm in a series of trials.

Factor	N	Trials							
		1		2		3		4	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	12	19.18	12.67	19.47	12.93	24.88	15.45	24.97	14.51
Black	6	22.32	9.1	21.49	9.11	20.76	8.46	18.12	12.26
Albino	19	22.07	112.22	20.96	12.07	27.42	19.31	34.96	34.04
Gender									
Female	14	23.22	10.07	24.35	7.55	28.06	16.47	38.27	25.51
Male	14	18.55	12.75	16.51	13.63	21.75	14.41	15.87	14.36
Genotype									
BACE pKO; PDAPP	12	18.62	12.18	18.35	13.34	21.03	10.06	17.21	16.97
PDAPP	16	22.58	11.1	22	10.12	27.81	18.39	34.47	25.09
ALL	28	20.88	11.52	20.43	11.53	24.9	15.52	27.07	23.3

Source Factor(s)	F	DF	p-value
Gender	1.5	4	0.24
Genotype	0.33	4	0.86

Tables 6.3a-b Descriptive and One-Way ANOVA statistics for performance on the constant speed rotorod in Study 011B mice by gender and genotype.

Factor	N	Trials													
		1		2		3		4		5		6		7	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	12	11.45	7.26	15.65	12.73	20.56	16.51	25.62	15.42	24.25	18.77	30.05	21.46	24.47	25.88
Black	6	11.16	5.49	11.77	9.12	13.33	12.16	10.38	5.99	22.54	17.65	25.85	16.71	19.2	8.03
Albino	10	18.98	8.15	19.04	13.33	17.91	11.54	23.13	14.16	22.7	18.45	30.06	17.88	29.65	16.55
Gender															
Female	14	16.07	8.39	18.54	11.36	20.94	14.47	24.77	14.39	29.62	20.48	37.25	17.77	30.62	15.18
Male	14	12.08	7.21	13.52	12.84	15.18	12.98	18.16	14.07	17.03	12.25	21.06	16.36	20.13	22.17
Genotype															
BACE pKO; PDAPP	12	13.65	7.38	15.07	9.74	12.09	7.39	18.45	12.31	17.64	11.49	24.54	16.05	23.11	15.54
PDAPP	16	14.4	8.56	16.75	13.99	22.54	15.9	23.73	15.73	27.59	20.63	32.61	20.02	26.84	22.6
ALL	28	14.07	7.94	16.03	12.17	18.06	13.8	21.47	14.36	23.33	17.76	29.15	18.68	25.18	19.51

Source Factor(s)	F	DF	p-value
Gender	0.63	7	0.73
Genotype	1.03	7	0.45

Tables 6.3c-d Descriptive and One-Way ANOVA statistics for performance on the accelerating speed rotorod in Study 011B mice by gender and genotype.

Seizure Phenotypes

6.4 PTZ-Induced seizures

Study 011B mice were ultimately tested for their responses to the seizure-inducing drug pentylenetetrazole (PTZ). The PTZ seizure induction model of epilepsy is commonly used in mice to detect either predisposition to seizures in genetically-modified animals or in tests of anti-epileptic compounds that promote resistance to seizures. Mice given 60 mg/kg of PTZ intraperitoneally typically display a particular pattern of seizure activities, described in section 2.3, with no spontaneous seizures observed prior to seizure induction. Post-PTZ, mice begin with mild partial clonic seizures (twitches) that expand to whole body tremors (general clonus) and finally severe tonic seizures (tonic) in which muscles are rigorously clenched and forelimbs dramatically extended. Animals with partial BACE deletions tested for spatial memory phenotypes in Study 006 had few observations of spontaneous seizure compared to homozygous BACE KO mice in Study 001, and these PTZ induced-seizure experiments were designed to profile the propensity to seizure in these animals. Specifically, it was of interest to determine whether partial BACE gene deletion proffered protection against seizure kindling on the PDAPP background in the manner it appeared to ameliorate PDAPP spatial memory impairments.

Study 011B mice by genotype did not differ on any measure of seizure kindling induced by PTZ except for that BACE pKO; PDAPP mice had greater resistance to developing moderate severity general clonic seizures ($F=6.84$, df 1/25, $p=0.015$; Tables 6.4.2a-b Figure 6.4.2b). Data from latency to partial clonus and latency to death were indistinguishable between PDAPP and BACE pKO; PDAPP mice, with 81.25% and 75% mortality in PDAPP and BACE pKO. PDAPP mice (Tables 6.4.1a-b, Figure 6.4.1a-b). Neither composite seizures scores nor scores separated by seizure type were appreciably different in PDAPP and BACE pKO; PDAPP mice (Figure 6.4.1c, Figure 6.4.2). Overall it appears that the partial deletion of the BACE gene reduces the propensity to have moderate strength seizures following PTZ administration in mice carrying the PDAPP transgene.

There were gender-specific differences in general clonic seizures, death latency and overall seizure scores (Tables 6.4.1-6.4.2a-b, Figures 6.4.1-6.4.2a-c). These differences were based in the greater susceptibility to seizures in male mice, but without the ability to perform interaction analyses on these animals it is difficult to assess the impact of this finding. Indeed, there did not appear to be a gender-related difference in the animals observed to have spontaneous clonic-tonic seizures in Studies 001 or 006 (Table 6.4.3).

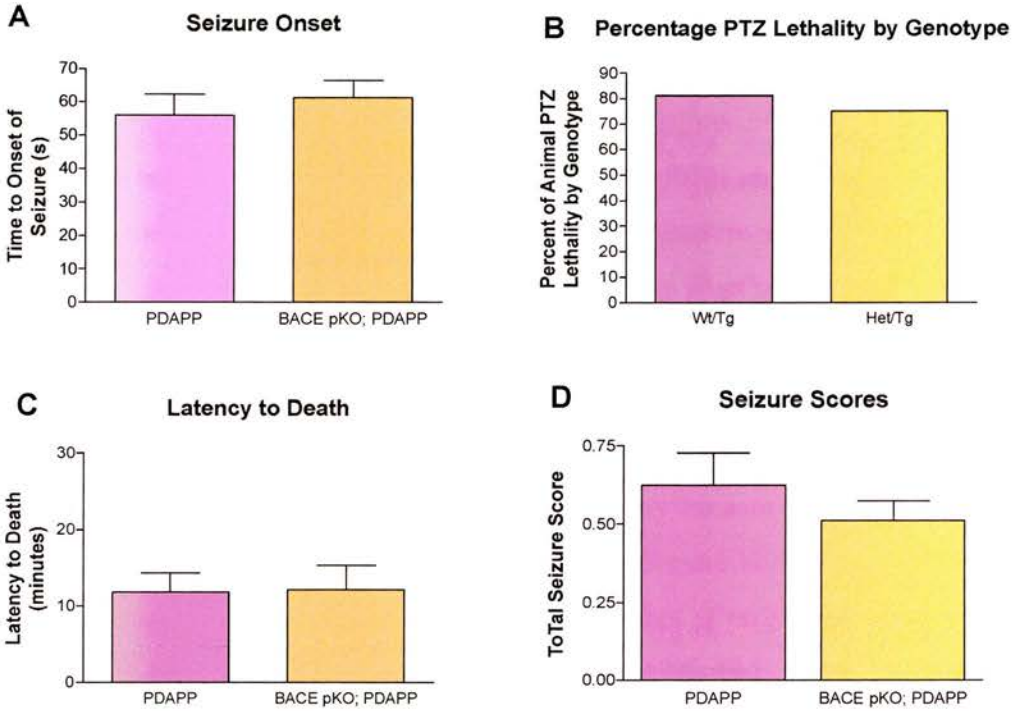


Figure 6.4.1 PTZ-induced seizure activity in 5mo Study 011B mice. Overall there is little to distinguish BACE pKO; PDAPP seizure response from that of PDAPP mice. A: Latency to first seizure observation, mild clonus, is similar between both genotypes of Study 011B. B: Percentage of mice that die within 30 minutes of 60mg/kg i.p. PTZ administration is equivocal between BACE pKO: PDAPP and PDAPP mice. C: Time to death in PTZ-treated mice is similar in Study 011B mice. D: Cumulative seizure scores that include mild and general clonus as well as severe tonic seizures were statistically similar between Study 011B mice by genotype.

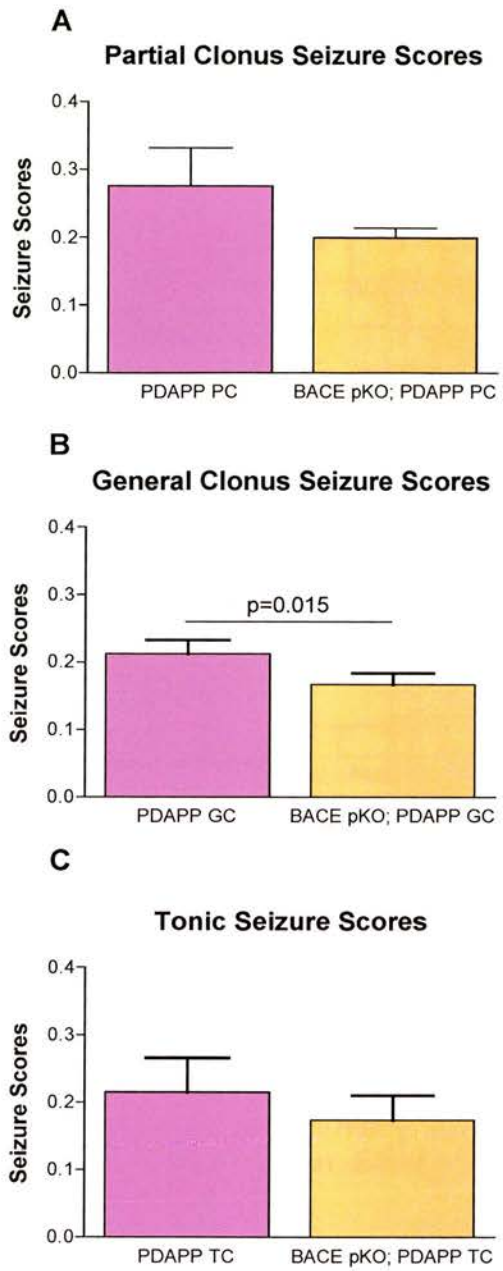


Figure 6.4.2 PTZ-induced component seizure scores in Study 5mo 011A mice. There is a non-significant trend for BACE pKO; PDAPP mice to have lower seizure scores (based on latency to that specific seizure type) than PDAPP mice. A: Mild or partial clonic seizures response in Study 011B mice is similar. B: BACE pKO; PDAPP mice have greater resistance to kindling general clonic seizures than PDAPP mice in response to PTZ treatment. C: BACE pKO; PDAPP and PDAPP mice have similar levels of severe tonic seizures in response to PTZ treatment.

Factor	Seizure Latency (min)			Death Latency			Seizure Score		
	N	Mean	Std	N	Mean	Std	N	Mean	Std
Color									
Agouti	12	0.85	0.26	11	7.36	4.09	12	0.69	0.46
Black	6	0.89	0.25	6	8.62	4.47	6	0.53	0.11
Albino	10	1.2	0.32	5	4.62	2.21	10	0.46	0.25
Gender									
Female	14	1.05	0.34	9	8.3	4	14	0.46	0.16
Male	14	0.92	0.28	13	6.24	3.89	14	0.69	0.44
Genotype									
BACE pKO; PDAPP	12	1.06	0.26	9	6.2	3.27	12	0.51	0.22
PDAPP	16	0.93	0.35	13	7.7	4.42	16	0.62	0.42
ALL	28	0.98	0.32	22	7.08	3.98	28	0.58	0.34

	Source Factor(s)	F	DF	p-value
Seizure Latency	Gender	3.53	1	0.072
	Genotype	3.39	1	0.077
Death Latency	Gender	N/A	1	0.028
	Genotype	N/A	1	0.98
Seizure Score	Gender	6.36	1	0.018
	Genotype	3.71	1	0.066

Table 6.4.1a-b Descriptive and One–Way ANOVA statistics for seizure responses and lethality in Study 011B mice by gender and genotype.

Genotype	Female		Male		All	
	Number of Mice	n (%) that died	Number of Mice	n (%) that died	Number of Mice	n (%) that died
BACE pKO; PDAPP	3	1 (33.3)	9	8 (88.9)	12	9 (75.0)
PDAPP	11	8 (72.7)	5	5 (100.0)	16	13 (81.3)
ALL	14	9 (64.3)	14	13 (92.9)	28	22 (78.6)

	Source Factor(s)	P-Values
Lethality	Gender	0.16
	Genotype	1

Tables 6.4.1c Descriptive and Fisher's Chi-squared test statistics for PTZ-induced seizure lethality in Study 011B mice by gender and genotype.

Factor	Partial Clonus Score			General Clonus Score			Tonic-Clonic Score		
	N	Mean	Std	N	Mean	Std	N	Mean	Std
Color									
Agouti	12	0.3	0.25	12	0.22	0.08	9	0.23	0.17
Black	6	0.23	0.05	6	0.19	0.06	4	0.16	0.11
Albino	10	0.18	0.06	10	0.17	0.08	7	0.17	0.12
Gender									
Female	14	0.21	0.07	14	0.18	0.08	7	0.16	0.07
Male	14	0.28	0.24	14	0.21	0.07	13	0.22	0.16
Genotype									
BACE pKO; PDAPP	12	0.2	0.05	12	0.17	0.06	10	0.17	0.12
PDAPP	16	0.28	0.23	16	0.21	0.08	10	0.22	0.16
ALL	28	0.24	0.18	28	0.19	0.08	20	0.19	0.14

	Source Factor(s)	F	DF
PC Score	Gender	2.91	1
	Genotype	3.27	1
GC Score	Gender	5.66	1
	Genotype	6.84	1
TC Score	Gender	2.3	1
	Genotype	1.87	1

Tables 6.4.2a-b Descriptive and One–Way ANOVA statistics for seizure component scores in Study 011B mice by gender and genotype.

Calbindin and Amyloid Histology

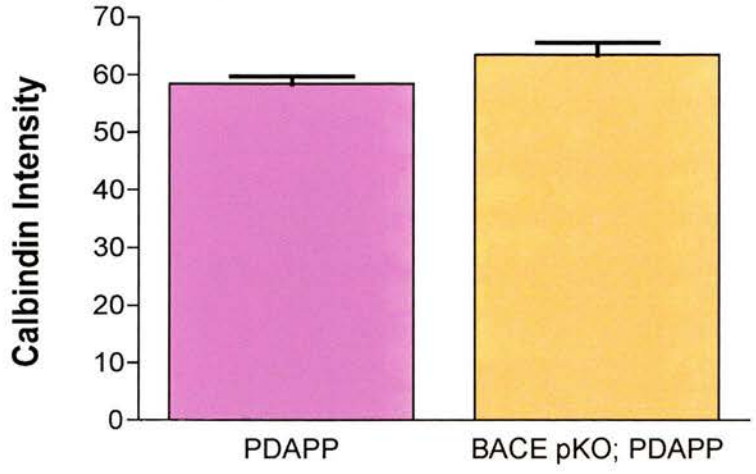
6.5 Calbindin histology in the hippocampal outer molecular layer

Following the behavioral phenotyping and PTZ treatment of Study 011B mice, mouse hippocampal tissues were immunochemically examined for Calbindin protein levels. Calbindin is a ubiquitiously expressed Ca^{++} binding protein found in cerebral neurons that is involved in homeostatic regulation of functioning neurons. CB has been also linked to neurogenesis in epileptic animals, as other researchers have reported that CB levels increase in response to seizure activity, including epileptic activity due to PTZ administration. If BACE pKO mice have experienced spontaneous seizures at a greater rate than PDAPP mice, then analysis of CB levels would provide another means to detect any functional differences related to seizure between the two genotypes of Study 011B.

Calbindin (CB) immunostaining in the OML of the hippocampus revealed that BACE pKO; PDAPP mice had a tendency towards having more CB than PDAPP mice ($p=0.072$; Table 6.5a-b, Figure 6.5a-c). While the statistical significance of this difference was not meaningful, even a tenuous trend towards greater CB in BACE pKO; PDAPP would represent one of the few measures in which the two genotypes in Study 011B even approached distinction from each other. This finding was more deeply explored in section 6.6 regarding the correlational analyses of general behavioral observations, PTZ-induced seizure profiles and CB levels. These analyses revealed a link between CB levels in BACE pKO; PDAPP mice and tonic seizures, in which CB was associated with resistance to severe seizure activity.

Antibody staining for APP-expression in Study 011B animals confirmed the genotypes of all mice, in accordance with the vendor's inventory. Animals carrying one copy of the PDAPP transgene typically develop sparse plaque-like amyloid deposits between 6-8mo, thus no confirmatory Aβ staining was performed as Study 011B mice were 4.75-5.25mo old, and negative results would be largely uninterpretable.

A **Calbindin Intensity in the Hippocampal OML**



B

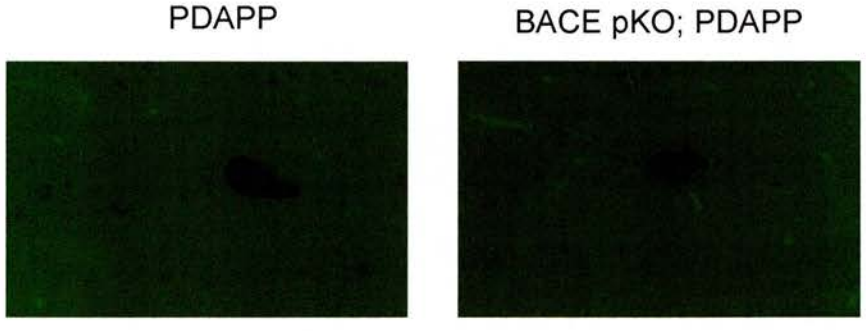


Figure 6.5 Calbindin (CB) immunoreactivity and images in the hippocampal outer molecular layer of 5mo Study 011B mice. A: There is no statistically significant difference in hippocampal CB levels in Study 011B mice. B: Images of the CB immunoreactivity, displaying sections with intensity values close to the genotype group average are also visually equivalent between BACE pKO; PDAPP and PDAPP mice.

Factor	N	Mean	STD
Color			
Agouti	11	61.24	6.87
Black	5	58.66	1.79
Albino	10	61.24	8.07
Gender			
Female	14	60.23	7.99
Male	12	61.34	4.87
Genotype			
BACE pKO; PDAPP	11	63.5	7.25
PDAPP	15	58.72	5.53
ALL	26	60.74	6.63

Source Factor(s)	F	DF	p-value
Gender	0.22	1	0.64
Genotype	3.56	1	0.072

Tables 6.5a-b Descriptive and One–Way ANOVA statistics for Calbindin immunoreactivity in Study 011B mice by gender and genotype.

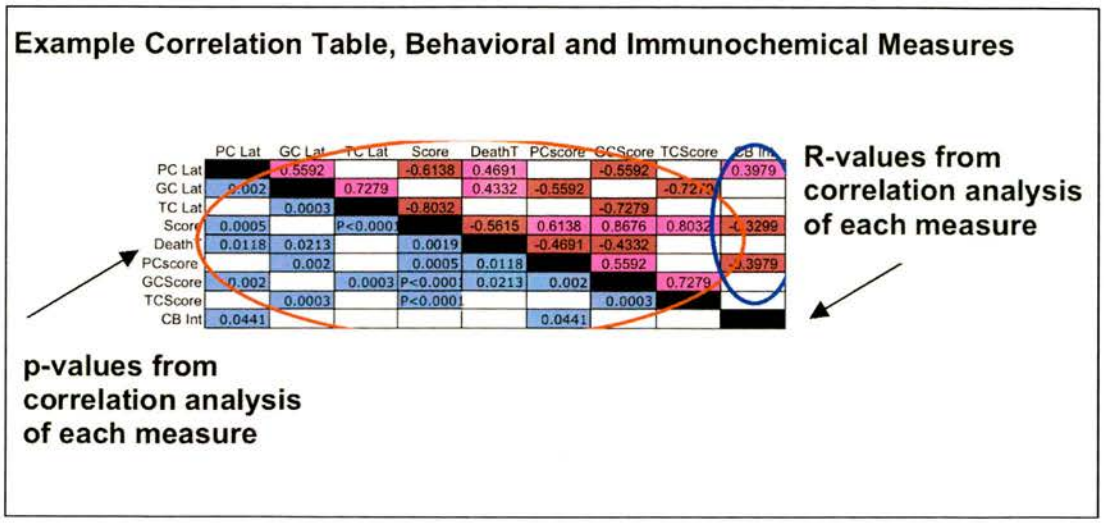
6.6 Correlation analyses of sensorimotor, seizure, and histological data

Correlation analysis cell key

R-Values Colorimetrics | P-Values Colorimetrics | Self-Correlation

Column Abbreviations

PC Lat =Latency to Partial Clonus
GC Lat= Latency to General Clonus
TC Lat = Latency to Tonic Seizure
Score = Composite seizure score
DeathT = Latency to death
PCscore= Partial clonus component of seizure score
GCscore= General clonus component of seizure score
TCscore = Tonic seizure component of seizure score
CB Int = Calbindin Intensity in the Hippocampal Outer Molecular Layer
GS1 = Grip strength day 1
GS2 = Grip strength day 2
GS3 = Grip strength day 3
2RR1 = Constant speed rotorod trial 1
2RR4 = Constant speed rotorod trial 4
3RR1 = Accelerating speed rotorod trial 1
3RR4 = Accelerating speed rotorod trial 4
3RR7 = Accelerating speed rotorod trial 7
SectX1 = Sector crossings, session 1
Rest1 = Complete rests in motion
Dist1 = Total distance traveled
Time1 = Total time spent in motion
Rear1 = Vertical activity counts
Stereo1 = Number of stereotypic movements
Open Dist1 = Total open field distance traveled
Open Time1 = Total time spent in motion in open field
Open Vert1 = Vertical activity counts



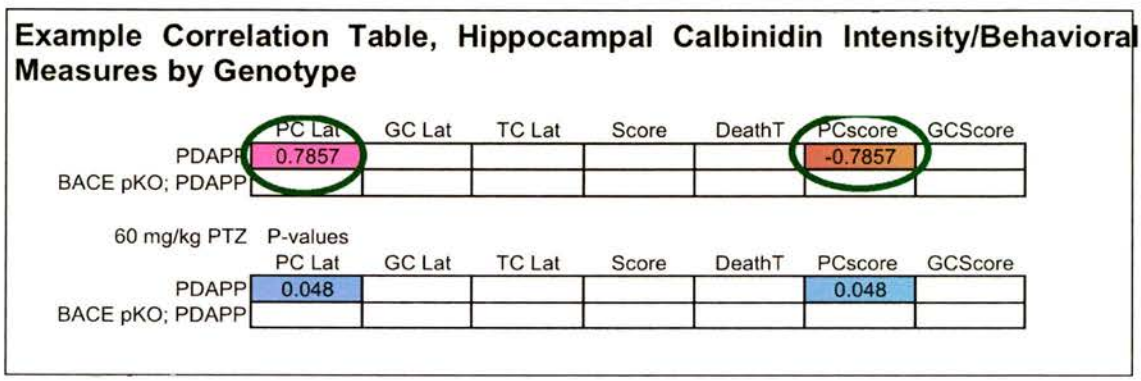


Figure 6.6.1a-b Example correlation tables. A: Correlation table of relationships between various behavioral measures, with R-values presented in upper diagonal section and p-values presented in lower diagonal section. Corresponding p-values and R-values are found in the same coordinate distance from the black diagonals separating the two types of values, with R-values at x_r,y_r coordinates, and p-values at y_p,x_p coordinates where $x_r=y_p$ and $y_r=x_p$ Values contained within red circle indicate significant intrameasure correlations, e.g. clonic to tonic seizure latency. Values contained within blue circles indicate significant intermeasure correlations, e.g. clonic seizure latency to Calbindin intensity. B: Correlation table of various behavioral measures and Calbindin intensity separated by genotype. R-value tables are above while P-values are below. Values in green circles indicate significant behavioral/Calbindin correlations.

All behavioral and histological data gathered from individual animals tested in Study 011B was subjected to Spearman's Correlational Test in an effort to uncover relationships between these varied measures. Measures were compared individually, with the expectation that correlations that may arise are due to underlying

relatedness of the brain processes that govern specific behaviors and/or anatomical features.

Correlational analyses of Study 011B behavioral and histological measures produced patterns of measure relationships that were reminiscent of those of Study 011A: there again were five groups of related measures that formed functional sets, and there also were certain sensorimotor measures that were predictive of seizure activity (Figure 6.6). Although there were some new features in Study 011B the five main sets of related measures were largely unchanged:

- Partial Clonic (PC)/General Clonic Seizures (GC)
- Grip Strengths
- Constant and Accelerating Rotorod, with broader relationships than in Study 011A
- Horizontal locomotor activity, with wider correlations to grip strength and rotorod data than in Study 011A
- Vertical/Stereotypic locomotor activity, including open field measures

Intrameasure relationships

Between Study 011A and 011B there was a shift in related measures from GC/TC to PC/GC respectively. This may be due to the fact that death latencies in Study 011B PDAPP mice in response to PTZ were about 1/3 shorter overall that in Study 011A, shortening the amount of time between PC and GC seizures while the time between GC and TC seizures remained the same (Figures 5.4b, 6.4b). In Study 011B the greater relatedness of the sensorimotor data values would allow for the grouping of grip strength, rotorod performance and all the spontaneous activity monitoring measures into one larger functional group. One interpretation of this change towards greater interrelatedness in the motor data measures could be attributed to the overall lack of distinction between PDAPP and BACE pKO; PDAPP mouse phenotypes. The indistinguishable genotypic mouse data from Study 011B statistically is based on similarities in data between genotypes, and thus less variation in the total

Table 6.6 Correlation of pharmacologic, behavioral and histological measures, with resulting R- and p-values of 5mo Study 011B mice. Table is located in pocket at back cover of document.

This table of correlation values underlines several intrameasure and intermeasure relationships.

Intrameasure values are highly correlated within each set, as performance on one measure is likely to be functionally related to performance on a similar measure. These intrameasure correlations are circled in red, and are closest to the table diagonal separating R- and p-values. Thus many measures within PTZ-seizure induction, trial performances over days of testing in tasks like grip strength and the rotorod, and the general and open field activity monitoring have high degrees of correlations within each set.

Intermeasure relationships occur between different task measures and are circled in blue, typically distant from the R-/p-value diagonal. Correlations between these metrics suggest that although the tasks vary methodologically, the underlying functional and/or anatomical bases for their performance are similar, and even predictive of one another. In particular, resistance to partial clonic seizure response appears to be correlated to higher CB intensity, longer post-seizure and death latencies are associated with greater forelimb grip strength. The ability to stay for longer periods on the rotorod was also related to greater grip strength and exploration over a wide range of arena space (SectX1, number of sectors crossed).

correlational data set. Decreases in variation mathematically are akin to increasing the power of any analysis, and ultimately confers greater ability to detect patterns of correlations.

Intermeasure relationships

There were many statistically significant relationships between CB and the various seizure measures in Study 011B (Table 6.7). There were unexpected positive correlations between PC and TC seizure latency and Calbindin intensity, a finding that was opposite to that of Study 011A. Again it may be possible that the greater lethality and shorter times to death seen in Study 011B animals treated with PTZ could be affecting the data, since there would be less time to affect any changes in baseline levels of CB before tissue processing. To examine this more closely, the individual PDAPP and BACE pKO; PDAPP genotypes were separately compared to all other seizure and observational behavior measures. This individual analysis revealed that in BACE pKO; PDAPP mice, high levels of CB were still negatively correlated to composite seizure scores as seen previously in Study 011A ($R=-0.3909$). However, TC latencies were positively associated with CB levels in BACE pKO; PDAPP mice ($R=0.7258$, $p=0.0269$). This finding seems to go against the pattern of correlation between CB-Seizure score, but in BACE pKO; PDAPP mice TC scores account for less of the total seizure score than do PDAPP TC scores in either study (Figure 5.4d-e, 6.4d-e), so this finding could be less relevant from this perspective. It is also important to realize that any differences in the correlational patterns between Study 011A and 011B could be due to the ages of the mice tested (18 vs. 5mo), as any of these opposing patterns could be due to aging effects.

There were other measures in Study 011B that were correlated to seizure measures. Again, CB levels were associated with seizures, as there was a significant relationship between propensity to PC seizures and high levels of CB, while there was a trend towards significance with propensity to TC seizures and high CB levels ($R=0.3979$, $p=0.0441$ and $R=0.4281$ and $p=0.0763$ respectively). In addition, grip strength ratio data on the third day of testing was highly correlated to death latency,

suggesting that individual animals with lower grip strengths may have had seizure activity as evinced by lower time to death. Animals that overall had greater resistance to seizure and death from PTZ administration are those most likely to have had the least spontaneous previous seizure activity during their lives. In addition to this concept of resistance to severe seizure through lack of prior experiences, limb weakness is one of the most common of the seizure sequelae, which taken together makes this grip strength and death latency correlation intuitively sensible.

Lastly, when CB correlations to all other measures were separated by genotype, a novel relationship between TC and CB in BACE pKO; PDAPP mice was detected (Table 6.7). In BACE pKO; PDAPP CB intensity was significantly correlated to lower TC activities, ($R=0.726$, $p<0.05$). In addition CB was related to Stereo1 values in a way in which greater CB intensity was correlated to lesser repetitive motor activity, which itself is a reliable predictor of seizure propensity ($R=-0.607$, $P<0.025$; Table 6.7). Finally, CB levels in BACE pKO; PDAPP mice were related to increased activity in open field areas, and a lack of anxiety phenotypes. This last finding is in opposition to the PDAPP CB/open field correlation data from Ch. 5. If CB is truly anything like a biomarker for functional cognitive status in mice, this genotypic discrepancy implies that the relationship is not absolute. Indeed this CB-function relationship may be age-dependent as Ch. 5 focused on aged mice and Ch.6 described young animal subjects, and there was an apparent age-related shift in CB across the PDAPP, BACE KO, and BACE KO; PDAPP mice in Study 001 (Figure 3.6.1).

Overall these Study 011B results with CB help support the concept that partial deletion of the BACE gene on a PDAPP background in certain circumstances may indeed be beneficial to the functioning nervous system, improving anxiety and seizure activity profiles. The next study will examine these findings in aged mice which have more direct comparability to Study 011A mice, in hopes of expanding this hint of functional improvement of PDAPP mice conferred by partial BACE gene deletion.

Table 6.7 Correlation of Calbindin (CB) to all other measures, R- and P-values of 5mo Study 011B mice. Table is located in pocket at back cover of document.

Specific assessment of CB correlations to behavioral and pharmacological metrics was done by genotype to discern wider patterns of predictive functional relationships. The only significant correlations with CB were in BACE p KO; PDAPP mice, which had lesser severe seizure activity, less repetitive movements, and spent more time in the open field with high CB levels. As repetitive or stereotypic movements in mice are predictive of seizure response to PTZ in mice, CB appears to be a biomarker for seizure protection in mice with partial BACE gene deletion. Anxiety phenotypes in mice are often represented by avoidance of open field areas, but BACE pKO; PDAPP mice with higher CB levels explored these areas more, suggesting that partial BACE KO ameliorates anxiety phenotypes in PDAPP mice.

Ch.7 Study 011C: General Behavioral Phenotyping and Response to Dose-Scaled Seizure Induction in 18mo Hemizygous BACE pKO x PDAPP mice

In Study 011C aged (18mo) hemizygous BACE pKO x PDAPP mice were assessed for their sensorimotor and seizure phenotypes using tests that measured limb strength, spontaneous and involuntary motor function, as well as activity profiles in response to a seizure inducing agent (Table 7.0a-b). Previous analysis using the same tests in aged homozygous BACE KO x PDAPP mice revealed a deficiency in motor coordination in BACE KO mice, and lesser resistance to chemically-induced severe seizures in mice carrying the PDAPP transgene. BACE KO; PDAPP mice were severely impaired in spatial memory processes even compared to PDAPP mice, suggesting that the lack of the BACE gene product exacerbated the PDAPP spatial memory phenotype. In addition, BACE KO; PDAPP mice in Study 011A appeared to have the least resistance to developing severe seizures in response to treatment with an epileptic kindling agent (PTZ). While Study 011B BACE pKO; PDAPP mice were indistinguishable from PDAPP mice on most measures, correlative data suggested that partial deletion of BACE genes confer some level of protection from seizure phenotypes. These Study 011B results gathered in young mice (5mo) needed further examination in aged animals.

In addition to examining the sensorimotor phenotypes of 18 mo BACE pKO, PDAPP mice for more direct comparison to aged homozygous BACE KO; PDAPP mice, Study 011C was also designed to detect differential responses between BACE pKO; PDAPP and PDAPP mice to induction of mild and severe seizures. Partial deletion of the BACE gene on a PDAPP background in Study 006 appeared to alleviate some of the spatial memory deficits of PDAPP mice, and it was of interest to see if this spatial memory rescue extended to protection from various types of seizure activity. Total N for this study was 32 mice (Table 7.0a).

The overall performance of Study 011C mice is summarized below (Table 7.0b). One-way ANOVA tests were conducted on all measures except for grip strength and the rotorod, which was subject to MANOVA testing, and the Lethality measure which was analysed with Fisher's exact Chi-squared test. Due to the scarcity of animals across all gender and genotype

groups, statistical analysis by color was not possible, nor was interaction analysis between dose, gender and genotype. Descriptive statistics are presented for all factors.

	Female				Male				ALL			
Factor	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL	Agouti	Black	Albino	ALL
Dose												
25 mg/kg PTZ	8	2	0	10	6	0	0	6	14	2	0	16
60 mg/kg PTZ	7	1	1	9	5	1	1	7	12	2	2	16
Genotype												
BACE pKO; PDAPP	8	2	0	10	6	0	0	6	14	1	1	16
PDAPP	7	2	0	9	5	1	1	7	12	3	1	16
ALL	15	4	0	19	11	1	1	13	26	4	2	32

Table 7.0a Study 011C mice, all aged 18mo.

Overall even with aged mice there was little to distinguish BACE pKO; PDAPP from PDAPP mice. There were no genotypic significant differences in Study 011C, and by gender the only difference was that female mice had shorter latencies to mild seizure signs than males. The most important factor impacting performance was dosage of penetylenetetrazole (PTZ), which was given at 25 and 60 mg/kg to induce mild and severe seizures respectively. Animals that were grouped into low and high PTZ dose cohorts were not selected until after they had completed other sensorimotor testing. Post-hoc analysis of PTZ dose group sensorimotor performance revealed no significant differences; no graphical data by dose is presented except for the seizure data.

	P-Values		
Measure	Gender	Dose	Genotype
Masses	0.14	0.18	0.21
Positional Tone	0.6	0.28	0.69
Crossings	0.75	0.34	0.15
Distance	0.92	0.33	0.11
Rests	0.64	0.63	0.33
Movement Time	0.97	0.28	0.099
Vertical Actvity	0.54	0.69	0.54
Stereotypy	0.61	0.56	0.33
Open Field Distance	0.91	0.36	0.13
Open Field Time	0.5	0.13	0.54
Open Field Rears	0.4	0.97	0.37
Rotorod Constant Speed	0.46	0.17	0.059
Rotorod Accelerating	0.82	0.77	0.59
PC Latency	0.012	0.0002	0.73
GC Latency	0.35	N/A	0.22
TC Latency	0.8	N/A	0.23
PC Score	0.036	0.017	0.73
GC Score	0.43	N/A	0.25
TC Score	0.85	N/A	0.46
Seizure Score	0.19	P<0.0001	0.61
Percentage Lethality	1	P<0.0000	1
Death Latency	0.61	P<0.0001	0.72
Calbindin Intensity	0.33	0.59	0.52

Table 7.0b Statistical summary of factor significance in Study 011C.

Mass and Muscular Function

7.1 Body Mass and Positional Tone

Forelimb muscular tone was measured in Study 011C mice, as this is often reduced in the aftermath of severe seizure activity. The original observations of spontaneous clonic-tonic seizures in homozygous BACE KO x PDAPP mice motivated concern that experimental mice were having unobserved seizures throughout life, which may have contributed to the early death phenotype of these animals (section 3.4-3.5). While it was not possible to continuously monitor all the BACE KO x PDAPP mice in their homecages long-term to record any spontaneous seizure activity, it was possible to perform simple behavioral tests to determine if animals had been experiencing spontaneous seizures. Analysis of forelimb muscular strength was one method employed to make judgments about the spontaneous seizure status of BACE pKO; PDAPP mice.

In Study 011C, manual measurements were made to assess the muscle tone of the mice, where each mouse was tested for its ability to resist being upended by gentle force at the level of the foreshoulder, with a score ranging 0 (normal) -3 (upended) given to animals in response to a gentle push (section 2.2.2). Analysis of these positional sense scores revealed no differences in resistance to upending gender or genotype ($F<1$ Gender, $F<1$ Genotype; Tables, 7.1c-d Figure 7.1b). BACE pKO; PDAPP also appeared to be impaired in positional sense relative to PDAPP mice, but this was not a statistically significant finding. Body masses between BACE pKO; PDAPP and PDAPP mice separated by gender were indistinguishable, as they were in Study 011B (Figures 6.1a, 7.1a, Tables 7.1a-b).

While positional sense is related to grip strength as they both rely on forelimb muscular tone, positional sense has a strong component of motor coordination as well. Thus the tendency towards deficiencies observed in BACE pKO; PDAPP mice in positional sense as well as their rotorod motor coordination deficits described in section 5.3, and later in section 7.3 of this chapter appear to support a concept of a general deleterious motor phenotype in aged BACE KO mice.

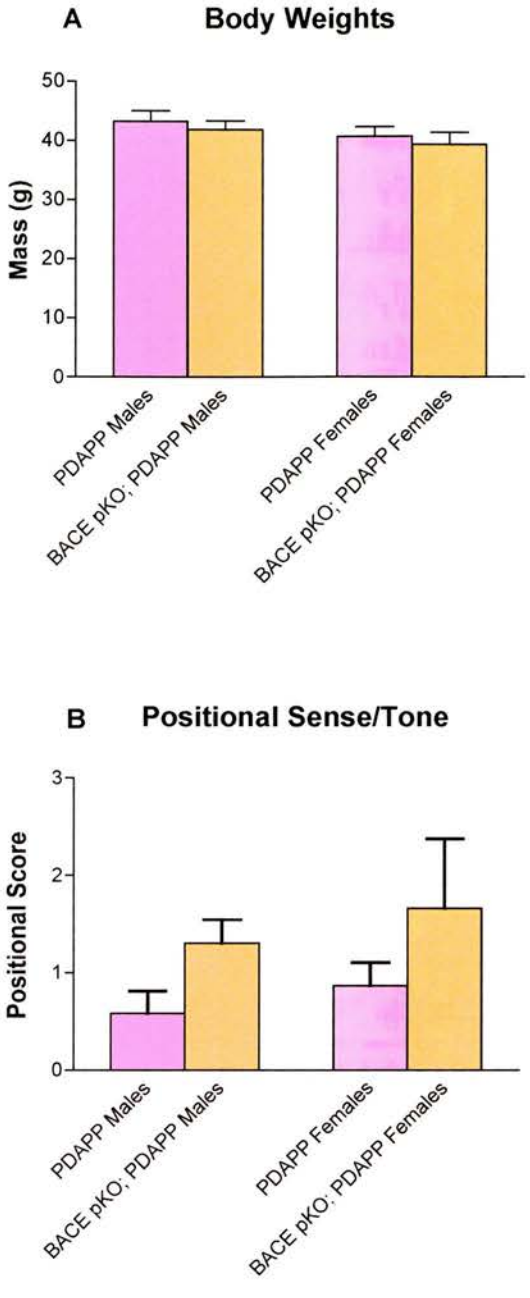


Figure 7.1 Body mass and positional sense of 18mo Study 011C mice by gender and genotype. A: Study 011C mice do not differ in body mass by either gender or genotype. B: Positional sense/tone scores above zero indicate reduced ability to remain afoot when gentle force is applied to the shoulder area, which can be based in poorer righting reflex and/or decreased forelimb muscular tone. Male BACE pKO; PDAPP mice have significantly higher positional scores than PDAPP mice, with a similar but non-significant trend in female mice, suggesting that the partial BACE gene deletion confers a motor deficit.

Factor	N	Mean (g)	STD
Color			
Agouti	24	42.25	1.46
Black	4	38.22	1.07
Albino	2	40.47	5.18
Gender			
Female	14	44.12	1.49
Male	18	39.37	1.66
Dose			
25 mg/kg PTZ	16	43.65	1.66
60 mg/kg PTZ	16	39.25	1.59
Genotype			
BACE pKO; PDAPP	16	39.34	1.45
PDAPP	16	43.56	1.82
ALL	32	41.45	1.2

Source Factor(s)	F	DF	p-value
Gender	2.03	1	0.14
Dose	1.75	1	0.18
Genotype	1.61	1	0.21

Table 7.1a-b Descriptive and One-Way ANOVA statistics for body masses in Study 011C mice by gender and genotype.

Factor	N	Mean	STD
Color			
Agouti	24	1.33	0.19
Black	3	0	0
Albino	2	0.5	0.5
Gender			
Female	18	1.29	0.23
Male	11	0.91	0.28
Dose			
25 mg/kg PTZ	14	1.43	0.25
60 mg/kg PTZ	15	0.87	0.24
Genotype			
BACE pKO; PDAPP	16	1	0.22
PDAPP	13	1.31	0.29
ALL	29	1.138	0.18

Source Factor(s)	F	DF	p-value
Gender	0.51	1	0.6
Dose	1.29	1	0.28
Genotype	0.37	1	0.69

Table 7.1c-d Descriptive and One-Way ANOVA statistics for grip strengths in Study 011C mice by gender and genotype.

Motoric Phenotypes

7.2 Spontaneous Locomotor Activity Monitoring

Analyses of spontaneous locomotor activity of Study 011C mice on the horizontal and vertical planes were made with an automated monitoring system as described in section 2.2.1. In Study 011C mice were assessed for these measures, including distance traveled, time spent in motion, number of sector crossings, number of rests in movement, vertical activity (rears) and stereotypic movements, as well as open field activity over two 15min sessions. These measurements are useful in any characterization of transgenic mice as they can provide quantitative information on the exploration and anxiety phenotypes of experimental mice. In general mice in activity monitoring arenas explore novel environments by ambulating around the perimeter of the area, with occasional forays in the central regions of the arena. Animals with reduced explorative characteristics will travel shorter distances, with lesser travel time than control animals. Animals with anxious phenotypes will also explore less, both horizontally and vertically, especially in the open central region of the arena. Finally, repetitive or stereotypic movements like circling, grooming and tremors are captured in spontaneous activity monitoring, as they are often related to propensity to seizures.

There were no significant differences in spontaneous motor activity in the BACE pKO; PDAPP and PDAPP mice of Study 011C (Tables 7.2.1a-b-7.2.2a-b, Figures 7.2.1-7.2.3). Previously, in aged mice homozygous for BACE gene deletion, greater vertical activity had been associated with faster conversion to severe tonic seizures, making vertical activity one of the most interesting spontaneous measures in this study (Figure 5.6).

In all other direct comparisons of BACE pKO; PDAPP and PDAPP general activity measures there were no significant differences, although there was again a trend towards BACE pKO; PDAPP being less horizontally explorative than PDAPP mice, as in younger mice of these genotypes (Table 7.2.1a-b, 7.2.2a-b, Figure 7.2.1-7.2.2). BACE pKO; PDAPP mice tended to have greater vertical activity and more stereotypic movements than PDAPP animals, but overall little can be said about the general spontaneous motor activity levels differences between these mice.

When the Study 011C mice were assessed for activity in the open central region of the monitoring arena, there were again no statistically meaningful differences between BACE pKO; PDAPP and PDAPP mice (Tables 7.2.3a-b, Figure 7.2.3). Overall the lack of significant inter-genotype differences between BACE pKO; PDAPP and PDAPP mice, suggesting that partial removal of the BACE gene product has no effect on general spontaneous motor or anxiety behavior in aged PDAPP mice.

		Total Distance (cm)		Total Time (s)		Rests in Motion	
Factor	**N**	Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	26	287.22	277.99	34.75	29.94	62.08	19.01
Black	4	223.43	94.36	30.1	14.03	75.5	34.16
Albino	2	182.9	5.09	25.3	0.99	63	16.97
Gender							
Female	19	277.83	252.36	33.56	27.78	65.58	23.94
Male	13	265.25	264.96	33.59	27.89	61.23	15.78
Dose							
25 mg/kg PTZ	16	228.59	199.46	28.35	23.46	65.75	16.71
60 mg/kg PTZ	16	316.86	297.87	38.8	30.65	61.88	24.69
Genotype							
BACE pKO; PDAPP	16	345.28	333.67	41.69	34.95	67.56	19.57
PDAPP	16	200.17	100.5	25.46	13.65	60.06	21.99
ALL	32	272.72	253.37	33.58	27.37	63.81	20.83

	Source Factor(s)	F	DF	p-value
Distance	Gender	0.01	1	0.92
	Genotype	2.65	1	0.11
	Dose	1	1	0.33
Time	Gender	0	1	0.97
	Genotype	2.91	1	0.099
	Dose	1.2	1	0.28
Rests	Gender	0.22	1	0.64
	Genotype	0.92	1	0.34
	Dose	0.23	1	0.63

Tables 7.2.1a-b Descriptive and One-Way ANOVA statistics for locomotor activity monitoring measures (distance, movement time, and rests) in Study 011C mice by dose, gender and genotype.

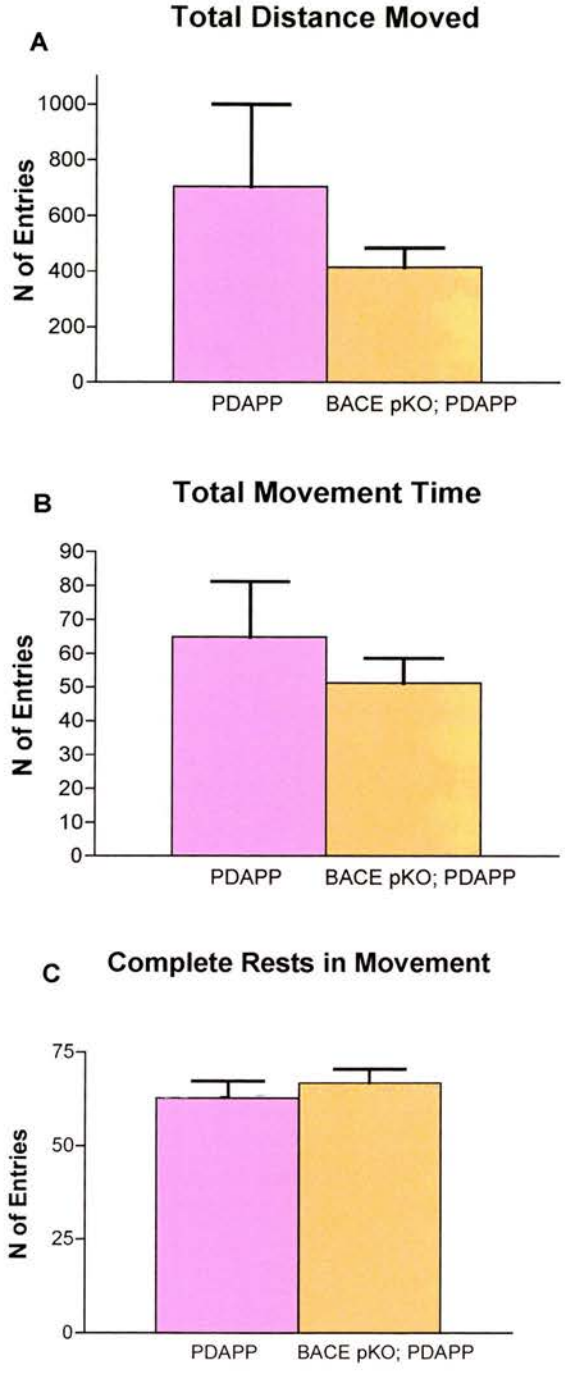


Figure 7.2.1 Spontaneous locomotor activity monitoring in 18mo Study 011C mice. A: While not a significant finding, BACE pKO; PDAPP mice had a tendency towards lesser exploration than PDAPP mice. B: BACE pKO; PDAPP mice tended to spend less time in motion than PDAPP mice. C: Study 011C mice were equivalent in the number of movement stoppages.

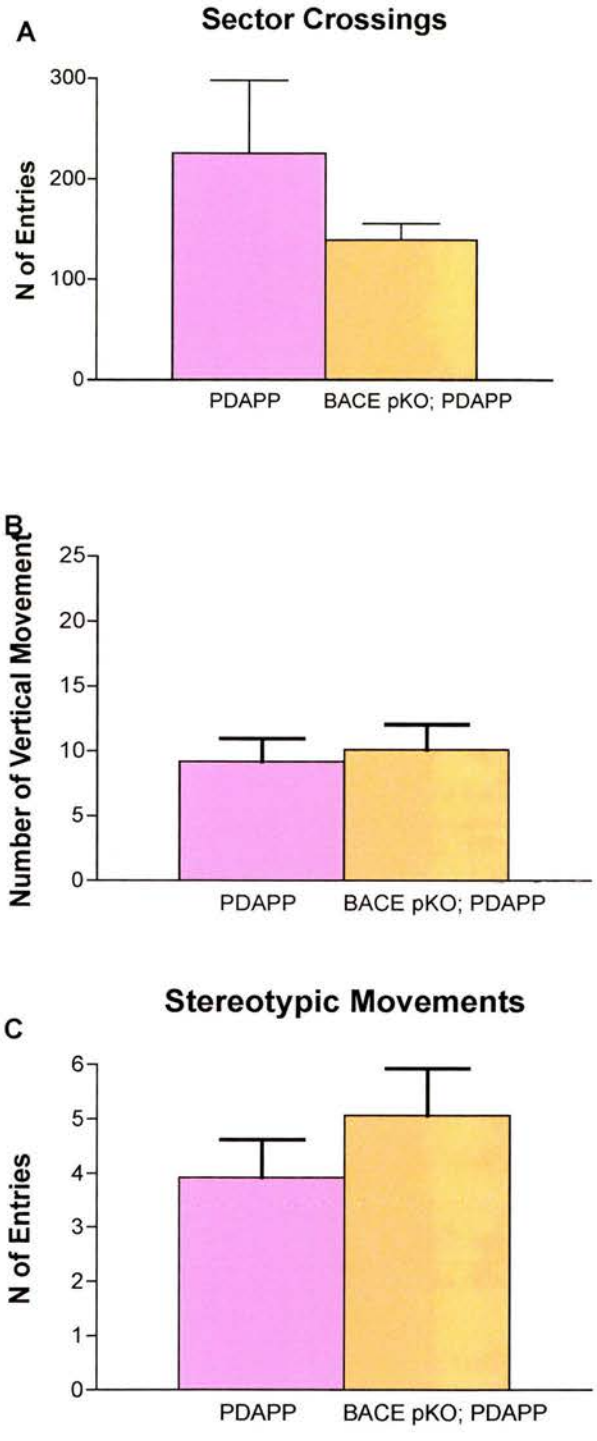


Figure 7.2.2 Spontaneous locomotor activity monitoring in 18mo Study 011C mice. A: BACE pKO; PDAPP mice had a non-significant tendency to explore a smaller area than PDAPP mice. B: Study 011C mice were equivalent in the number of vertical movements or rearings. C: BACE pKO; PDAPP mice had a greater tendency to have repetitive or stereotypic movements than PDAPP mice.

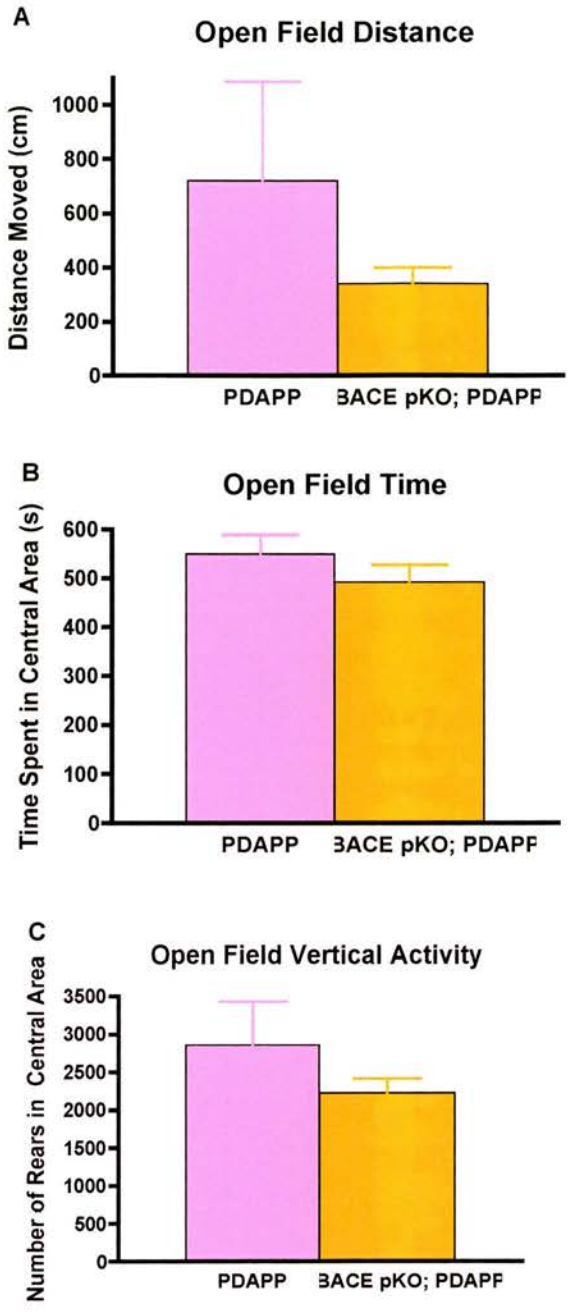


Figure 7.2.3 Open field activity in 18mo Study 011C mice. Locomotor activity in an open field area is a measure of anxiety in rodents, as anxious animals are disinclined to enter the central region of any novel environment. A: BACE pKO; PDAPP mice tend to explore less in an open field area than PDAPP mice. B: BACE pKO; PDAPP mice appear to spend slightly less time in the open field area than PDAPP mice. C: BACE pKO; PDAPP mice have less vertical activity in the open field area than PDAPP mice. These trends in aged mice are similar to results in young BACE pKO; PDAPP mice from Study 011B (Figure 6.2.3), and they collectively suggest that partial BACE gene deletion may confer an anxious phenotype on the PDAPP mouse background.

Factor	N	Sector Crossings		Vertical Rearings		Stereotypy	
		Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	26	142.12	81.46	11.42	11.29	4.77	4.11
Black	4	129	51.39	14	9.31	3.25	2.06
Albino	2	100	18.38	14.5	4.95	8.5	2.12
Gender							
Female	19	141.79	77.72	10.95	8.66	4.53	3.64
Male	13	132.08	75.47	13.38	13.28	5.23	4.42
Dose							
25 mg/kg PTZ	16	125.06	62.03	11.06	8.43	4.38	3.93
60 mg/kg PTZ	16	150.63	87.51	12.81	12.7	5.25	3.99
Genotype							
BACE pKO; PDAPP	16	157.94	97.46	13.06	11.38	5.5	4.24
PDAPP	16	117.75	38.64	10.81	10.09	4.13	3.58
ALL	32	137.84	75.73	11.94	10.64	4.81	3.92

	Source Factor(s)	F	DF	p-value
Crossings	Gender	0.11	1	0.75
	Genotype	2.2	1	0.15
	Dose	0.96	1	0.34
Rearings	Gender	0.39	1	0.54
	Genotype	0.38	1	0.54
	Dose	0.17	1	0.69
Stereotypy	Gender	0.26	1	0.61
	Genotype	1	1	0.33
	Dose	0.34	1	0.56

Table 7.2.2a-b Descriptive and One-Way ANOVA statistics for locomotor activity monitoring measures (crossings, rears and stereotypic activity) in Study 011C mice by dose, gender and genotype.

Factor	N	OF Distance (cm)		OF Time (s)		OF Rearing	
		Mean	Std	Mean	Std	Mean	Std
Color							
Agouti	26	312.21	304.59	516.1	182.81	2231.9	1012.2
Black	4	265.83	171.07	675.4	71.48	2076	962.03
Albino	2	129.65	9.97	447.3	282.56	2171	1801.71
Gender							
Female	19	301.43	286.96	548.3	156.04	2346.5	955.42
Male	13	285.61	287.07	507.5	220.33	2007.2	1095.6
Dose							
25 mg/kg PTZ	16	248.94	213.37	482.7	176.31	2210.6	761.88
60 mg/kg PTZ	16	341.07	338.84	580.7	180.85	2206.6	1238.73
Genotype							
BACE pKO; PDAPP	16	373.88	366.77	553	161.01	2385.3	1145.3
PDAPP	16	216.13	130.67	510.5	205.02	2032	858.29
ALL	32	295	282.44	531.7	182.61	2208.6	1011.61

	Source Factor(s)	F	DF	p-value
OF Distance	Gender	0.01	1	0.91
	Genotype	2.49	1	0.13
	Dose	0.87	1	0.36
OF Time	Gender	0.46	1	0.5
	Genotype	0.38	1	0.54
	Dose	2.45	1	0.13
OF Rears	Gender	0.73	1	0.4
	Genotype	0.83	1	0.37
	Dose	0	1	0.97

Tables 7.2.3a-b Descriptive and One-Way ANOVA statistics for open field locomotor activity monitoring measures (OF distance, time and rears) in Study 011C mice by dose, gender and genotype.

7.3 Rotorod Motor Coordination

To complete the motoric phenotyping of the Study 011C BACE pKO x PDAPP mice, animals were tested for involuntary motor coordination on a rotorod apparatus. Mice were placed on a rod capable of turning at constant or accelerating speeds, and measured for latency to fall. Over several trials mice generally will learn to improve their motor performances and lengthen their falling latencies. Study 011C mice were tested for 4 trials on two consecutive days in which performance on the static (10rpm) rotorod was tested on the first day and performance on the accelerating rotorod (0-40rpm) was tested on the second day (section 2.2.3).

Aged BACE pKO; PDAPP mice had tendency to perform poorly on the accelerating rotorod compared to aged PDAPP mice, while they were indistinguishable on the constant speed rotorod paradigm (Accelerating Rotorod: F=2.64, df 1/268, p=0.059Tables 7.3a-d; Figure 7.3a-b). In both constant and accelerating rotorod tests, BACE pKO; PDAPP mice showed little improvement in their performance over a number of trials, a finding that was similar to that of similarly aged mice with homozygous BACE KO genotypes in Study 011A. However, before claiming that BACE gene deletions predispose mice to poorer motor coordination, one important caveat about the comparability of animals from different lines must be mentioned. PDAPP mice of Study 011A were superperformers with the longest latencies to fall and greatest rates of improvements, while Study 011C PDAPP mice had performance levels that were far below these high standards. It must be stated that while these mice are genotypically identical with respect to the PDAPP transgene, they are in fact from different colonies with divergent breeding schemes, which could affect the comparability of inter-group behavioral testing (Figure 2.11). Mice of both genotypes in Study 011C had notably very poor rotorod performances, which were easily the shortest falling latencies in accelerating tests of animals tested in either Study 011A or 011B. This said, it still appears that certain patterns of motor performance in BACE KO mice over the course of 2 separate studies have been replicated, suggesting that both complete and partial BACE KO confers a motor coordination deficit in aged mice.

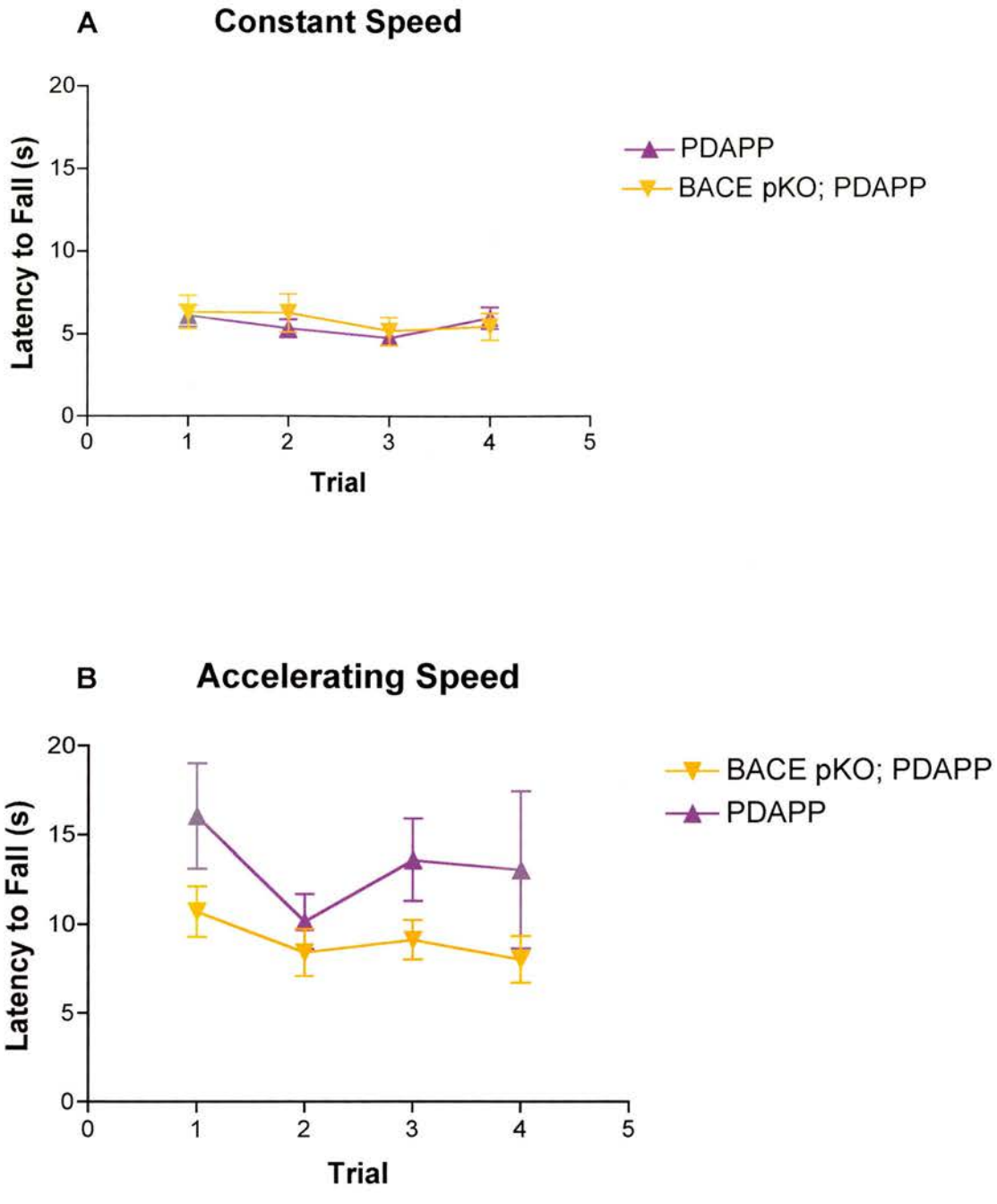


Figure 7.3 Motoric rotorod coordination in 18mo Study 011C mice. A: Aged Study 011C mice do not have genotypic differences in their performance on the constant speed rotorod. B: BACE pKO; PDAPP mice have a tendency to fall sooner from the accelerating rotorod compared to PDAPP mice. Animals of both genotypes are exceedingly poor performers on this task compared to younger BACE pKO; PDAPP and PDAPP mice (Figure 6.3) and do not display any improvement over trials, suggesting either/or a major motor coordination phenotype and/or deficit in motor skills learning in both genotypes at this age.

Factor	N	Trials							
		1		2		3		4	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	26	6.3	4.21	6.51	4.35	4.59	3.05	5.48	3.23
Black	4	4.82	2.62	2.71	2.32	4.74	1.87	4.62	1.17
Albino	2	7.76	3.62	6.27	0.42	2.57	2.62	3.87	2.83
Gender									
Female	19	6.48	4.93	6.05	5.28	4.39	3.42	4.83	2.66
Male	13	5.82	2.16	5.94	1.98	4.6	2.02	5.88	3.42
Dose									
25 mg/kg PTZ	16	6.62	5.01	6.7	5.36	5.01	3.32	5.08	2.25
60 mg/kg PTZ	16	5.76	2.54	5.26	2.29	3.91	2.3	5.47	3.7
Genotype									
BACE pKO; PDAPP	16	5.99	5.24	6.09	5.44	4.14	3.61	4.5	2.26
PDAPP	16	6.41	2.4	5.92	2.65	4.8	2.05	5.99	3.47
ALL	32	6.2	3.97	6	4.17	4.48	2.88	5.27	3

Source Factor(s)	F	DF	p-value
Gender	0.38	1	0.82
Genotype	0.72	1	0.59
Dose	0.45	1	0.77

Tables 7.3a-b Descriptive and One-Way ANOVA statistics for performance on the constant speed rotorod in Study 011C mice by dose, gender and genotype.

Factor	N	Trials							
		1		2		3		4	
Color		Mean	Std	Mean	Std	Mean	Std	Mean	Std
Agouti	26	12.54	10.36	8.41	6.82	13.94	9.75	12.74	19.8
Black	4	13.11	7.54	10.4	7.22	6.27	2.1	5.33	1.18
Albino	2	6.8	2.44	4.88	0.42	2.51	1.1	5.09	2.43
Gender									
Female	19	14.26	11.16	9.04	7.84	12.65	11.21	13.61	22.62
Male	13	9.04	5.85	7.49	4.17	11.51	6.26	7.62	4.42
Dose									
25 mg/kg PTZ	16	9.87	6.72	7.8	3.79	13.48	6.83	9.61	6.95
60 mg/kg PTZ	16	14.77	11.82	9.13	8.81	10.85	11.8	13.09	25.18
Genotype									
BACE pKO; PDAPP	16	9.25	4.51	7.15	4.12	8.4	4.19	7.82	6.34
PDAPP	16	15.05	12.31	9.65	8.28	15.78	11.65	14.55	24.19
ALL	32	12.24	9.69	8.44	6.62	12.21	9.49	11.29	17.97

Source Factor(s)	F	DF	p-value
Gender	0.94	1	0.46
Genotype	2.64	1	0.059
Dose	1.75	1	0.17

Tables 7.3c-d Descriptive and One-Way ANOVA statistics for performance on the accelerating speed rotorod in Study 011C mice by dose, gender and genotype.

Seizure Phenotypes

7.4 PTZ-Induced Seizures

Pharmacologic seizure studies were conducted on Study 011C mice, based on the premise that prior seizure experience and/or genetic propensity to kindle seizures would produce differential epileptic response profiles (section 2.3). Previous experiments with aged homozygous BACE KO x PDAPP mice revealed that BACE KO; PDAPP mice had reduced resistance to seize compared to seizure-prone PDAPP mice (section 5.4). Testing of younger BACE pKO; PDAPP mice showed no such differences in seizure propensity compared to the PDAPP response to seizure, but this result could have been due to effects of age, as these mice were 5mo old and may have had little cumulative seizure activity if they were so disposed (section 6.4). In Study 011C aged BACE pKO; PDAPP were tested to provide a better frame of comparison to the homozygous BACE KO; PDAPP mice of Study 011A.

In addition, the design of Study 011C allowed for the examination of BACE pKO; PDAPP responses to lower doses of PTZ (25mg/kg) which elicit mild partial clonic seizures (Tables 7.4.1a-b, Figure 7.4.1a-b). Mice treated with 25mg/kg of PTZ had a range of mild seizure activity consisting of sporadic localized head or tail twitches over a period of 30min, in which all animals survive. There was no statistical difference between BACE pKO; PDAPP and PDAPP mice in the onset or scores for partial clonic seizure response to a low dose of PTZ (Genotype: $F<1$; Figure 7.4.1a-b).

At 60mg/kg, treatment of Study 011C mice was almost uniformly lethal, with 100% death in BACE pKO; PDAPP and 87.5% death in PDAPP mice (data not shown). The increase in lethality in response to PTZ could be based on the change of drug lots between Study 011B and 011C, as over a year had elapsed to allow for the aging of animals and the prior lot had been replaced. Seizure latencies by type in mice treated with 60mg/kg PTZ were not significantly different between genotypes (Genotype: $F<1$; Tables 7.4.1a-b, Figure 7.4.2a). There was a trend towards longer latencies in the more severe types of seizures in BACE pKO; PDAPP mice, suggesting that these mice had experienced lesser spontaneous seizure activity throughout life and could resist kindling severe convulsions. Analysis of time to death in Study 011C mice given high doses of PTZ revealed a longer latency in BACE pKO;

PDAPP that bordered on statistical significance, also hinting that animals with BACE pKO had greater seizure resistance than PDAPP mice ($p=0.056$, Figure 7.4.2c). In composite seizure scores as well as seizure scores by type, there was no statistically meaningful difference between BACE pKO; PDAPP and PDAPP mice (Tables 7.4.2a-b). It is worth mentioning that BACE pKO; PDAPP mice did have a tendency towards lesser composite scores than PDAPP mice in Study 011C, a finding which was reproduced in Study 011B (Figures 6.4d, 7.4.3a).

Finally, seizure response to PTZ in Study 011C animals was largely dose-dependent, as animals receiving 60 mg/kg of PTZ generally had significantly shorter latencies to partial clonic seizures than animals treated with 25mg/kg of PTZ (Dose: $F=19.69$, df 1/31, $p=0.0002$; Table 7.4.2a-b, Figure 7.4.3b). BACE pKO; PDAPP mice appeared have lesser seizure responses than PDAPP mice at high PTZ doses, suggesting that partial BACE gene ablation may confer resistance to seizures on an hAPP background. Overall, higher doses of PTZ produced the expected profile of more severe seizure activity in shorter times, in which dose-based differences in seizure response within genotypes was highly significant ($F=152.3$, df 3/31, $p<0.001$; Tables 7.4.1a-b; Figure 7.4.3a). The seizure data from Study 011C suggests that BACE pKO on a PDAPP background has a protective effect on seizure resistance, but it is parsimonious to say that at a minimum partial deletion of BACE in this experiment did not amplify propensity to seizure, and did not uncover any sign of greater spontaneous seizure activity in these mice.

There were significant differences in gender response to PTZ seizure induction as aged Study 011C female mice had shorter latencies to the first signs of mild seizure compared to males (Tables 7.4.1a-b). This finding is the opposite of the gender effect seen in Study 011B, as young male mice from the BACE pKO x PDAPP line had shorter general clonic seizure and death latencies and higher overall composite seizure scores. This age-related shift was similar to that seen upon observation of spontaneous seizures in Study 001 and 006 (Table 7.4.3). While this it is not unexpected that there could be gender differences in response to PTZ treatment, this age-related change in seizure pattern by gender is unusual, and the mechanistic basis, if any, is unknown.

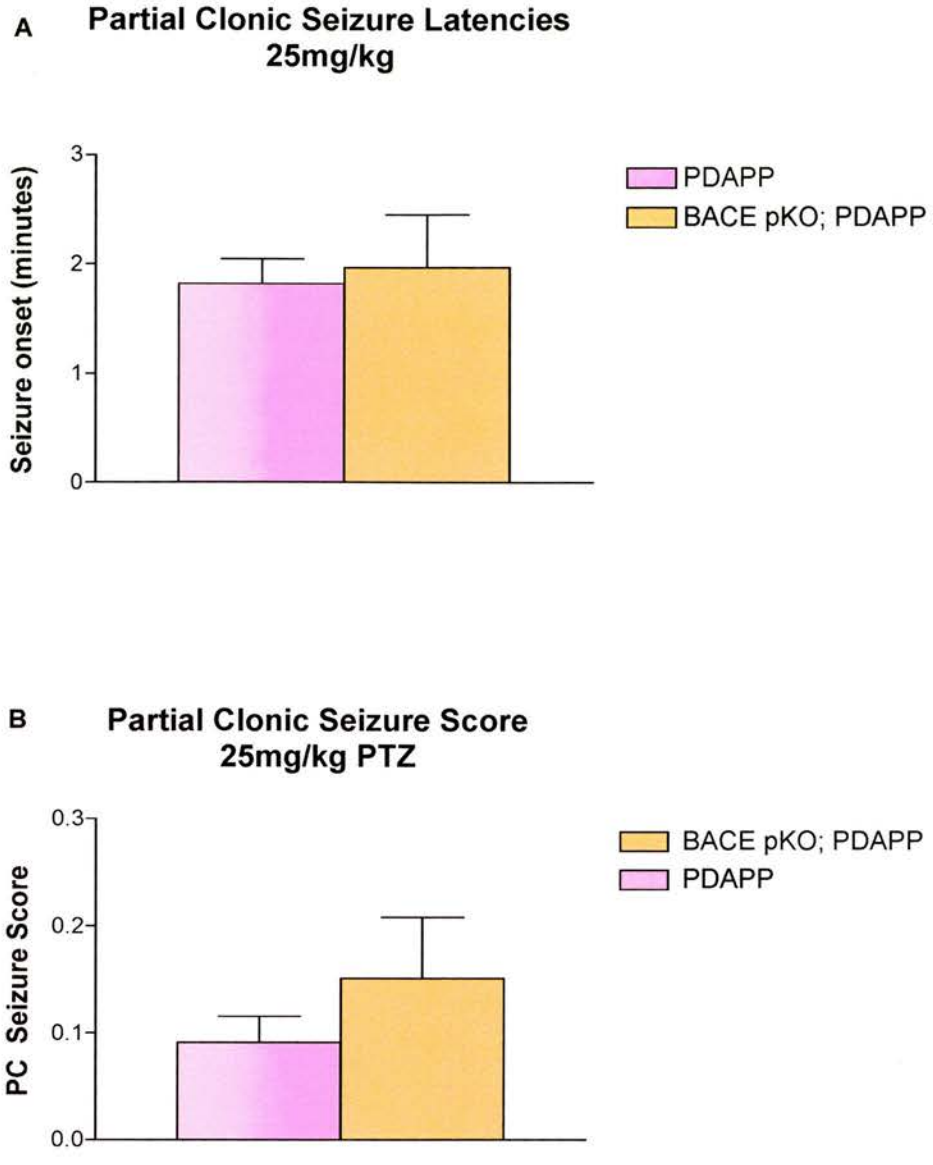


Figure 7.4.1 Low dose PTZ-induced seizures in 18mo Study 011C mice. A: Study 011C mice treated with 25 mg/kg i.p. PTZ do not differ appreciably in the latency to mild clonic seizure activity. B: Aged BACE pKO; PDAPP mice have a tendency to have higher partial clonic seizure scores than PDAPP mice.

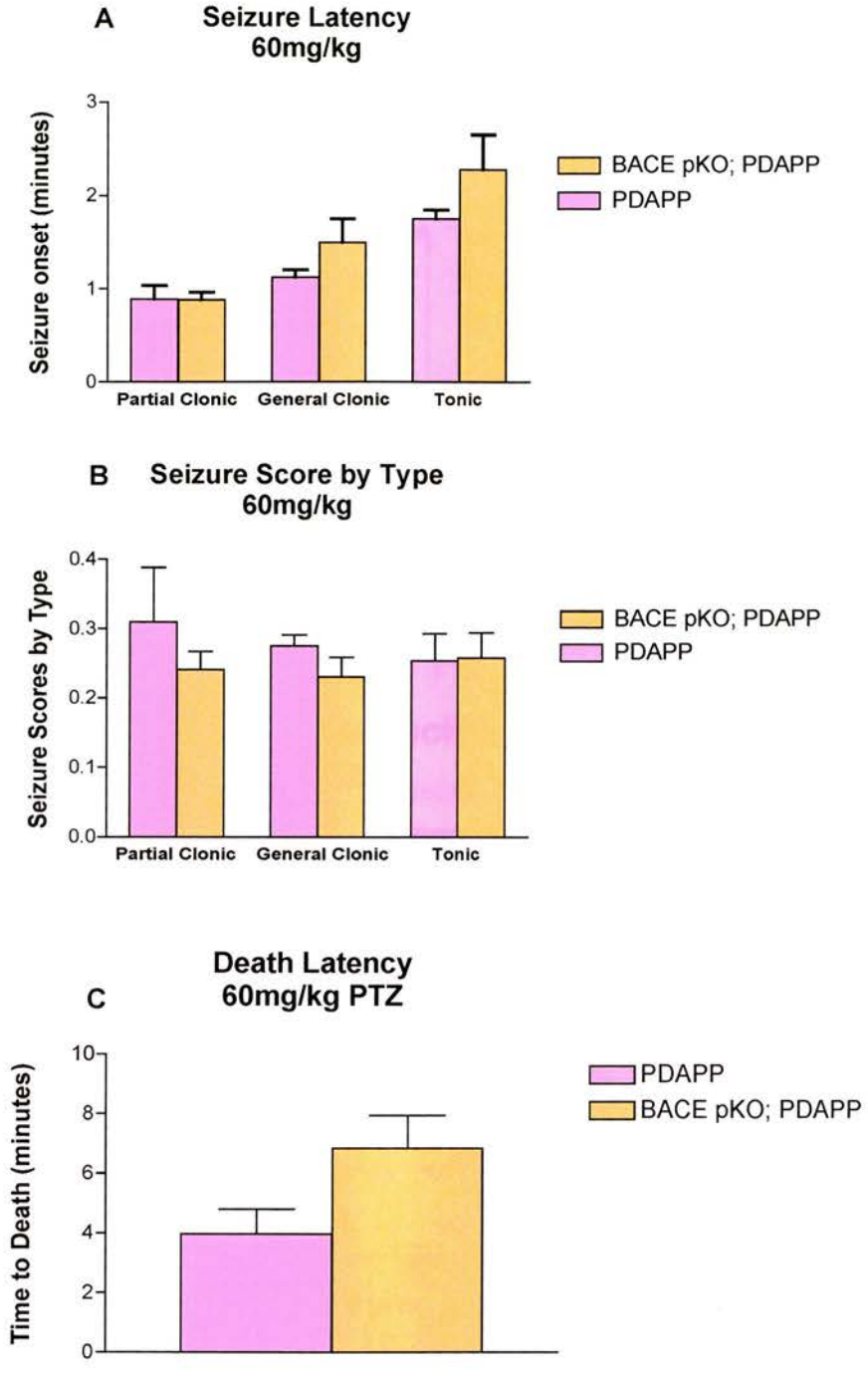
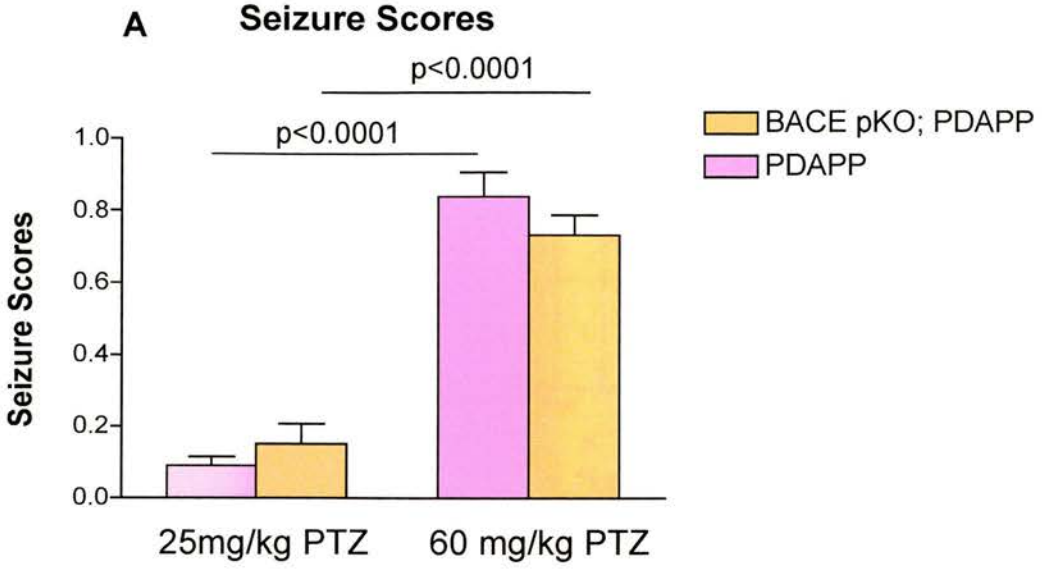


Figure 7.4.2 High dose PTZ-induced seizures in 18mo Study 011C mice. A: Given 60 mg/kg of PTZ, BACE pKO; PDAPP mice tend to resist developing moderate (general clonic) and severe (tonic) seizures compared to PDAPP mice. B: PDAPP mice appear to have slightly higher contributions from mild and moderate seizures than BACE pKO; PDAPP mice, suggesting a greater overall seizure liability in these mice. C: Upon high dose treatment with PTZ, BACE pKO; PDAPP mice have a nearly significant tendency to survive for longer periods of time than PDAPP mice (p=0.056).



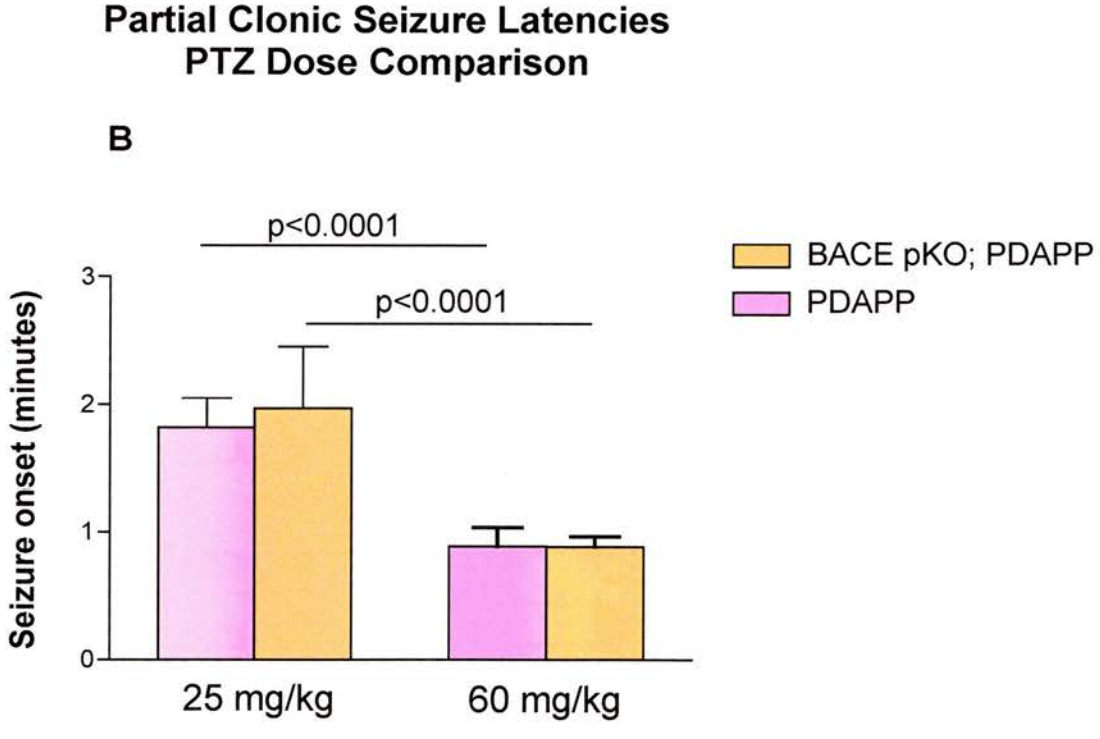


Figure 7.4.3 Comparison of low and high dose PTZ-induced seizures in aged 180mo Study 011C mice. A: Composite seizure scores in both BACE pKO; PDAPP and PDAPP mice increase in a significantly dose-dependent fashion following treatment with 25 or 60 mg/kg of PTZ. B: Study 011C mice treated with high doses of PTZ develop partial clonic seizures significantly faster than similar mice treated with a low dose of PTZ.

Factor	Seizure Onset (min)			Death Latency			Seizure Score		
	N	Mean	Std	N	Mean	Std	N	Mean	Std
Color									
Agouti	23	1.43	0.89	12	5.5	3.38	26	0.4	0.35
Black	4	1	0.8	2	4.22	2.99	4	0.62	0.46
Albino	2	1.08	0.42	2	5.97	1.13	2	0.87	0.13
Gender									
Female	17	1.1	0.66	9	5.92	3.84	19	0.46	0.39
Male	12	1.69	1	7	4.74	1.65	13	0.44	0.36
Dose									
25 mg/kg PTZ	13	1.9	0.97	0	NA	NA	16	0.12	0.12
60 mg/kg PTZ	16	0.89	0.32	16	5.4	3.05	16	0.79	0.18
Genotype									
BACE pKO; PDAPP	15	1.39	1.02	8	6.84	3.12	16	0.44	0.34
PDAPP	14	1.29	0.66	8	3.96	2.35	16	0.47	0.41
ALL	29	1.34	0.85	16	5.4	3.05	32	0.45	0.37

	Source Factor(s)	F	DF	p-value
Seizure Latency	Gender	7.29	1	0.012
	Genotype	0.12	1	0.73
	Dose	19.69	1	0.0002
Death Latency	Gender	N/A	1	0.72
	Genotype	N/A	1	0.61
	Dose	N/A	N/A	P<0.0001
Seizure Score	Gender	1.79	1	0.19
	Genotype	0.27	1	0.61
	Dose	152.5	1	P<0.0001

Table 7.4.1a-b Descriptive and One–Way ANOVA statistics for seizure responses and lethality in Study 011C mice by dose, gender and genotype.

Factor	Female		Male		All	
	Number of Mice	n (%) that died	Number of Mice	n (%) that died	Number of Mice	n (%) that died
Dose						
25 mg/kg PTZ	10	0 (0%)	6	0 (0%)	16	0 (0%)
60 mg/kg PTZ	9	9 (100%)	7	7 (100%)	16	16 (100%)
Genotype						
BACE pKO; PDAPP	10	5 (50.0)	6	3 (50.0)	16	8 (50.0)
PDAPP	9	4 (44.4)	7	4 (57.1)	16	8 (50.0)
ALL	19	9 (47.4)	13	7 (53.9)	32	16 (50.0)

	Source Factor(s)	P-Values
Lethality	Gender	1
	Genotype	1
	Dose	P<0.0001

Tables 7.4.1c Descriptive and Fisher's Chi-squared test statistics for PTZ-induced lethality in Study 011C mice by dose, gender and genotype.

Factor	PC Score			GC Score			TC Score		
	N	Mean	Std	N	Mean	Std	N	Mean	Std
Color									
Agouti	23	0.2	0.13	12	0.25	0.07	11	0.26	0.08
Black	4	0.36	0.28	2	0.25	0.07	2	0.27	0.03
Albino	2	0.2	0.08	2	0.3	0	2	0.37	0.05
Gender									
Female	17	0.26	0.19	9	0.24	0.08	8	0.27	0.09
Male	12	0.16	0.07	7	0.27	0.05	7	0.27	0.08
Dose									
25 mg/kg PTZ	13	0.15	0.12	0	NA	NA	0	NA	NA
60 mg/kg PTZ	16	0.28	0.16	16	0.25	0.07	15	0.27	0.08
Genotype									
BACE pKO; PDAPP	15	0.21	0.12	8	0.23	0.08	8	0.26	0.1
PDAPP	14	0.23	0.19	8	0.28	0.05	7	0.29	0.04
ALL	29	0.22	0.16	16	0.25	0.07	15	0.27	0.08

Source	Factor(s)	F	DF	p-value
PC Score	Gender	4.93	1	0.036
	Genotype	0.12	1	0.73
	Dose	6.51	1	0.017
GC Score	Gender	0.67	1	0.43
	Genotype	1.47	1	0.25
	Dose	NA	NA	NA
TC Score	Gender	0.04	1	0.85
	Genotype	0.58	1	0.46
	Dose	NA	NA	NA

Tables 7.4.2a-b Descriptive and One–Way ANOVA statistics for seizure component scores in Study 011C mice by dose, gender and genotype.

	3 mo	13 mo	18 mo
Gender	Mice Removed (% of Total N)	Mice Removed (% of Total N)	Mice Removed (% of Total N)
Study 001			
Male	3 (7.3%)	3 (7.1%)	7 (11.6%)
Female	5 (7.7%)	2 (4.8%)	5 (11.4%)
Study 006			
Male	2 (6.9%)	0	0
Female	0	0	2 (17.2%)

Table 7.4.3 Descriptive statistics of spontaneous tonic-clonic seizures observed in Study 001 and 006 mice by gender.

Calbindin and Amyloid Histology

7.5 Calbindin Histology in the Hippocampal Outer Molecular Layer

Calbindin (CB) is a Ca^{++} binding protein found in neurons that has been shown to increase in experimental conditions that include seizure activity. As CB has been implicated as a marker for hippocampal neuron function, it was particularly interesting to analyse BACE pKO; PDAPP brain tissues for CB intensity levels following treatment with both low and high doses of a pharmacologic seizure-inducing agent. While treatment of PTZ at 60 mg/kg in young hemizygous BACE KO mice and aged homozygous BACE KO mice proved to be equivocal in CB levels, it is possible that analyzing data from aged groups given low doses of PTZ could provide information on any shifts in CB levels as a response to increasing doses of PTZ. Study 011C mouse brain tissues were immunostained with an antibody for CB with fluorescent intensity levels captured using a confocal microscope and quantified as described in section 2.4.1 using the NIH Image program.

At low doses of PTZ, levels of CB immunoreactivity were statistically equivalent between BACE pKO; PDAPP and PDAPP mice (Figure 7.5a). At 60 mg/kg of PTZ, CB was lower in PDAPP mice relative to BACE pKO; PDAPP mice ($F=2.614$, df 3/29, $p=0.05$; Figure 7.5a). It appears that the response of mice from each genotype is different with escalating doses of CB, as PDAPP mice have somewhat reduced levels of CB going from 25 to 60mg/kg doses of PTZ, while BACE pKO; PDAPP mice had static levels of CB with the same doses. These shifts in CB level by genotype are difficult to interpret, although there are certainly possible explanations.

CB is a marker for Ca^{++} homeostasis in neurons, and is considered a functional marker because normal living neurons have high requirements for Ca^{++} trafficking. Previous interpretations of correlational analysis of aged hemizygous BACE KO mice implied that high CB levels were associated with cognitive function (table 4.8.3). It may be that in the PTZ-untreated mouse high levels of CB are synonymous with behavioral function because CB is being properly used by the neurons and are thus reflective of activity. When low doses of PTZ are applied there is large range of responses in PDAPP mice, with a much smaller variability in BACE pKO; PDAPP mice. It may be that this difference is due to depletion of

CB rather than divergence in the original level of CB. Indeed it is possible that BACE pKO; PDAPP mice were better able to utilize their CB and regulate their intracellular Ca^{++} trafficking, so that they have a more static level of CB even in the face of neuronal challenge, allowing them to resist seizure onset and death for longer periods of time. In fact there was a trend towards CB levels increasing with higher doses of PTZ in BACE pKO; PDAPP animals, suggesting that they retain cellular responsiveness to severe neuronal activation stressors.

Immunostaining with an antibody to hAPP to confirm the presence of the PDAPP transgene in these mice revealed no mistyped animals.

Factor	N	Mean	STD
Color			
Agouti	25	86.35	16.9
Black	3	73.64	5.19
Albino	2	82.54	4.14
Gender			
Female	17	87.39	18.29
Male	13	81.47	12.1
Dose			
25 mg/kg PTZ	15	86.78	19.78
60 mg/kg PTZ	15	82.87	11.27
Genotype			
BACE pKO; PDAPP	15	82.97	11.54
PDAPP	15	86.68	19.64
ALL	30	84.82	15.94

Source Factor(s)	F	DF	p-value
Gender	0.98	1	0.33
Genotype	0.43	1	0.52
Dose	0.3	1	0.59

Tables 7.5a-b Descriptive and One–Way ANOVA statistics for Calbindin immunoreactivity in Study 011C mice by dose, gender and genotype.

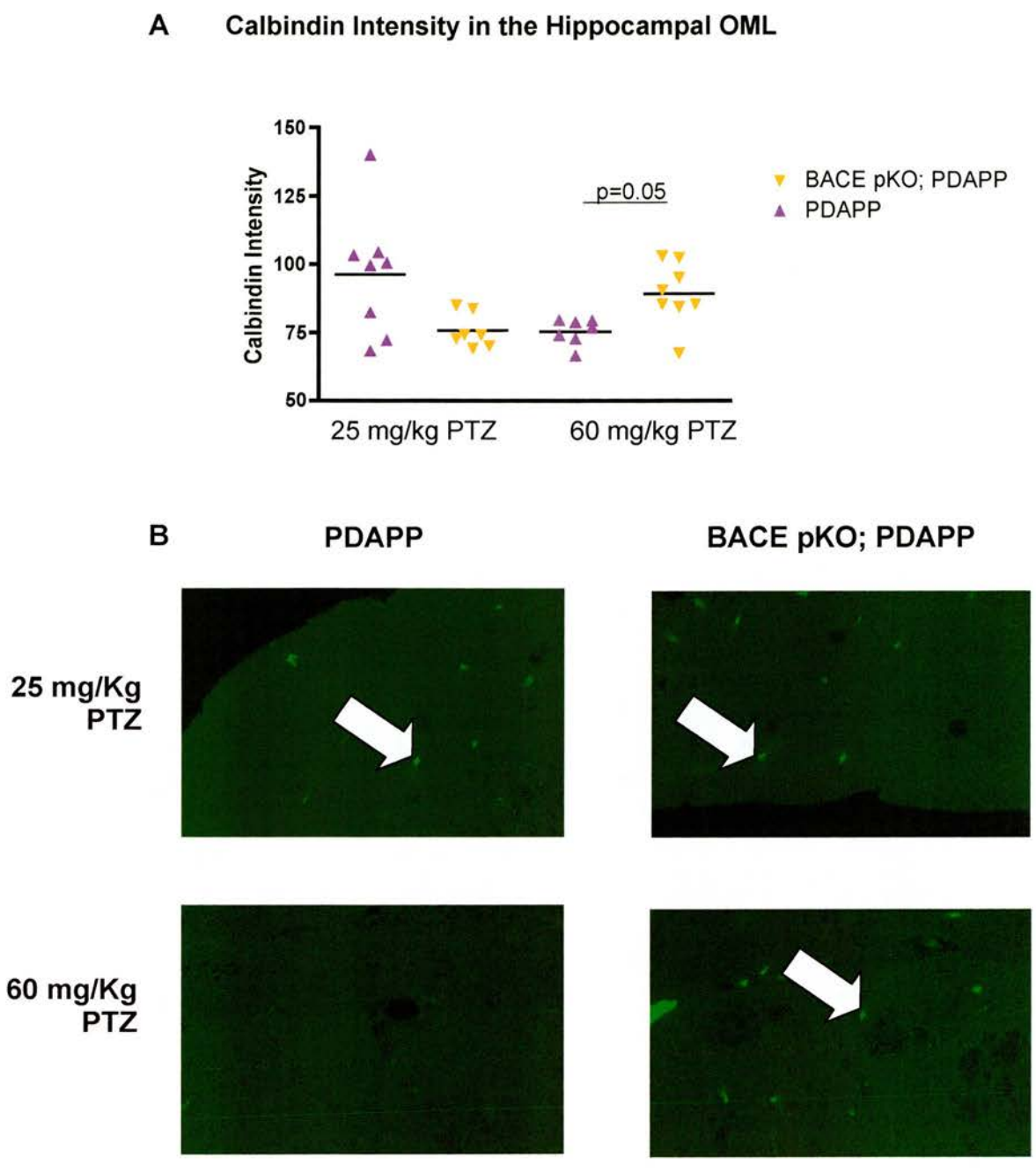


Figure 7.5 Calbindin (CB) immunoreactivity and images in the hippocampal outer molecular layer of 18mo Study 011C mice. A: After treatment with 25 mg/kg of PTZ, BACE pKO; PDAPP mice have statistically equivalent CB levels as PDAPP mice. PDAPP mice treated with 60 mg/kg PTZ have significantly lower CB levels than BACE pKO; PDAPP mice. B: Images of the CB immunoreactivity, displaying sections with intensity values close to the genotype group average are also visually equivalent between BACE pKO; PDAPP and PDAPP mice. White arrows point to brightly stained blood vessel artifacts.

7.6 Correlation Analyses of Behavioral and Histological Data

Correlation Analysis Cell Key

R-Values Colorimetrics P-Values Colorimetrics Self-Correlation

Column Abbreviations

PC Lat =Latency to Partial Clonus
GC Lat= Latency to General Clonus
TC Lat = Latency to Tonic Seizure
Score = Composite seizure score
DeathT = Latency to death
PCscore= Partial clonus component of seizure score
GCscore= General clonus component of seizure score
TCscore = Tonic seizure component of seizure score
CB Int = Calbindin Intensity in the Hippocampal Outer Molecular Layer
PositTone = Positional sense/tone
2RR1 = Constant speed rotorod trial 1
2RR4 = Constant speed rotorod trial 4
3RR1 = Accelerating speed rotorod trial 1
3RR4 = Accelerating speed rotorod trial 4
SectX1 = Sector crossings, session 1
Rest1 = Complete rests in motion, session 1
Dist1 = Total distance traveled, session 1
Time1 = Total time spent in motion, session 1
Rear1 = Vertical activity counts, session 1
Stereo1 = Number of stereotypic movements, session 1
Open Dist1 = Total open field distance traveled, session 1
Open Dist2 = Total open field distance traveled, session 2
Open Time1 = Total time spent in motion in open field, session 1
Open Time2 = Total time spent in motion in open field, session 2
Open Vert1 = Vertical activity counts, session 1
Open Vert2 = Vertical activity counts, session 2

Example Correlation Table, Behavioral and Immunochemical Measures

R-values from correlation analysis of each measure

p-values from correlation analysis of each measure

Example Correlation Table, Hippocampal Calbinidin Intensity/Behavioral Measures by Genotype

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscore	GCScore
PDAPP	0.7857					-0.7857	
BACE pKO; PDAPP							

60 mg/kg PTZ P-values

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscore	GCScore
PDAPP	0.048					0.048	
BACE pKO; PDAPP							

Figure 7.6.1a-b Example correlation tables. A: Correlation table of relationships between various behavioral measures, with R-values presented in upper diagonal section and p-values presented in lower diagonal section. Corresponding p-values and R-values are found in the same coordinate distance from the black diagonals separating the two types of values, with R-values at x_r,y_r coordinates, and p-values at y_p,x_p coordinates where $x_r=y_p$ and $y_r=x_p$ Values contained within red circle indicate significant intrameasure correlations, e.g. clonic to tonic seizure latency. Values contained within blue circles indicate significant intermeasure correlations, e.g. clonic seizure latency to Calbindin intensity. B: Correlation table of various behavioral measures and Calbindin intensity separated by genotype. R-value tables are above while P-values are below. Values in green circles indicate significant behavioral/Calbindin correlations.

Correlational analysis provides a mathematical method for finding associations between diverse behaviors, pharmacologic responses and histological markers. These associations are tested with the premise that measures with values that vary with respect to each other are linked by some underlying neuronal processes. Using individual measure data collected from Study 011C animals were analysed against all other measures with Spearman's Correlational Test that non-parametrically assigns log ranks to each value within a measure set. The resulting correlation R values and statistical significance p-values were then arranged into a table format, in which diagonal cells have self-values which by definition have R=1 and $p<0.0001$. Accordingly, each half of the tables is reflexive, having R-values that are columns in one direction and p-values that are rows in the other, intersecting with identical or self correlations in black boxes that run diagonally across the table.

Intra- and intermeasure relationships

Correlational analysis of all Study 011C mice revealed a continuation of relationship patterns seen in Study 011A and 011B. Again there were several groups of behavioral measures that formed functional sets, as they were highly correlated to each other. These behavioral intersets were not much different from the previous two seizure studies:

- General Clonic (GC)/Tonic Seizures (TC)
- Constant and Accelerating Rotorod, with broader relationships to seizure measures than in Study 011A
- Horizontal locomotor activity, and subset open field measures
- Vertical/Stereotypic locomotor activity

Overall, a quick glance at the correlational maps of Study 011A and 011C reveal visual patterns of similarities, possibly due to their similarity in age (Tables 5.6, 5.7, 7.6, 7.7). There was again a strong relationship between GC and TC measures. GC latency and scores were associated to rotorod performance such that poor performance in the rotorod seemed to be predictive of higher scores in GC seizures (GC/TC: $R=0.520$, $p<0.05$; 3RR1/GC Score: $R=-0.576$, $p<0.025$). GC latencies and Scores were also correlated to vertical activity, as increased values in Rears were associated with lower latencies to GC seizures, a finding that

Table 7.6 Correlation of pharmacologic, behavioral and histological measures, with resulting R- and p-values of 18mo Study 011C mice. Table is located in pocket at back cover of document.

This table of correlation values underlines several intrameasure and intermeasure relationships.

Intrameasure values are highly correlated within each set, as performance on one measure is likely to be functionally related to performance on a similar measure. These intrameasure correlations are circled in red, and are closest to the table diagonal separating R- and p-values. Thus many measures within PTZ-seizure induction, trial performances over days of testing in the rotorod, and the general and open field activity monitoring values have high degrees of correlations within each set.

Intermeasure relationships occur between different task measures and are circled in blue, typically distant from the R-/p-value diagonal. Correlations between these metrics suggest that although the tasks vary methodologically, the underlying functional and/or anatomical bases for their performance are similar, and even predictive of one another. In particular, resistance to general clonic seizure response and longer times to death post-seizure appear to be correlated to higher CB intensity and performance on the constant speed rotorod. Resistance to general clonic seizure is also seen in animals with high levels of vertical activity, while resistance to the mildest (partial clonic)forms of seizure is associated with greater locomotor activity. Lastly, longer times spent in the open field is correlated in aged Study 011C mice to superior performance in the constant speed rotorod task.

makes intuitive sense as pathological repetitive behaviors are often post-seizure sequelae. In addition, dose of PTZ was strongly correlated to PC Lat, PC Score, and TC Score, which replicates the findings of section 7.4 using a dramatically different statistical analysis, and lends credence to the power of correlational analysis to uncover sensible relationships between measures.

The entire set of horizontal locomotor measurements were so highly related that they could almost be considered interchangeable, as $R>0.607$ and $p<0.001$ between all combinations of SectX1, Rest1, Dist1 and Time1. In addition, Study 011C animals had values in Stereo1 that were also strongly related to Rear1, as seen before in Studies 011A and 011C, but also to horizontal measures like Time1 and Dist1 (Rear1/Stereo $R=0.653$, $p<0.001$; Dist1/Stereo1 $R=0.468$, $p<0.01$; Time1/Stereo1 $R=0.491$, $p<0.005$). Measures like PositTone, 2RR1, and 2RR4 did not have any significant relationships to any other measures. Unlike the results from Studies 011A and 011B, there were few significant relationships between open field measures and other tasks, as open field time correlated to performance on the first day of the accelerating rotorod ($R=0.47$, $p<0.01$).

When separated by genotype and dosage of PTZ treatment, correlations between CB intensity in the hippocampal outer molecular layer and other measures become apparent. BACE pKO; PDAPP animals treated with lower doses of PTZ had positive correlations between CB intensity and performance on the rotorod such that higher latencies to fall off the accelerating rotorod were associated with higher CB levels ($R=0.786$, $p<0.05$). At the higher doses of PTZ however, there were no relationships between CB and behavioral or pharmacologic measures in BACE pKO; PDAPP mice. In PDAPP mice given 60 mg/kg of PTZ, there was a positive mathematical correlation between PC measures and CB intensity, as though higher CB levels were related to resistance to developing PC seizures ($R=0.786$, $p<0.05$). This pattern was reminiscent of that seen in Study 011B with BACE pKO; PDAPP mice, which had the same positive correlations between CB and TC seizure activity (Table 6.7). While these are singular relationships, it appears that there is some kind of pattern in animals given high doses of PTZ, such that CB levels may indicate protection against development of seizure activity. As the statistical significance of these correlations were tenuous, this analysis would

ultimately need to be reproduced for any strong statements about CB levels in the hippocampus as a predictor for protection against seizure activity.

However, the correlation data from these studies using aged BACE KO or BACE pKO mice consistently argue that performance on the rotorod is predictive of seizure propensity. Given the poorest rotorod performers were aged animals with some kind of BACE KO, this also suggests that homozygous deletion of BACE does confer a deleterious seizure-related phenotype (Figures 5.3, 7.3). There are biological possibilities for a lack of motor strength and coordination in BACE-deficient animals, as BACE1 distribution patterns are altered in patients with Inclusion Body Myositis (IBM), a muscular degeneration disease that features amyloid deposits in vacuolated muscular fibres (Askanas V, 1992; Vattemi et al., 2001; Vattemi et al., 2003). As BACE1 is implicated in the development of neuromuscular junctions, it is possible that BACE KO mice over time develop an IBM-like phenotype. Earlier analysis of BACE KO mice did not reveal any muscular pathology, but these studies were performed in young mice, which would preclude discovery of an age-related muscular phenotype.

Table 7.7 Correlation of Calbindin (CB) to all other measures, R- and P-values of 18mo Study 011C mice. Table is located in pocket at back cover of document.

Specific assessment of CB correlations to behavioral and pharmacological metrics was done by genotype to discern wider patterns of predictive functional relationships. In Study 011C mice treated with a low 25 mg/kg dose of PTZ, BACE pKO; PDAPP animals, high levels of CB are associated with superior performance in the accelerating rotorod paradigm. However this relationship shifts in animals treated with higher 60 mg/kg doses of PTZ, as Study 011C PDAPP mice with high CB levels are poor performers on the constant speed and accelerating rotorod. However, this inverse relationship between function and CB levels is not broadly applicable as PDAPP mice with high CB levels do have greater resistance to developing mild seizures. Taken together these correlations suggest at the minimum that if CB is truly a biomarker for hippocampal function, the relationship to other functions is not a simple one.

Ch.8 Discussion and Summary

8.1 Phenotypic characterization of BACE KO x PDAPP mice

The behavioral and histological characterization of the BACE KO x PDAPP mouse line was conducted to test the straightforward hypothesis that removal of the rate-limiting enzyme β-secretase on a mutant human APP expressing background would positively impact the deleterious cognitive and histological phenotypes present in the PDAPP transgenic mouse. Although amelioration of PDAPP deficits represents the most supportive outcome for the BACE inhibition strategy of Alzheimer's Disease, there are in fact four possible outcomes on any given phenotypic measure from the crossing of BACE KO and PDAPP mice, which were outlined in Ch.1 on p. 66-7 (these outcomes also could be age-related and evolve over time):

- Deletion of BACE on a background overexpressing Aβ could rescue the mouse cognitive deficits associated with the PDAPP transgene.
- Deletion of BACE and subsequent loss of β-CTF and Aβ could worsen the phenotype if these metabolites and/or some other substrate of BACE are required for normal learning and memory as well as general neuronal activity regulation in mice.
- Deletion of BACE could produce an intermediate phenotype that improves/worsens the cognitive phenotype of PDAPP mice, dependent on the dosage of the gene.
- Deletion of BACE could have no effect the PDAPP mouse phenotype.

Given the diverse array of spatial memory, sensorimotor, seizure induction and histological analyses performed on these mice, it is somehow not surprising that depending on the measure being tested, all of the possible outcomes above are observed in BACE KO, BACE pKO; PDAPP and BACE KO; PDAPP mouse phenotypes. These results within each type of experimental paradigm used in this dissertation are reviewed in the next sections, followed by a discussion focusing on the normal role of APP metabolism in the nervous system and ultimately implications for the risks and rewards of BACE inhibition strategy for AD.

8.2 Spatial memory phenotypes of BACE KO and BACE KO; PDAPP mice

PDAPP mice have been previously shown to be deficient in several aspects of water maze spatial memory performance relative to non-transgenic control mice, and the experiments of Studies 001 and 006 reproduce these findings (Table 8.1) (Chen et al., 2000). While the accumulation of cerebral Aβ has been implicated as a causal factor in the development of AD, BACE KO mice that are unable to produce this APP metabolite also have a spatial memory phenotype that is limited to impairment in serial learning and memory capacity (Figures 3.2.1-3.2.3, 3.3.1). This BACE KO memory capacity deficit suggests that long-term synaptic plasticity may require a functional BACE enzyme. When the total spatial memory phenotype of the BACE KO mouse relative to the PDAPP mouse is considered, it seems that the BACE KO phenotype is milder than that of the PDAPP mouse, which in addition to lesser relative capacity and serial learning deficits at all ages, also has no perseverative impairment. The fact that BACE KO mice are fully viable, fertile and have longer lifespans than mice with a single copy of the PDAPP transgene suggests that the normal system is capable of compensating for even a complete lack of the BACE1 enzyme, although the mild spatial memory phenotype suggests it is somehow involved in normal cognition (Table 3.4, 8.1).

Study 001	Spatial Memory			
	Acquisition	Perseveration	Serial Learning	Capacity
Young	Platform Location 1	Location 2	Average TTC 1-3	N Platforms learned
PDAPP	no Δ	no Δ	-	--
BACE KO	no Δ	no Δ	-	-
BACE KO, PDAPP	-	no Δ	---	---
Middle-aged				
PDAPP	-	no Δ	--	---
BACE KO	no Δ	no Δ	no Δ	-
BACE KO, PDAPP	no Δ	no Δ	--	---
Old				
PDAPP	no Δ	-	--	-
BACE KO	no Δ	no Δ	-	-
BACE KO, PDAPP	no Δ	-	---	---

Table 8.1 Spatial memory phenotypes of Study 001 BACE KO x PDAPP mice. All animal performances for each measure are compared to Control (non-transgenic) and/or PDAPP mice of the same age. The (-) denotes a performance that was significantly poorer than that of Control mice, (--, ---) denotes performance that was significantly poorer than that of Control and PDAPP mice, and "no Δ" denotes no statistical difference in performance.

However, the spatial memory phenotype of the BACE KO; PDAPP mouse was anything but mild. The BACE KO; PDAPP mouse was the most impaired of all animals tested, with significant deficits in acquisition, perseveration, serial learning, and memory capacity at various ages (Table 8.1). In addition, the depth of the impairment of BACE KO; PDAPP mice suggests that it is the cumulative effect of both BACE KO and PDAPP mouse phenotypes (Figures 3.2.1-3.2.3, 3.3.1). This represents a singularly intriguing finding, as it would suggest that there are two distinct mechanistic pathways by which APP metabolism impacts learning and memory processes. Closer examination of the deficits between PDAPP, BACE KO, and BACE KO; PDAPP mice reveals that old animals with PDAPP transgenes in particular have difficulties forming new memories (perseveration at spatial location 2), while all the mice have lesser spatial memory capacity.

The perseverative deficit shared by PDAPP and BACE KO; PDAPP mice draws attention to the distinction between APP metabolism in each genotype. Animals of both genotypes overexpress mutant hAPP, but only the PDAPP mouse has the ability to generate Aβ40/42 fragments. One explanation for a deficit in perseveration in both mice is that simple overexpression of hAPP in the PDAPP transgene regardless of further metabolism is itself toxic to synaptic plasticity. There is some evidence to support this possibility, as earlier neuroanatomical analysis of PDAPP mice showed a marked hippocampal and callosal atrophy which was correlated to spatial reference and working memory (Dodart et al., 2000). Unpublished analysis of BACE KO; PDAPP mouse brains by Elan researchers shows that the PDAPP-associated hippocampal atrophy is not rescued by BACE gene deletion, so it is also possible that these phenotypes are the result of developmental transgene-associated brain shrinkage. It must be noted also that both types of mice would overproduce the APP intracellular domain (AICD) fragment, which has remained largely unexplored compared to Aβ40/42 but is reportedly involved in apoptotic nuclear signaling and regulation of APP itself (Passer et al., 2000; von Rotz et al., 2004). It is possible that this shared phenotype is the product of AICD-induced altered nuclear signaling and/or upregulation of APP itself.

Another explanation for this shared perseverative phenotype is that the PDAPP and BACE KO; PDAPP mice develop a similar impairment via divergence in their pathways. PDAPP mice feature an overproduction of Aβ40/42, β-CTF, sβAPP and AICD, while BACE KO;

PDAPP mice overproduce Aβ17-40/42, α-CTF, sαAPP, and AICD (Figures 1.4a-b). While several players in the amyloidogenic pathway have been implicated in neurological disease processes, the non-amyloidogenic pathway has been investigated and associated with rescue of cognitive deficits and reduction of amyloid pathology. It may be that the absolute lack of amyloidogenic fragments is deleterious to cognitive and synaptic function in the same way that excess is, not just in BACE-deficient but also in γ-secretase-deficient animals (Furukawa et al., 1996; Colciaghi et al., 2002; Postina et al., 2004; Saura et al., 2004). This serves to support the concept that amyloidogenic pathway is involved in normal cognitive processes.

As a whole, these deleterious spatial memory phenotypes in the BACE KO and BACE KO; PDAPP mouse do not completely agree with the results of Ohno et al. (2004) who characterized the BACE1 -/-; Tg2576 mouse line (Ohno et al., 2004). These authors noted that on the background of the Swedish human APP mutation, deficits in social recognition and Y-maze exploration were ameliorated with BACE gene deletion. The BACE KO mouse itself had a memory deficit in social recognition, while the BACE1 -/-; Tg2576 mice had near normal cholinergic physiology compared to Tg2576 control mice. While these results seem to be in opposition to the exceedingly poor spatial memory performance of the BACE KO; PDAPP mouse, there are many points in which the two experiments and mouse lines differ. It may be that the two sets of tasks are too divergent to fairly compare the lines. BACE KO; PDAPP mice perform very poorly on spatial memory tasks that rely more specifically on hippocampal function, but Y-maze and social recognition require significant frontal cortex involvement so deficits in these tasks could exist in the same animal. Water maze testing of the BACE1 -/-; Tg2576 mice would shed light on this disparity. In addition, the differences between the animals themselves could factor into the opposing directions of these results. While Ohno and colleagues (2004) tested 4-6mo old animals, this dissertation examined animals from 3-18mo of age, and it may be that 4-6mo is too early a time point for any age-related BACE-/-; Tg2576 deficit to be detected.

One global interpretation of the BACE KO x PDAPP phenotypes is that memory capacity is governed by more than one set of amyloid-driven pathways, while acquisition and flexibility of memory are processes sensitive to the accumulation of APP and/or AICD. These hypotheses would be best explored by a new experiment in which conditional knockout of

BACE is achieved on a hAPP transgenic mouse background, in which spatial memory, amyloid metabolism and electrophysiological parameters are examined. For example, if impairment in capacity for both BACE KO and hAPP mice was related to deficits in late-LTP and all deficits in acquisition were directly related to levels of AICD these data would support the global interpretation stated above.

8.3 Spatial memory phenotypes of BACE pKO; PDAPP mice

Spatial memory testing of PDAPP and BACE pKO; PDAPP mice with a serial water maze task revealed few distinctions between the performance of the two genotypes until they were 13 or 18mo old (Figures 4.2.1-4.2.3, 4.3.1). In most measures collected in young mice it appears unlike complete BACE gene deletion, partial BACE deletion does not exacerbate any existing PDAPP spatial memory deficits. However an age-related shift in phenotype was observed in BACE pKO; PDAPP mice. At 13mo, BACE pKO; PDAPP mice had ameliorated spatial memory deficits compared to PDAPP mice in acquisition, perseveration and serial learning. (Figure 4.2.2c, 4.2.3a, Table 8.2). However, by 18mo BACE pKO; PDAPP mice were severely impaired in perseveration, serial learning and memory capacity relative to PDAPP animals. Thus is appears that the beneficial effects of BACE gene reduction rescues the PDAPP mouse deficits only within a certain range of age-related Aβ accumulation.

Study 006	Spatial Memory			
	Acquisition	Perseveration	Serial Learning	Capacity
BACE pKO, BACE	Platform Location 1	Location 2	Average TTC 1-3	N Platforms learned
Young	no Δ	no Δ	no Δ	no Δ
Middle-aged	+	+	+	no Δ
Old	no Δ	---	---	---

Table 8.2 Spatial memory phenotypes of Study 006 BACE pKO x PDAPP mice. All animal performances for each measure are compared to PDAPP mice of the same age. The (+) and (-) symbols denote performances that were significantly better or worse than that PDAPP mice, and "no Δ" denotes no statistical difference in performance. The number of (+) and (-) symbols ranges from 1,2 or 3 and is indicative of the magnitude of the differences between the two groups.

This age-related shift in cognitive phenotypes suggests again that there may be multiple ways in which alteration of APP metabolism can deleteriously impact spatial learning and memory. While 18mo BACE pKO; PDAPP animals have far less amyloid accumulation than PDAPP

mice, the inferior spatial memory in the mice with partial BACE gene deletion status argues that some other aspect of BACE activity is needed for normal cognitive function. Decreasing the productivity of the β-secretase pathway ostensibly increases processing in the α-secretase pathway, but these α–metabolites have been shown to be neuroprotective themselves, which suggests another sources for this age-related impairment.

In part the data from Study 006 also agrees with that presented by Singer et al. (2005), in which the authors performed behavioral testing on hAPP mice (London and Swedish mutations) and saw cognitive rescue upon 4 weeks of cerebral lentiviral delivery of small interfering RNA targeting BACE1 sequences. These 11mo old animals exhibited restoration of dendritic and synaptic markers like MAP-2, and Synaptophysin, with dramatic reduction in Aβ production and plaque deposition. However, although the works of Singer et al. (2005 and the 13mo Study 006 suggest that BACE1 inhibition may result in beneficial cognitive outcomes, the 18mo Study 006 data casts a specter of caution on the long-term ability of BACE1 activity reduction to prevent cognitive impairment in AD. This issue could be examined more closely with in vivo experiments with effective BACE inhibitor compounds, or alternatively long-term studies with animals treated with BACE1 siRNA or conditional BACE1 gene knockout animals.

8.4 Sensorimotor phenotypes of BACE KO x PDAPP mice

8.4.1 Visual Cued Navigation

Performance in the visual cued navigation (VCN) aspect of the water maze requires a basic sensorimotor functionality to swim and use intramaze cues to locate the platform. However, there is also some aspect of procedural (motor) and associative learning involved as well. In Studies 001 and 006 there was no major difference in VCN trial latencies between Control mice and those of other genotypes – except for 18mo BACE KO, PDAPP mice that took significantly longer to navigate to the visible platform (Figures 3.1.1-3.1.2, Table 8.3). At both 3 and 18mo of age PDAPP and BACE KO; PDAPP mice swam at slower speeds in the VCN task (Figure 3.1.2a,c). Although this slower swim speed phenotype could influence the analysis of hidden platform navigation, the criterion for performance was set at locating the

platform in 21s or less. Given the size of the pool (150cm), BACE KO; PDAPP mice released at even a site most distant from a platform would be able to swim to the platform well within the 21s time limit, as the slowest swim speed was 22 cm/s (Figure 3.1.1c, 3.1.2c). So despite this slow swimming phenotype, the poor performance of the BACE KO; PDAPP on the spatial memory task was most likely determined by memory and not motor status.

Finally, it must be noted that at 13mo both PDAPP and BACE KO mice both had significantly shorter swim latencies than Control mice on Day 1 (Figure 3.1.1b). While these findings were statistically meaningful, it may be that these are spurious findings, as Control mice at this age took an uncharacteristically long time to navigate to the visible platform (56s vs. 29-45s) when there was no difference between swim speeds at this age (Figure 3.1.2b). There were no VCN differences between any of the Study 006 mice, featuring the PDAPP and BACE pKO, PDAPP mice (Table 8.3).

Study 001	Non-Spatial Water Maze	
	Visual Cued Navigation	Visual Cued Navigation
Young	Latency	Swim Speed
PDAPP	no Δ	no Δ
BACE KO	no Δ	no Δ
BACE KO, PDAPP	no Δ	-
Middle-aged		
PDAPP	no Δ	no Δ
BACE KO	no Δ	no Δ
BACE KO, PDAPP	no Δ	no Δ
Old		
PDAPP	no Δ	no Δ
BACE KO	no Δ	no Δ
BACE KO, PDAPP	-	-

Study 006	Non-Spatial Water Maze	
	Visual Cued Navigation	Visual Cued Navigation
BACE pKO, BACE	Latency	Swim Speed
Young	no Δ	no Δ
Middle-aged	+	no Δ
Old	no Δ	no Δ

Table 8.3 Visual cued navigation phenotypes of Study 001 and 006 mice. All animal performances for each measure are compared to Control (Study 001) or PDAPP (Study 006) mice of the same age. The (+) or (-) symbols denote a performance that was significantly better or poorer than that of Control mice (Study 001), or PDAPP mice (Study 006), and "no Δ" denotes no statistical difference in performance.

8.4.2 Mass and muscular strength observations of BACE KO x PDAPP mice

Simple analysis of body masses of aged Study 011A, 011B, and 011C mice revealed that male BACE KO; PDAPP weighed less on average than male Control mice (Figure 5.1a, Table 8.4). Young Study 011B mice displayed no body mass differences between genotypes. Before one can conclude that BACE KO; PDAPP mice that swim slowly and weigh less than other mice are simply weak or sickly animals, the muscular strength testing results must be considered. Aged PDAPP mice had greater grip strengths than Control mice, with a similar trend in BACE KO; PDAPP mice (Figure 5.1c). This finding suggests that mice carrying the PDAPP transgene have a dominant fine motor and strength phenotype, but it can also be interpreted as an anxiety characteristic as anxiety in rodents is associated with increased forelimbs grips forces (Benaroya-Milshtein et al., 2004). At the same time, young BACE KO; PDAPP mice had equivalent positional sense as PDAPP mice (Figure 7.1b, Table 8.4). These PDAPP grip strength findings and trends are in agreement with the rotorod performance data.

Study 011A	Mass & Muscular Observations	
Old	Body Mass	Muscle Strength
PDAPP	-, males	+, males
BACE KO	no Δ	no Δ
BACE KO, PDAPP	-, males	no Δ
Study 011C		
BACE pKO, BACE	no Δ	no Δ
Study 011B		
Young		
BACE pKO, BACE	no Δ	no Δ

Table 8.4 Mass and muscular observations of Study 011A, 011B and 011C mice. All animal performances for each measure are compared to Control (Old BACE KO x PDAPP mice) or PDAPP (BACE pKO x PDAPP) mice of the same age. The (-) denotes a body mass that was significantly less than that of PDAPP mice, (+) denotes performance that was significantly better than that of Control mice, and "no Δ" denotes no statistical difference in performance.

8.4.3 Spontaneous sensorimotor activity in BACE KO x PDAPP

Analysis of the locomotor activity of BACE KO x PDAPP mice revealed few significant differences between any of the genotypes, although there were trends towards lowered horizontal plane activity in aged PDAPP mice from Study 001 (Figure 5.2.1a-b, 5.2.2, Table

8.5). The only statistically meaningful finding was that BACE KO; PDAPP mice displayed more vertical activity (rearings) than PDAPP animals (Figure 5.2.2b). This excessive rearing behavior, is part of a range of movements including repetitive movements that are part of a seizure- phenotype.

Subset analysis of the aged Study 001 mice in the central "open field" of the monitoring arena showed that BACE KO; PDAPP mice spent much less time in the open area compared to both Control and PDAPP animals, while BACE KO mice had a strong tendency for the same (Figure 5.2.3b). Avoidance of the open field is a classic test for anxiety, and if this is interpreted as an anxiety phenotype in animals will BACE gene ablation, then this reproduces the findings of other researchers (Delbarre et al., 1970; Britton and Britton, 1981; Harrison et al., 2003). Harrison et al. (2003) also reported that their BACE KO mice had deficits in open field exploration, and decreases in the levels of 5HT, which plays a role in anxiety as well as other affective disorders. However, the analysis of Study 011A BACE KO mice did not agree with the decreased exploration phenotype also presented by Harrison and colleagues. This may simply be due to differences between the age of animals tested (10 weeks versus 18mo) and the fact that only a few Study 011A BACE KO mice were examined (N=3), and all were female.

A more reasonable assessment of a possible BACE-related hypoactivity phenotype can be drawn from the young and aged BACE pKO; PDAPP analysis done in Studies 011B and 011C. While there again were no significant differences between the involuntary locomotor activity of PDAPP and BACE pKO; PDAPP mice, there were trends towards decreased horizontal plane activity in BACE pKO; PDAPP mice at both ages tested (Figures 6.2.1a-b, 7.2.1a-b, Table 8.5). In addition there were similar tendencies for reduced open field activity in BACE pKO; PDAPP mice, suggesting that the BACE gene deletion does confer not only a hypoexploratory but also an anxiety phenotype (Figures 6.2.3, 7.2.3).

	Spontaneous Locomotor Activity			
Study 011A Old	Horizontal activity	Vertical activity	Stereotypy	Open Field
PDAPP	no Δ	no Δ	no Δ	no Δ
BACE KO	no Δ	no Δ	no Δ	no Δ
BACE KO, PDAPP	no Δ	+, vs. PDAPP	no Δ	-- vs Control and PDAPP
Study 011C				
BACE pKO, BACE	no Δ	no Δ	no Δ	no Δ
Study 011B Young				
BACE pKO, BACE	no Δ	no Δ	no Δ	no Δ

Table 8.5 Spontaneous locomotor observations of Study 011A, 011B and 011C mice. All animal performances for each measure are typically compared to Control (Old BACE KO x PDAPP mice) or PDAPP (BACE pKO x PDAPP) mice of the same age. The (--) denotes a performance that was significantly poorer than that of comparator mice, (+) denotes performance that was significantly better than that of PDAPP comparator mice, and "no Δ" denotes no statistical difference in performance.

8.4.5 Rotorod performance in BACE KO x PDAPP mice

While the grip strength tests largely measured fine motor control, locomotor performance measured on the rotorod is more a readout of gross motor coordination. Male mice harboring the PDAPP transgene in Study 011A had supranormal grip strengths, but in the rotorod all aged BACE KO and BACE KO; PDAPP mice had poor rotorod performances regardless of gender (Figure 5.3.1, Table 8.6). Similarly, aged BACE pKO; PDAPP mice in Study 011C also did poorly on the accelerating rotorod (Figure 7.3b). This is in agreement with the trends towards poorer positional tone phenotype seen in aged BACE pKO; PDAPP mice of Study 011C as well, as this task has elements of both forelimb strength and motor coordination (Figure 7.1b). It may also be that poor motor coordination in BACE-deficient mice is a dominant phenotype, as Study 011A PDAPP mice had superior rotorod performances, while BACE KO; PDAPP mice performed as poorly as BACE KO mice.

Any BACE KO muscular findings could represent a phenotype with an explainable mechanism. Although AD is the most prevalent of the amyloid accumulation diseases, Inclusion Body Myositis (IBM) is a disease in which patients present with progressive muscular atrophy, with vacuolization of muscle tissue. Examination of the neuromuscular

junction of IBM patients has revealed the pathogenic presence of amyloid, as well as alterations in the levels of BACE1 and BACE2 enzyme (Askanas V, 1992; Vattemi et al., 2001; Vattemi et al., 2003). Like AD, IBM is an age-related disorder, and the muscle wasting strikes both distal and proximal muscle groups, but most notably in the large muscles of the leg. Although no muscular pathology was seen in young mice lacking BACE, the seemingly poorer locomotor coordination phenotype of the aged mice with BACE gene ablations could be related to an IBM-like muscular disorder that is possibly age-related, and for this reason neuromuscular side effects of BACE inhibition that must be considered (Roberds et al., 2001). Alternatively, it is possible that poor BACE KO; PDAPP performance on the rotorod is influenced by a deficit in procedural motor skills learning, just as the BACE-deficient mice had impairments in spatial learning and memory.

Study 011A **Old**	Motor Coordination	
	Rotorod static	Rotorod accelerating
PDAPP	no Δ	no Δ
BACE KO	no Δ	no Δ
BACE KO, PDAPP	+, vs. PDAPP	+, vs. PDAPP
Study 011C		
BACE pKO, BACE	no Δ	no Δ
Study 011B **Young**		
BACE pKO, BACE	no Δ	no Δ

Table 8.6 Rotorod motor coordination of Study 011A, 011B, and 011C mice. All animal performances for each measure are typically compared to Control (Old BACE KO x PDAPP mice) or PDAPP (BACE pKO x PDAPP) mice of the same age. The (+) denotes performance that was significantly better than that of PDAPP comparator mice, and "no Δ" denotes no statistical difference in performance.

8.5 PTZ-induced seizure responses of BACE KO x PDAPP mice

After observing spontaneous tonic-clonic seizures in Study 001 mice during water maze testing, there was a concern that BACE gene deletion may predispose animals to seizure activity. In two subsequent experiments aged BACE KO x PDAPP and BACE pKO x PDAPP mice were tested at 60 mg/kg of the seizure inducing agent pentylenetetrazole (PTZ) in an effort to produce major seizures (Studies 011A, 011C). In addition young BACE pKO; PDAPP mice were tested with 60 mg/kg of PTZ (Study 011B), and old BACE pKO; PDAPP

mice were tested with a lower 25 mg/kg dose of PTZ which would cause minor seizures (Study 011C). The results of Study 011A showed that PTZ treatment was more lethal to genetically modified animals, and PDAPP mice had the least resistance to seizure onset, as presented by partial clonus scores (Figure 5.4.1a, 5.4.2a, Table 8.7). However, the major finding was that BACE KO; PDAPP mice had the greatest severe tonic-clonic seizure activity, suggesting that their lack of resistance was due to significant prior seizure activity or neuronal dysfunction in brain areas that regulate epileptiform kindling (Figure 5.4.2c). Unfortunately, there were too few BACE KO mice to make assessments of their seizure susceptibility. Fortunately, there were enough aged BACE pKO; PDAPP mice to perform a similar severe seizure experiment, and compared to PDAPP mice, mice with a partial BACE gene deletion had a tendency to survive seizure induction for longer (7.4.2c, Table 8.7). Along with this finding, it appears that BACE pKO; PDAPP mice treated with a high dose of PTZ displayed trends towards greater resistance to developing seizures overall (Figure 7.4.2a,b). a similar trend towards lesser seizure activity was present in young BACE pKO; PDAPP mice of Study 011B compared to PDAPP mice given high doses of PTZ, as these animals had significantly less moderate severity general clonic seizures (Figure 6.4.1d, 6.4.2a,-c).

When given lower doses of PTZ to examine resistance to lesser seizures, aged BACE pKO; PDAPP mice appeared to have somewhat higher mild clonus scores, which is actually similar to a Control-like response to PTZ treatment (Figure 5.4.2a, 7.4.1b). Comparison of aged PDAPP and BACE pKO; PDAPP seizure responses to 25 and 60mg/kg of PTZ revealed a strong-dose dependency, with significantly more total seizure activity and faster onset to mild seizures with higher doses (Figure 7.4.3).

These seizure data suggest a gene dosage effect of BACE on seizures, as absolute loss of BACE was associated with severe seizures, while partial loss of BACE appeared to rescue some of the seizure phenotype, both on the PDAPP background. The fact that this trend is also present in young BACE pKO; PDAPP mice argues that BACE plays a role in the regulation of neuronal activity, which if perturbed can result in seizures. There is ample evidence tying modifications in the APP processing pathway to seizure activity and neuronal regulation, in both humans and animals (Holcomb et al., 1998; Steinbach et al., 1998; Del

Vecchio et al., 2004). Clonic seizures are indeed part of the clinical profile of AD patients themselves, and have been linked to specific mutations in the γ-secretase complex activity, Presenilin 1 (PS1) (Petersen, 1998; Ezquerra et al., 1999; Devi et al., 2000; Furuya et al., 2003; Velez-Pardo et al., 2004). Loss of function between BACE and PS1 would both result in deficiency of Aβ, although loss of PS1 would also result in accumulation of β-CTF peptides. Also, previous research with transgenic animals that either are APP-nulls or overproduce APP that lacks an α-secretase cleavage site develop spontaneous seizure activities as well (Steinbach et al., 1998; Moechars et al., 1999). These experiments all implicate seizures vis-à-vis neuronal activity dysregulation as the outcome for deviation from a "normal" APP metabolite production.

Old	PTZ-Induced Seizure Response					
60 mg/kg PTZ dose	Partial Clonus	General Clonus	Tonic-Clonic	Seizure Onset	Death Latency	Lethality
PDAPP	-	no Δ	no Δ	no Δ	no Δ	+
BACE KO	no Δ	no Δ	no Δ	no Δ	no Δ	+
BACE KO, PDAPP	-	+ vs PDAPP	+	no Δ	no Δ	+
BACE pKO, BACE	no Δ	no Δ	no Δ	no Δ	no Δ/+	no Δ

Old		
25 mg/kg PTZ dose	Partial Clonus	Seizure Onset
BACE pKO, BACE	no Δ	no Δ

Young	PTZ-Induced Seizure Response					
60 mg/kg PTZ dose	Partial Clonus	General Clonus	Tonic-Clonic	Seizure Onset	Death Latency	Lethality
BACE pKO, BACE	no Δ	-	no Δ	no Δ	no Δ	no Δ

Table 8.7 PTZ-induced seizure responses of Study 011A, 011B and 011C mice. All animal responses are compared to Control (BACE KO x PDAPP mice) or PDAPP (BACE pKO x PDAPP) mice of the same age. The (-) denotes a seizure response that was significantly less than that of Control and PDAPP mice, (+) denotes a seizure-based response that was significantly greater than that of Control or PDAPP comparator mice, and "no Δ" denotes no statistical difference in performance.

8.6 Histological analysis of BACE KO X PDAPP Mice

One of the major analytical endpoints of interest in the generation of BACE KO x PDAPP mice was the examination of effects on amyloid deposition pathology. While BACE1 was thought to be the primary neuronal β-secretase activity, it was possible that BACE2 or some other activity could become the compensatory central APP β-cleaving enzyme (Bennett et al., 2000; Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001). Later assessments of BACE

KO tissues and gene expression experiments revealed that there was no compensatory enzyme activity as Aβ was not detected and levels of BACE2 were unaltered (Basi et al., 2003; Luo et al., 2003). Most notably, when Tg2576 mice were crossed to BACE1 KO animals, plaque-like amyloid deposition was abolished (Ohno et al., 2004).

There has been a wide array of analysis of the BACE KO x PDAPP mouse, which has not been published in peer-reviewed journal format, but is currently in preparation by other Elan researchers. The major findings have been replicated and produced by qualitative investigation in this thesis and will be discussed in the following sections on APP, Aβ and Calbindin (CB) immunostaining.

8.6.1 hAPP immunoreactivity in BACE KO x PDAPP mice

The antibody 8E5 is used to detect the presence of the PDAPP transgene in mouse brains, and is also used to quantitate the neuritic plaque burden in aged mice with the PDAPP transgene (Table 8.8) (Games et al., 1995). In Studies 001, 006, 011A, 011B, and 011C, this antibody was used only to confirm the genotype of animals (Figure 3.7.1, 4.7.1, Sections 5.5, 6.5, 7.5) In one case the genotype was found to be in conflict with the vendor's report and that animal was removed from analysis (Section 5.5). Other researchers working on the BACE KO x PDAPP mouse lines found that there were indeed no neuritic plaques in Study 001 BACE KO; PDAPP brains, as there was no amyloid deposition of any kind (Figure 3.7.1. panel D) (McConlogue et al., 2003). Study 006 mice which had only one functioning BACE gene had a significant decrease in neuritic plaque burden at 13 and 18mo compared to PDAPP mice of the same age (Figure 4.7.1 panel B) (McConlogue et al., 2003).

8.6.2 Aβ immunoreactivity in BACE KO x PDAPP Mice

Diffuse total amyloid plaques are typically visualized with 3D6 immunostaining. Plaque-like depositions were abolished in BACE KO; PDAPP Study 001 mice, while diffuse plaque burden was reduced in BACE pKO; PDAPP mice (Figure 3.7.2 panel D, 4.7.2 panel B, Table 8.9). (McConlogue et al., 2003). Interestingly, plaque reductions in mice at 13mo mirrored the behavioral rescue of perseveration deficits in the water maze in BACE pKO; PDAPP

mice. These findings replicate those reported by Ohno et al. although data regarding the amyloid deposition status of partial BACE deletion on an hAPP overexpressing background have not been reported elsewhere.

Study 001	Amyloid Histology
Young	hAPP
PDAPP	present
BACE KO	none
BACE KO, PDAPP	present
Middle-aged	
PDAPP	Neuritic plaques
BACE KO	none
BACE KO, PDAPP	present
Old	
PDAPP	Neuritic plaques
BACE KO	none
BACE KO, PDAPP	present

Study 006	Amyloid Histology
BACE pKO, BACE	hAPP
Young	no Δ
Middle-aged	+
Old	++

Table 8.8 hAPP immunoreactivity of Study 001 and 006 mice. All immunoreactivity levels for each measure are compared to Control (Study 001) or PDAPP (Study 006) mice of the same age. The "present" denotes the presence of hAPP, "none" indicates no hAPP was detected, "Neuritic plaques" indicates that accumulations of the 8E5 antibody were present in the hippocampus and/or prefrontal cortex, (+,++) denotes the degree of amelioration of neuritic plaque burden compared to PDAPP mice of the same age, and "no Δ" denotes no visually obvious difference in hAPP.

Study 001	Amyloid Histology
Young	Aβ
PDAPP	-
BACE KO	none
BACE KO, PDAPP	none
Middle-aged	
PDAPP	--
BACE KO	none
BACE KO, PDAPP	none
Old	
PDAPP	---
BACE KO	none
BACE KO, PDAPP	none

Study 006	Amyloid Histology
BACE pKO, BACE	Aβ
Young	+
Middle-aged	+
Old	++

Table 8.9 hAPP immunoreactivity of Study 001 and 006 mice. All genetically modified immunoreactivity levels for each measure are compared to PDAPP mice of the same age. The "none" indicates no 3D6+ plaques were detected, (-,--,---) denotes the degree of accumulation of diffuse plaque burden compared to PDAPP mice of the same age, (+,++) and denotes the degree of amelioration of diffuse plaque burden compared to PDAPP mice of the same age.

8.6.3 Calbindin immunoreactivity in BACE KO x PDAPP Mice

The Calbindin (CB) analysis performed in these experiments were motivated by reports that CB levels in hAPP J20 mouse dentate gyrus were depleted, and spatial memory performance in the water maze correlated to levels of this Ca^{++} binding protein (Palop et al., 2003). It was postulated that decreases in CB bespoke of impaired neuronal Ca^{++} homeostasis and that CB could be a biomarker for cognitive impairment. Indeed, as CB is also reported to be decreased in the cortex of AD patients, this seemed a reasonable hypothesis (Lally et al., 1997; Geula et al., 2003). However, histological analysis in Study 001 and 006 dentate gyrus revealed a pattern much more complex than simply amyloid-driven CB depletion.

In Study 001 there appeared to be a PDAPP-linked depletion of CB relative to all other genotypes at 3mo, but at 13mo all CB levels were similar (Figure 3.6.1a-b). However by 18mo, there appeared to be a BACE KO-related increase in CB dentate gyrus levels, which is in conflict with the concept that CB depletion is a surrogate for spatial memory performance in the water maze (Figure 3.6.1c, Table 8.10). The results of Study 006 suggest CB are similar, as 18mo BACE pKO; PDAPP mice have both much lower levels of CB and poorer spatial memory performance than PDAPP animals, (Figure 4.6.1c). These patterns suggest that the relationship, if any, between CB and hippocampal memory function is dynamic.

Study 001	Histology
Young	Calbindin
PDAPP	-
BACE KO	no Δ
BACE KO, PDAPP	-
Middle-aged	
PDAPP	no Δ
BACE KO	no Δ
BACE KO, PDAPP	no Δ
Old	
PDAPP	no Δ
BACE KO	+
BACE KO, PDAPP	no Δ

Study 006	Histology
BACE pKO, BACE	Calbindin
Young	+
Middle-aged	no Δ
Old	-

Table 8.10 Calbindin immunoreactivity of Study 001 and 006 mice. All genetically modified immunoreactivity levels for each measure are compared to Control (Study 001) or PDAPP (Study 006) mice of the same age. The (-) or(+) denotes a deficit or increase in CB levels compared to Control or PDAPP mice, and "no Δ" denotes no statistical difference in performance.

Hippocampal CB levels in BACE KO x PDAPP mice driven to seizures by PTZ treatment are for the most part equivocal except for one notable exceptions. Old BACE pKO; PDAPP mice given a high dose of PTZ have higher levels of CB relative to PDAPP mice (Figure 7.5a, Table 8.11). Similarly, young BACE pKO; PDAPP mice have a trend towards higher CB levels compared to PDAPP mice when administered a high dose of PTZ (Figure 6.5). The significance of the age-specific genotypic CB phenotype seen in Study 001 and 006 suggest that levels of CB are changing over time, and this may play a role in the disparity between CB levels in response to different doses of seizure-inducing drug. Previous authors have found similarly confusing CB levels in response to severity of seizure. Gary et al. found that there was an association between neuronal protection from seizure and CB levels but this relationship is seen prior to excitotoxic insult, such that CB levels after injury are not informative as to the protective role CB may play in neurons vulnerable to seizure (Gary et al., 2000).

Old	Histology
60 mg/kg PTZ dose	Calbindin
PDAPP	no Δ
BACE KO	no Δ
BACE KO, PDAPP	no Δ
BACE pKO, BACE	+

Old	Histology
25 mg/kg PTZ dose	Calbindin
BACE pKO, BACE	no Δ

Young	Histology
60 mg/kg PTZ dose	Calbindin
BACE pKO, BACE	no Δ

Table 8.11 Calbindin immunoreactivity of Study 011A, 011B and 011C mice. All genetically modified immunoreactivity levels for each measure are compared to Control or PDAPP (BACE pKO; PDAPP) mice of the same age. The (+) denotes and increase of CB compared to PDAPP mice of the same age, and "no Δ" denotes no statistical difference in performance.

While the relationships between CB levels, APP processing and response to seizure kindling are on the surface difficult to comprehend, it is possible that these interactions are important

to the pathological phenotypes of the BACE KO x PDAPP mice. One possible explanation for these findings is that unobserved seizure activity is affecting the levels of dentate gyrus CB. There are several reports linking seizure activity to CB levels, as one of the neuronal consequences of chemically-induced seizures is increased neurogenesis in the hippocampus (Parent et al., 1997; Yang et al., 1997; Gary et al., 2000; Lee et al., 2002; Jiang et al., 2003). Newly-born neurons have high energy requirements and Ca^{++} trafficking in these cells is increased, such that CB measurements in the various brain regions are routinely measured in neurogenesis experiments (Lally et al., 1997; Yang et al., 1997; Eriksson et al., 1998; Nilsson et al., 1999; Gary et al., 2000; Lee et al., 2002; Geula et al., 2003; Shetty, 2004). One intriguing connection between neurogenesis and the hippocampus is that the formation of new neurons could be an important mechanism for learning and memory, as evidence indicates that learning can drive birth of highly plastic new neuronal cells (Eriksson et al., 1998; Doetsch et al., 1999; Nilsson et al., 1999; Schinder and Gage, 2004; Schmidt-Hieber et al., 2004). One additional dimension of complexity to this is that amyloid itself has been reported to disrupt the process of neurogenesis – which in turn may be a contributor to the poor spatial memory and decreased synaptic plasticity of hAPP mice (Bondolfi et al., 2002; Haughey et al., 2002; Wen et al., 2004). While these CB analyses have generated more questions than answers, it is possible that electrophysiological methods can help determine whether CB is a functional biomarker for hippocampal function or simply an uninformative red herring.

8.7 Correlational relationships for BACE KO x PDAPP mice phenotypic measures

To generate a phenotypic profile for novel transgenic animals, several different types of behavioral, histological and other assessments are performed. The readouts of these measurements typically are described as unitary findings, but in many cases the neural bases of cognitive function and neuroanatomy for seemingly divergent phenotypes are in fact related. For instance, there are reports that link performance in the water maze to levels of cerebral amyloid (Chen et al., 2000; Kotilinek et al., 2002; Westerman et al., 2002). While relationships between amyloid levels and spatial memory performance at this point have sufficient experimental support and are to a certain degree "expected", there may be many

other important unexpected functional relationships that have yet to be discovered. The correlational analyses of the results of each phenotypic measure in the BACE KO x PDAPP operate in this manner, as these analyses described mathematical relationships between similar types of tests (water maze acquisition and memory capacity) and revealed linkages between dissimilar measures that may have shared underlying functional anatomy (rotorod performance and resistance to developing severe tonic-clonic seizures).

Among the many "expected" intratask relationships were correlations between water maze measures, open field and general horizontal activity monitoring, and sequential rotorod performances (Tables 3.8.1, 4.8.1, 5.6, 6.6, 7.6). Conversely, even among the related measures there were relationships that appeared to be counterintuitive, like the lack of correlation between water maze acquisition and perseveration in Study 001 and 006 mice, which suggests a distinct neural basis for the two spatial memory functionalities (TTC 1 and TTC2, Table 3.8.1, 4.8.1). However, the most interesting correlations came from performing analyses on measures that on the surface do not appear to be similar, such that they formed intertask or intermeasure relationships (e.g., resistance to severe seizures and rotorod performance in aged animals) (Table 5.5, 7.5). These "discovered" correlations were numerous and derived value from their ability to identify potential surrogate assessments for partner tests that are difficult or otherwise onerous to conduct, like vertical activity monitoring or rotorod testing in lieu of lethal seizure testing.

Another valuable feature of correlation analysis was that larger general patterns of function and dysfunction can be discerned. The relationship between hippocampal CB levels and behavioral function in Studies 001 and 006 is such that at young ages high CB levels are not correlated to spatial memory performance, but that high CB levels in aged mice are associated with poorer performances in Study 006. In seizure studies with young and aged BACE pKO x PDAPP mice (Studies 011B and 011C), and to a lesser degree in aged BACE KO x PDAPP mice (Study 011A), higher CB is associated with seizure protection Tables 5.6-5.7, 6.6-6.7, 7.6-7.7). These correlational findings suggest that absolute levels of CB are not linearly predictive of cognitive function, as the experimental context like age or neuronal trauma appears to be important. The fact that the dentate gyrus is immunoreactive for CB, simply means that it is present, not that the proteins are functional, and can shepherd Ca^{++}

ions when stimulated by normal or excessive neuronal activity. It is thus possible that the higher levels of CB in aged BACE KO and BACE KO; PDAPP mice may represent a pool of non-functional proteins that are unable to traffic Ca^{++}. Alternatively, if this CB pool is functional, then it may be a biomarker for dysfunction in some other aspect of neuronal activity, as the concentration of CB may be elevated as a compensatory mechanism.

One model that captures the duality of the correlations between dentate gyrus CB immunoreactivity and cognitive function is based on a U-shaped curve (Figure 8.1). In such a model, increasing levels of CB only correspond to normal cognitive function, which includes spatial memory and resistance to seizure induction, within a certain range, as excessively high or low concentrations both relate to cognitive impairment. This model does not specifically address the experimental contexts that produce CB depletion or enrichment, but it is possible that age-related synaptic loss and/or irregular patterns of neuronal activity may mechanistically influence CB levels.

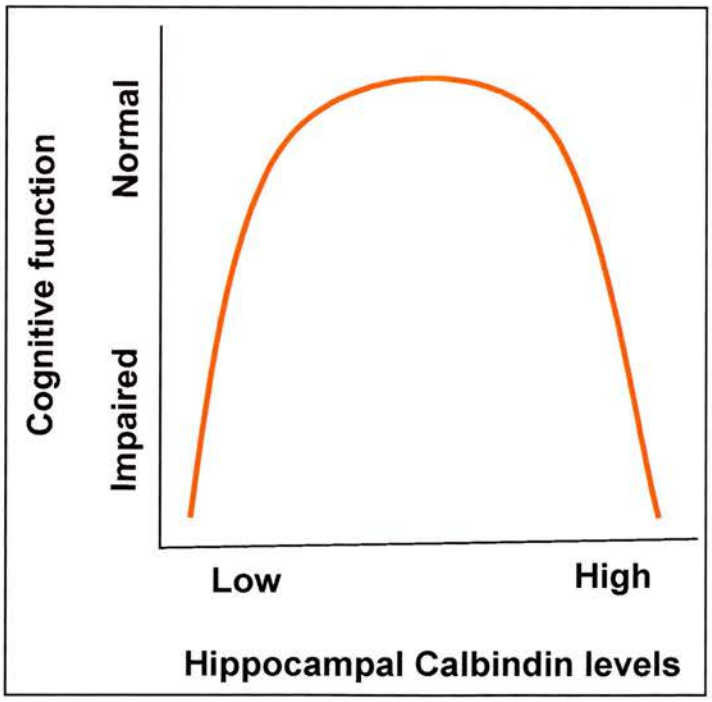


Figure 8.1 U-shaped curve models a possible relationship between hippocampal Calbindin levels and cognitive function.

8.8 Role of the amyloid processing pathway in cognitive function

The Amyloid Cascade hypothesis in one form or the other is a central framework for much of the AD basic and therapeutic research today. Whether deposited in compact or diffuse plaques, soluble or insoluble, fibrilized or not, Aβ is routinely described as an entity toxic to synapses, neurons, and ultimately cognitive function. What is not underlined or equally examined is the normal role of amyloid in the nervous system. *In vitro* experiments like that of Kamenetz and colleagues cast amyloid in a different light, as an important regulator of basal neuronal activity (Kamenetz et al., 2003). Numerous *in vivo* experiments in which Aβ is genetically depleted yield animals that have poor cognitive performance in spatial memory tasks and are often prone to seizure activity (Steinbach et al., 1998; Saura et al., 2005). While there is strong evidence that accumulation of Aβ40/42 is indeed deleterious to neuronal and subsequently cognitive function, the findings reported in this dissertation aver that the converse is also true, in that over time loss of function of amyloid is as just as damaging to the nervous system as amyloidogenic gain of function (Table 8.13).

Given the links between regulation of basic neurotransmission and amyloid both *in vitro* and *in vivo*, it is perhaps not too surprising that alterations to the APP processing pathway via BACE deletion should give rise to the motor and spatial memory phenotypes seen in this dissertation (Wang et al., 2000; Pettit et al., 2001; Boncristiano et al., 2002; Doraiswamy, 2002). However, modification of BACE genes are simply a means of effecting the final product of the APP cleavage, Aβ. As no other substrate for BACE has yet been found that is implicated in cognitive function, the consequences of BACE gene deletion must be discussed with regard to perturbation to the APP processing pathway. This pathway is highly regulated and modulation of the substrates, products and enzymes involved (whether APP, Aβ, P3, BACE, α- or γ-secretase) appears to yield deleterious effects on cognition, although the mechanism by which these perturbations impact function is unclear (Figure 8.2).

Perhaps the more appropriate context in which to examine this question of how the APP pathway affects cognitive function is at the most basic level, at the synapse. Soluble Aβ has been shown in non-transgenic rodent tissue to bind to cortical ACh receptors, inhibit LTP, and APP overexpression causes overall synaptic depression in hippocampal excitatory

synapses (Wang et al., 2000; Freir et al., 2001; Pettit et al., 2001; Kamenetz et al., 2003). If Aβ exists as an inhibitory modulator of basic neuronal activity, then several pieces of evidence immediately agree with this concept.

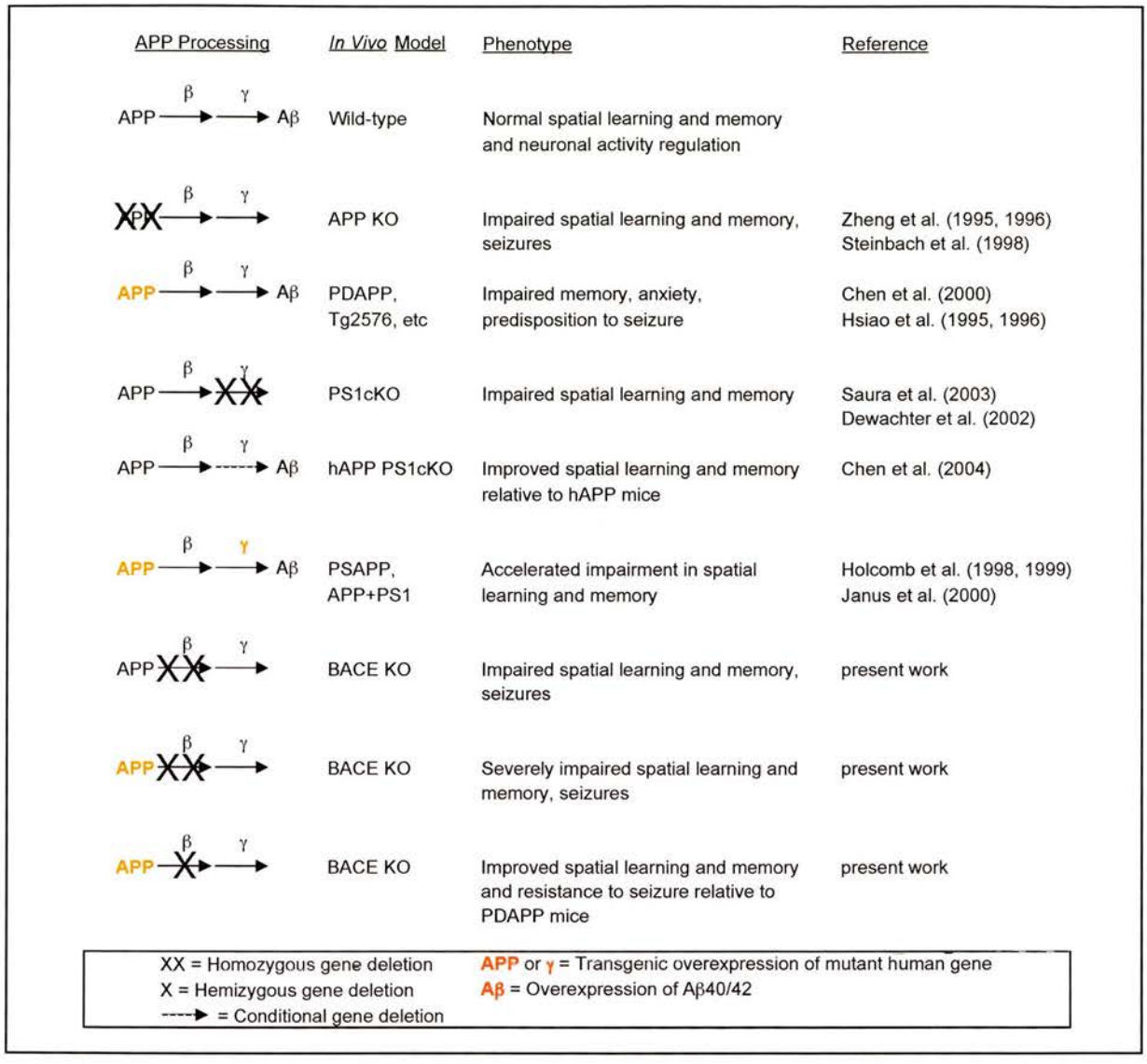


Figure 8.2 Cognitive phenotypes of genetically modified *in vivo* mouse models of APP processing pathway alterations. Diagrams feature a biochemical schematic of various modifications to the normal amyloid processing pathway in genetically altered mice, and their *in vivo* outcomes. While the genetic modifications may differ in target (APP, β- or γ-secretase) the resulting features of these animal models are similar, as many of them have seizure activity and spatial memory impairments.

In a context like AD or in hAPP transgenic mice, excess hAPP and Aβ could conceivably cause the following synaptic and functional consequences:

- The uncoupling of neuronal firing to events in the environment cause activity-dependent reductions in the number of synapses, which may be a contributing factor to the loss of synapses in areas like the hippocampus and cortex with heavier accumulations of amyloidogenic processing.
- Synaptic remodeling that is not driven by event-related activity results in disorganized neural networks, which is worsened by the neuritigenic properties of amyloid.
- The depleted and disorganized synaptic environments can allow strong activity in a small subset of excitatory cells to have disproportionately broad effects on the existing neural network, resulting in seizure activity.
- Excessive Aβ causes neuronal depression, leading to impaired memory function via the inability to generate lasting LTP activity.

Conversely, similarly deleterious consequences on neuronal and general cognitive function can arise due to lack of inhibitory modulatory control by the APP processing pathway:

- Synapses are improperly strengthened by timely but random firing events, resulting in a disorganized neural network.
- Non-meaningful neuronal connectivity impedes the performance of memory-based tasks that rely on formation of event contingent synapses.
- Impaired inhibitory control of synapses results in excitotoxic neuronal activity conducive to seizure kindling.

Finally, the exacerbated spatial memory impairment seen in all BACE KO; PDAPP and 18mo BACE pKO; PDAPP mice suggests that if it is due to additive deficits from two separate mechanisms, then it may be that this phenotype is the consequence of simultaneous loss- and gain-of-function dysfunction. It is reasonable to conclude that if an entity regulates a vital function, then perturbations to this entity will also cause changes to the function it regulates. Perhaps this is why Aβ appears to be so toxic to synapses in AD, and why AD patients with accumulations of Aβ have such widespread

progressive mental disorders. The lack of patients with naturally occurring deficiencies in amyloid may explain why so little focus is placed on its normative role, as the deleterious gain-of-function effects of Aβ take such a heavy toll on the aged population in the form of AD. However, if the medical research community is willing and ready to embrace amyloid modifying treatments, specifically with BACE inhibition, then the theoretical risks and benefits must be examined.

8.9 Implications for BACE inhibition treatment in Alzheimer's Disease

The results of the characterization of the BACE x PDAPP animals described previously in this dissertation suggest that absolute loss of BACE on an amyloid overexpressing background acts to worsen the spatial memory status and seizure propensity of PDAPP mice. While this may sound like a damning statement for BACE inhibition as an AD therapy, there is still reason to believe to continue to examine this strategy as a potential source of meaningful benefit to AD patients.

The first major piece of evidence to support BACE inhibition for AD is that partial loss of BACE on a PDAPP background did improve spatial memory function with respect to acquisition, perseveration, and general serial learning in middle-aged animals. While this may seem like a limited positive finding, two things must be considered. The first is that the ability to acquire, rewrite and serially encode information is critical to learning and memory, and is a key feature of synaptic plasticity. These cognitive improvements could translate to major improvements in daily living functions if they could be applied to AD patients. The second item to consider is that the age in which the spatial memory was improved relative to PDAPP mice suggests that progressive cognitive dysfunction in the presence of amyloid plaques can be arrested within a specific context. This result echoes the findings of other researchers in amyloid-modifying treatment studies using other transgenic hAPP mice, as lowering amyloid levels in younger animals seems to be more effective than "curing" cognitive pathology in aged animals (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002; Austin et al., 2003; Jensen et al., 2005). While this trend in hAPP treatment studies may be telling us that prophylactic AD treatments will be the most successful, it may also be that these models are limited by their greater relationship

with end-stage AD disease than the disease at the time of clinical diagnosis. In either case, the improvement of spatial memory deficits in 13mo BACE pKO; PDAPP mice is important.

This is not to say that deleterious spatial memory phenotypes were not also uncovered with partial BACE gene deletion in Study 006. The increase in spatial memory deficits in 18mo BACE pKO; PDAPP mice suggests that while excess Aβ in the PDAPP mouse produces one set of memory impairments, another set of cognitive deficiencies can occur when there is a reduction of BACE activity in the context of aged PDAPP mice. The neurodegeneration present in the PDAPP mouse is a dynamic process that undergoes dramatic changes over time, and it is difficult to draw definitive parallels between disease states in 13mo and 18mo old mice with that of mild, moderate and highly symptomatic AD. These data suggest that the *in vivo* effects of BACE inhibition on an AD disease state must be examined over time, preferably from a number of different intervention time points to best understand the dynamic between cognitive benefit and collapse derived from BACE reduction.

Underlying non-spatial neuronal dysfunction was also notably ameliorated in even older 18 mo BACE pKO; PDAPP mice, as they were more resistant to and survived longer after high dose PTZ-induced seizure induction. This finding is equally as exciting as the improved spatial memory function at 13mo, as this demonstrated that despite lifelong reduction of BACE enzyme activity, BACE pKO; PDAPP animals were still able to respond to an acute neuronal challenge in a more typically normal manner. Thus it appears that while complete BACE KO and partial BACE KO on an aged PDAPP background is doubly damaging to spatial memory, potentially by two distinct mechanisms, other cognitive benefit can be achieved by addressing one mechanism via BACE reduction alone.

Aside from the spatial memory and seizure phenotypes, there were also notable anxiety and motor coordination deficits in the BACE KO x PDAPP mice. In the case of the anxiety characteristics, it is possible that this seemingly dominant PDAPP phenotype is the result of developmental abnormalities in the PDAPP mouse line caused by a lifetime

of hAPP expression. Alternatively, this unchanged phenotype may be separate from that of the PDAPP mouse, but this would have to be explored by use of other *in vivo* models that would specifically allow for normal development without excess hAPP.

The motor coordination impairment phenotype is however, possibly indicative of a true mechanistic toxicity induced by removal of BACE1. Impaired motor coordination was a dominant phenotype of BACE KO; PDAPP animals, with a trend towards a similar deficit in aged mice with a partial BACE gene deletion. The fact that this phenotype appeared to be age-related, as only 18mo BACE KO, BACE KO; PDAPP and BACE pKO; PDAPP mice had poorer rotorod performances, suggests a link to the amyloid neuromuscular disease IBM.

Cognitive improvements and impairments, motor deficits and anxiety have been identified in this initial phenotyping of BACE KO; PDAPP mice, but there are limitations to using genetically-modified animals to understand the implications of BACE inhibition for AD. The BACE KO x PDAPP model incorporates three separate transgenic lines that each have their own flaws as *in vivo* AD research tools. These three lines will be examined separately, beginning with the PDAPP mouse.

The PDAPP mouse as a model of AD produces mutant human Aβ at a level 5-14x that of endogenous Aβ from birth, but the majority of AD patients are not carriers of genetic mutations that result in lifelong overexpression of cerebral amyloid. As the PDAPP mouse has a high level of hAPP from a very young age, its pathology is accelerated compared to that most AD patients, as these mice have less than 50% of the total lifespan of Control mice (Figure 3.5a) . This early expression also adversely impacts neonatal brain development, as PDAPP mice have hippocampal and callosal atrophy (Dodart et al., 2000). Although PDAPP mice develop AD-like cognitive impairments it is difficult to determine if these are direct effects of excess amyloid on synapses or secondary effects of amyloid on development. From this perspective it must be emphasized that the PDAPP mouse is an attempt to approximate FAD, but it fails to capture all the nuances of the disease (lacking neurofibrillary tangles and neuronal loss), and it also includes a disease evolution that is not within the scope of sporadic AD.

The purpose driving the creation of animals that absolutely lack the BACE1 enzyme is to determine the most extreme repercussions of removing that gene product from the living system. BACE KO mice had overall milder phenotypes than PDAPP mice, and lived longer on average, although they have motor coordination problems (Table 3.4). While there is no apparent change in α-secretase or BACE2 expression as a result of BACE1 KO in these mice, it is possible that BACE KO mice were influenced by other compensatory shifts in gene expression that could negatively affect memory or motor function (Basi et al., 2003). These mild spatial memory BACE KO-affiliated phenotypes may be developmental or progressive with age, but they are not the most accurate way to gage how BACE reductions will affect an AD patient, as indeed most AD patients have elevated BACE1 levels (Fukumoto et al., 2002; Gatta et al., 2002; Fukumoto et al., 2004; Li et al., 2004).

BACE KO; PDAPP mice are a better approximation of theoretical BACE reduction on an AD-like background, but it shares the same drawbacks of both PDAPP and BACE KO mouse models, and seemingly suffers an additive combination of their deficits, including a lifespan that is only 30% of Control animals. The most valuable theoretical model of BACE inhibition is the BACE pKO; PDAPP mouse, as this animal has had the benefit of some functional BACE1 enzyme throughout development. It is indeed very interesting that the BACE pKO; PDAPP mouse line was either better than or no worse than PDAPP mice for the 13mo age in all measures tested, but much worse at 18mo in spatial memory. The arbitrary 50% reduction of the BACE gene product was seemingly well-tolerated, as these mice were fertile and lived longer than PDAPP littermates (Table 4.5).

Certain of the limitations inherent in phenotyping traditional genetically-modified animals could be addressed by the development of a conditional BACE KO animal on a PDAPP background. Thus the animals would have the full benefit of BACE throughout development, by employing different conditional knockout systems, different levels and regions of BACE reduction could be achieved. This experimental paradigm could be a fruitful method to answer some of the theoretical questions about how much of the BACE KO phenotype was a direct or indirect consequence of aberrant development. However,

use of a BACE conditional knockout would introduce the novel limitation of not being able to know the breadth of effect of BACE tissues on the entire system as the enzyme would only be lessened locally in the brain.

Knockdown of BACE gene transcription by cerebrally-injected RNA interference (RNAi) sequences that inhibit transcription from DNA to RNA, goes a step further towards understanding the effects of BACE inhibition for AD (Fire et al., 1998). Like conditional BACE KO, BACE-directed RNAi would allow the developing nervous system full access to BACE gene products. While both conditional BACE KO and RNAi would have spatially restricted local effects, RNAi has the additional advantage of allowing the experimenter to specifically choose the time of BACE reduction (current brain-specific conditional knockout systems begin to activate at a few weeks after birth), and is also reversible if the cerebral RNAi infusions are stopped depending on the delivery system (Hommel et al., 2003; Beglopoulos and Shen, 2004). While this technology has several advantages over more traditional in vivo genetic manipulation, the added complication of intracerebral infusions and the fact that BACE is still being reduced at the level of the gene rather than at the enzyme activity level like a pharmacologic treatment does mitigate the predictive value of this model. The positive initial findings reported by Singer et al (2005) using siRNA targeting BACE1 on a hAPP mouse model with accelerated amyloid deposition are indeed heartening, as these animals have ameliorations in amyloid-driven neuropathology as well as behavioral improvements. These results are similar to that of 13mo BACE pKO; PDAPP mice, but it was not until 18mo that these animals displayed a severe cognitive impairment. It is of critical importance that BACE RNAi experiments be done over a long period of time and from various starting times to best determine if this BACE reduction paradigm will be continue to be beneficial to cognition within the context of progressive aging.

Ultimately, the best way to truly gauge the effects of BACE inhibition on AD patients would be to have an actual compound that could be given to aged rats or transgenic hAPP mice. Repeated drug dosing would inform as to whether cognitive deficit reversals are possible in aged animals, or if neuromuscular phenotypes evolve with consistently reduced BACE activity. If BACE inhibition is efficacious in reducing amyloid and

memory impairments, and the majority of the BACE KO non-memory phenotypes are in reality developmental defects, then BACE inhibition would have earned its place as one of the most exciting potential therapies for AD. If BACE inhibitor drugs are biochemically and mnemonically efficacious, but do cause IBM-like conditions, BACE reduction can still be a viable strategy for AD if a suitable therapeutic window between efficacy and side effects can be found. It is unlikely that BACE inhibition could cause catastrophic cognitive collapse and motor problems like the BACE KO; PDAPP mouse phenotype as the chances of developing a systemically-administered drug that could reduce BACE activity levels in acidic cellular compartments to 0-50% are vanishingly small. Indeed results from deletion of one BACE gene allele in the BACE pKO; PDAPP mice suggests that even long-term loss of 50% of BACE activity could be well-tolerated and cognitively efficacious in dosing regimes, but must be scrutinized very carefully at older ages.

In summary, the characterization of the BACE x PDAPP mouse line has shown that while complete deletion of the BACE gene causes a mild but progressive spatial memory phenotype, absolute BACE deletion on an hAPP background causes severe cognitive deficits. These findings underscore the importance of the APP processing pathway in normal neuronal activity regulation and memory processes, building upon the body of knowledge that suggests loss of function in this pathway is as deleterious to the nervous system as Aβ gain of function. In addition to these findings, the cognitive rescue at middle ages and the cognitive deterioration at older ages in mice with partial deletion of BACE on the PDAPP background serves as both the strong endorsement and caution for BACE inhibition as a strategy for treatment of AD. Definitive information on whether β-secretase activity reduction would be a benefit or detriment to AD patients will likely only come from in vivo experiments done with an efficacious BACE inhibitor compound.

Ch. 9 References

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Ch. 10 Appendix

10.1 Behavioral phenotypes of other genetically modified mouse models relevant to AD

Transgenic Model	Gene, Mutation, Promoter	Behavioral Phenotypes	Age of Phenotype	Reference
TgCRND8	hAPP K670N,M671L V717I, Hamster PrP promoter	Low survival rates	50% death by 3 mo	Chisthi et al. (2001), J Biol Chem
		WM Spatial Memory	11 w	
C3H/B6		Retention, Acquisition		
		Auditory Startle	6-7, 10-12, 12-14, 15-17w (progressive)	McCool et al. (2003), Brain Res
		Paired Pulse Inhibition	6-8, 12-14, 15-17 w	
J20	hAPP K670N, M671L V717F, PDGF promoter	WM Spatial Memory	6-7 mo	Palop et al. (2003), PNAS
		Retention, Acquisition		
DBA/2J C57Bl6J				
TAS10	hAPP K670N,M671L Thy-1 promoter	WM Spatial Memory	6 , 12, 18mo	Richardson et al. (2003), Neurosci
		Retention, Acquisition		
		Y-Maze Alternation	12, 18 mo	
APP KO	APP-null by Homologous Recombination	Decreased locomotion	14w	Zheng et al. (1996), Ann NY Acad
		Lesser forelimb grips	14w	
		Decreased body mass	14w	
		Spontaneous seizures	14w	
APP/RK (FVB/N)	hAPP K670N, M671L V717I, Thy-1 Pr	Premature death	1 yr	Moechars et al. (1996), EMBO J
		Aggression	3mo	Moechars et al. (1999) Neurosci
		Hyperactivity	3mo	Moechars et al. (2000) JBC
		Spontaneous seizures	6mo (progressive)	
ADAM10xAPP (FVB/N)	**hAPP V717I, hADAM10** Thy-1 Pr	**Rescue of WM spatial memory deficits** relative to hAPP mice	**6-10 mo**	Postina et al. (2004) J Clin Invest
BACE1 KO		Less exploratory in	6-7 w	Harrison et al. (2003), Mol Cell Neurosci
		Holeboard, open field	6-7 w	
		Greater limb tone	6-7 w	
		Greater Righting Reflex	6-7 w	
hBACE1 Tg	hBACE1 CAMKIIa Pr	Bold exploratory phenotype in Holeboard, open field	6-7 w	Harrison et al. (2003), Mol Cell Neurosci
		Lesser body mass	6-7 w	
BACE1 -/-/Tg2576+	**BACE1 -/- hAPP Tg2576**	**Rescued social recognition Memory relative to Tg2576**	**4-6mo**	**Ohno et al. (2004), Neuron**
		Rescued Y-Maze Alternation Relative to Tg2576	**4-6mo**	

WM = water maze
(progressive) indicates a behavioral phenotype that is age-related

Table 10.1 Behavioral Phenotypes of Other Transgenic Mouse Models Related to AD, genetic models with modifications to hAPP, α- and β-secretase.

Transgenic Model	Gene, Mutation, Promoter	Behavioral Phenotypes	Age of Phenotype	Reference
PS1 cKO	PS1-deficient By cre/lox aCAMKII promoter	WM Spatial Memory Retention, Reference	5, 8 mo	Yu et.al. (2001), Neuron
PS1 Tg	PS1 L235P	Object Recognition	6mo	Huang et.al. (2003), Exp Neurol
PS1 Tg	PS1 L286V	Object Recognition	6mo	Janus et.al. (2000), Neurobiol Aging
hPS2 Tg	PS2 N141I Dominant Negative	WM Spatial Memory Retention, Reference	12mo 12mo	Hwang et.al. (2002), FASEB J
APP+PS1	hAPP Tg2576, hPS1 M146L	Y-Maze Alternation Y-Maze entries Open Field String Agility Test WM Spatial Memory, Acquisition Radial Arm WM, Working Memory	12-14 w, 6, 9 mo 5-7 , 15-17 mo 15-17 mo (progressive) 15-17 mo (progressive) 15-17 mo (progressive) 4-5, 14.5-16.5, 15-17mo	Holcomb et.al. (1998), Nat Med Arendash et.al. (2001), Brain Res Gordon et.al. (2001), Neurobiol Aging
PSAPP	hAPP Tg2576, hPS1 A246E	Contextual Fear Conditioning WM Spatial Memory Retention, Acquisition	5, 9mo 14mo	Dineley et.al. (2002), J Biol Chem Liu et.al. (2003), Neuroreport
APPxPS1(-/-)	hAPP V717I, Conditional KO of PS1, cre/lox, LacZ promoter	Object recognition	3-6mo	Dewachter et.al. (2002), J Neurosci
PS2APP	hAPP K670N, M671L, hPS2 N141I	Lesser body mass Hyperactivity High shock thresholds Weaker grip strengths Improved rotorod performance WM Spatial Memory Retention, Acquisition Active Avoidance'	4, 16 mo 4, 16 mo 4, 16 mo 4, 16 mo 4, 16 mo 8, 16mo (progressive) 8, 16mo (progressive)	Richards et.al. (2003), J Neurosci
PS1 cKO x hAPP	**PS1-deficient By cre/lox CAMKIIa promoter, hAPP J20**	**Rescue of WM spatial memory deficits** relative to J20 mice	**3-4mo Lesser rescue at 15.5-17.5mo ages**	Saura et.al. (2005), J Neurosci

WM = water maze
(progressive) indicates a behavioral phenotype that is age-related

Table 10.2 Behavioral Phenotypes of Other Transgenic Mouse Models Related to AD, genetic models with modifications to γ-secretase.

10.2 Animal use protocol approved for pentylenetetrazole-Induced seizure experiments in BACE KO x PDAPP mice

Taken from an original internal Elan Pharmaceuticals animal use protocol submitted for review and approval by our Internal Animal Care and Use Committee (IACUC) by Dione Kobayashi. This protocol details the activity monitoring, grip strength, rotorod and seizure tasks upon which the analysis of the BACE KO x PDAPP mice was based. This animal use protocol, was approved 4/23/03 after expedited review by George Shopp Ph.D. (IACUC Chairman) and Hermann Bonasch D.V.M. (Consulting Veterinarian).

Please Check One
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Renewal__ _

Protocol No. MO-PH-18-03

1. **Principal Investigator:** Dione Kobayashi

1. **Work Phone:** 650 794-4343

3. **Emergency Phone:** 650 274-4343

3. **Project Associate(s): Tracy Cole**

3. **Work Phone:** 650 866-2870

3. **Project Title:**

   Pilot Behavioral and Pharmacologic Assessment of Spontaneous Activity, Motor Coordination, Muscular Strength and Epileptiform Activity in Mice.

7. **Species identification and source of animals:** DBA 2J and C57BL6J mice, male and female, of no more than 3mo of age. Prefer vendor source that can provide both strains, as DBA/2J is not as commonly ordered as C57Bl6 and inter-vendor variability between strains is should be avoided if at all possible.

8. **Proposed number of animals/year:** 80, 16 C57Bl6J mice and 64 DBA/2J mice.

9. **Estimated starting date:** April 2003

10. **Building/Room Locations of Animals: 800 Building, Room 199, later Room 140B.**

10. **Describe experimental goals in appropriate terminology in order that the IACUC committee members will understand the purpose of the experiment and why the research requires the use of live animals.**

    In the analysis of genetically manipulated (GM) or pharmacologically-treated (PT) animals in therapeutic research, it is often useful to determine whether the animals

have behavioral alterations that are indicative of greater systemic effects. Changes in appearance, motor activity and dexterity, and functional musculature in PT or GM animals are critical observations that can provide information in mechanisms of action or drugs or genes, and can help predict the array of adverse reactions that may arise from therapeutics. In addition, PT and GM animals used in neurological therapeutic research must also be assessed for perturbations of the nervous system, including changes in anxiety and fear responses, epileptiform and pain thresholds, and cognition. To help provide a basis of analysis for our experimental animals, a set of representative tasks must be developed in-house to address the effects of specific gene manipulations or drug treatments on these basic functions. Specifically, the establishment of these tasks could provide useful data for BACE1 KO characterizations and BACE inhibitor treatment studies in PDAPPs.

These pilot studies performed on mice from the background strains (C57Bl6J an DBA/2J) of our GM and PT mice will serve as historical strain controls for future experiments, thereby eliminating the need to perform serial strain controls and greatly reducing the numbers of animals required for studies. To ensure that best and most informative use of these pilot study animals, a complex set of behavioral features must be collected. The relative subjectivity/objectivity and thus rigor of each task set is variable. However, even the more subjective tasks based on grading of observed behaviors (Functional Observational Battery, FOB) are valuable and gain rigor through multifactor analysis, in which composite FOB scores can be distinctly grouped based on results from similar tests, e.g. spontaneous locomotion, gait and rotorod performance.

In addition this protocol uses many important behavioral screening techniques. The listed PA will gain experience from observing FOB data collection, gaining proficiency for future solo testing. Also, efforts will be made to create a video record of stereotypic FOB behaviors and scores to assist experimenters learning the tasks and to help make grading more uniform.

Summary: Objective 1: to develop tasks for future screening of PT and GM animals, Objective 2: to generate a set of historical control data as a basis for comparison. Objective 3: To expose Project Associate to a wide array of behavioral tasks and collect visual information for more uniform behavioral scoring.

12. **Are there alternative methods available to reduce or replace the use of live animals in this research effort?**

To gauge the effects our therapeutics may have on human patients, we need an intact mammalian system. However, one of the main purposes of performing these studies is to reduce future animal use based in animals generated from the C57Bl6 and DBA 2J strains.

13. **Provide a justification for the need for the total number of animals required for this experiment.**

Some of the methodologies used in this protocol, like the rotorod and PTZ-induction of seizure require Ns of at least 8 per group to overcome the inherent interanimal variability. This must also be done with sufficient numbers of male and female animals to account for experimental deviations due to sex differences. At least two experiments are planned, totaling 80 animals.

The Experiment 1 will utilize 8 animals per strain, 8 of each gender for a total of 32 animals. This will be to determine whether there is significant by-gender differences, and the results will play into the gender of the animals for Experiment 2, whether it will be mixed in gender or one gender or another. The second experiment will be a PTZ dose-finding experiment with 12 animals per dose arm, of 4 doses, bringing the

pilot total to 80.

Summary:

Experiment 1: 2 strain comparision, gender comparison, N=8 per strain, gender, total N-=32
Experiment 2: Dose-response experiment with seizure-inducing agent PTZ, 3 doses of PTZ plus vehicle control N=12 each dose, total N=48. Mixed/one gender will be used depending on the results of Experiment 1; strain used will be DBA/2J.

14. **Describe the experimental methods to be performed on the laboratory animals.**

Please note that testing will be performed in the order of tests described, from least to most distressful, culminating in euthanasia of animals.

Only one experimenter will be allowed to perform the FOB testing, assign behavioral scores and submit descriptions for the induced seizure profiling to promote uniformity of scoring and collection of meaningful data. This experimenter is experienced in handling mice in a number of behavioral paradigms, familiar with the behavioral responses of unaltered mice described in the FOB, has observed a variety of murine seizure activities, and also is proficient in administering intraperitoneal and subcutaneous injections to mice. All experimenters named on this protocol have experience and training in basic handling of mice, so that the grip strength, automated locomotion testing, and motoric coordination tasks may be effectively conducted by all listed experimenters.

SHIRPA Functional Observation Battery (SHIRPA acronym is derived from the laboratories in which the battery was developed: SmithKline Beecham Pharmaceuticals, Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit, Imperial College School of Medicine at St Mary's Royal London Hospital, St Bartholomew's and the Royal London School of Medicine. Phenotype Assessment)

Animals will be removed from their home cages and be manually handled or otherwise placed in empty observation cages.

General condition: Note general body thinness, grooming status and stained fur, vocalization when handled or in cage, noting if animals appears hunched, dehydrated or has edema.

The severity of various abnormalities (see below) should be ranked according to the q-scale:

0 = normal
1 = slight
2 = moderate
3 = severe

Splay reflex: Animal is lifted near base of tail; a normal (0) animal splays and extends hind limbs.

Reduced: not out to sides and not fully extended, held close to abdomen And/or unable to extend legs.
(NB: animals with reduced splay reflex may also show gait abnormalities and reduced foot withdrawal reflex.)

Visual Placing: lift mouse by the tail and slowly move it downward towards the benchtop edge. A normal (0) animal extends the forelimbs and attempts to seize

the edge as soon as it is within reach. A reduced response for is scored as 1 –animal extends and grips bench after contact of whiskers and/or nose, 2 – no response even after nose contact with bench.

Rearing: Animal is placed in standard open field enclosure and observed for 2 minutes. The number of rears, i.e. front legs lifted completely off the benchtop but does not have to raise itself up, is counted. Include when animal uses the side of enclosure for support and lifts paws for grooming. No acclimation time is provided and counting should commence within ca 10 seconds of transfer. The previous animal should be removed from the open field before transferring a new animal. A disposable lab liner should be used in the enclosure and replaced as needed to prevent animal distractions from previous voided excreta.

Activity: Animal is transferred to the standard open field and observed briefly. Normal activity is to explore the new environment, this may be decreased or increased and is scored on a 3 point scale. Decreased activity may range from walking around occasionally but noticeably less than control (-1), walks only when stimulated (-2) or will not walk even when stimulated (-3). Increased activity may range from constant movement with normal gait (1), constant movement (2) with rapid gait and animal may try to escape from cage, to (3) animal runs about and tries to escape, gait and posture are slightly abnormal. Stereotypic or bizarre movement e.g. circling, repetitive grooming, head flicking, head searching, walking backwards, rolling over, back flipping, paw flicking, etc should be noted in the free text

Prostration – Animal lies on the bottom of the cage (ventral or lateral recumbancy) and appears powerless and does not respond to stimulation.

Sedation – State of decreased functional activity and reduced response to external stimuli, e.g. sound or touch.

Comatose – Animal is unconscious and there are no reflex responses e.g. pinna reflex although respiration is detectable.

(If animal is comatose for more than 15 observed minutes, it will be euthanized.)

Gait: observe animal moving about the open field, gently prod the animal if it doesn't move. Look at the movement of all four limbs in relation to one another and the saggital plane of the body. The normal animal moves opposing limbs simultaneously and remains steady.

Ataxia: staggering, wobbly gait, i.e. muscular coordination failure even though power for movement remains. May range from slight loss of equilibrium as indicated by an irregular gait (1), marked loss of equilibrium - animal can walk a straight line but gait is very irregular (2) to extreme - animal can hardly walk and there is almost complete loss of coordinated movement (3).
Splayed - Hindlimbs may be splayed or point to the side of the body
Tiptoe – walking on toes, i.e. the heels of the feet are elevated or perpendicular to the surface

Paralysis: The hind or fore limb function may be affected from slight (1) difficulty using limb when walking to severe (3) muscles of limb stiff and not able to be used, animal drags limb or for forelimbs, 'snow plows'. The affected limbs (fore or hind) should be noted and, if unilateral, side noted.

Posture: observe animal while moving about – normal posture is straight back and pelvis off the surface. Abnormalities should be classified (see below) and severity ranked.

Hunched: back raised
Low: pelvis is low, severe is when the pelvis is flat on the surface

Fur: normal fur is sleek and unstained, abnormalities include: sparse - (alopecia), stained, ungroomed, or piloerection (hair standing on end)

Tremor: rhythmic, involuntary movement of a muscle, limb or whole body caused by repeated alternating contractions of flexor and extensor muscles. Tremors can often be observed without handling the animal but detection of fine tremors may require grasping the animal around the back. 0 – no tremors, 1 – occasional fine tremors, 2 – continuous but not pronounced, 3 - coarse tremors, easily observed and where the ability to walk may be affected. Tremors also include fasciculations (irregular contractions of a muscle block) and writhing (animal twists its whole body and tail from side to side).

Convulsion: more severe than tremors in that a convulsion is always a whole body response and animal can not maintain an upright posture during a convulsion. Severity and type (see below) should be noted

Clonic type: alternative contraction and relaxation of the voluntary muscles

1) **C = Clonic** - co-ordinated, unsymmetrical convulsion and natural, purposeful like movements, e.g. running, sometimes preceded by a running excitement (Rn)
2) **Cs = Clonic symmetrical** - repetitive symmetrical jerks or twitches of the limbs
3) **Rn = Running excitement** - often accompanied by mild clonus or leading to a severe convulsion
4) **Ch = Champing** - clonus of the jaws only
5) **P = Popcorn** - seizure where animal repeatedly "pops" into the air
6) **A = Asphyxia** - a terminal clonic or clonic-tonic convulsion resulting from respiratory failure.

Tonic type: persistent contraction and spasm of a set of voluntary muscles.

1) **T = Tonic** - sustained extension of hindlimbs, usually preceded by tonic flexion **(Tf)** This is used if tonic flexation occurs without extension
2) **Op = Opisthotonus** - head, body and limbs are rigidly extended and arched backwards.
3) **Em = Emprosthonus** - opposite of Op i.e. extended forward.

Miscellaneous Type

1) **Rr = Rock and roll** - animal is prostrate on its back and rocks from side to side in a seeming effort to right itself, occasionally rolling over (overshooting) and continuing to rock again.
2) **Su = Sitting up** - sits upright on hindlimbs during the seizure
3) **Pr = Praying** - sitting up seizure in which forelimbs are held together or crossed in attitude resembling prayer.

If convulsions appear to be initiated by stimulation, i.e. touch, this should be noted.

It is not expected that animals will have seizure or convulsant activity endogenously without administration of agen PTZ. Howver, animals will be euthanized if observed to have seizures of any kind for more than 15 minutes in the FOB.

Tail: the posture of the tail is observed and abnormalities may range from extremely limp and dragging around (-1), normal (0), stiff (1) but not close to body, rigid (2) and S shaped, or Straub tail (3): rigid and held vertical or arched over the back.

Unusual movement of the tail, i.e. lashing back and forth, is noted in the free text

Urine - signs of excessive urination are noted by wet fur around the vulva/penis. Urinary incontinence is indicated by wetness of the lower abdomen and may be associated with hindlimb paralysis and is noted in the free text. Abnormal color is also noted in the free text

Feces - if different from normal scored for severity 1 – slight, 3 – severe/ marked the color and state should be noted, diarrhea is soft to liquid feces.

Respiration: The character and rate of breathing is compared to control animals. The depth of respiration may be decreased or increased as well as the rate. Labored breathing, dyspnea, is deep respiration with movement of the thorax, gasping is deep respiration with mouth wide open. Noisy respiration may range from wheezing, whistling to croaking

Approach Response: Approach the animal from the front with a blunt rod and record whether it evades (E), ignores (I) or attacks (A) the rod. Normal (N) behavior is to orient to the rod and may investigate it.

Righting Reflex: The animal is laid on its back and the time and effort to turn itself over is noted. A normal animal immediately rights itself while a reduced reflex may range from a slight, few second delay (-1), rights itself after struggle (-2) to an inability to right and stays on its back (-3).

Handling Behavior: fearful, aggressive, or normal

Abdominal Tone: Gently press the abdomen until firm resistance is felt. Tone may be increased (1) or decreased (-1) compared to control (0) animal.

Cyanosis: color of the extremities, e.g. ears and feet may indicate the degree of perfusion and blood oxygenation. Scored from normal (0), through paleness (1), to blue (3). Other changes in skin color, i.e. jaundice (yellow), should be noted in the free text. Erythema, i.e. redness, should be noted in the free text.

Salivation: the wetness of the mouth and surrounding fur is evaluated for signs of increased salivation. Normal is scored as 0, fur immediately around mouth is wet (1), a definite wet area ca 3 – 10 mm (2) all the way to the chin and throat wetness (3). Signs of dried saliva should be noted.

Lacrimation: Eyes are examined for presence of colorless fluid, normal condition (score 0) is slightly wet, cornea reflects light. Excess fluid may range from slight, noted as a minimal accumulation at the lower eyelid (1) to severe (3) where the adjacent fur is wet. Dry eye (scored –1) is noted as a dry, dull appearance of the eye. Chromodacryorrhea is red – dark fluid around the eyes and should be noted and ranked.

Toe Pinch: The foot is pinched with fingers or cushioned forceps (carefully, without breaking bones or dislocating joints). Normal animals try to vigorously withdraw foot. An abnormal response may be caused by analgesic effect (animal cannot feel stimulus) or motor nerve damage (feels stimulus but unable to withdraw foot). Scored as normal (0), slight (more pressure is needed to elicit normal response) to no response to firm pressure (-2).

Palpebral Closure : Opening of the eyelids is noted as reduced (i.e. ptosis, scored as –1) or normal (0)

Eye Prominence: Compared to control animals, eyes may appear to protrude (exophthalmos) when viewed from a front on position (1) or to be recessed into the sockets, endophthalmos (–1). If only one eye is effected note right or left.

Pupil Size: The animal is restrained and the eye observed for constriction of the pupil (miosis, -1) or dilation (mydriasis, 1) under normal room light illumination. It is difficult to examine a mouse eye pupil so this observation is not routinely conducted for mouse studies.

Corneal Reflex: The animal may need to be lightly restrained, the cornea is gently touched with a probe (stick with soft hair attached) taking care to not touch the eyelid or eyelashes. Normal animals blink (scored as 0), abnormal response is absent (-2) or reduced (-1).

Pinna Reflex: The animal is held behind the shoulders but not scruffed since free head movement is needed to respond, and the ear canal probed. A normal response is a head twitch and is scored as present (0) or absent (-1).

Acoustic Startle: loud, short-lived noise e.g. click or clap. A normal response (0) to stimulus is short lived and animal increases alertness and may cease activity. An exaggerated reaction (score 1) may jump, bite or attack and response may be prolonged while decreased reaction (-1) is no response.

Geotaxis: Place animal horizontal on screen tilted 45°. Note direction of mouse movement, i.e. rotate and walks up (U), across (A) or down (D) the screen.

Inverted Screen: Place animal on screen and invert. Note if animal drops off (2), hangs on for 60 sec or slowly climbs to top (1) or readily climbs to top within 20 sec (0). Repeat for a total of 3 chances or until score = 0 and note number of trials.

Grip Strength Test

Using a San Diego Instruments Grip Strength apparatus, mice will be tested on both fore- and hindlimbs. The apparatus consists of an acrylic platform with space for force gauges at either end to which animal grip yokes may be attached. Mice will grasp the triangular grip yoke with their fore- or hindlimbs while the experimenter grasps the animal's tail 3/4 from the base, moving the animals in the x-axis away from the strength gauge until it releases the yoke. The average force recorded from 3 successive trials will be taken as the grip strength value.

Minimal distress is expected from this manipulation, the level will be similar to what occurs during basic handling to remove an animal from its home cage.

Motor Activity

Using an automated system to quantitate spontaneous motor activity, animals will be placed in an open plexiglass arena. After an unmeasured period of acclimatization of about 10m, animals will be followed by the detection system for various periods of time, measuring patterns of ambulation, rearing, grooming and other repetitive behaviors. These time periods will not last longer than 2h, as the animal is bereft of food and water during this observation.

Motor Coordination with a Rotorod Apparatus

The measurement of motoric coordination in rodents is commonly done using a mechanized rotorod apparatus, which causes minimal distress to animals when applied properly with a sensible protocol. The apparatus itself consists of the test box, which houses 4 40cm x 10cm x 40 cm rotation chambers, the driver for the

rotorod on the side of the chamber boxes, and 2 instrumentation boxes beneath the test box which control the timing, speed, and electrical shock grid. Using either rats or mice, test animals are placed in on a motorized rod 40mm or 70mm in diameter in an enclosed chamber. An individual trial begins with the rotation of the rod and ends when the rodent falls from the rod (a distance of 30 cm) to the metal grid below, giving the primary measure of latency to fall. The rod can be programmed to rotate at specific speeds over time, giving information on the motor capacity of the animals at various speeds. The metal grid below the rotation chambers is electrified with a 3-5mA current, which is noxious but not painful to touch, and serves to prevent the rodents from prematurely leaving the rod without respect to its motor capabilities. After trials animals are returned to their home cages to recoup before their next trial in 10 minutes.

In this type of study, each experimental group or arm consists of N=15-20 per strain or genotype, to provide sufficiently powered statistical analysis. There are three stages to the rotorod testing performed at Elan to assess motor coordination and capacity, comprising a 3-day test regime. On the first day, animals are acclimated to the test chambers, in which they are placed on the rod for 30s for 4 trials with 10 minutes intertrial breaks. One the second day, animals are placed on a rod that rotates at 10rpm for 4 90s trials, in which animals that are unable to stay on the rod for 2 trials at the maximum 90s are considered impaired. With the information gained from this phase of testing, impaired animals can be removed from the third phase of testing, so that the third phase of testing can be used to assess the finer gradations of motor capacities in the remaining animals, and to spare incapable animals from further stress. On the third day of testing, animals are subjected to 7 trials of 240s maximum duration with rotation increasing steadily from 0-40rpm. Primary measurements are made from calculating average latencies over trials from constant and accelerating speed tests.

Typical Rotorod Protocol Schedule

Day 1	non-moving rotorod acclimatization	Trial 1
(optional)	animals will be placed on rod until	Trial 2
	they can stay on for 30s	Trial 3
	Electrified grid on intensity 3-5	Trial 4
Day 2	slow-moving rotorod training	Trial 1
	animals will be placed on rod at	Trial 2
	10 rpm for 90s, 10m intertrials	Trial 3
	Electrified grid on intensity 3-5	Trial 4
Day 3	Increasing-speed rotorod testing	Trial 1
	animals to be placed on rod	Trial 2
	that increases from 0-40 rpm	Trial 3
	over 240s, 10m intertrials	Trial 4
	Electrified grid on intensity 3-5	Trial 5
		Trial 6
		Trial 7

Seizure

Pentylenetetrazole (PTZ, from Sigma Chemicals) is a commonly used seizure-inducing drug that acts via the GABAA/benzodiazepine receptor complex, possibly by blocking Cl- influx. By employing intraperitoneal (i.p.) or subcutaneous (s.c.) at varying doses in rodents, seizures of a range of strengths and durations can be initiated. A typical experiment involves removal of an animals from its home cage to a larger observation area. Animals are dosed i.p. with PTZ at 20, 40, 60, and/or 80 mg/kg made with 5-10ml/kg dose volume in 0.9% saline solution and observed for seizure profile.

This profile includes observations of the time to onset, the severity and description of seizure (see above text regarding seizures in FOB section), number of seizures and duration of seizure activity. Observation follows in two phases with an initial Phase 1 in which animals are watched for the onset of seizure for 30 minutes, with a Phase 2 15 minute observation to gain descriptive information about the seizures. Alternatively, 85 mg/kg PTZ can be given s.c. at 85 mg/kg with a 30 minute window to observe appearance of seizure profiles. After 30 minutes of post-injection observations has passed, animals are euthanized. As the purpose of this part of the study is to build a wide range of seizure activity profiles, animals will not be immediately euthanized upon seizure unless they meet certain criteria, since with many kinds of seizure activity resolves itself within the 30 minute window:

- Animals displaying asphyxic clonic seizures will be euthanized immediately.
- Animals displaying "popcorn" clonic seizures will be euthanized if they continuously display this activity for more than 5 minutes
- Animals displaying continuous running, clonic symmetric or general clonic seizure activity for more than 5 minutes will be euthanized.
- Animals displaying more than 3 episodes of opisthotonic or emprosthonic seizures irregardless of duration will be euthanized
- Animals displaying episodes of partial clonus characterized by brief head twitches or vocalizations will not be euthanized prior to the 30 minute timepoint unless they progress to a sufficient level to any of the seizure criteria described above.

In the first experiment with the male and female animals (N=32) a single dose will be tested, 60 mg/kg, which should be sufficient to generate seizure activity in a majority of animals. The second experiment will entail dosing at the 20, 40, 60, and 80 mg/kg levels for a total of 12 animals per dose, N=48 to generate dose-response curves.

15. **Is a surgical procedure contemplated?**

_____ Yes ___x__ No

Building/Room location for surgery

16. **Describe the surgical procedures.**

17. **Describe post surgical care procedures.**

18. **Describe dosages/route of administration of anesthetics, analgesics and/or tranquilizers.**

19. **Will animals be used in more than one protocol.**

No.

20. **PAIN/DISTRESS - check C, D, or E, which is most appropriate for this protocol.**

C.___ The procedures to be performed on animals in this protocol does not involve pain or distress (excluding routine injections and venipuncture).

D.____ The procedures to be performed on animals in the protocol does involve pain or distress, and will be ameliorated by drugs described in #18.

E.__x__Pain and/or distress will be experienced by animals in this protocol and drug will not be used. A justification is required for non-use of pain ameliorating drugs.

Justification:

Some subset of the animals will develop tonic seizures which may result in death, but this is one of the central purposes of the protocol, to broadly explore the deleterious neurological side effects that could arise from our therapeutic research either via drug or gene manipulation. The time of distress will be kept to a minimum with euthanasia following induction of seizure at 30 minutes. Also, the second experiment proposed will be a dose-response study with PTZ-seizure induction to find the minimum effective dose that can elicit seizure responses in 95% of the DBA 2J and C57/Bl6J mice.

19. **Describe euthanasia techniques.**

Animals will be euthanized using carbon dioxide asphyxiation.

21. **Does this procedure duplicate previous experiments? If yes, complete justification.**

No.

22. **Agreement of compliance**

We agree to conduct our experiments according to this protocol and conform with the policies of Elan Pharmaceuticals, the N.I.H. Guide for the Care and Use of Laboratory Animals (revised 1985) and OPRR guidelines (revised Sept. 1986). Changes in the protocol can only be implemented by submitting an amendment to the protocol for IACUC review.

Further, as Principal Investigator for this protocol, I have established that the project associates involved with this study have adequate training and experience to conduct the methods required.

Principal Investigator_____ Date _____

23. **Expedited Review - requires two IACUC members signature.**

Signature_____ Date

Signature_____ Date

24. **Institutional Animal Care and Use Committee (IACUC) review.**

Date _____

Approved _____

Signature of Chairman _____

25. **USDA Category (if applicable).**

26. References:

1. Macdonald RL, Greenfield LJ (1997) Mechanisms of action of new antiepileptic drugs. Current Opinion in Neurology 10:121-128.

2. Kosobud AE, Crabbe JC (1990) Genetic correlations among inbred strain sensitivities to convulsions induced by 9 convulsant drugs. Brain Research 526:8-16.

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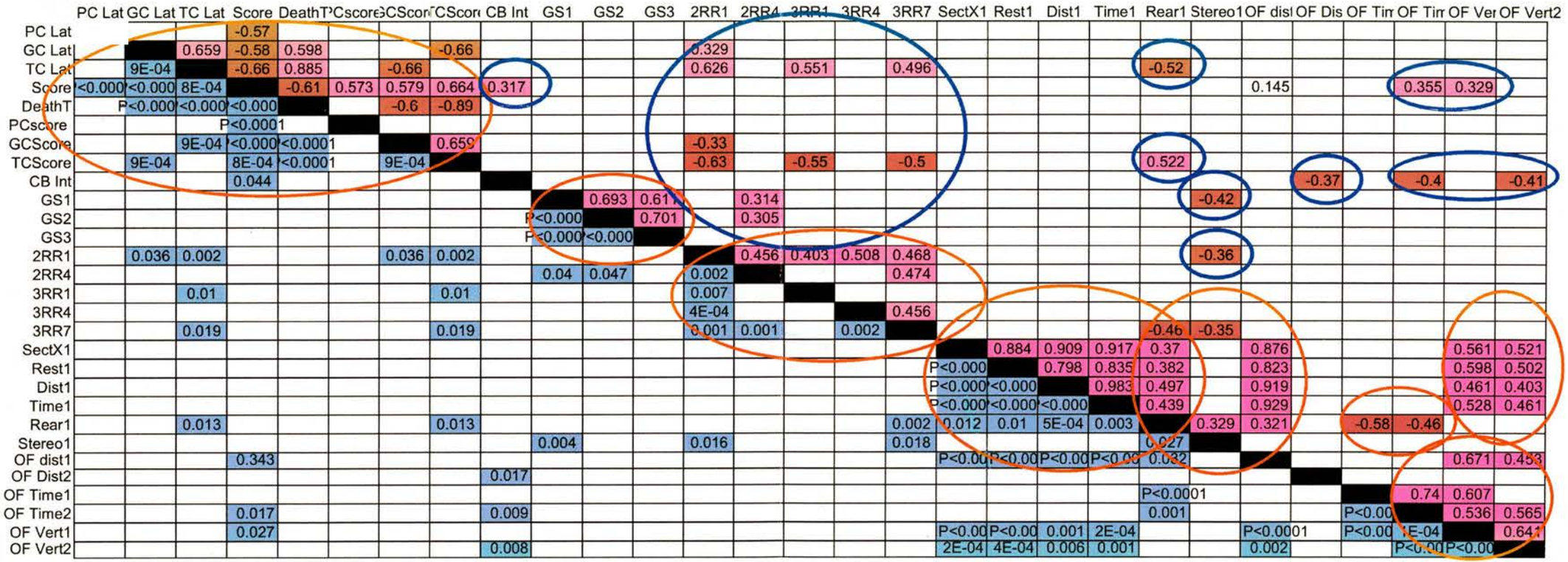
4. Meyer OA, Tilson HA, Byrd WC, Riley MT. (1979) A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav Toxicol. 1979 Fall;1(3):233-6.

5. Forster MJ, Lal H. (1999) Estimating age-related changes in psychomotor function: influence of practice and of level of caloric intake in different genotypes. Neurobiol Aging. 1999 Mar-Apr;20(2):167-76

6.Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome. 1997 Oct;8(10):711-3.



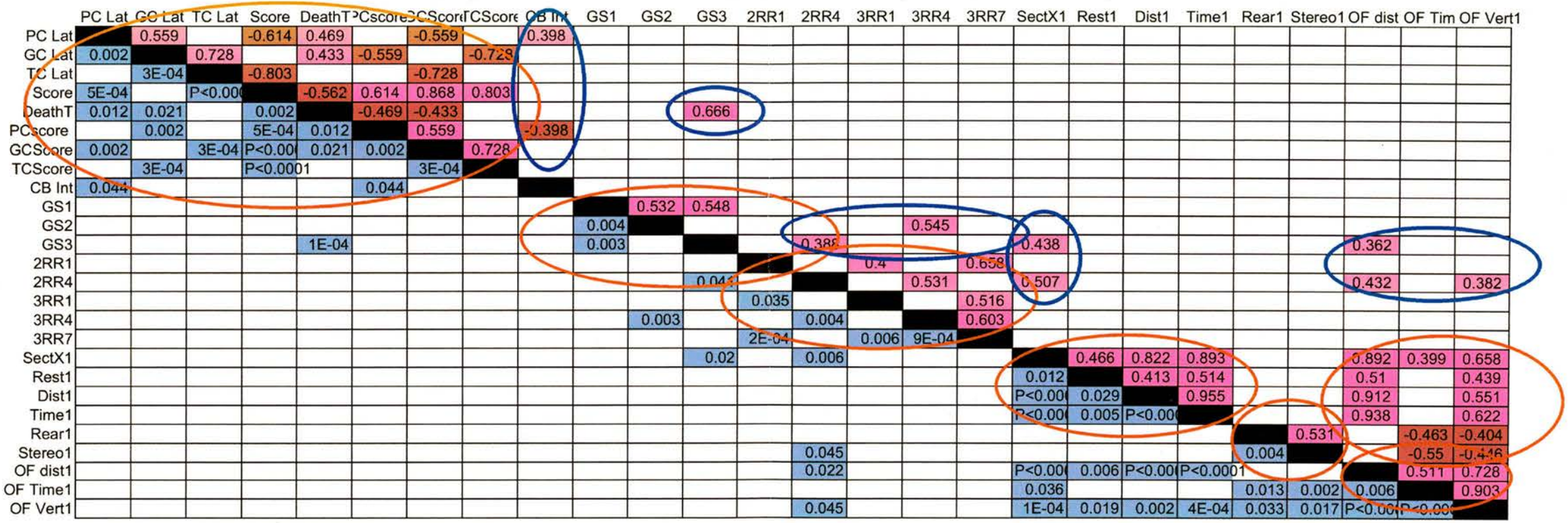
	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscore	GCScor	TCScor	CB Int	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dis	OF Tim	OF Tim	OF Ver	OF Vert2
PC Lat				-0.57																									
GC Lat			0.659	-0.58	0.598			-0.66					0.329																
TC Lat		9E-04		-0.66	0.885		-0.66						0.626		0.551		0.496					-0.52							
Score	<0.000	<0.000	8E-04		-0.61	0.573	0.579	0.664	0.317															0.145			0.355	0.329	
DeathT		P<0.000	<0.000	<0.000			-0.6	-0.89																					
PCscore				P<0.0001																									
GCScore			9E-04	<0.000	<0.0001			0.659					-0.33																
TCScore		9E-04		8E-04	<0.0001		9E-04						-0.63		-0.55		-0.5					0.522							
CB Int				0.044																					-0.37		-0.4		-0.41
GS1											0.693	0.61		0.314									-0.42						
GS2									P<0.000			0.701		0.305															
GS3									P<0.000	<0.000																			
2RR1		0.036	0.002				0.036	0.002						0.456	0.403	0.508	0.468						-0.36						
2RR4										0.04	0.047		0.002				0.474												
3RR1			0.01					0.01					0.007																
3RR4													4E-04				0.456												
3RR7			0.019					0.019					0.001	0.001		0.002						-0.46	-0.35						
SectX1																			0.884	0.909	0.917	0.37		0.876				0.561	0.521
Rest1																		P<0.000		0.798	0.835	0.382		0.823				0.598	0.502
Dist1																		P<0.000	<0.000		0.983	0.497		0.919				0.461	0.403
Time1																		P<0.000	<0.000	<0.000		0.439		0.929				0.528	0.461
Rear1			0.013					0.013									0.002	0.012	0.01	5E-04	0.003		0.329	0.321		-0.58	-0.46		
Stereo1										0.004			0.016				0.018					0.027							
OF dist1				0.343														P<0.00	P<0.00	P<0.00	P<0.00	0.032						0.671	0.453
OF Dist2									0.017																				
OF Time1																						P<0.0001					0.74	0.607	
OF Time2				0.017					0.009													0.001				P<0.00		0.536	0.565
OF Vert1				0.027														P<0.00	P<0.00	0.001	2E-04			P<0.0001		P<0.00	1E-04		0.641
OF Vert2									0.008									2E-04	4E-04	0.006	0.001			0.002			P<0.00	P<0.00	

Table 5.6 Correlation of Histological and Behavioral Measures, R- and P-values of All Study 011A Mice

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscor	GCScc	TCSco	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo	OF dist	OF Dis	OF Tim	OF Tim	OF Ver	OF Vert2
Controls																												-0.58
PDAPP																								-0.71		-0.63		
BACE KO; PDAPP																												

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscor	GCScc	TCSco	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo	OF dist	OF Dis	OF Tim	OF Tim	OF Ver	OF Vert2
Controls																												0.029
PDAPP																								0.003		0.013		
BACE KO; PDAPP																												

Table 5.7 Correlation of Calbindin to All Other Measures, R- and P-values of Homozygous BACE KO x PDAPP 011A Mice



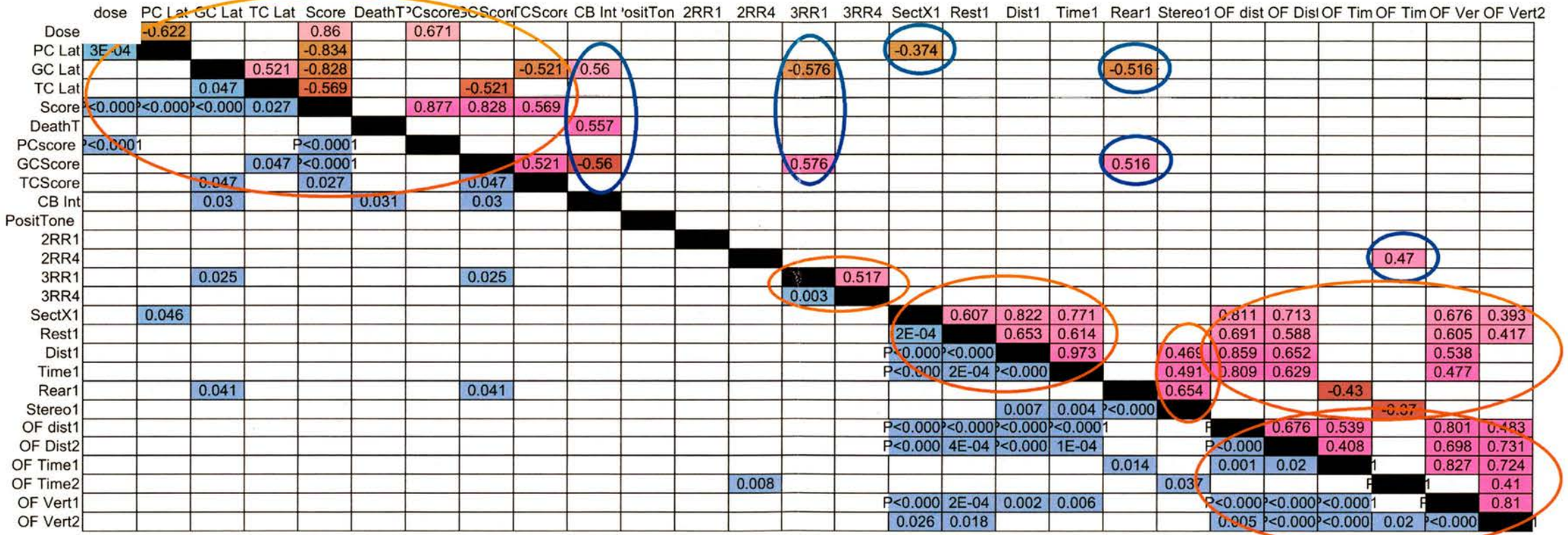
	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscore	GCScore	TCScore	CB Int	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Tim	OF Vert1
PC Lat		0.559		-0.614	0.469		-0.559		0.398																	
GC Lat	0.002		0.728		0.433	-0.559		-0.728																		
TC Lat		3E-04		-0.803			-0.728																			
Score	5E-04		P<0.000		-0.562	0.614	0.868	0.803																		
DeathT	0.012	0.021		0.002		-0.469	-0.433					0.666														
PCscore		0.002		5E-04	0.012		0.559		-0.398																	
GCScore	0.002		3E-04	P<0.000	0.021	0.002		0.728																		
TCScore		3E-04		P<0.0001			3E-04																			
CB Int	0.044					0.044																				
GS1											0.532	0.548														
GS2										0.004						0.545										
GS3					1E-04					0.003				0.388				0.438						0.362		
2RR1															0.4		0.658									
2RR4												0.04				0.531		0.507						0.432		0.382
3RR1													0.035				0.516									
3RR4											0.003			0.004			0.603									
3RR7													2E-04		0.006	9E-04										
SectX1												0.02		0.006					0.466	0.822	0.893			0.892	0.399	0.658
Rest1																		0.012		0.413	0.514			0.51		0.439
Dist1																		P<0.00	0.029		0.955			0.912		0.551
Time1																		P<0.00	0.005	P<0.00				0.938		0.622
Rear1																							0.531		-0.463	-0.404
Stereo1														0.045								0.004			-0.55	-0.416
OF dist1														0.022				P<0.00	0.006	P<0.00	P<0.0001				0.511	0.728
OF Time1																		0.036				0.013	0.002	0.006		0.903
OF Vert1														0.045				1E-04	0.019	0.002	4E-04	0.033	0.017	P<0.00	P<0.00	

Table 6.6 Correlation of Histological and Behavioral Measures, R- and P-values of All Study 011B Mice

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscor	GCScor	TCScor	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Tim	OF Vert1
PDAPP																									
BACE pKO; PDAPP			0.726					-0.726														-0.697		0.627	0.636

	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscor	GCScor	TCScor	GS1	GS2	GS3	2RR1	2RR4	3RR1	3RR4	3RR7	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Tim	OF Vert1
PDAPP																									
BACE pKO; PDAPP			0.027					0.027														0.017		0.039	0.035

Table 6.7 Correlation of Calbindin to All Other Measures, R- and P-values of PDAPP and BACE pKO; PDAPP 011B Mice



	dose	PC Lat	GC Lat	TC Lat	Score	DeathT	PCscore	GCScore	TCScore	CB Int	PositTon	2RR1	2RR4	3RR1	3RR4	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dist	OF Tim	OF Tim	OF Ver	OF Vert2
Dose		-0.622			0.86		0.671																				
PC Lat	3E-04				-0.834											-0.374											
GC Lat				0.521	-0.828				-0.521	0.56				-0.576						-0.516							
TC Lat			0.047		-0.569			-0.521																			
Score	<0.000	<0.000	<0.000	0.027			0.877	0.828	0.569																		
DeathT										0.557																	
PCscore	<0.0001				P<0.0001																						
GCScore				0.047	<0.0001				0.521	-0.56				0.576						0.516							
TCScore			0.047		0.027			0.047																			
CB Int			0.03			0.031		0.03																			
PositTone																											
2RR1																											
2RR4																									0.47		
3RR1			0.025					0.025							0.517												
3RR4														0.003													
SectX1		0.046															0.607	0.822	0.771			0.811	0.713			0.676	0.393
Rest1																2E-04		0.653	0.614			0.691	0.588			0.605	0.417
Dist1																P<0.000	<0.000		0.973		0.469	0.859	0.652			0.538	
Time1																P<0.000	2E-04	<0.000			0.491	0.809	0.629			0.477	
Rear1			0.041					0.041													0.654			-0.43			
Stereo1																		0.007	0.004	<0.000					-0.37		
OF dist1																P<0.000	<0.000	<0.000	<0.0001				0.676	0.539		0.801	0.483
OF Dist2																P<0.000	4E-04	<0.000	1E-04			P<0.000		0.408		0.698	0.731
OF Time1																				0.014		0.001	0.02		1	0.827	0.724
OF Time2													0.008								0.037			P	1		0.41
OF Vert1																P<0.000	2E-04	0.002	0.006			P<0.000	<0.000	<0.000	1	P	0.81
OF Vert2																0.026	0.018					0.005	<0.000	<0.000	0.02	<0.000	

Table 7.6 Correlation of Histological and Behavioral Measures, R- and P-values of All Study 011C Mice

25 mg/kg PTZ -values

	PC Lat	GC Lat	TC Lat	Score	DeathT	Cscore	CScor	CScore	ositTon	2RR1	2RR4	3RR1	3RR4	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dist	OF Tim	OF Tim	OF Ver	OF Vert2
PDAPP																									
BACE pKO; PDAPP												0.786													

25 mg/kg PTZ -values

	PC Lat	GC Lat	TC Lat	Score	DeathT	Cscore	CScor	CScore	ositTon	2RR1	2RR4	3RR1	3RR4	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dist	OF Tim	OF Tim	OF Ver	OF Vert2
PDAPP																									
BACE pKO; PDAPP												0.028													

60 mg/kg PTZ -values

	PC Lat	GC Lat	TC Lat	Score	DeathT	Cscore	CScor	CScore	ositTon	2RR1	2RR4	3RR1	3RR4	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dist	OF Tim	OF Tim	OF Ver	OF Vert2
PDAPP	0.786					-0.786					-0.786		-0.847												
BACE pKO; PDAPP																									

60 mg/kg PTZ -values

	PC Lat	GC Lat	TC Lat	Score	DeathT	Cscore	CScor	CScore	ositTon	2RR1	2RR4	3RR1	3RR4	SectX1	Rest1	Dist1	Time1	Rear1	Stereo1	OF dist	OF Dist	OF Tim	OF Tim	OF Ver	OF Vert2
PDAPP	0.048					0.048					0.048		0.024												
BACE pKO; PDAPP																									

Table 7.7 Correlation of Calbindin to All Other Measures, R- and P-values of PDAPP and BACE pKO; PDAPP 011C Mice