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Effects of Long-Term Administration of Caffeine in a Mouse Model for Alzheimer's Disease

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Effects of Long-Term Administration of
Caffeine in a Mouse Model for Alzheimer's Disease

by

William Schleif

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Biology
College of Arts and Sciences
University of South Florida

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**Effects of Long-Term Administration of Caffeine in a
Mouse Model for Alzheimer's Disease**

William Schleif

ABSTRACT

A recent epidemiological study suggested that higher caffeine intake reduces the risk of Alzheimer's disease (AD). Caffeine, a widely consumed stimulatory drug, is a non-selective adenosine receptor antagonist that has been shown to increase plasma adenosine levels in rodents. To determine any long-term protective effects of caffeine in a controlled longitudinal study, caffeine was added to the drinking water of APP_{sw} transgenic (Tg) mice between 4 and 9½ months of age, with behavioral testing done during the last 6 weeks of treatment. The average daily intake of caffeine per mouse (1.5 mg) was the human equivalent of 5 cups of coffee/day. Across multiple cognitive tasks of spatial learning/reference memory, working memory, and recognition/identification, Tg mice given caffeine (Tg+Caff) performed significantly better than Tg control mice and similar to non-transgenic controls. Discriminant Function Analysis involving multiple cognitive measures clearly showed the superior overall cognitive performance of Tg+Caff mice compared to Tg controls. Analysis of A β in the hippocampus by ELISA revealed Tg+Caff mice had significantly less soluble A β ₁₋₄₀ and insoluble A β ₁₋₄₂. In a follow-up study involving neurochemical analysis only, caffeine was added to the drinking water of 17 month old APP_{sw} mice for 18 days. In this study, Tg+Caff mice also

showed a significant reduction of insoluble A β ₁₋₄₂ in the hippocampus. In contrast to the reduced extracellular brain levels of adenosine in Tg controls, caffeine treatment normalized brain adenosine levels in Tg mice to that of non-transgenic controls. Analysis of amyloidogenic secretase activity revealed the reduction in A β is likely because of a reduction in γ -secretase activity as a result of increased SAM silencing of PS1 expression. This study suggest that a modest, long-term caffeine intake of approximately 500 mg per day (5 cups of coffee) may reduce considerably the risk of AD by decreasing amyloidogenesis.

I. Alzheimer's Disease

With exponential growth in the population of aged individuals in industrialized countries comes the increasing prevalence of age-related disorders. Foremost amongst these disorders is Alzheimer's disease (AD), which is currently the leading cause for dementia in the elderly and afflicts an estimated 4.5 million people in the United States alone. This number is expected to triple over the next fifty years if a cure is not found (Hebert et al., 2003). The onset of Alzheimer's is as tragic as it is devastating; progressively disrupting areas of the brain responsible for memory and motor skills that ultimately leave the patient comatose and unable to communicate or relate with their environment before they succumb to death. This continual decline of the patient's lifestyle leaves them in a state that is a strain on their family and is costly to society.

Behavior Characterization

The initial onset of AD is often times difficult to pinpoint. During the latent period of the disease, the underlying disruptions in normal brain physiology begin to occur decades before any cognitive decline is noticed. Some patients develop a condition termed MCI (Mild Cognitive Impairment), which is characterized by the progressive decline of short-term memory and indicates a substantial risk for developing AD. An estimated 10-15% of MCI patients progress into AD per year, yet it is also worth mentioning that not all MCI subjects develop AD (Petersen et al., 2001). MCI represents a transitional state where patients have a noticeable decline in memory different from that

of normal aging but are not yet diagnosable as having AD. The short-term memory impairment found in MCI is similar to AAMI (Age-Associated Memory Impairment), but cognitive testing can distinguish between the two. Some MCI patients exhibit neuropsychiatric symptoms as well. A recent case study found over 30% of MCI patients exhibited symptoms of depression, aggression, anxiety, apathy and irritability. These symptoms follow a similar trend seen in early AD patients and may serve as a clinical indicator of MCI severity (Feldman et al., 2004).

Early AD is marked by a moderate loss in working memory that is frequently accompanied by depression and the symptoms mentioned earlier. Typically language difficulties also appear with the patient struggling with tests that involve word-finding and recall. Patients progress into moderate AD when the severity of these symptoms increase. In addition to a significant decrease in short term memory, the language difficulties seen in early AD worsen. Moderate AD patients often times create spatially disordered writing and rely on simpler grammatical sentences to express themselves (Forbes et al., 2004). Long term memory loss also develops, and moderate AD patients are prone to wandering and hallucinations as well. The progressive and debilitating decline in cognition eventually leads to the nearly absolute loss of intellect in the advanced stage of AD preceding death. The advanced stage of AD also sees the initial deterioration of motor and sensory skills. Patients in this stage live in a vegetative, bedridden state, unable to care for themselves in any function.

Pathological Characterization

The prominent lesions characteristic of AD brain pathology, neuritic plaques and neurofibrillary tangles, were first described by Alois Alzheimer in 1906 but the exact

chemical compositions of these lesions were only discovered in the past four decades. The extracellular neuritic plaques, primarily found in the cerebral cortex and hippocampus, are composed mainly of amyloid β -peptide ($A\beta$), a protein that accumulates in various degrees during normal aging. Several genetic mutations affect the onset of AD by increasing the rate of $A\beta$ production, through mechanisms that will be discussed later. Neurofibrillary tangles consist of intracellular paired helical filaments that are left behind as “tombstones” when neurons degenerate.

Aberrant processing of the amyloid precursor protein (APP) leads to the two forms of $A\beta$ found in neuritic plaques that differ in their amino acid lengths. $A\beta_{42}$, the principal component of the core in neuritic plaques, is more susceptible to aggregation than the slightly smaller form of $A\beta_{40}$ (Selkoe, 2001). Recognized as a potential precursor to neuritic plaques, diffuse plaques are composed solely of $A\beta_{42}$ and generally lack any signs of dystrophic neurons. These diffuse plaques are found in significant numbers in the typical areas associated with AD but also appear in brain areas that will never develop mature neuritic plaques. Diffuse plaques are also found in the brains of healthy patients that never show any signs of dementia. Aggregation of $A\beta_{40}$ with $A\beta_{42}$ leads to the more compact, fibrillar neuritic plaques associated with AD.

The accumulation of fibril $A\beta$ in the extracellular space activates microglia within and surrounding neuritic plaques, which attempt to clear $A\beta$ by phagocytosis and release free radicals and the cytokines IL-1 β and TNF- α (Schubert et al., 2000). Astrocytes are found in a concentric ring around the cores of mature plaques, and are activated by the proinflammatory cytokines released by microglia. Astrocytes expedite the rate of amyloid

deposition and plaque formation by releasing the inflammatory proteins α_1 -antichymotrypsin (ACT) and apolipoprotein ϵ (APOE) (Potter et al., 2001). Dystrophic neurites are also found amid mature amyloid deposits, likely as a result of the free radicals and inflammatory mediators released by microglia and astrocytes. This neuroinflammatory cascade that results from the accumulation of A β forms the central tenant of the amyloid hypothesis as the causative agent for the cognitive decline seen in AD.

Neurofibrillary tangles (NFT) accrue when the microtubule-associated protein tau is phosphorylated by intracellular kinase(s). The hyperphosphorylation of tau causes it to uncouple from microtubules and form intracellular paired helical filaments (PHF) that constitute the molecular makeup of NFTs. The subsequent formation of neurofibrillary tangles from PHFs disrupts normal cell trafficking and may lead to neuronal death. Neurofibrillary tangles in AD are typically found in limbic and association cortices of the brain, often times in association with mature amyloid plaques (Selkoe, 2001). Although NFTs are considered a pathological hallmark of AD, they are also associated with several other neurodegenerative diseases and occasionally a late-stage AD patient may exhibit low levels of these tangles upon autopsy.

Also associated with the insidious nature of AD is a significant decline in synapses between neurons, as well as the atrophy of neuronal populations in the cerebral cortex and hippocampus. Axonal transport is crucial for synapse viability and accumulation of β -amyloid has been shown to inhibit fast axonal transport in cultured rat neurons (Hiruma et al., 2003), giving a plausible explanation for the significant loss of synapses seen in AD. Exposure of human cortical neurons to β -amyloid *in vitro* also

disrupts calcium regulation, leading to an increase in intracellular calcium stores that may enhance neuronal loss via glutamate-induced excitotoxicity (Mattson et al., 1992). This combined loss of synapses and neurons leads to a progressive loss of neurotransmitter systems that are responsible for both short and long term memory, reflecting the behavioral symptoms seen in AD.

Genetics of Alzheimer's Disease

The two types of Alzheimer's disease, sporadic and familial, differ only in the age of onset and share similar pathological and behavioral hallmarks. The sporadic form of AD typically has an age of onset over 65 years and represents the majority (>95%) of confirmed Alzheimer's patients. Usually the sporadic, or late-onset, form of AD has an unidentifiable cause aside from the possible presence of various risk factors. Patients with a genetic disposition to develop AD represent the familial form and constitute only a small percentage of all AD cases. These patients may be diagnosed with AD as early as their thirties, and no later than their sixties, depending on the nature of the mutation. Familial AD, or FAD, is linked to autosomal-dominant mutations in three causative genes of interest. Each of these mutations disrupts the normal processing of APP, resulting in increased production of β -amyloid species.

The APP gene is located on chromosome 21, and was initially linked to AD due to the similar neuropathology between AD and the genetic disorder trisomy 21, where the extra copy of APP leads to its increased expression and deposition of $A\beta$ (Hardy and Selkoe, 2002). APP is a transmembrane protein with a long extracellular N-terminus and a much shorter cytoplasmic C-terminus. During normal APP processing, the protease α -secretase cleaves APP in the middle of the $A\beta$ domain generating non-amyloidogenic

APP fragments. The less common proteolytic cleavage of APP by β - and γ -secretases generates the β -amyloid species found in diffuse and neuritic plaques (Zekanowski et al., 2004). The FAD mutations linked to the APP gene change APP processing so that β - and γ -secretases are more likely to cleave APP and produce amyloidogenic fragments, thus accelerating the onset of the disease.

Specific mutations in the APP gene were only discovered in the past decade. A double missense mutation in the 670 and 671 amino acids of APP was isolated from several Swedish families that exhibited early onset of AD. This double point mutation directly favors the cleavage of APP by β -secretase and increases levels of $A\beta_{40}$ and $A\beta_{42}$ (Mullan et al., 1992). Additional missense mutations in the APP gene have also been identified from families of other nationalities that increase the likelihood of γ -secretase cleavage. In particular, a mutation at amino acid 717 of APP was found in a London family. This mutation increases the cleavage of APP by γ -secretase and raises the levels of $A\beta_{42}$, the β -amyloid species that is especially prone to aggregate.

Mutations in the APP gene are relatively rare however, and subsequent genetic screening of early onset AD patients found that the majority of FAD cases can be linked to missense mutations in a presenilin gene, PS1. Rarer FAD cases are seen with mutations in another presenilin PS2. Located on chromosomes 14 and 1 respectively, missense mutations in these genes have been linked to FAD cases in hundreds of families worldwide. The exact molecular actions of the presenilin genes have been difficult to ascertain, but it is widely accepted that they influence the γ -secretase cleavage site on APP. It is therefore not surprising that mutations in the presenilins linked to FAD

increase γ -secretase activity, leading to elevated levels of $A\beta_{42}$ (Tandon and Fraser, 2002). Patients with presenilin mutations linked to FAD exhibit a 1.5 -to 3-fold increase in neuritic plaques compared to late-onset AD cases (Selkoe, 2001). In addition to being the most common cause of FAD, mutations in the PS1 gene also lead to the earliest age of onset and most aggressive form of the disease.

Diagnosis of Alzheimer's Disease

The importance of diagnosing AD at its earliest stage is critical because of its implications in the efficacy of the limited pharmaceuticals and treatments currently used to treat AD. Diagnosing any form of Alzheimer's disease with complete certainty is nearly impossible however until the patient dies and an autopsy can be performed to identify the pathological hallmarks of AD discussed earlier. There are difficulties in recognizing the initial manifestations of Alzheimer's symptoms and differentiating between the cognitive decline associated with aging and other diseases that cause dementia. Often times the initial diagnosis of AD is made only after other forms of dementia are eliminated and at this point diagnosis is probable at best. Recent work has failed to yield a test battery that can distinguish definitively between the cognitive decline of normal aging, other forms of dementia, and the initial clinical manifestations of early Alzheimer's disease but many recent advances have increased the accuracy in diagnosing AD in more advanced cases.

Clinical diagnosis of AD is dependent on several criteria: gradual onset of dementia in the absence of other potential dementia-causing disorders between 40 -90 years of age, impaired daily activities, behavioral alterations, family history, neuroimaging, and several biological markers. Some patients have relatively intact daily

functioning with cognitive deficits without full-blown dementia. These people fall into the broader categories of MCI, cognitive impairment no dementia, questionable dementia, isolated memory impairment and minimal AD (Nestor et al., 2004).

Dementia is diagnosed after a poor score on the Mini-Mental State Examination (MMSE), a test given during a clinical evaluation that can reveal a decline in memory. Other cognitive deficits seen in word naming and calculations are also seen after further screening. Tests are conducted to rule out common potential causes of dementia such as a vitamin deficiency, hyperthyroidism, neurosyphilis, or stroke before a diagnosis of probable AD is made. Additional support for an AD diagnosis may come from a positive family history, especially if AD is present in first-degree relatives. DNA diagnostic testing can confirm a familial basis of the disease and is useful for screening the risk of family members who are asymptomatic. Commercial tests are readily available for the more common causative mutations in the presenilin genes and for variations in the APOE gene that may increase susceptibility for sporadic AD, yet a clinical test for APP remains undeveloped. The presence of one or two copies of the ApoE4 allele with accompanying dementia also lends support for a more definitive AD diagnosis, as this particular allele confers an increased risk to developing the disease by reasons that will be discussed later (Gaskell et al., 2004).

Genetic confirmation of AD using clinical DNA testing is accurate, yet it is limited in its application by the relatively small number of AD cases that have a familial basis. Therefore, the development of biological markers that can contribute to the early diagnosis of sporadic AD is underway but has yet to reach widespread clinical application. Currently the most widely used markers for AD are amyloid- β proteins and

both tau and hyperphosphorylated tau protein levels collected from the CSF, but there are drawbacks to using either marker. AD patients are characterized by low levels of $A\beta_{42}$ in both blood plasma and the CSF, but there is significant variability in $A\beta_{42}$ levels between individuals. Low levels of $A\beta_{42}$ may also be found in other diseases, such as depression. This makes it difficult to set the standard range for clinical AD in the overall population using this marker, yet longitudinal studies on individuals are useful (Sobow et al., 2004). The mechanism responsible for a decrease in $A\beta_{42}$ concentrations is still open to debate. A recent study found a correlation between lowered CSF $A\beta_{42}$ levels and the decreased brain volumes and enlarged ventricles found in AD patients (Wahlund and Blennow, 2003), indicating a possible dilution effect and/or decreased production of $A\beta_{42}$. It is more likely that the brain acts as a sink and increased deposition of $A\beta_{42}$ into plaques reduces the concentration of $A\beta_{42}$ remaining to diffuse into the blood.

The use of phosphorylated-tau protein (p-tau) in the CSF as a diagnostic marker for AD has proven to be far more accurate than $A\beta_{42}$. P-tau is consistently seen in high levels in the CSF of AD patients, and is far more specific than $A\beta_{42}$ or total tau levels for differentiating AD from other relevant diseases that may cause dementia. It is also useful for differentiating between geriatric depression and AD, even when there is significant overlap of clinical symptoms (Buerger et al., 2003). Unfortunately, the sampling technique used to collect CSF is highly invasive and this limits the application of both $A\beta_{42}$ and p-tau as CSF biomarkers in some circumstances. When applicable and in conjunction with a thorough neurological evaluation and imaging techniques, the use of

these biological markers is very powerful in increasing the accuracy of AD diagnosis however.

Some of the more powerful diagnostic markers for AD involve imaging the medial temporal lobe and monitoring any alterations in particular brain areas. Repeated MRI studies reveal significant atrophy in this region, specifically in the volumes of the hippocampus and entorhinal cortex, in early to late stage AD patients (Jack et al., 1997, Xu et al., 2000). MRI has also been found important in identifying the early conversion of MCI patients to mild AD patients (DeToledo-Morrell et al., 2004), a potentially instrumental finding in diagnosing early AD patients at a period where current and future treatments can exert their most dramatic effects.

An additional tool in the arsenal of AD diagnostics is PET imaging. PET scans traditionally utilize functional imaging to detect changes in brain metabolism by using a radioactively labeled isotope that reveals areas of glucose metabolism. This type of PET scan unfortunately is only useful in detecting changes in the medial temporal lobe in more advanced AD cases (Ishii et al., 1998), yet this type of imaging is useful in studying physiological aspects of AD. Recently a radioactive ligand was developed that binds to A β plaques and allows imaging of areas of the brain with large numbers of neuritic plaques (Klunk et al., 2004). This new technique opens the door for easier evaluation of anti-amyloid therapies and as another tool in increasing the accuracy of AD diagnosis. Currently, radioligands for PET imaging of NFT's remain to be developed yet research into this area is underway (Mathis et al., 2004).

Risk Factors for Alzheimer's Disease

The initial onset and later progression of AD is moderated by the presence or absence of various risk factors. The most profound and unavoidable risk factor for AD is aging, yet not all of the elderly will develop AD. Another important risk factor for both late-onset and familial AD is inheritable, the ApoE4 allele (mentioned earlier as a marker used in genetic screening). The ApoE4 allele has a gene dose effect: with two copies of the allele conferring greater risk than possession of one copy of ApoE4 (Veurink et al., 2003). Inheritance of this allele enhances susceptibility for sporadic AD and also decreases the age of onset of the disease. ApoE is involved in cholesterol transport and cholesterol is linked to amyloid deposition and deposition. Therefore, the propensity for AD due to possession of ApoE4 is believed to be as a result in a deficiency in amyloid clearance and subsequent increased deposition of beta-amyloid in plaques (Selkoe, 2001). It is also not surprising that high blood cholesterol (LDL) is also a risk factor for AD.

The neuronal cell losses characteristically seen in AD patients are typically associated with markers for oxidative stress caused by the body's inflammatory response to neuritic plaques and NFTs. A diet poor in antioxidants, such as Vitamin E or others found in vegetables and fruit, lowers the body's ability to respond and protect itself from oxidative stress. Low dietary uptake of antioxidants exacerbates neuronal oxidative damage and increases the risk for developing AD (Polidori, 2004). High blood levels of homocysteine are also associated with an increased risk of AD, but it may be as a result of a folic acid deficiency (Quadri et al., 2004).

Unlike the associated risks for AD mentioned thus far, an environmentally enriched lifestyle provides protection against AD and decreases the risk of developing

dementia with increasing age (Fratiglioni et al., 2004). Such an environment includes exposure to intellectually stimulating activities, as well as profound social and physical activities that are usually absent from most nursing homes. The cognitive benefit from these activities are believed to share the same pathway and all seem to reduce stress, increase both cognitive reserve and blood flow to the brain (Fratiglioni et al., 2004).

Treatments for AD

Current treatments for AD are based on treating the symptoms rather than the disease itself. The commonly prescribed acetylcholinesterase inhibitors (donepezil, rivastigmine, and reminyl) have some efficacy in slowing down the inevitable onset of neuropsychiatric symptoms in patients with mild to moderate AD (Holmes et al., 2004). These drugs compensate for the decline in memory due to the deficits in the acetylcholine neuronal network in the medial temporal lobe. They increase synaptic concentrations of acetylcholine by blocking the enzyme acetylcholinesterase, which is responsible for breaking down the neurotransmitter acetylcholine. This increases the efficiency of remaining cholinergic neurons that have yet to be disrupted from AD progression. As AD advances these drugs no longer have an affect in preserving memory however. The acetylcholinesterase inhibitors have a relatively short window of effectiveness, typically between 1-2 years.

The only other FDA approved drug for treating AD symptoms, Memantine, was only recently approved for treating moderate to severe AD patients. Memantine is a non-specific NMDA receptor antagonist that reduces glutamingeric-caused excitotoxicity that may be present in the pathogenesis of AD. Used in conjunction with donepezil, memantine has been shown to make significant improvements in cognitive function and

daily activities in even severe AD patients (Tariot et al., 2004), but like the acetylcholinesterase inhibitors memantine has a relatively limited duration of effectiveness.

II. Animal Models of AD

The complex etiology of Alzheimer's disease requires a practical model that closely resembles the ontogeny of the disease and can provide insight into possible therapeutic preventions. Human testing is generally out of the question due to moral implications in testing novel treatments, so researchers turned to animal models. In particular, the mouse lines PDAPP, APP_{sw}, and APP/PS1 have been genetically altered to replicate behavioral and pathological aspects of AD. These mice are manipulated by randomly inserting a wild-type or mutant AD transgene into the genome of a fertilized mouse egg. Utilizing a strong promoter that overexpresses the gene in brain-specific areas, these transgenic mice enable the impact of the gene of interest to be evaluated. The short life span of mice (1 to 2 years), while beneficial for research, requires that the promoter overexpress the transgene of interest so the same symptoms that may take decades to occur in humans will develop in mice (Seabrook and Rosahl, 1999). Numerous mouse lines have been developed, each with their own assets and drawbacks in replicating the disease in humans.

PDAPP Model

Pathology. The PDAPP mouse model incorporates a platelet-derived growth factor (PDGF)- β promoter in neurons in the brain to drive overexpression of a human APP minigene associated with the London type mutation (APP_{717V_EF}) found in some familial AD cases. When compared to wild type littermates, this genomic alteration results in a well characterized, age-dependent neuropathology exhibiting extracellular levels of A β and amyloid deposits similar to that found in AD. Diffuse and mature A β deposits are seen in the hippocampus, corpus callosum, and cingulate cortex as early as 3-4 months old in PDAPP mice (Dodart et al., 2000). Plaque density increases with age in these regions and by 6-10 months numerous plaques are seen (Games et al., 1995; Irizarry et al., 1997). In aged PDAPP mice, increasing plaque burden is associated with the neuritic dystrophy, cytoskeletal alterations, synaptic degeneration, and gliosis that are also found in AD patients (Schenk et al., 1997; Larson et al., 1999). PDAPP mice do not exhibit any neurofibrillary tangles (Masliah et al., 1996) and do not develop the characteristic neuronal loss associated with AD however. These mice do show marked hippocampal atrophy by 3 months but this is possibly due to developmental abnormalities, independent of increased A β deposition (Dodart et al., 2000).

Behavior. Important correlations have been made between the pathological consequences of progressively overexpressing this mutant hAPP gene in PDAPP mice and the behavioral results that ensue. These mice can be behaviorally tested at various time points to link cognitive deficits to disruptions in specific brain areas due to increasing amyloid plaque burden. Initial testing found PDAPP mice develop an age-dependent decline in object recognition that the authors linked to the increased levels of

A β plaques in cortical structures in mice older than 6 months (Dodart et al., 1999). The same study found deficits in spatial memory in 3, 6, and 9-10 month old PDAPP mice using an 8-arm radial maze. Deficits in spatial memory at the early 3 month time point suggest an age independent decline because this is well before significant amyloid deposition has occurred. In a later paper, the authors attributed this decline in spatial memory to the overexpression of human APP and subsequent abnormal hippocampal formation during development rather than increased amyloid deposition (Dodart et al., 2000). A different study using a novel water maze task revealed an age-related decline in working spatial memory. These PDAPP mice were found cognitively intact at 6-9 months, but were impaired at 13-15 months and progressively worsened by 18-21 months (Chen et al., 2000). These authors found this age-dependent decline did correlate with increased β -amyloid plaques and linked it to interruption of synaptic transmission by β -amyloid burden.

Further support for an age-related decline in spatial memory in the PDAPP mouse line came from a recent study in 2004. This study utilized a PDAPP mouse model that does not exhibit abnormal hippocampal atrophy during development. Using a full 6 week battery that examines sensorimotor skills and cognition, Nilsson et al. (2004) found no differences between 2 month old PDAPP mice and the non-transgenic controls. Another group of PDAPP mice were tested at 16 months of age and these animals were impaired in Morris water maze and in overall radial arm water maze performance (RAWM) (Nilsson et al., 2004). Further statistical analysis of 15-16 month old PDAPP mice found a significant correlation between deficits in Morris water maze, platform recognition, and RAWM performance with the deposition of A β in the hippocampus and cerebral cortex

(Leighty et al., 2004). Such correlations provide evidence that the PDAPP mouse line develops impaired working memory because of accruing levels of A β .

APP_{sw} and APP23 Models

Pathology. The APP_{sw} and APP23 mouse models for AD both incorporate a human APP gene with the ‘Swedish’ double mutation (K670N/M671L). These mutations enhance cleavage of APP by β -secretase which favors A β ₄₂ production. These APP_{sw} and APP23 models differ however because each one utilizes a different promoter that overexpresses APP by varying degrees. This difference leads to important distinctions between the two models in both pathology and behavior.

Initially described by Hsiao et al. (1996), the APP_{sw} mouse line uses a hamster prion protein promoter limited to neurons in that brain that drives five to sixfold higher expression of a human APP695 insert when compared to expression of the endogenous mouse APP gene. The consequences of overexpressing this insert leads to abnormal changes in the pathology of these mice. Given the nature of the mutations in the insert, it is not surprising that APP_{sw} mice show increasing levels of total A β in the brain. Specifically, Hsiao et al. (1996) found that the concentration of A β ₄₀ was 5-times higher and the concentration of A β ₄₂ was 14-times higher when comparing APP_{sw} mice between 2-8 months and 11-13 months of age. Rare plaques of insoluble A β begin appearing in small quantities as early as 6-7 months in the hippocampus and entorhinal cortex, with diffuse and compact plaques increasing to significant numbers by 12 months of age in the hippocampus and cortex of the brain (Hsiao et al., 1996; Kawarabayashi et al., 2001) eventually resembling similar levels found in AD patients.

Importantly, amyloid plaques in the CA1 area of the hippocampus of 16-month old APP_{sw} mice were found in association with gliosis and neuritic dystrophy (Irizarry et al., 1997). Other studies have also found activated microglia in response to amyloid plaques in aged APP_{sw} mice (Frautschy et al., 1998; Benzing et al., 1999). Another study found an accumulation of prostaglandin E₂ (PGE₂) and tumor necrosis factor α (TNF α), both mediators of inflammation from gliosis, in APP_{sw} mice older than 10 months of age (Quadros et al., 2003). A further study using 21-25 month old APP_{sw} identified areas of oxidative damage in association with amyloid plaques, likely because of the release of free radicals during the inflammatory process (Pappolla et al., 1998). The presence of these inflammatory proteins and A β -induced oxidative damage makes this mouse line an attractive model for testing therapeutics that target the inflammatory cascade seen in AD.

Although the APP_{sw} mouse line replicates several aspects of AD, namely amyloidosis, vascular angiopathy, free radical formation, and inflammatory mediators in association with amyloid plaques, it is an incomplete model of AD because these mice fail to develop neurofibrillary tangles and no global neuronal or synaptic losses have been reported. Even in aged APP_{sw} mice, where extensive plaque formation is associated with inflammatory mediators and pro-oxidant activity, neuronal populations remain relatively consistent with neuronal numbers in non-transgenic controls in areas of the brain such as the hippocampus, where large numbers of neurons atrophy during the progression of AD in humans (Irizarry et al., 1997). With no clear abnormalities in the brain structures associated with learning and memory in these mice, some researchers have suggested that the increasing soluble levels of A β are to blame for the increasing behavioral impairments that APP_{sw} mice exhibit as they grow older. In fact, one study found severe

impairment in *in vitro* and *in vivo* long-term potentiation (LTP) in neurons of APP_{sw} mouse hippocampus that correlate with cognitive impairment and rising levels of both soluble and insoluble A β (Chapman et al., 1999).

The other mouse model that incorporates the ‘Swedish’ double mutation, the APP23 transgenic mouse, employs a murine Thy-1 promoter element to overexpress a mutant APP751 gene in neurons in the brain. These mice have a seven fold higher expression of the mutant APP insert than normal mouse APP (Dodart et al., 2002). APP23 mice develop A β plaques in the cortex at 6 months, much sooner than APP_{sw} mice (Sturchler-Pierrat et al., 1997). A β plaque numbers increase with age and spread extensively in this model to the neocortex, hippocampus, white matter, and thalamus. Associated with these plaques are dystrophic neurons, activated microglial and astrocytes. These mice also develop congophilic angiopathy, where large deposits of A β ₄₀ are found in conjunction with the brain vasculature (Calhoun et al., 1999), a trait found in some 90% of AD patients (Vinters, 1987). In striking dissimilarity to the APP_{sw} mouse model, APP23 mice exhibit a 14% loss of CA1 pyramidal neurons in the hippocampus in 14-18 month old mice, although no losses are seen in the neocortex (Calhoun et al., 1998). The authors attribute this loss directly to the formation of dense A β plaques. This model also fails to develop neurofibrillary tangles.

APP_{sw} Behavior. An important component of the behavioral characterization of transgenic mice is an assessment of any sensory or motor deficits that may influence the performance of the mice in cognitive-based tasks. Accordingly, the APP_{sw} mouse failed to show any deficits in a visible platform tasks at either 6 or 9 months of age, indicating that visual acuity is not impaired in these mice (Holcomb et al., 1999). Further

sensorimotor evaluation, assessed through the use of various agility tasks and a Preyer reflex test, also failed to detect any significant disturbances (Holcomb et al., 1999; Holcomb et al., 1998). In a later study, APP_{sw} mice at 3, 9, 14, and 19 months of age were subjected to a full battery of sensorimotor tasks. The authors concluded that younger APP_{sw} mice are not impaired in motor function overall, and older transgenic mice show impairment similar to non-transgenic controls (King et al., 2002). Recently, Arendash et al., (2004) reported that 6 month old APP_{sw} are impaired in a balance beam task, but perform normally in the elevated maze task for anxiety and other sensorimotor tasks, such as string agility. Additionally, Leighty et al. (2004) found no correlation between the balance beam task, a measure of sensorimotor skills, and cognitive performance. The lack of any sensory or motor abnormalities in APP_{sw} mice, or significant correlation with cognitive performance, validates linking any performance issues in cognitive-based tasks to disruptions in memory, not to the physical attributes of the mice.

The Y-maze alternation task has elucidated deficits in mnemonic processing in the APP_{sw} mouse, but inconsistently so. Hsiao et al. (1996) first found 3 month old APP_{sw} mice performed similar to controls, yet 9 month old Tg⁺ mice showed significant impairment in spontaneous alternation in this task. King et al. (2002) found APP_{sw} mice aged 3, 9, 14, and 19 months had an “overall” reduced alternation in the Y-maze task when all age groups were analyzed collectively. In a later study, Arendash et al. (2004) found APP_{sw} mice were impaired overall in spontaneous alternations at 5 and 8.5 month time points collectively. On the other hand, Holcomb et al. (1999) reported APP_{sw} mice were impaired in alternation behavior at 3 months yet were unimpaired at 9 months of

age. This task is likely not as sensitive as some of the other cognitive-based tasks because Y-maze impairment remains inconsistent in this mouse model, although differences in methodologies and genetic backgrounds may play a role in these discrepancies.

Not surprisingly, 15-17 month old APP_{sw} mice also showed significant T-maze alternation impairment (Chapman et al., 1999). The authors correlated the poorer T-maze impairment to their *in vivo* findings of impaired long-term potentiation in both the CA1 and dentate gyrus regions of the hippocampus from the same mice and suggest that rising A β levels in these aged mice acts directly to infer synaptic deficiencies evident in reduced LTP. Unfortunately at this late age, the authors could not discriminate between the effects of soluble or insoluble A β , although the authors hint diffusible forms of A β are most likely responsible. This group also showed the APP_{sw} mouse model lacks deficits in sensorimotor attributes, but only used an open field task to claim this.

Initial cognitive testing of APP_{sw} mice using the Morris water maze revealed no differences in learning and memory in spatial reference tasks between non-transgenic and transgenic mice at 3 months of age (Hsiao et al., 1996). At a later age in this study however, 9 month old APP_{sw} were found impaired in their escape latency in the same task and also spent less time in the platform's quadrant in the probe trial, indicating these mice did not learn the location of the platform. Recently in 2004, however, Arendash et al. found impaired acquisition and retention performance in the Morris water maze as early as 5.5 months of age, suggesting the initial appearance of small A β oligomers are responsible for impairment of both reference learning and memory. A previous study done by King and Arendash (2002) reported their mice were unimpaired in Morris water maze even up to 19 months of age. These contrasting results from the same lab were

suggested to be caused by the different background strains of the mice used in these studies or by changes in their genetic backgrounds caused by multiple generations of inbreeding. These differences enabled the effects of the transgene to become more evident in the later study. Further evidence for the role of cognitive disturbances by small A β oligomers came from a study that reported impaired reference memory in the Morris water maze task at 6 months of age in APP_{sw} mice (Westerman et al., 2002). Although one study using the Morris water maze failed to replicate cognitive impairment in 6 or 9 month old APP_{sw} mice, this is likely because of differences in testing paradigms and the genetic backgrounds that exist between mouse colonies in different labs (Holcomb et al., 1999). In summary, the consensus of the behavioral studies thus far indicate Morris water maze impairment in APP_{sw} mice begins around 6 months of age.

Using a circular platform task adopted from rat behavioral studies, King et al. (2002) found 3, 9, 14, and 19 month old APP_{sw} mice perform comparably to non-transgenic age-matched controls, with both groups showing a learning effect that reduced the number of errors made over the 7 day testing period. Arendash et al. (2004) reported no differences between 6 month old APP_{sw} mice and non-transgenics in circular platform performance as well. Testing by Pompl et al. (1999) revealed impairment in 7 month old APP_{sw} mice during a reversal learning phase of the circular platform task however. APP_{sw} mice learned the location of the escape hole in relation to various cues as well as non-transgenic mice; however, when the escape hole was moved, transgenic mice showed increased errors and escape latency that failed to improve. This is a different variation of the task however, and the literature is consistent in reporting a lack of impairment of APP_{sw} mice in the standard circular platform task at any age.

Additionally, APP_{sw} mice repeatedly show poor performance in other cognitive tasks. Hsiao et al. (1996) reported impaired platform recognition latency in 9-10 month old APP_{sw} mice that coincided with increases in soluble A β . King et al. (2002) also showed APP_{sw} mice at 9 months of age were significantly slower in locating a visible platform, and both of these studies performed the platform recognition task after conducting the Morris water maze task. Recently, a similar behavior paradigm using the platform recognition task identified impairment even earlier in 6 month old APP_{sw} mice (Arendash et al., 2004). In contrast, Westerman et al. (2002) found unimpaired platform recognition performance throughout the 2 year life span of the mice, but this test was performed before Morris water maze testing. Impaired escape latency for APP_{sw} mice in the platform recognition task thus has been suggested to reflect difficulties in switching between the spatial (cued) strategy used in Morris water maze to a search/recognition strategy that ignores the previously learned extra-maze spatial cues. This explains why APP_{sw} mice are impaired in the platform recognition task after they have already learned the platform location in the Morris water maze task, but have not been impaired in the platform recognition task if it is done prior (Arendash et al., 2004, Westerman et al., 2002).

The radial arm water maze (RAWM) is a sensitive working-memory task used to evaluate both short term memory and delayed memory recall. The first testing of APP_{sw} mice in the RAWM task found 15.5 month old mice made significantly more errors in the trial 5 component of the task when compared to non-transgenic controls, indicating these mice were deficient in working memory (Morgan et al., 2000). RAWM testing of younger 6.5-7 month old APP_{sw} mice revealed that these animals made more errors

overall in both the trial 4 and trial 5 components of the task, further highlighting the deficits these mice have in working memory (Arendash et al., 2004). The consistent working memory impairment these mice show in this task by 6.5 months of age indicates the oligomeric form of A β is likely responsible for their cognitive impairment, as these young mice have yet to develop amyloid plaques.

In summary, APP_{sw} mice do not develop any gross sensorimotor deficits that would otherwise impair performance in cognitive tasks. These mice consistently develop significant impairment in reference learning/memory that is apparent in the Morris water maze as early as 5.5-6.5 months and is likely caused by the increasing amounts of soluble A β oligomers. The difficulties in switching escape strategies are also apparent for 9 month or older APP_{sw} mice in the platform recognition task, which is reflected in their increased escape latencies. APP_{sw} mice also exhibit working memory impairment in the RAWM task at 6.5 months of age, as these mice make more errors in the trial 4 and 5 components of the task. Based on these overall behavioral findings, it is reasonable to expect 8-9 month old APP_{sw} mice will have impaired reference learning/memory, impaired recognition/identification, and impaired working memory evident in the tasks discussed thus far.

The APP23 mouse model has undergone behavioral characterization as well. A behavioral characterization of the APP23 model was done by Kelly et al. (2003) at 3, 18, and 25 months of age using passive avoidance tasks, platform recognition, and small and large Morris water maze tasks. Age-related impairments in passive avoidance and in small pool acquisition were reported, while APP23 mice were found to be impaired at every time point in the larger Morris water maze during acquisition testing. The authors

also reported visual deficiencies were not responsible for this impaired spatial learning by using a platform recognition task in the larger pool. An age-related decline in Morris water maze and probe trial performance was reported also at the early age of 3 and 6 months, while 2 month old animals were unimpaired when compared to non-transgenic controls (Van Dam et al., 2003). Further studies have also reported progressive deficits in acquisition (learning) of the Morris water maze task in both 16 month (Lalonde et al., 2002) and 2 year old APP23 mice (Dumont et al., 2004). In addition, increased exploratory behavior was detected in 2 year old APP23 mice through the use of the open field task, while anxiety was decreased in the open arm (+) maze task (Dumont et al., 2004). The repetitive results of impaired spatial memory in APP23 mice in the Morris water maze indicates these animals represent an effective model for behavioral testing in AD, yet the behavior of these mice in working memory tasks, such as the RAWM, that examine long term memory remains to be determined.

PSAPP Model

Pathology. The PSAPP, or APP_{sw}/PS-1, mouse model is a combination of two mutations associated with familial Alzheimer's disease. The same "Swedish" mutation used in APP_{sw} mice that enhances β -secretase activity on APP and a PS-1 mutation that favors the enzymatic cleavage of APP by γ -secretase are both overexpressed in this model resulting in an exponential increase in A β levels with aging, even when compared to that of transgenic APP_{sw} mice (Holcomb et al., 1998). Studies have shown that PSAPP mice begin showing A β deposits in both the hippocampus and cortex between 3-6 months of age (Takeuchi et al., 2000), with significant numbers present by 9-12 months of age (Borchelt et al., 1997; Holcomb et al., 1998). Although deposits of A β resemble

those found in AD, compact plaques of fibrillar A β have been reported to form before diffuse plaques in the PSAPP model, whereas this trend is opposite in human AD (Gordon et al., 2002). Nevertheless, by 12 months of age the compact plaques in PSAPP mice are surrounded by dystrophic neurites, reactive microglia and GFAP-expressing astrocytes, indicating that a neuroinflammatory response is present (Gordon et al., 2002). In depth analysis of the inflammatory response in PSAPP mice found that amyloid plaques increase continuously with age up to the latest age studied, 25 months (Matsuoka et al., 2001). These authors found cyclooxygenase-2 (an inflammatory response protein) in conjunction with the astrocytes surrounding amyloid plaques. Complement component 1q was identified with the microglia associated with amyloid plaques, which indicates microglia may be attempting to clear amyloid via the complement pathway.

Like the other AD animal models discussed thus far, the PSAPP mouse line also fails to develop neurofibrillary tangles. Takeuchi et al. (2000) reported a non-significant loss in cortical and CA1 neurons of the hippocampus in 12 month old PSAPP mice, yet this study used a low sampling number. A further study of 22 month old PSAPP found a 38.5% reduction in CA1 pyramidal neurons (Sadowski et al., 2004). Deficits in LTP have also been found as early as 3 months in PSAPP mice, when amyloid plaques and deficits in short term memory first appear (Trinchese et al., 2004).

PSAPP Behavior. The first study to examine behavioral aspects of PSAPP mice found that by 3-4 months these mice had significantly impaired alternation performance in the Y-maze task, months before the initial deposition of A β has been detected (Holcomb et al., 1998). The same lab found that this deficit in Y-maze performance persisted with increasing age, as PSAPP mice were also impaired at 6 and 9 months of

age (Holcomb et al., 1999). This same study also failed to detect any deficits in spatial working memory at 6 or 9 months of age using the Morris water maze. These results show that behavioral deficits are likely due to the presence of soluble A β . Using a full behavioral battery, a later study found that PSAPP mice showed no change in spontaneous Y-maze alternations at either 5-7 or 15-17 months of age (Arendash et al., 2001). This same study also showed normal Morris water maze performance at 5-7 months of age but an aged-related impairment in this task was found later at 15-17 months. Another study investigating the effects of a lifelong immunization with human A β found unvaccinated PSAPP mice were impaired in both Morris water maze and the radial arm water maze (RAWM) at 4-6 months and 15-16 months of age (Jensen et al., 2005). Similar impairments in RAWM at 15.5 months were reported in another study of PSAPP mice (Morgan et al., 2000). The findings of cognitive impairment at such an early age in this same colony of mice was attributed by the authors to multiple generations of crossbreeding that enhances the susceptibility of the mice to the effects of mutant APP overexpression.

Early PSAPP working-memory deficits at 3-4 months of age in the RAWM and impaired reference-memory in the Morris water maze at 6-8 months were also reported by Trinchese et al. (2004). The working-memory impairment was evident in the number of errors the PSAPP mice made in the RAWM that strongly correlated with A β levels and amyloid burden in these mice. A further correlation was made between RAWM errors and synaptic deficiencies in LTP at 6-8 months of age. The authors propose this lends additional support to the idea that increasing A β levels and amyloid burden impair LTP, thus reducing the animals' performance in tasks based on cognition.

Limits of Animal Models for Alzheimer's disease

In addition to the various pathological aspects of AD that fail to materialize in AD transgenic mice (such as neurofibrillary tangles or global neuronal loss that were mentioned earlier for each specific model) are differences in mouse and human pathology that possibly may skew the generalizations made after a potential therapeutic is tested. Specifically, A β plaques in both APP23 (Kuo et al., 2001) and APP_{sw} mice (Kalback et al., 2002) were found to be soluble in SDS-containing buffers whereas human A β plaques are not, indicating mouse plaques are less dense. This distinction is important because it is believed to be responsible for the weaker complement response to A β plaques and the resultant weaker inflammatory cascade found in APP23 mice (Schwab et al., 2004). Knowing that human fibrillar A β plaques are neurotoxic from *in vitro* studies (Lorenzo et al., 2000), a less compact form of these plaques in the mice may also explain the lack of neuronal losses in animal models of AD.

Transgenic mice are also incomplete in their behavioral replication of the disease. Many of the behavioral aspects of early Alzheimer's disease are difficult to ascertain in humans even through the use of cognitive tests such as the MMSE that examine various aspects of memory. Semantic memory, such as word or event recall, is usually the first apparition signaling Alzheimer's disease in humans and cannot be assessed in a mouse animal model. Obviously the language difficulties which surface in moderate AD are also problematic to examine in mice. Mouse animal models are thus limited to behavioral measures that examine spatial learning/memory, recognition/identification, exploratory behavior, sensorimotor skills, and anxiety, which may only give some insight into the onset of depression, motor skills, or memory impairment of this disease in mice.

Given these limits in replicating all aspects of Alzheimer's disease, transgenic mice still have been paramount in our understanding of the disease and the possibility of developing therapeutics that lay the foundation for future clinical testing looks promising through the use of these mice.

III. Caffeine Consumption and Alzheimer's Disease

There is little doubt that the widespread consumption of caffeine by the global population makes it an important consideration when evaluating dietary influences on the etiology of human diseases. Caffeine can be found in popular foods and beverages such as tea, coffee, cocoa, chocolate, and soft drinks that constitute dietary items consumed sometimes as a chronic staple of the Western diet. The average caffeine consumption in the US is equivalent to 1-2 cups of coffee a day, or 168 mg/person/day (Fredholm et al., 1999). Caffeine may also be found in certain aspirins and other over-the-counter drugs, although these would not represent a source of long-term caffeine ingestion.

Caffeine's stimulatory effects on behavior and attention have been known for some time, but its effects on other aspects of human neurobiology have only recently begun to be explored. Epidemiological studies indicate that sufficient daily caffeine intake throughout life may be neuroprotective in both Parkinson's disease (Ross et al., 2000) and Alzheimer's disease (Maia and Mendonca, 2002). Notably, Maia and Mendonca found that AD patients consumed markedly less caffeine (74 mg/day) during the twenty years preceding diagnosis of AD when compared to age-matched controls

(199 mg/day). Daily caffeine intake included any potential sources of caffeine and was not solely restricted to caffeine ingested from coffee. This information suggests that chronic caffeine intake during the middle years of life may delay or even prevent altogether the onset of the behavioral symptoms seen in sporadic Alzheimer's disease, an important finding.

The molecular mechanism(s) that grants caffeine's neuroprotective effects remains to be clearly elucidated, but many possibilities have been suggested. Once this mechanism has been identified, caffeine-based therapeutics could become useful in treating neurodegenerative diseases because of their availability and general lack of long-term side effects.

Pharmacological Profile of Caffeine

Whether taken orally or administered intravenously, the pharmacokinetics of caffeine are identical in humans and animals (Arnaud, 1993). Likewise, the gastrointestinal absorption and bioavailability of caffeine reaches 99-100% within 45 minutes of ingestion in both human and animal models (Bonati et al., 1984, Blanchard and Sawers, 1983a). Peak plasma level of caffeine is reached between 15 and 120 minutes after ingestion in humans, with a half-life that ranges from 2.5 to 4.5 hours in humans (Arnaud, 1987) and only 0.7 to 1.2 hours in rats and mice (Bonati et al., 1984). It is also known that the plasma half-life of caffeine remains relatively unchanged in both young adults and the elderly (Blanchard and Sawers, 1983b). On average, a typical cup of coffee provides a caffeine dose of 0.4 to 2.5 mg/kg which gives a peak plasma concentration of caffeine of 1 to 10 μ M (Fredholm et al., 1999). This information, once accounting for weight and metabolic differences between animal models and humans,

allows for interpolations for dose-dependent effects between caffeine studies in animal models and humans.

Caffeine's hydrophobic nature allows for rapid absorption through all biological membranes, hence its rapid absorption into the bloodstream. This same tendency also allows caffeine to freely pass through the blood brain barrier (Tanaka et al., 1984), an obstacle that limits the design and size of many other potential neurotherapeutics. Once absorbed into the blood stream, caffeine is also broken into its main derivatives by the liver. Caffeine's major metabolites in rodents and humans with bioactivity include 1,3-dimethylxanthine (theophylline) and 1,7-dimethylxanthine (paraxanthine). Both of these compounds were found to mimic some of caffeine's effects in the CNS and which will be discussed in greater detail later on (Benowitz et al., 1995). Theophylline in particular is of interest because of its common use as a treatment for asthma and other diseases that increase bronchial constriction.

Molecular Actions of Caffeine

Caffeine has been used experimentally for some time to study its inhibitory effect on cyclic nucleotide phosphodiesterase isozymes of the brain and other tissues of the body (Vernikos-Danellis and Harris, 1968). The caffeine dose needed in order to achieve this effect, however, is within the millimolar range which far exceeds typical human plasma levels of caffeine after ingestion. This same range is also required to release intracellular calcium stores via activation of ryanodine receptors (McPherson et al., 1991). In addition, caffeine plasma levels in excess of 500 μM , or more than 50 cups of coffee, are toxic, likely because of caffeine's blockade of GABA_A receptors at this extreme range (Fredholm et al., 1999). Therefore, it is reasonable to assume that neither

of these actions of caffeine can be attributed to its neuroprotective effects that are being investigated. The only other molecular action of caffeine that can be achieved within physiological doses in the CNS is adenosine receptor antagonism, a mechanism that is shared by both theophylline and paraxanthine. In fact, theophylline's affinity for adenosine receptor antagonism is three to five times higher than caffeine (Fredholm et al., 1999). Theophylline has also been shown to enhance hippocampal LTP, yet the dose needed for this effect is in the 100 to 1000 μ M range which is beyond that of typical caffeine consumption (Tanaka et al., 1990).

Adenosine. As the main constituent of ATP, adenosine is present both intracellularly and extracellularly throughout the body. Although adenosine is an important modulator of neurotransmission, it is not considered a classical neurotransmitter because it is neither stored in vesicles nor dependent on Ca^{+} for release (Fisone et al., 2004). Instead, adenosine is transported between the cytoplasm and extracellular space through equilibrative nucleoside transporters. The direction of this transport is dependent on the adenosine concentration gradient in both sides of the membrane (Gu et al., 1995), which under normal conditions facilitates the intra-cellular transport of extracellular adenosine as adenosine is incorporated into AMP intracellularly via the enzyme adenosine kinase. During times of physiological stress, such as ischemia or hypoxia, intracellular levels of adenosine rise due to hydrolysis of ATP which subsequently fuels an increase in the extracellular concentration of adenosine (Wallman-Johansson and Fredholm, 1994). Aside from adenosine efflux from the cytoplasm, a second minor source of adenosine exists in the extracellular space that involves the

conversion of released adenine nucleotides into adenosine by several enzymes (Zimmerman and Braun, 1999).

Rising extracellular levels of adenosine are thus viewed as a protective response from metabolic injury and various therapeutics have been proposed recently to specifically raise extracellular adenosine levels. In particular, the drug propentofylline has been forwarded as a potential treatment for Alzheimer's disease, and it showed promising results in a clinical study (Kittner et al., 1997). One role of this drug is blockade of the equilibrative adenosine transporters, which leads to an increase in extracellular adenosine levels because of the conversion of adenine nucleotides into adenosine mentioned earlier. The elevation of extracellular adenosine levels downregulates activated glial cells and leads to the restoration of altered calcium homeostasis (Ringheim, 2000). Likewise, the drug dipyridamole blocks adenosine uptake and inhibits the enzyme adenosine deaminase that breaks down adenosine, thus leading to increased extracellular concentrations and has been shown to block the enhanced vasoconstriction induced by soluble A β by increasing cGMP activity (Paris et al., 1999). Adenosine itself is also administered commonly by paramedics as a treatment to slow atrial tachycardia by increasing conduction time through the AV node. Finally, it has also been found that long-term ingestion of caffeine in rats leads to significantly increased levels of adenosine in blood plasma in a dose-dependent manner through an unknown mechanism (Conlay et al., 1997). Although adenosine does not cross the blood brain barrier, it can be assumed a rise in brain adenosine levels should accompany rises in plasma adenosine levels because caffeine can enter the brain from plasma and exert similar effects on adenosine receptors there as well.

Adenosine Receptors. Currently, four G-protein-coupled receptor (GPCR) subtypes have been identified to have adenosine affinity. These subtypes, named A₁, A_{2A}, A_{2B}, and A₃, are expressed throughout the brain in particular and in both the central and peripheral nervous system (Fredholm et al., 1994); they are collectively referred to as the P1 (adenosine selective) receptors. The high affinity receptors A₁ and A_{2A} are typically of interest because they are most likely responsible for the neuromodulatory effects of adenosine at physiological levels, while the lower affinity A_{2B} receptors have been relatively ignored because they are most likely only activated when adenosine levels are increased (Fredholm et al., 1999; Fisone et al., 2004). The low affinity A₃ receptors are sparsely distributed in humans and are little affected by caffeine or its metabolites in rats (Fredholm et al., 1999). Although A_{2B} and A₃ receptors cannot be completely excluded from having a neuroprotective effect in relation to caffeine, this is simply because evidence that might support such a role is lacking so these receptors are not included in this thesis. Refer to Figure 1 at the end of this section for an overview of caffeine-mediated effects by adenosine receptor antagonism.

The A₁ type adenosine receptors have high affinity for both caffeine and adenosine. These receptors are widely distributed in the brain, with higher expression in the cerebral cortex, cerebellum, hippocampus, and in the dorsal horn of the spinal cord (Ribeiro et al., 2003), while lower expression levels of this receptor are also found in the basal ganglia (Rivkees et al., 1995). These receptors (along with the A₃ receptors) activate inhibitory G-proteins (both G_i and G_o) which may lead to the inhibition of adenylyl cyclase, closure of Ca²⁺ channels (MacDonald et al., 1986), and activation of K⁺ channels (Trussel and Jackson, 1985). Inhibition of adenylyl cyclase ultimately leads

to decreased production of cAMP, thus activation of these receptors is generally thought to provide an inhibitory effect on secondary messenger systems while stabilizing the cellular membrane by blocking Ca^{2+} influx.

The A_1 receptor is present on the membranes of neurons, microglia, and astrocytes (Ongini and Schubert, 1998). The majority of neuronal adenosine A_1 receptors are located on presynaptic nerve terminals where they provide inhibition of neurotransmitter release after activation by adenosine (Fisone et al., 2004), although some post synaptic A_1 receptors may also exert an inhibitory effect (Ribeiro et al., 2003). Most importantly, activation of A_1 receptors has been shown to limit neuronal release of the excitatory amino acid glutamate (Flagmeyer et al., 1997) and decreases in acetylcholine release have also been reported (Brown et al., 1990). Activation of A_1 receptors in the striatum was also linked to inhibition of the D_1 receptor-mediated increase in adenylyl-cyclase activation (Abbracchio et al., 1987), giving an explanation for the reports of decreases of striatal extracellular dopamine levels following A_1 receptor activation (Okada et al., 1996). Caffeine administration would disinhibit these neurons, leading to an increase in neurotransmitter release (see Figure 1).

The inhibitory effect of adenosine A_1 receptor activation on glutamate release and Ca^{2+} influx likely is responsible for the neuroprotection (particularly in hippocampal regions) seen when animal models of cerebral ischemia are administered A_1 selective adenosine receptor agonists (Rudolphi et al., 1989). The stabilization of Ca^{2+} homeostasis after A_1 receptor activation raises the depolarization threshold needed to remove the Mg^{2+} blockade in NMDA receptors (present in large numbers in hippocampal neurons) leading to a reduced risk of excitotoxicity (Ongini and Schubert, 1998). Treatment with an A_1

selective adenosine receptor antagonist on the other hand, is shown to exacerbate damage in the hippocampus during ischemia because of the increased exposure to excitotoxicity (Rudolphi et al., 1997).

The A_{2A} type adenosine receptors also have high affinity for caffeine and are activated by the nanomolar concentrations of adenosine present during normal physiological conditions in the brain (Fisone et al., 2004). These receptors are highly expressed in the basal ganglia, particularly in the striatum, and the olfactory bulb, while much lower levels of this receptor are found in the hippocampus and cortex (Sebastiao and Ribeiro, 1996). The A_{2A} receptors activate stimulatory G-proteins (G_s in the periphery and G_{olf} in the striatum) which in turn activate adenylyl cyclase (Herve et al., 2001) and may also activate voltage-sensitive Ca^{2+} -channels in certain cells (Fredholm et al., 1999). It has been shown in some hippocampal neurons that A_{2A} receptor activation upregulates Ca^{2+} uptake via class A calcium channels, through the activation of adenylyl cyclase signaling (Goncalves et al., 1997). Notably, A_{2A} adenosine receptors sometimes are located with A_1 receptors on the same cells, indicating these different receptor types may have opposing roles when on the same cell. Indeed, in the striatum (a brain area where A_{2A} receptors are high and A_1 receptors are low) activation of the A_{2A} receptor leads to a desensitization of the A_1 receptor (Dixon et al., 1997). The end result of adenosine receptor activation thus may depend on the densities of each receptor type in that particular brain area.

A_{2A} receptors are located on both neurons and microglia, but not on astrocytes (Fiebach et al., 1996). A_{2A} receptors are also present on coronary arterial walls and upon activation cause vasodilation (Linden, 2001). In addition, A_{2A} receptors are also located

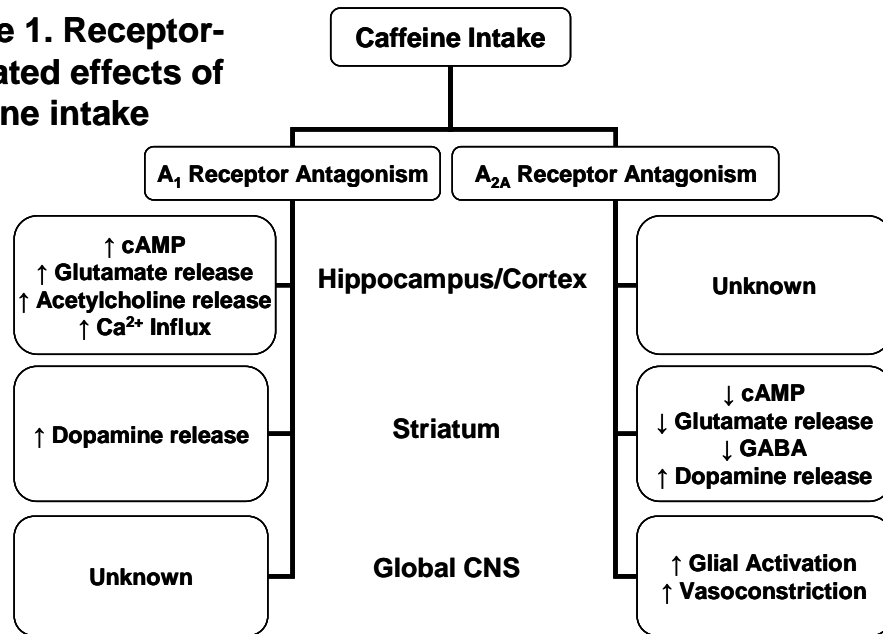
in the periphery on bone-marrow derived cells; such as neutrophils, monocytes, macrophages, platelets, and mast cells, where *in vitro* activation of these receptors has been shown to reduce the release of reactive oxygen species and the pro-inflammatory cytokine TNF- α (Sullivan, 2003). An *in vivo* study confirmed that genetic inactivation of A_{2A} receptors in the periphery results in the loss of a negative feedback mechanism that would otherwise limit a systemic inflammatory response and reduce tissue damage (Ohta and Sitkovsky, 2001). This suggests that A_{2A} receptor activation potentially might reduce the pro-inflammatory component of the amyloid cascade in the brains of Alzheimer's afflicted patients. Expression of A_{2A} receptor mRNA on glial cells in the brain is limited however (Schiffman et al., 1991), thus their role in any A_{2A}-modulated actions is unlikely to be significant. Therefore, blockade of glial A_{2A} receptors by caffeine would also be limited so it should not have a noticeable effect on inflammation in Alzheimer's disease (see Figure 1).

Activation of A_{2A} receptors by selective A_{2A} receptor agonists has shown significant increases in extracellular glutamate levels in the striatum (Popoli et al., 1995) and cerebral cortex (O'Regan et al., 1992), providing evidence that activation of striatal A_{2A} adenosine receptors gives a detrimental effect during times of ischemia and/or excitotoxicity in direct contrast with A₁ receptor-mediated protection. Accordingly, A_{2A} receptor blockade by selective A_{2A} adenosine receptor antagonists has been found to reduce neuronal striatal damage induced by the excitotoxin quinolic acid (Popoli et al., 2002) and A_{2A} receptor KO mice have been reported to be protected against MPTP-induced neuronal damage in the striatum (Chen et al., 2001). With this information in mind, any neuroprotective effects of A_{2A} antagonists against Alzheimer's are likely due

to vasodilatation in the periphery, rather than in the brain itself because excitotoxic damage is not indicated in striatal areas in patients with this particular disease.

Particularly in Parkinson's research, neuronal A_{2A} receptors have received intense interest because of their roles in dopaminergic transmission in the dopamine rich areas of the striatum. The majority of striatal A_{2A} expression is found in association with the inhibitory dopamine activated D₂ receptors on the GABAergic medium-sized neurons of the indirect pathway that project from the striatum to the globus pallidus and subthalamic nucleus (Ferre et al., 1997). Under normal conditions, dopamine is the major modulator of this pathway, with A_{2A} receptors playing an opposing role to activation of the inhibitory D₂ receptors. When dopamine levels diminish in Parkinson's disease, this GABAergic/globus pallidus/subthalamic nucleus pathway is overactive, contributing to the motor abnormalities present in this disease. Thus A_{2A} specific antagonists have been forwarded to potentially correct this imbalance, in addition to their neuroprotective effect mentioned earlier. While this information is important for potential therapeutics against the degeneration of the dopaminergic system seen in Parkinson's disease, it is unlikely to have much bearing in Alzheimer's disease.

Figure 1. Receptor-mediated effects of caffeine intake



Immediate Effects of Caffeine Intake

Physiological Changes. The initial absorption of caffeine into the bloodstream creates widespread changes throughout the CNS. Acute doses of caffeine lead to increases in cerebral energy metabolism in areas of the brain responsible for motor activity and the sleep cycle, and this response is not abolished by any appearance of tolerance (Fredholm et al., 1999). Surprisingly, this increase in cerebral metabolism is also accompanied by a decrease in cerebral blood flow due to the cerebral vasoconstriction induced by caffeine seen during rest (Higashi et al., 2004). Caffeine’s effect on alertness is positive however, so it is likely that any increases in metabolism with reduced blood flow are met with increased glucose utilization. Interestingly, 1-5 mg/kg doses of caffeine in rats leads to widespread increases in glucose utilization throughout the brain, including areas of the cortex, hippocampus, and central components of the nigrostriatal dopaminergic system (Nehlig and Boyet, 2000). A caffeine-induced

increase in metabolism coupled with decreased blood flow could potentially lead to increased levels of extracellular adenosine in an ischemic-like response.

In the absence of caffeine, adenosine levels increase during prolonged wakefulness which suppresses cortical activity by activating the inhibitory A₁ receptors on mesopontine cholinergic neurons (Rainnie et al., 1994). The increase in cortical activity following caffeine intake evident in the electroencephalogram (EEG) as decreases in delta and theta waves and an increase in alpha and beta waves (Patat et al., 2000) is thus linked to caffeine's antagonism of A₁ receptors on these particular neurons. Caffeine also decreases GABA release in the globus pallidus, leading to increased activity in this region as well (Fredholm et al., 1999). Furthermore, a microdialysis study with rats found that caffeine administration dose-dependently raised dopamine and acetylcholine concentrations in the prefrontal cortex (Acquas et al., 2002). The authors also reported the increases in dopamine concentrations were lost after tolerance developed to chronic caffeine treatment, while caffeine's effects on acetylcholine were not. This information lends support to the reported positive affect of caffeine on alertness that is not lost in chronic caffeine users.

Behavioral Effect. Although acute use of lower doses of caffeine is known to promote locomotor effects in caffeine intolerant rodents, the exact mechanism for this action remains to be clearly pinpointed to one receptor. Acute doses of caffeine given in mice in particular are reported to decrease exploratory behavior in the open field (Meyer and Caston, 2004) and increase anxiety in the plus-maze task (Silva and Frussa-Filho, 2000). Given that A_{2A} receptors are found in particular abundance in the striatum (an area of the brain profoundly involved in locomotion) it has generally been assumed that

blockade of A_{2A} receptors following caffeine administration is mainly responsible for the changes in locomotor activity (see Table 1). Concordantly, it is not surprising that A_{2A} receptor KO mice are resistant to the stimulatory effects of caffeine (Halldner et al., 2004).

Caffeine also is reported both anecdotally and experimentally to provide an immediate positive effect on learning and memory. It is interesting to note that a study found an acute dose of caffeine administered to caffeine-naïve rats after training in the Morris water maze improved their reference memory in the probe trial at doses of 0.3-10 mg/kg, whereas a higher dose of 30 mg/kg had no effect on performance (Angelucci et al., 2002). Acute caffeine doses of 0.1-0.3 mg/kg also were also found to reverse memory disruption in the two-way active avoidance task induced by MPTP injections in rats (Gevaerd et al., 2001). Additional studies found that administration of an A₁ specific receptor antagonist MDL102503 to rats reverses scopolamine-induced memory impairment in the water maze. In addition, another A₁ specific receptor antagonist (KFM19) also improved performance in other cognitive based tasks, such as the Y-maze (Jacobson et al., 1996). Castellano (1976) also found a caffeine dose of 1 mg/kg to mice improved the learning and consolidation processes in a Y water maze task.

Although these studies give evidence for a potential positive effect on memory by an acute dose of caffeine in rodents, they don't provide much insight into the effect a long-term treatment of caffeine might have on memory and learning in rodents. Furthermore, it is also difficult to tell if improved performance in these cognitive tasks following acute doses of caffeine is simply related to increased alertness in these mice and not an increase in memory or learning. For instance, caffeine doses of 2.5 and 5 mg/kg had no effect on

avoidance learning of mice in the shuttle-box avoidance test, while caffeine doses of 10 mg/kg actually impaired their performance (Sansone et al., 1994). Similar results were found by Izquierdo et al (1979), who found mice administered 29.9 mg/kg of caffeine were significantly impaired in their memory retention in the passive avoidance task. It is likely alertness is less of a component in mouse performance in the avoidance tasks, because these tasks utilize a conditioned response rather than the reliance on spatial cues for orientation in the Morris water maze. Lastly, a study using wild type rats found that acute doses of caffeine improve tracking performance, indicating improved alertness (Evenden et al., 1993) (see Table 1 for a summary of these results).

Controlled human studies have produced mixed results. James (1998) reported no effects of caffeine on performance were seen when administered on either an acute or chronic basis. This study did find participants were more alert and less tired following acute intake of caffeine, but felt less alert following chronic exposure to it. A study that stratified the dose-dependent effects of caffeine found caffeine administered at a lower dose (250 mg) produced pleasant subjective feelings and positive effects on performance whereas a higher dose (500 mg) produced unpleasant subjective feelings and decreased performance (Kaplan et al., 1997). Differences in performance were also reported between studies using low dosages of caffeine. A low dose of caffeine (100mg) failed to have an effect on short or long-term memory retrieval in middle to elderly men and women (Schmitt et al., 2003). A study utilizing a CANTAB battery found 60 mg of caffeine sped up reaction times in pattern recognition, delayed match to sample, and match to sample visual searches (Durlach et al., 1998). Both of these studies are similar however because they included habitual caffeine users forced to abstain from caffeine for

the study purposes. This is significant because another study reported the caffeine-induced increase in a sustained attention task was abolished when the moderate caffeine users were tested after no longer being deprived of caffeine (Yeomans et al., 2002). The authors go on to theorize that caffeine's beneficial affect on memory is mainly due to withdrawal reversal, a likely suggestion supported by information gleaned from animal studies dealing with acute or chronic doses of caffeine.

Table 1. Immediate Effects of Moderate Caffeine Intake

<u>Physiological</u>	<u>Receptor Action</u>
Cerebral Vasoconstriction	Global vasculature A _{2A} antagonism
Increased glucose utilization	Unknown
Increased cortical EEG activity	Mesopontine Cholinergic A ₁ antagonism
Neurotransmitter Disinhibition	A ₁ antagonism
<u>General Behavior</u> (Humans and Rodents)	
Increased alertness	Mesopontine Cholinergic A ₁ antagonism
Increased anxiety	Striatal A _{2A} antagonism
<u>Rodent Cognition*</u>	
Improved reference memory (Morris water maze)	Unknown
Improved learning/consolidation (Y water maze task)	Unknown

* Improved cognition may be due to increased alertness

Long-Term Effects of Caffeine Intake

Behavioral Effect (Rodents). The locomotor disturbances induced by acute doses of caffeine disappear after chronic caffeine administration. For instance, rats given an acute dose of caffeine show decreased exploratory behavior in the open field task and also show increased anxiety in the plus-maze test; yet after 21 days of chronic caffeine administration both of these disturbances disappear (Bhattacharya et al., 1997). Another study reported that tolerance to A₁ receptor blockade is indicated for the lack of consistent locomotor disturbances after caffeine intake in caffeine tolerant individuals

(Karcz- Kubicha et al., 2003). Overall, these animal studies point to a minimizing or complete loss of the motor effects of caffeine once tolerance develops.

The chronic administration of caffeine to rodents leads to adaptive changes that abolish the cognitive changes seen during acute treatment of caffeine as well. Chronic administration of the A₁ selective receptor antagonist CPX for only 9 days resulted in a tolerant adaptation that caused CPX to have no effect on the spatial learning and memory of mice tested in the Morris water maze (Von Lubitz et al., 1993). Chronic caffeine treatment for 15 days also had no effect on prevention of memory loss in trained rats (Molinengo et al., 1994). Prior studies in wild-type rodents thus have shown that administration of caffeine or specific adenosine receptor antagonists for approximately two weeks results in no obvious effects on memory or learning, although no truly chronic caffeine administration studies (that might relate to caffeine use in humans) have been done investigating cognitive measures.

Behavioral Effect (Humans). Large population studies done to examine the long-term effects of caffeine use on human memory have been controversial because of the many variables that must be taken into account. For instance, Hameleers et al. (2000) found in a cross-sectional study of 1875 adults participating in the MAAS study (the Maastricht Aging Study) and ranging in age from 24-81 years that higher habitual caffeine intake is significantly associated with faster response speed and improved long-term memory, yet no differences in short-term memory were found. In contrast, a longitudinal study of the same MAAS group found no relationship between habitual caffeine intake and verbal memory performance (van Boxtel et al., 2003). An earlier study of 9003 British adults reported that aged individuals appear to be more receptive to

caffeine's cognitive boosting affects than younger individuals (Jarvis, 1993), thus suggesting that the inclusion of younger adults in the MAAS studies may have provided the confounding results.

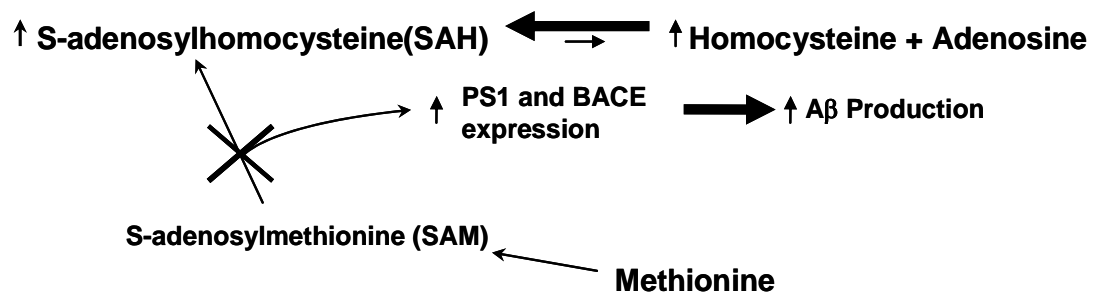
Physiological Changes of Long-Term Caffeine Intake. The study by Maia and Mendonca (2002) that suggested caffeine intake is associated with a lower risk for AD has driven several studies to investigate caffeine's antagonism of specific adenosine receptors in the CNS, the more obvious effect of caffeine intake (Dall'Igna et al., 2004; Dall'Igna et al., 2003) . These studies fail to fully account for the typical patterns of caffeine consumption in humans however. Knowing that caffeine's half-life in humans can be less than 3 hours, it is reasonable to assume that the short term effects of caffeine intake only exist for brief durations during the typical day. This makes it difficult to attribute an overall protection against AD (which is progressively and continually disrupting the normal brain physiology decades before any symptoms appear) to the immediate blockade of adenosine receptors. It is therefore relevant to look to any long term effects of caffeine as the main agent granting protection from AD. From this standpoint, caffeine's neuroprotective effect can be elucidated from several studies indicating an effect of caffeine on adenosine modulation separate from acute receptor antagonism. These are significant because relationships between impaired adenosine levels and Alzheimer's disease have already been implicated but not completely explored.

It is particularly noteworthy that increased plasma levels of homocysteine, a known risk factor for AD, are associated with decreased adenosine formation in plasma taken from AD patients (Selley, 2004). Interestingly, increased levels of homocysteine

are associated with normal aging, decreased physical activity, and diets high in animal protein yet deficient in fruits and vegetables (Miner et al., 1997). These are all also risk factors for AD.

At the molecular level, high levels of homocysteine interfere with the intracellular production of adenosine by forcing the normal conversion of S-adenosylhomocysteine (SAH) into adenosine and L-homocysteine to occur in reverse, thus sequestering adenosine as SAH inside the cell (Fredholm et al., 1999; Fig.2). Indeed, high levels of SAH have recently been identified in the brains of AD patients, and elevated levels of SAH in AD patients have been shown to inhibit important methyltransferases in the brain (Kennedy et al., 2004). Furthermore, the enzyme S-adenosylmethionine (SAM), which is involved in the continuous conversion of methionine to homocysteine (Miner et al., 1997), is found in decreased concentrations in Alzheimer's disease (Morrison et al., 1996; Morris, 2003; Mizrahi et al., 2003). The SAM enzyme has recently been identified as a methyl donor to a promoter site on the PS1 gene resulting in decreased PS1 expression (Scarpa et al., 2003), and also is involved in the down-regulation of *BACE* (β -secretase) (Fuso et al., 2005) (Fig. 2).

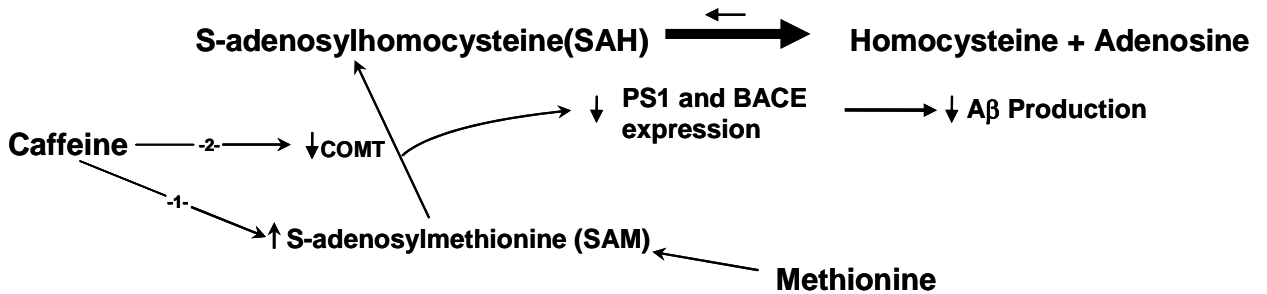
Figure 2. Impact of elevated levels of homocysteine on SAM in AD



If high levels of homocysteine are present, either because of diet, vitamin B₁₂/folate deficiency, or advancing age; then it is likely that SAM methylation of PS1 and BACE is blocked by the increased concentrations of SAH. Thus, decreasing methylation by SAM may result in a rise in expression of the PS1 and BACE genes, leading to increases in A β production. It has also been found through *in vitro* studies that a folic acid deficiency and elevated homocysteine levels disrupt DNA repair in hippocampal neurons, which sensitizes them to the toxic effects of amyloid accumulation (Kruman et al., 2002). This information implies that increased homocysteine levels may play both a direct and indirect role in Alzheimer's disease, and represents a potential target for the long-term effects of caffeine intake (see Figure 3).

Interestingly, a recent *in vivo* experiment found extracellular levels of adenosine were elevated 8 hours after caffeine administration in rats (Conlay et al., 1997). The authors proposed this increase may be a result of adenosine receptor blockade. However, caffeine is the downstream precursor of a biosynthetic process in tea leaves that begins with SAM (Koshiishi et al., 2001). Thus, caffeine intake may elevate SAM levels when it is degraded. It is also possible that caffeine's blockade of A_{2A} receptors on astrocytes leads to inhibition of COMT (catechol-O-methyltransferase), the enzyme responsible for conversion of SAM to SAH. The elevation of SAM would lead to the decreased expression of the genes that would otherwise lead to AD (see Figure 3) and might be evident by increased adenosine levels as SAH was hydrolyzed to adenosine and homocysteine.

Figure 3. Proposed impact of caffeine on the SAM/SAH cycle in AD



Tolerance to Caffeine

The long-term administration of caffeine results in the disappearance of the behavioral side effects associated with caffeine intake (e.g. tolerance). Concordantly, this should be the result of neuromolecular changes because of the fluctuating presence of caffeine and elevation of adenosine levels. Indeed, numerous studies involving rodents have reported that chronic treatment with caffeine, ranging from 4-28 days, results in increased numbers of A₁ receptors in both cortical (Tsutsui et al., 2004; Shi and Daly, 1999; Shi et al., 1993) and hippocampal neurons (Rudolphi et al., 1989; Johansson et al., 1993). The mechanism for this increase remains unresolved, as none of these studies reported changes in mRNA for the A₁ receptor. Many of these studies examined A_{2a} receptors as well, but no changes were seen in receptor counts or mRNA levels (Shi and Daly, 1999; Johansson et al., 1993; Shi et al., 1993). These findings suggest A₁ receptors are responsible for the development of tolerance to the behavioral and physiological side effects of caffeine. Importantly, this increase in A₁ receptor levels has implications for potentially reversing trends seen in AD as well. Additionally, a 4-day treatment of caffeine to mice also found an approximate 17% increase in the density of cortical L-type

calcium channels, but the authors failed to suggest a mechanism for this and its implications remain unknown (Shi et al., 1993).

The considerable 8 hour delay between caffeine administration and increases in adenosine first noticed by Conlay et al. was later replicated and traced to the blockade of A₁ receptors (Andresen et al., 1999). The authors suggested the time lag between receptor antagonism and increases in adenosine may be caused by new protein synthesis, post-translational modifications, or reductions in the synthesis of key enzymes responsible for metabolizing adenosine, such as adenosine deaminase. The authors go on to propose that A₁ receptors monitor extracellular levels of adenosine and when activated, influence adenosine levels by potentially modulating the activity of adenosine deaminase. Therefore the losses in hippocampal A₁ receptors reported in patients with dementia (Deckert et al., 1998) and Alzheimer's disease (Ulas et al., 1993) suggests impaired monitoring of adenosine function in AD. If adenosine is being trapped intracellularly in the form of S-adenosylhomocysteine, then the reduction of extracellular adenosine could potentially lead to the reductions in its receptor as well. Given the likely role of APP in axonal transport (Kamal et al., 2001) and accumulating A β interferes with fast anterograde and retrograde axonal transport (Hiruma et al., 2003), it is also possible to surmise impaired axonal transport may lead to the decrease in adenosine A₁ receptors seen in AD cases.

Diminishment of A₁ receptor function would impair an innate neuronal pathway that monitors Ca²⁺ homeostasis, which is known to be disrupted in neurons after long term exposure to the A β ₁₋₄₂ isoform found in AD. As mentioned earlier, NMDA receptors are found in abundance in the hippocampus making neurons in this area particularly

sensitive to excitotoxic damage during pathological conditions. Therefore, it is likely that the progression of AD leads to decreases in A₁ receptor-mediated protective mechanisms against excitotoxicity in both the cerebral cortex and hippocampus in combination with the toxic effects of A β , creating a hostile environment for neurons in these brain areas. An increase in A₁ receptors following long term treatment of caffeine thus may reverse this trend restoring A₁ receptors to normal or above physiological levels.

Health Risks of Caffeine Intake

The impact of moderate caffeine intake on health has been scrutinized through epidemiological studies with no clear detrimental effects. Caffeine's relationship with blood pressure is of particular concern, yet most studies report no association between caffeine consumption and blood pressure in tolerant individuals (Chou and Benowitz, 1994; Robertson et al., 1984; Bertrand et al., 1978). Other studies have found an association between caffeine and changes in blood pressure, but these findings are considered controversial because they fail to distinguish between infrequent caffeine users and habitual caffeine users (Rachima-Maoz et al., 1998). It has also been suggested that hypertensive patients are more prone to the pressor effects of caffeine, although this sensitivity was not found in a case study (Robertson et al., 1984). Moderate caffeine intake is known to raise blood pressure in caffeine-naïve subjects and in subjects abstaining from caffeine long enough to lose their tolerance to caffeine, yet this pressor effect is only in the range of 2-3 mm Hg and this effect is lost within 24 hours after tolerance appears (Myers, 2004). High intake of caffeine also can lead to tachycardia, heart palpitations, and a small decrease in heart rate, but again these effects are minimized after tolerance develops within days (Fredholm et al., 1999).

The Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure expressed their view that restriction of caffeine intake is not a recommended procedure on reducing blood pressure (Chobanian et al., 2003), yet some doctors continue to recommend the elderly or people already prone to hypertension avoid caffeine. If caffeine does have neuroprotective properties, it would be prudent instead to recommend a moderate, sustained caffeine intake throughout life to reduce any of the cardiovascular effects discussed previously that could appear if tolerance to caffeine is lost during any abstinence from the drug.

Caffeine, as well as other methylxanthines, is a well known diuretic and natriuretic that inhibits proximal tubular reabsorption by antagonizing renal A₁ receptors (Rieg et al., 2004). Finally, caffeine also raises the respiratory rate in the same, but yet not as effective, mechanism as theophylline (the common bronchodilator prescribed for asthmatics) (Benowitz, 1990). Caffeine also has weak self-reinforcing properties when compared to the drugs of abuse, such as cocaine or heroine, and is not described as an addictive substance in the *Diagnostic and statistical manual of mental disorders* (4th ed.). Although symptoms such as headache and fatigue may accompany caffeine withdrawal when caffeine intake is reduced significantly (Griffiths et al., 1990), none of the side effects of prolonged, moderate caffeine intake have been linked conclusively to any adverse health conditions.

Summary

Although blockade of A₁ and A_{2A} adenosine receptors produces some potential immediate neuroprotective effects, it is likely that caffeine's biggest contribution to neuroprotection in Alzheimer's disease may be the reinforcement of SAM methylation of

PS1 and BACE. Given the nature of moderate caffeine consumption and its metabolism, the short-term neuroprotective effects of caffeine would certainly not exist long enough to have any lasting contribution against the lifelong onslaught of AD. Although it has yet to be determined exactly how caffeine raises SAM levels, several possibilities exist. First, caffeine is a xanthine derivative of SAM, and once metabolized may provide extra substrate for the synthesis of SAM, leading to the increased formation of this compound. Caffeine may also inhibit COMT activation, which would decrease astrocytic homocysteine synthesis (Huang et al., 2005) and reduce SAM conversion to SAH.

Regardless, the chronic use of caffeine generates a rise in SAM that likely leads to diminished expression of genes that are known to be overexpressed in familial Alzheimer's disease and are likely askew in sporadic AD, resulting in decreased production of A β and prevention of the corresponding changes in neuropathology. Ideally, changes in expression of these genes is minimal but sufficient enough to slow down A β production thus avoiding many of the negative side effects of other therapeutics designed to minimize expression of these genes. It is also possible that diminished production of A β allows for innate protective mechanisms in the body to equilibrate the clearance and production of A β . Caffeine may also be effective in reversing the decreases in hippocampal A $_1$ receptors seen in AD and aging through mechanisms that develop as tolerance in response to chronic caffeine intake, ultimately restoring the endogenous protective pathway against alterations in Ca $^{2+}$ or excitotoxicity.

IV. Specific Aims

Overexpression of the hAPP gene with the “Swedish” double mutation in APP_{sw} mice results in impaired reference/learning memory and impaired working memory by 8-9 months of age that closely mimics the cognitive decline seen in Alzheimer’s patients. These behavioral impairments are certainly linked to the appearance of A β species, but other pathological changes must also be taken into consideration as well when evaluating the full scope of the disease. The decrease in A₁ adenosine receptors found in the brains of late Alzheimer’s patients may be a marker for other important disruptions in the brain, especially considering adenosine’s relationship with homocysteine/SAH/SAM and its potential indirect impact on enzymes reported to decrease expression of genes involved in AD. SAM-related changes have yet to be examined in animal models for Alzheimer’s disease, so it is therefore relevant to see if there are any differences between APP_{sw} and non-transgenic mice.

Caffeine use has been implicated with the occurrence of Alzheimer’s disease, with moderate caffeine intake decreasing the risk for the disease. Additionally, caffeine use has been shown to increase cortical and hippocampal A₁ receptors and raise extracellular adenosine levels in blood plasma. If this occurs in the brain, it may further support the link between impaired SAM function and Alzheimer’s disease. I propose that APP_{sw} mice will have decreased cortical and hippocampal A₁ receptors and subsequently, will also have reduced extracellular brain adenosine and S-adenosylmethionine levels in

the brain. These APP_{sw} mice will also be cognitively impaired because of increased levels of soluble A β . Furthermore, long-term administration of a moderate caffeine dose to APP_{sw} mice will reverse these effects, ultimately reducing amyloid load in these mice and restoring cognitive function.

The specific aims of my research proposal are:

- To behaviorally characterize a group of APP_{sw} and Tg- control mice in a full battery of sensorimotor and cognitive tasks to determine the degree of behavioral impairment these mice develop at 8 months of age.
- To determine the behavioral effect a 4 month treatment of oral caffeine (beginning at 4 months of age) has on a similar group of age-matched 8 month old APP_{sw} transgenic mice.
- To explore any differences in SAM and adenosine between non-transgenic and APP_{sw} mice.
- To evaluate the relationship between long-term caffeine administration in a mouse model of Alzheimer's disease and any changes in amyloidogenic processing in the CNS as a result of caffeine treatment.
- To determine the relationships between brain A β levels, brain adenosine receptor levels, and cognitive performance in APP_{sw} and caffeine-treated APP_{sw} transgenic mice.

V. Materials and Methods

Animals

57 mice were included in this study. Each mouse had a mixed background of 56.25% C57, 12.5% B6, 18.75% SJL, and 12.5% Swiss-Webster. All of these mice were derived from a cross between P (parental generation) heterozygous male mice carrying the mutant APP_{K670N, M671L} gene (APP_{sw}) with F1 PS1 (transgenic line 6.2) female mice to obtain an F2 generation consisting of APP/PS1, APP_{sw}, PS1, and non-transgenic mice. After weaning, the mice were genotyped with only APP_{sw} and non-transgenic mice selected for behavioral testing and/or caffeine administration. These mice were then group housed in cages with rodent chow and water or caffeinated water *ad libum*. All mice were maintained in a 10 hour dark and 14 hour light cycle at all times, and all behavioral testing was performed during the light cycle.

Effects of Long-Term Caffeine Administration in Young Adult APP_{sw} Mice

General Protocol

A total of 41 single transgenic APP_{sw} mice and 16 non-transgenic (NT) littermates were randomly selected for this study. 14 APP_{sw} mice were randomly selected from the APP_{sw} group and were administered caffeine treatment in drinking water beginning at 4 months of age. The remaining 27 transgenic mice, as well as 16 non-transgenic littermates, were provided normal water to serve as Tg and NT controls. All mice were removed from group housing and moved to single housing two weeks before behavioral

testing began. Behavioral testing began at 8 months of age (four months into caffeine treatment) and consisted of a 6-week battery that was composed of 3 sensorimotor-based tasks, one anxiety-based task, and five cognitive-based tasks. These tasks were performed in the following order: open field activity, balance beam, string agility, Y-maze, elevated plus maze, Morris water maze, circular platform, platform recognition, and radial arm water maze. All mice were euthanized following completion of behavioral testing and brains were removed for further analysis. This analysis consisted of quantification of brain soluble/insoluble A β levels, determination of both α - and β -CTFs, and assessment of brain adenosine receptor densities. Fig. 1 depicts a timeline for this study. Mice were weighed every two weeks to ensure no changes in weight occurred from the caffeine treatment.

Study Timeline

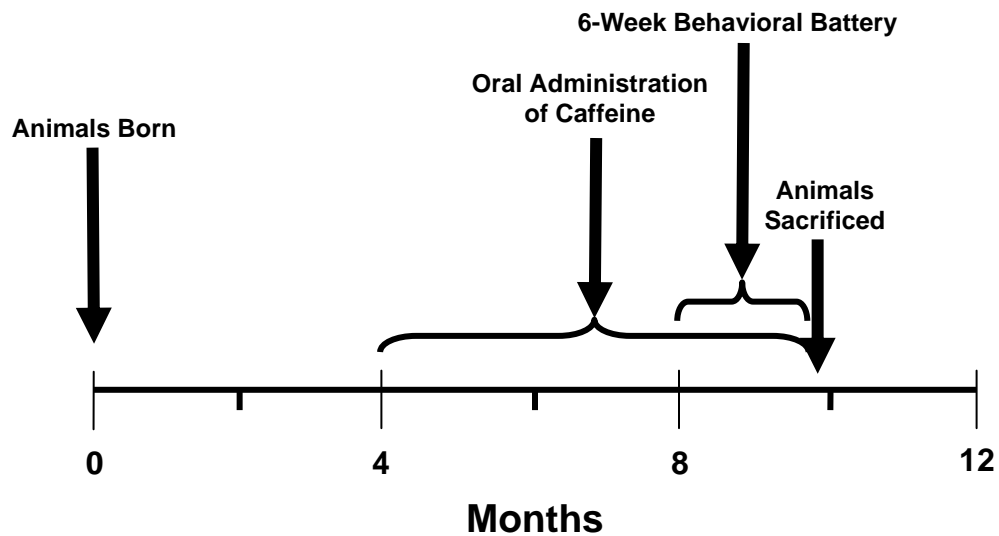


Fig 4. General protocol time line for long-term caffeine administration study.

Caffeine Treatment

At 4 months of age, 14 APP_{sw} group housed mice were given *ad libum* access to only water with 0.3 mg/mL caffeine (*Sigma*) dissolved in it. On average, mice drink 5 mL per day, giving a daily dose of 1.5 mg of caffeine to each mouse. Given that metabolic rate (MR) = $\text{Mass}^{-1/4}$, the MR of mice (average weight = 0.025kg) is 7.2 x greater than humans (average weight = 68kg). Thus, a 1.5 mg daily dose in a mouse is equivalent to an approximately 500 mg daily caffeine intake (~5 cups of coffee) by a human. The caffeinated water was changed two times a week to ensure caffeine remained fully dissolved at the appropriate concentration. Control APP_{sw} and NT mice were given *ad libum* access to untreated tap water that was also changed twice weekly to ensure freshness. Mice were kept under these conditions for 3½ months, at which point they were separated into single housing, two weeks before behavioral testing began (Fig. 1). Caffeine treatment was continued throughout this time and during behavioral testing. The weights of the animals were monitored throughout this study to ensure no significant weight reductions occurred.

Behavioral Assessment

Over a 6-week time course, mice were behaviorally tested for characterization of their sensorimotor, anxiolytic, and cognitive functions utilizing the following tasks and in the order described:

Open-Field Task. This test assessed exploratory behavior and activity by placing mice into an unfamiliar open black box (81 x 81 cm) with 28.5-cm walls. The bottom of the box was marked by 4 horizontal and 4 vertical lines, dividing the surface into 16 squares. The task consists of a single trial, wherein a single mouse is placed in the

center of the field and allowed to explore for 5 minutes. During this period, each line crossing was recorded. Before each trial, the surface of the box was sprayed with a diluted vinegar solution to erase any scent cues.

Balance Beam Task. The balance beam consists of a 1.1-cm-wide dowel beam suspended 43 cm above a padded surface. Flanking each end of the 51-cm-long dowel are 14 x 10.2 cm platforms. Each animal was placed perpendicular to the dowel at the center of the beam and released for an interval of 60 seconds. The duration the animal was able to stay on the balance beam was recorded. If the animal remained on the balance beam for the full time and/or escaped to one of the platforms, the maximum score of 60 seconds was recorded. Each mouse's balance and general motor function was evaluated by subjecting the animals to 3 trials, with the overall average indicating the best approximation of the mouse's performance.

String-Suspension Task. This task is an additional sensorimotor test used to characterize the agility and grip strength of mice. Animals were allowed to grip the string with only their forepaws and released for a single trial of 60 seconds. Each animal was scored on a 0-5 rating system (0=animal was unable to stay on the string; 1=animal was able to hang onto the string for 60 s by only two forepaws; 2=was given if the animal was able to hang onto the string by two forepaws and one hind limb; 3= animal remained on the string for 60 s and gripped the string by two forepaws and both hindpaws, 4=animal was able to grip the string with four paws and its tail; 5=was given if the mouse escaped from the string to one of the support columns.

Y-Maze Task. This task was used to assess basic mnemonic processing (by spontaneous percent alternation) and exploratory activity (by total number of arm

choices) of mice placed into a black Y-maze. The arms of this maze were 21 cm-long and 4 cm-wide with 40 cm-high walls. Each mouse was placed in the center of the maze, facing the arm designated number two and allowed one five minute trial of free exploration of the three alleys in the maze. The number of total arm choices and sequence of arm choices were recorded. Alternation percentage is defined by the proportion of arm choices that differ from the last two choices. For instance, if a mouse selected the following sequence of arm choices (1,2,3,1,3,1,2,1), the total number of alternation opportunities would be six (total entries minus two) and the percentage alternation would be 50% (three of six). Before each trial, the interior of the maze was spray with a diluted vinegar solution to erase any scent cues.

Elevated Plus-Maze. The elevated (+)-maze was used to assess anxiety in mice. The task has four arms (30 x 5 cm) attached to a 5 x 5 cm central area, all made of plywood and painted black. Two opposite facing arms were unenclosed and open to the surrounding environment. The other two opposite facing arms were enclosed by black aluminum sheet walls (15-cm height). This entire structure sits on a wooden pedestal, elevated 82 cm above the floor level. Each mouse was placed into the center area facing a closed arm and allowed to explore the plus-maze for a single five minute trial. The number of closed and open arm entries, and the amount of time spent in open arms was recorded. Before each trial, the maze was cleaned with a diluted vinegar solution to erase any scent cues.

Morris Water Maze. This water-based task was used to evaluate spatial reference learning/memory of the mice. A 100-cm circular inflatable pool was divided into four equal quadrants by black lines drawn on the floor of the pool; an indiscernible

9-cm platform was submerged 1.5 cm below the water's surface in quadrant two. The environment surrounding the pool was decorated with eye-catching visual cues to aid the mice in orientating themselves with respect to the pool. Each mouse was subjected to four trials a day over a 10 day period. Each trial began by placing the mouse into a different quadrant and allowing it to swim freely for a maximum of 60 seconds. The same quadrant start pattern was used each day. After swimming to the platform (or being guided to the platform if the mouse was unable to locate the platform after 60 s), the animal was allowed to remain on the platform for 30 s before starting the next trial. The latency for each animal to locate the platform in all four trials and the average for all trials was recorded. After the tenth day of acquisition testing, a 60 s probe trial was performed the following day to determine memory retention. For this single trial, the submerged platform was removed and each mouse was placed into the quadrant opposite to the quadrant that formerly contained the platform in acquisition testing. The animal's swim path and number of annulus crossings were recorded on videotape; the percent of time spent in each quadrant, as well as average swim speed, were determined from these videotapes.

Circular Platform Task. This task tests reference learning/memory by placing the mice in a curtain-enshrouded 69-cm circular platform with 16 holes (4.5 cm diameter) equally placed 1.3 cm from the outside edge. The holes designated 4, 8, 12, and 16 allowed the placement of an escape box directly underneath the hole. Two-dimensional cues were placed on the inside walls of the enclosing curtain, as well as on the platform's walls. The aversive light and wind stimuli used to motivate mice to escape the platform was provided by two 150-W lamps placed 76 cm above the platform and a high-speed fan

placed 15 cm above the platform. The first day of the circular platform task consisted of shaping (wherein mice were placed into the center of the platform and gently guided to the location of their escape platform), with the following eight days designated for actual testing. The mice were subjected to a single trial each day that consisted of placing each mouse in the center of the platform and allowing it a 5 minute maximum to locate and enter the escape box. During the testing period, the total number of errors (head pokes into non-escape holes) and the latency to escape from the platform were recorded. The surface of the circular platform was cleaned after each trial with a dilute vinegar solution and the escape box location was moved to a different location after each mouse's trial to control for any scent cues (although the box remained in the same location for each particular animal over the eight days of testing).

Platform Recognition Task. This water-based task characterizes the ability of the mice to escape to a visible platform in changing locations placed in the same 100-cm inflatable water pool used in the Morris water maze. A delayed latency to escape indicates a potential deficit in visual acuity or, more likely, an impaired ability to switch strategies from a spatial cued strategy used in the Morris water maze to a search/recognition strategy needed for this test. In this task, a 9-cm circular escape platform was elevated 0.8 cm above the surface of the water with an affixed 10 x 40 cm black and white visual cue to clearly mark it as the escape platform. Performance was evaluated over four days of testing, with 4 trials per day. Each day, the mice were placed in the pool at the same location for each trial, but the platform was moved successively to a new quadrant for each of the four trials. If a mouse was unable to locate the platform in the 60 s provided, it was gently guided to the platform. A 30 s rest period was given to

each mouse on the platform. Latency to find the platform was recorded over all 4 trials each day, which were averaged for statistical evaluation.

Radial Arm Water Maze (RAWM). This final water-based task requires the mice to have intact working (short-term) memory and is the most stringent for determining cognitive deficiencies. The RAWM maze incorporated the same 100-cm circular pool used previously, but also used an aluminum insert that creates 6 equal-sized radial arms surrounding a central open swimming area 40 cm in diameter. Each arm was 30.5 cm long and 19 cm wide, and a transparent 9 cm circular platform that rests 1.5 cm below the water's surface was placed near the end of the randomly assigned goal arm of the maze for each day. The same spatial cues used in the Morris water maze were provided on the walls surrounding the RAWM task throughout testing. Each day of RAWM testing consisted of four acquisition trials and one memory retention trial. RAWM testing was conducted over nine successive days. For each acquisition trial, a mouse was placed in the water at the entrance of a novel start arm of the maze for that day facing the central swimming area. This start arm was never the same arm that contained the submerged escape platform, and the start arm sequences and goal arm location were semi-randomly selected each day. The mouse was then allowed to navigate the maze for 60 seconds. During this 60 s, if the mouse chose the wrong arm (that did not contain the escape platform) it was gently guided back to the start arm to renew navigating the maze and an error was recorded. If a mouse fails to select an arm within 20 seconds, it was gently guided back to the start arm and an error was recorded. If the animal failed to find the platform at the end of 60 s, it was gently guided to it. Once locating the platform, the mice were allowed a 30 s rest period. The latency to escape the maze and the number of

wrong arm choices were recorded over all four successive acquisition trials. Upon completion of the fourth acquisition trial, the mice were returned to their home cage for a 30 minute interval before being returned to the pool for the fifth and final trial of the day, the memory retention trial. The last trial of the four successive trials (trial 4, T4) and the 30-minute delayed retention trial (trial 5, T5) are considered measures of working memory. Any mouse that did not make at least 3 choices during a trial and were unable to locate the escape platform had a penalty assessed for that trial. This penalty was calculated by averaging trial one errors for the first three days of testing for animals which could not find the escape platform but made more than three arm choices during those trials. For this study, the penalty error assessed was 7.1.

Brain Collection

Immediately following completion of behavioral testing, all mice were anesthetized with Nembutal (1mg/10 gm body weight), then pericardially perfused with 0.9% saline. Brains were then removed and split into halves by a single mid-sagittal cut. The left hemisphere was fixed in 4% paraformaldehyde for 24 hours at 4°C, followed by graded sucrose solutions (10, 20, and 30% (w/v) sucrose in 0.1 x Sorenson's phosphate buffer). The right hemisphere was dissected out into the following areas: cerebellum, anterior and posterior cortex, striatum, and hippocampus. These areas were immediately frozen on dry ice and stored at -80°C. For half the animals in each group, the right frontal cortex, striatum, and hippocampus were used to measure the densities of adenosine receptors. For the remaining half of animals in each group, the right hippocampus was used to determine levels of soluble/insoluble A β and combined right frontal + posterior cortex was used for analysis of α - and β -carboxyl terminal fragments.

Analysis of β - and α -CTFs (carboxyl terminal fragments)

This procedure was performed by Dr. Jun Tan and Kavon Rezai-zadeh. Frontal and posterior cortices were thawed, combined, and placed in ice-cold lysis buffer (20 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{mL}$ leupeptin) with 1 mM PMSF. Brain tissues were then sonicated on ice for approximately 3 mins, cooled on ice for 15 mins, and then centrifuged at 15,000 rpm for 15 mins. Following homogenization, aliquots corresponding to 50 μg of total protein were electrophoretically separated using 16.5% Tris-tricine gels. Electrophoresed proteins were then transferred to PVDF membranes (Bio-Rad), washed in dH_2O , and blocked for 1 hr at room temperature in Tris-buffered saline (TBS; Bio-Rad) containing 5% (w/v) non-fat dry milk. After blocking, membranes were hybridized for 1 hr at room temperature with primary antibody [APP-carboxyl-terminal antibody 369(1:1000)]. Membranes were then washed 3 times for 5 min each in dH_2O and incubated for 1 hr at room temperature with the appropriate HRP-conjugated secondary antibody (1:1,000, Pierce Biotechnology, Inc. Rockford, Illinois). All antibodies were diluted in TBS containing 5% (w/v) non-fat dry milk. Blots were developed using the luminol reagent (Pierce Biotechnology). Blot intensities were analyzed qualitatively and assessed a ratio of 2:1 or 1:1 depending on their intensities in relation to each other (β -CTF vs. α -CTF).

Adenosine Receptor Densities (Completed by Dr. Edward Jackson)

Western Blotting. The tissues were placed in eppendorf tubes with 0.1ml SDS buffer (50mMTris, ph 7.0, 2% SDS, 10% glycerol) containing protease inhibitors (2 $\mu\text{g}/\text{ml}$ antipain, 1 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 1 mg/ml phenylmethylsulfonyl

fluoride) and homogenized with a small plastic pestle in ice. The homogenate was centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatant was recovered. Protein in the supernatant was terminated by the copper bicinchoninic acid method. Laemmli buffer was added to samples, after which they were placed in boiling water for 5 min and then chilled immediately on ice. 15 µg protein/well samples were loaded onto a 7.5-10% acrylamide gel and subjected to SDS PAGE using the Bio-Rad minigel system. Proteins were then electroblotted onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% milk for 1h and incubated at 4°C overnight with the first antibody (anti-A1 1:1000 diluted in PBS containing 0.5% Tween 20; anti-A2a 1:1000 diluted in PBS containing 0.5% Tween 20, antibodies were from Santa Cruz). After three washes with PBS containing 0.5% Tween 20, the membrane was incubated at room temperature for 1h with HSP-conjugated secondary antibody (Amersham) at 1:10,000 dilution. The membrane was exposed to film and the signals were detected by a supersignal substrate kit (Pierce).

RT-PCR. Total RNA was isolated using Trizol reagent solution (GIBCO). By using the primer sequences listed in Table 2, 0.5 µg RNA was reverse transcribed and amplified using a Titanium One-Step RT-PCR Kit (Clontech). Each PCR cycle (a total of 30 cycles for A1 and 32 cycles for A2a) consisted of denaturing at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 60 s. The products were separated on a 1% agarose gel with EB staining and the signal was detected by UV.

Table 2. Mouse adenosine receptor PCR primers and cDNA sizes

Receptor	Accession No.	Primer	Nucleotides	Sequence 5'-3'	Product Size
A1	NM_009629	Forward	659	TAGGGCAACGCCTTTGGGAC	849
		Reverse	1507	ATGGGTGTCAGGCCTACCAC	
A2A	NM_009630	Forward	177	GCCATCACCATCAGCACTGG	734
		Reverse	910	TCAGGACGTGGGTTCGGATG	

The band densities were quantitatively measured using Scion-image software.

Background signals were obtained in each lane and subtracted from the band densities to correct for the background signal.

Effects of Caffeine Administration in Aged APP_{sw} Mice

General Protocol

At 17 months of age, 8 APP_{sw} mice and 3 non-transgenic (NT) littermates were randomly selected to determine the effect of caffeine administration on both extracellular brain levels of adenosine and soluble/insoluble brain A β levels.

Four APP_{sw} group-housed mice were given *ad libum* access to water with 0.3 mg/mL caffeine (*Sigma*) dissolved in it, as per the long-term study. Four APP_{sw} mice and three NT group-housed mice were not administered caffeine in their drinking water and thus served as controls. Mice were kept under these conditions for 18 days, at which point they were euthanized (Fig. 5) and brains were removed for quantification of extracellular brain adenosine levels, soluble/insoluble A β levels, γ -secretase activity, and S-adenosyl methionine (SAM) levels. The weights of the animals were monitored throughout this study to ensure no significant weight reductions occurred.

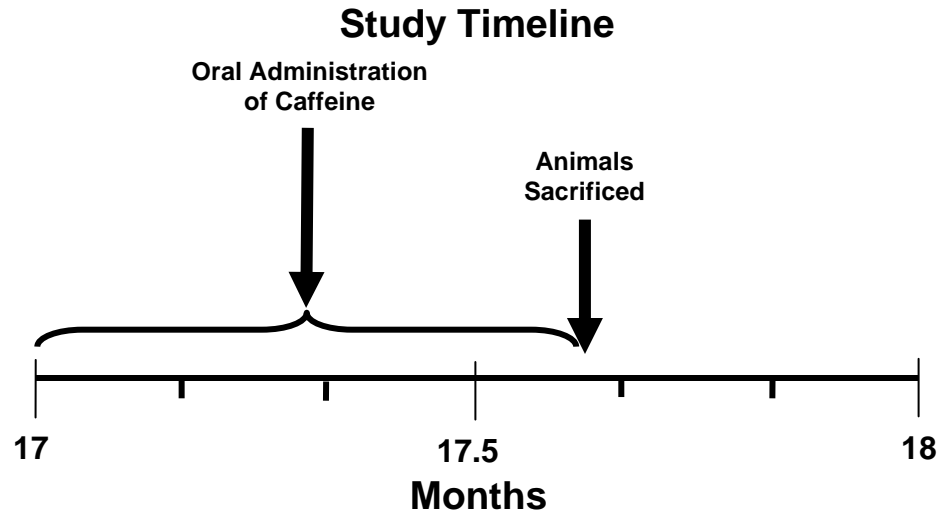


Fig. 5. General protocol time line for caffeine administration in aged mice.

Brain Collection

Immediately following caffeine treatment, all mice were anesthetized with Nembutal (1mg/10 gm body weight). Each mouse's skullcap was surgically removed and the brain was quickly excised and split into halves by a single mid-sagittal cut. The left hemisphere was rapidly snap-frozen in liquid nitrogen and reserved for measurement of extracellular brain adenosine levels through HPLC. This technique was done quickly to protect the tissue from ischemic conditions which would disrupt any adenosine measurements. The right hemisphere was dissected out into the following areas: cerebellum, anterior and posterior cortex, striatum, and hippocampus. These areas were immediately frozen in dry ice and stored at -80°C . Later, the hippocampus was used to measure the levels of soluble and insoluble $\text{A}\beta$ through ELISA. Combined anterior and posterior cortices were later analyzed for both γ -secretase activity and S-adenosyl methionine levels.

Measurement of Brain Adenosine Levels (Completed by Dr. Edward Jackson)

Sample Preparation. Half brains were weighed (50-60 mg) and washed with 500 μ L cold phosphate-buffer saline (PBS). Tissue was transferred to a centrifuge tube containing 500 μ L water and then boiled for four minutes to inactivate adenosine deaminase (and any other enzymes present in the sample). The tissue was then homogenized in a power homogenizer and centrifuged at 14,000 rpm for five minutes. The supernatant was drawn off and centrifuged a second time. The resulting supernatant was loaded onto centrifugal filter devices (Biomax-30, Millipore) and filtered to remove proteins. The filtrate was diluted 1:200 in water and internal standard (adenine 9- β -D arabinofuranoside) was added to a final concentration of 10 pg/ μ L. The standard curve was created in water and the samples were analyzed with an LCMS assay.

Mass Spectrometry. The assay was developed using a Thermofinnigan HPLC system coupled to a Thermofinnigan LCQ Duo ion trap mass spectrometer equipped with an electrospray ionization source (ESI). The mass spectrometer was operated in the ESI positive ion mode. The analytes were monitored using single ion monitoring; for adenosine and adenine 9- β -D arabinofuranoside (internal standard), the m/z was 268.

Measurement of γ -Secretase Activity (Collaboration with Kavon Rezai-zadeh)

Brain samples consisting of combined frontal and posterior cortices were placed in ice-cold lysis buffer (20 mM Tris, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/mL leupeptin). Brain tissues were then sonicated on ice for approximately 3 mins, cooled on ice for 15 mins, and then centrifuged at 15,000 rpm for 15 mins. No

PMSF was added to these samples as it may disrupt the activity of γ -secretase. Cell lysates from these samples were then analyzed for γ -secretase activity using a γ -secretase kit provided by R&D Systems, Inc.. Following the manufacturer's protocol, a secretase-specific peptide conjugated to reporter molecules EDANS and DABCYL was added to each cell lysate. Cleavage of this peptide by the γ -secretase present from each cell lysate results in a fluorescent signal that is proportional to the level of secretase enzymatic activity. Fluorescent signals were detected using a fluorometric reader. Protein concentrations were quantified to ensure equal protein levels were present in each sample.

SAM (S-adenosyl-methionine) Quantification (Collaboration with Kavon Rezai-zadeh)

The same cortical homogenates used for γ -secretase activity were also used for SAM quantification. SAM was quantified in these samples using a Bridge-It S-Adenosyl-Methionine (SAM) Fluorescence Assay provided by Mediomics, LLC. Following the manufacturer's protocol, 10 μ l of each sample were added to a polypropylene Eppendorf tube. 90 μ l of SAM assay solution was added to each sample and vortexed for ~1 second. Then, 90 μ l of the resultant mixture from each sample was loaded in a black microplate, covered and incubated in the dark for 30 minutes. The fluorescent intensity was then measured using a fluorescent microplate reader (settings: excitation ~485 nm; emission ~665 nm). By using the standard curve generated by the fluorescence of known SAM levels, the concentration of SAM in unknown samples was calculated by its fluorescent intensity. Protein concentrations were quantified to ensure equal protein levels were present in each sample.

Determination of Soluble/Insoluble A β Levels

Tissue Homogenizing/Extraction. To determine the levels of both soluble/insoluble A β (1-40) and A β (1-42), the hippocampi from mice in each group (6 Tg+caff, 6 Tg+, 1 NT from Study A and 3 Tg+caff, 3 Tg+, 1 NT from Study B) were homogenized in tissue homogenization buffer (THB) (250mM sucrose, 20mM tris-HCl, 1mM EDTA, 1 mM EGTA, and protease inhibitor). 0.4% diethylamine (DEA; in 100 mM NaCl) solution was added to each homogenate and each sample was then centrifuged at 100,000 x g for one hour. The supernatant from each sample was drawn off and stored at -80°C for later quantification of soluble A β levels. Formic acid (FA) was then added to the remaining pellet and samples were sonicated for one minute. Following sonification, samples were spun down at 100,000 x g for one hour. The intermediate phase was drawn off, and added to a FA neutralization solution. These FA-extracted solutions were designated for quantifying insoluble A β levels and stored at -80°C.

A β (1-40) and (1-42) ELISA. Sandwich ELISA kits for both A β (1-40) and (1-42) were utilized (Signet laboratories). All extracts were thawed and appropriately diluted to ensure results within the standard curve for each kit. Plates were coated with capture antibodies specific to the amino-terminus for either the A β peptide (1-40) or (1-42), depending on which kit was being used. Before loading the samples, each plate was washed three times in a wash diluent. The standard curve (consisting of samples with known amounts of A β peptide) and each brain sample extract were run in duplicate and averaged to obtain the most accurate results. After loading the samples, the plate was incubated at 4°C overnight and was washed three times the next day to remove unbound peptide. A primary antibody was then added that binds to the carboxy-terminus of the

peptide and allowed to incubate for two hours. After washing, a secondary antibody conjugated to horseradish peroxidase was added to bind to the primary antibody and allowed to incubate for another two hours. The plate was again washed and an O-phenylenediamine (OPD)-substrate was added to each well that visualizes the bound A β peptide by reacting with horseradish peroxidase. This reaction occurred for 45 minutes, at which point a stop solution was added and the plate was read at an absorbance of 490 nm. The optical density (OD) for each sample was measured and compared to the OD of the standard curve, allowing for the quantification of bound A β (either 1-40 or 1-42 depending on which kit was used) in each well.

Statistical Analysis

Behavior. Standard one-way ANOVAs were performed using *Statistica* software to determine inter-group behavioral comparisons between APP mice, caffeine-treated APP mice, and NT mice in the open-field, balance beam, Y-maze, elevated plus-maze, Morris water maze acquisition and probe trial, circular platform, platform recognition, and radial arm water maze (RAWM) tasks. *Post-hoc* pair-by-pair differences between groups (planned comparisons) were later determined by the Fisher LSD test. Two-way repeated measure ANOVAs were also performed to elucidate any behavioral differences in groups in the multi-day tasks across days (platform recognition) and blocks (3-day blocks for RAWM or 2-day blocks for Morris water maze acquisition). Mann-Whitney U-tests for non-parametric data were performed to compare performance of mice in the string agility task. RAWM swim speed was calculated by averaging overall T4+T5 latency and dividing by the average of overall T4+T5 errors. Any animals that were

consistently unable to complete any task (e.g., floaters, circlers) were eliminated from statistical analysis.

Pathology. Group comparisons for insoluble/soluble A β , adenosine receptor measurements, secretase activity, SAM levels, and brain extracellular adenosine levels were done by standard one-way ANOVAs. After ANOVA analysis, *post-hoc* pair-by-pair differences between groups were analyzed with the Fisher LSD test. Correlation analysis was also performed using Systat software to elucidate any potential relationships between the behavioral, A β pathology, and brain adenosine measures.

Factor/Discriminant Factor Analysis. Factor analysis (FA) was performed using Systat software to group all behavioral measures into common factors. This allows for the relationship between individual behavioral measures to be determined, and also might indicate potential performance in a task based on previous performance in another task. This FA was performed using all 19 behavioral measures from all groups (NT, APP_{sw}, and APP_{sw}+Caffeine) in this study.

To investigate if a relationship exists between behavioral and A β pathological measures, correlation analysis was performed using Systat software. Correlation analyses were performed between all 19 behavioral measures and all four A β measures. Correlation analyses were also performed between all 19 behavioral measures to determine if any inter-task relationships existed. Finally, correlation analysis was also performed to determine the relationship between extracellular brain adenosine levels and the four A β measures.

Additionally, discriminant factor analysis (DFA) was run using Systat software with two different DFA methods (direct entry and stepwise-forward) using all 19

behavioral measures and also only the 8 cognitive-based measures that loaded from Factor 1. These DFA's determined if the three groups of mice were behaviorally distinct from one another. The direct entry method used all 19 behavioral measures, or the 8 measures from Factor 1 of FA, while the stepwise-forward method started with 19 or 8 measures, respectively, but selected from these measures based on their variance to best differentiate between the three groups.

VI. Results

Weight Analysis. Throughout the study, mice in all three groups were weighed every two weeks to ensure no significant differences occurred from the four month caffeine treatment. Before the start of the caffeine treatment, there were no group differences in weight between the transgenic and non-transgenic mice. Additionally, there were no group differences between the NT, Tg, and Tg+Caff mice at the end of behavioral testing when the animals were euthanized. Repeated measures analysis revealed no group by time interaction [$F(13,260)=0.66$; $p=0.89$], indicating NT, Tg, and Tg+Caff mice all gained weight at a similar rate during the entire four month period of treatment.

Behavior- Sensorimotor Evaluation

Open Field and Y-Maze Entries. Tg mice exhibited increased open field activity (Fig. 6a) when compared to NT controls ($p<0.05$), while activity of Tg+Caff mice was not differ from NT mice at 8 months of age. Furthermore, both Tg and Tg+Caff mice

made significantly more total arm choices in the Y-maze task (Fig. 8a), another measure for activity/exploration, when compared to NT mice ($p < 0.02$ for both groups).

Balance Beam. In balance beam testing, there were no group differences between the mice (Fig. 6b), indicating that all mice had similar balance ability and intact general motor function.

String Agility. In the string agility test (Fig. 6c), the Tg+Caff mice were less agile than the Tg and NT mice ($p < 0.05$), although this difference was simply because less Tg+Caff mice escaped the string (score of 5) than the other two groups. As Tg+Caff mice averaged a ranking of 4 in this task, they managed to cling to the string with all limbs for the full 60 seconds and did not fall. Therefore, the difference does not imply a motor impairment in Tg+Caff mice. The treatment-related impairment was task specific and, moreover, did not influence performance in the cognitive-based tasks (see correlation analysis).

Elevated Plus-Maze Anxiety. There were no group differences in open and closed arm entries (Fig. 7b and c). Tg mice, but not Tg+Caff mice, spent more time in open arms when compared to NT mice ($p < 0.05$; Fig. 7a).

Behavior- Cognitive Evaluation

Y-Maze Alternation. In Y-maze testing for spontaneous alternation behavior, there were no group differences in percent alternation (Fig. 8b). Tg mice were unimpaired in this task, thus any protective effect from the long-term administration of caffeine was not possible.

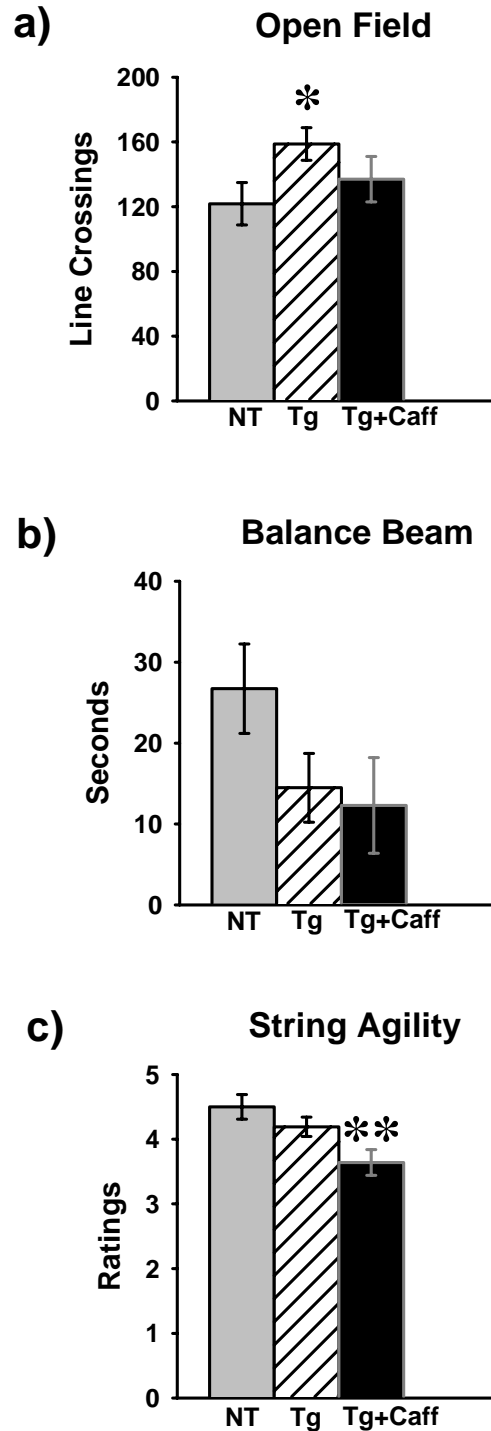


Figure 6. Open field, balance beam, and string agility performance. Tg mice showed increased activity in the open field task. Tg+Caff mice had decreased performance in the string agility task, but did not have any general motor impairment. * = significantly different from NT group at $p < 0.05$, ** = significantly different from both other groups at $p < 0.05$.

Elevated Plus Maze

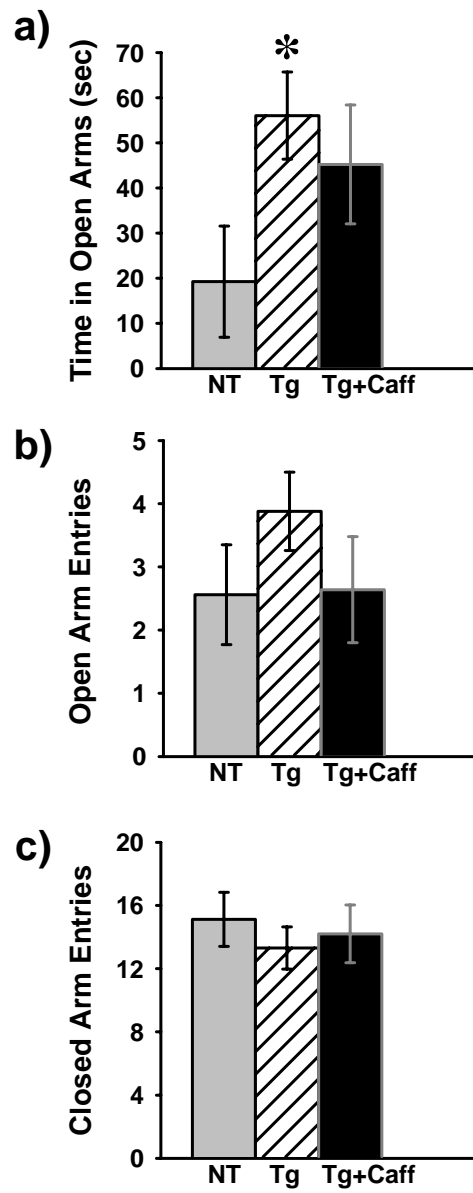


Figure 7. Elevated plus-maze performance. Tg mice spent significantly more time in the open arms than NT mice, while Tg+Caff mice were no different than NT mice. * = significantly different from NT group at $p < 0.05$.

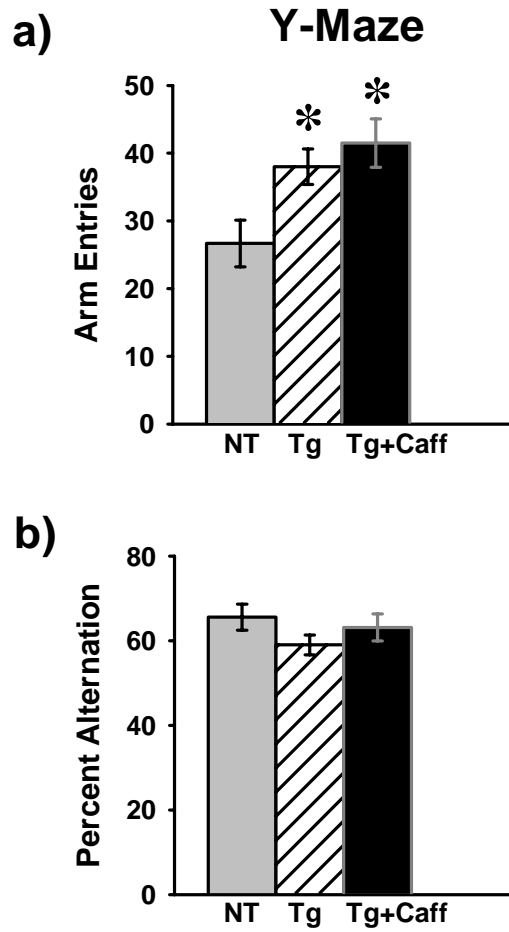


Figure 8. Y-maze performance. Both Tg and Tg+Caff mice had significantly more arm entries than NT mice. * = significantly different from NT group at $p < 0.02$.

Morris water maze acquisition. Escape latency data from this task were divided into five 2-day blocks. There was no overall group effect across all five blocks [F(2,50)=1.69; p=0.19] (Fig. 9). However, a strong group by block interaction was evident [F(8,200)=3.51; p=0.0008], which was clearly due to the inability of Tg mice to improve acquisitional performance after the second block of testing. *Post hoc* analysis of individual blocks revealed NT and Tg+Caff mice showed a strong learning effect in the last two blocks acquisition, resulting in significantly faster escape latencies when compared to Tg mice (Fig. 9). Not surprisingly, Tg escape latencies on the last day of testing were also significantly higher than NT and Tg+Caff escape latencies (p<0.5 and p<0.02, respectively). Thus, the long-term administration of caffeine protected against Morris maze acquisitional impairments that would otherwise be present in APP_{sw} mice at this age.

Results from the Morris maze probe trial are consistent with the protective effect of caffeine treatment in APP_{sw} mice during acquisition (Fig. 10). Tg mice showed no quadrant preference during this spatial memory retention phase of testing. By contrast, Tg+Caff mice showed an exclusive preference for the quadrant formerly containing the submerged platform (Q2) and spent significantly more time in this quadrant than Tg mice (p<0.025). NT mice showed only a partial preference for Q2, although their percent time spent in Q2 was not statistically different from that of Tg+Caff mice. Tg+Caff mice also made significantly more annulus crossings than Tg mice (p<0.005; Fig. 10), giving further support for a protective effect of caffeine treatment in spatial memory retention.

Morris Maze Acquisition

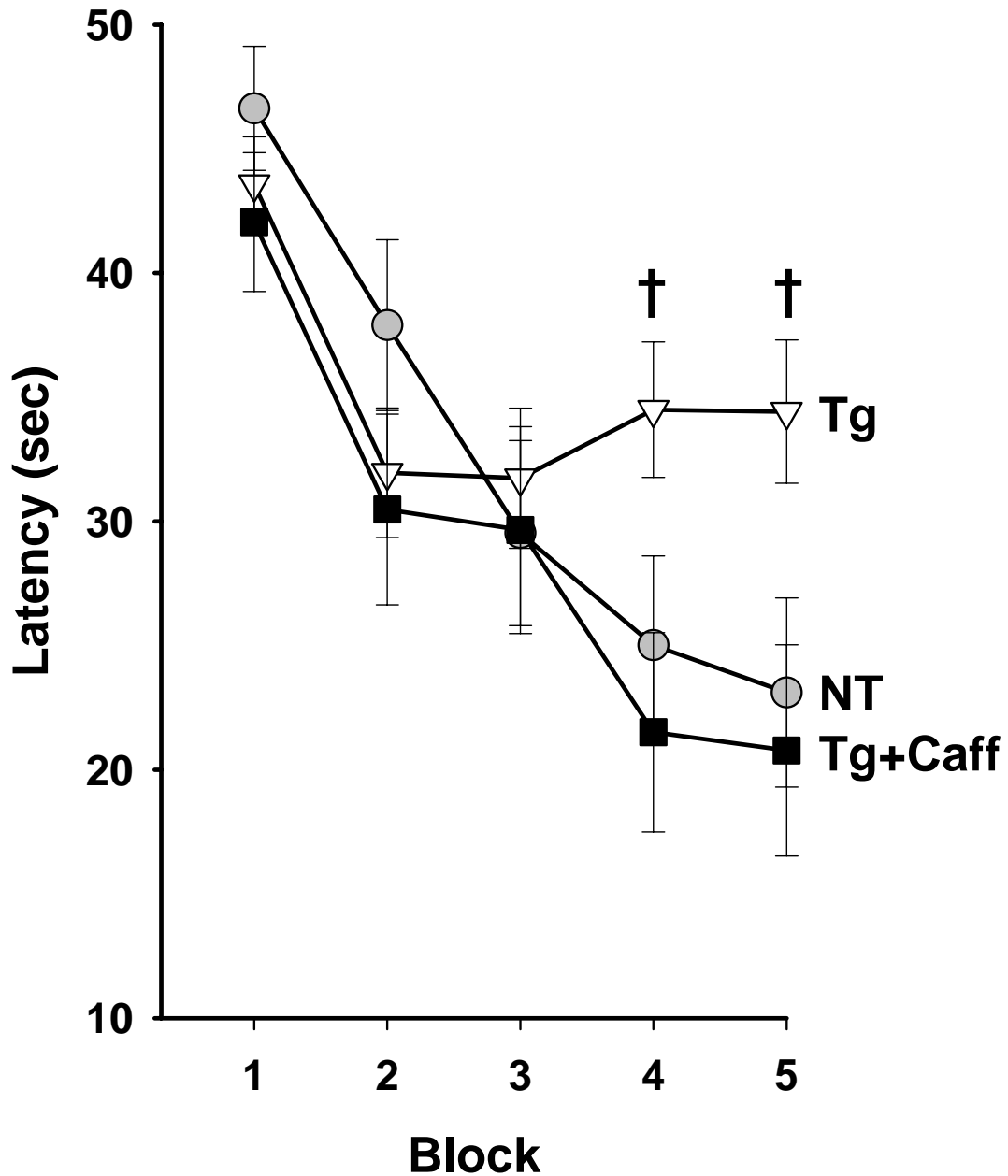


Figure 9. Morris water maze acquisition latencies. The 10 days of acquisition, as measured by latency to find a submerged platform, are presented in five 2-day blocks. Tg mice were significantly impaired in spatial reference learning in blocks 4 and 5, while Tg+Caff mice performed no different than NT mice. † = Tg mice had significantly higher escape latencies than both other groups at $p < 0.05$ or higher level of significance.

Morris Maze Retention

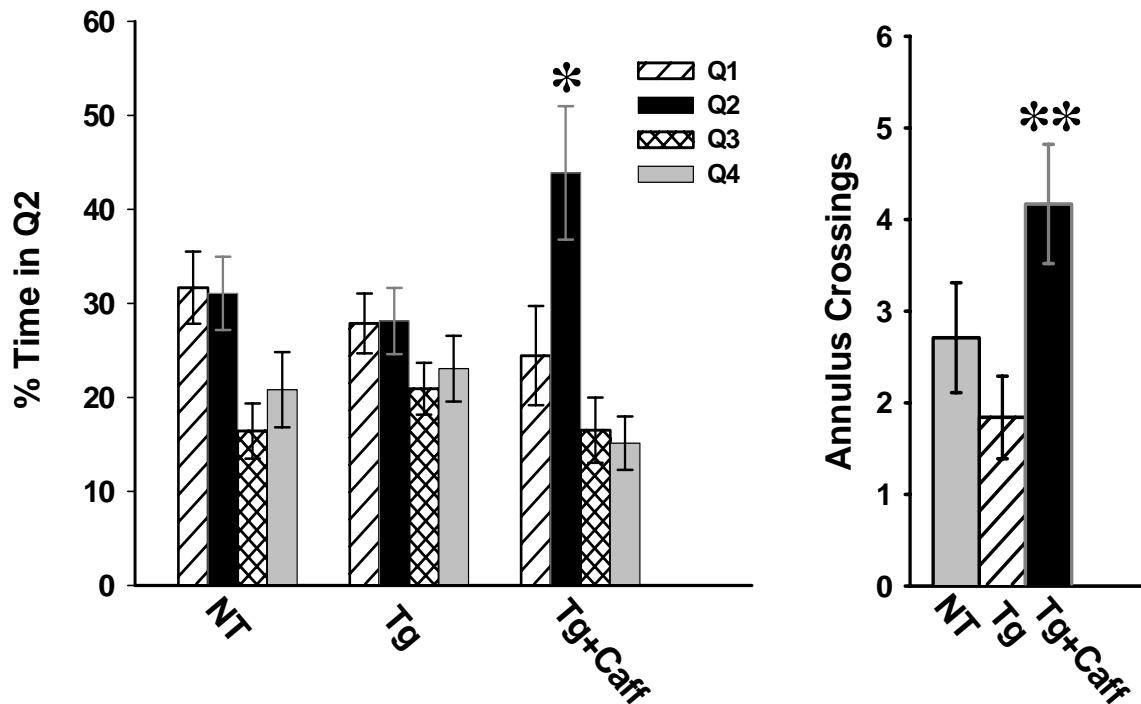


Figure 10. Probe trial testing for reference memory retention in the Morris water maze. Tg+Caff mice showed an exclusive preference for the quadrant formerly containing the submerged platform (Q2). This preference is also indicated by the significantly higher number of annulus crossings made by Tg+Caff mice when compared to Tg mice. * = significantly greater than all other quadrants at $p < 0.01$ and significantly greater than Q2 for Tg group at $p < 0.025$. ** = significantly different from Tg mice at $P < 0.005$.

Circular Platform. In this task of reference learning/memory, there was an overall groups effect over all eight days of testing [$F(2,51)=7.66$; $P=0.001$]. *Post hoc* analysis indicated that Tg mice had significantly higher escape latencies overall compared with NT and Tg+Caff mice ($p<0.005$; Fig. 11). Additionally, Tg escape latencies were significantly higher than NT and Tg+Caff escape latencies on the last day of testing ($P<0.025$ and $P<0.001$, respectively). Very similar group differences were observed when the number of errors was analyzed (data not shown). For all animals, there was an overall training (days) effect [$F(7,357)=37.33$; $p<0.00001$], indicating that all animals collectively improved their performance across days despite the impaired performance of Tg mice. Thus, the otherwise certain impairment of APP_{sw} mice in the circular platform task, evident in both their escape latencies and error making, was prevented by the long-term administration of caffeine.

Circular Platform

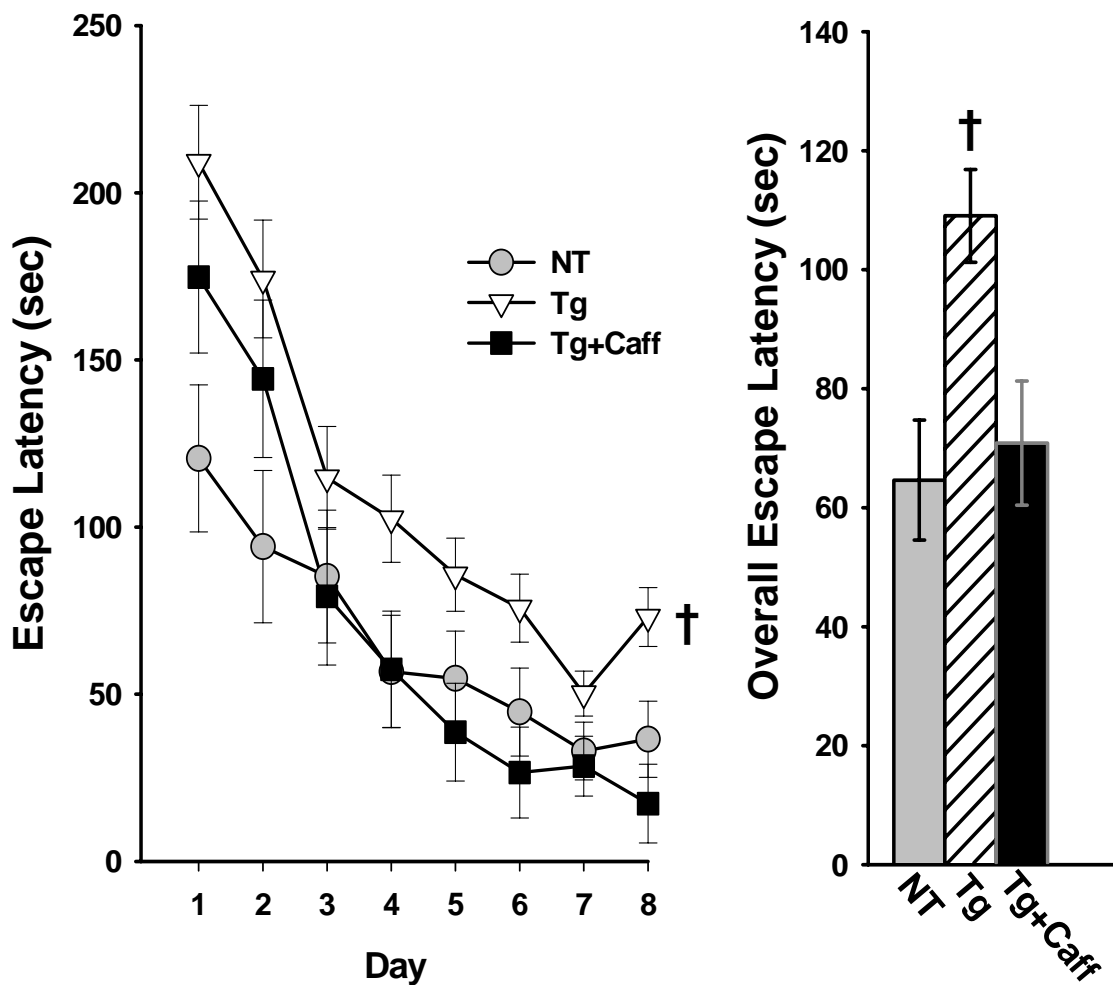


Figure 11. Circular platform performance. Tg mice were significantly slower in locating the escape hole over the eight days of circular platform testing, while Tg+Caff mice performed similarly to NT mice. † = Tg mice significantly higher escape latencies than both other groups at $p < 0.01$ or higher level of significance.

Platform Recognition. In platform recognition testing, an overall groups effect was present [$F(2,49)=7.44$; $p<0.002$], with *post hoc* analysis revealing that Tg mice had significantly higher escape latencies than NT mice ($P<0.0005$; Fig. 12). By contrast, Tg+Caff mice performed at the same level as NT mice and nearly had lower overall escape latencies compared to Tg mice ($p=0.065$). The benefits of caffeine administration were evident even on the second day of testing and were profound by the last two days of testing (Fig. 12) in that Tg+Caff mice performed identically to NT mice and significantly better than Tg mice on both of those final days. A strong group by day interaction was present [$F(6,147)=4.14$; $p<0.001$] due to the poor performance of the Tg group relative to the other two groups. The apparent difficulties that Tg mice have in switching between the spatial (cued) strategy used in Morris water maze to a search/recognition strategy in the platform recognition task are reflected in their high escape latencies. In sharp contrast, the long-term administration of caffeine protected against this “strategy switching” impairment.

RAWM. For RAWM statistical analysis, data was evaluated across three 3-day blocks for T1 (semi-randomized initial trial), T4 (final acquisition trial), and T5 (delayed retention trial). The beneficial effects of caffeine on working memory were immediately evident in block 1 of testing (Fig. 13). Tg+Caff mice were similar in performance to NT controls and already better than Tg mice ($p<0.05$) during T4, while Tg mice were impaired during both T4 ($p<0.0001$) and T5 ($p<0.00001$). In blocks 2 and 3 of testing, Tg mice had significantly higher escape latencies during working memory trials T4 and

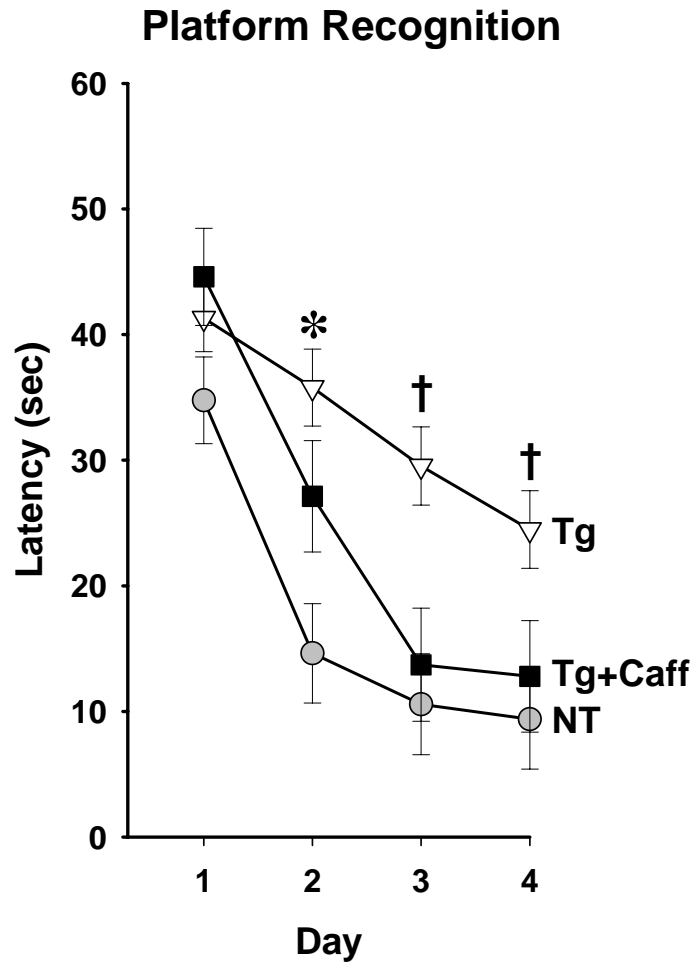


Figure 12. Platform recognition performance over four days of testing. By the third and fourth day of testing, performance of Tg+Caff mice was identical to NT mice and much better than Tg controls. * = Tg and Tg+Caff mice significantly worse than NT mice at $p < 0.05$, † = Tg mice significantly worse than both NT and Tg+Caff mice at $P < 0.05$ or higher level of significance.

RAWM

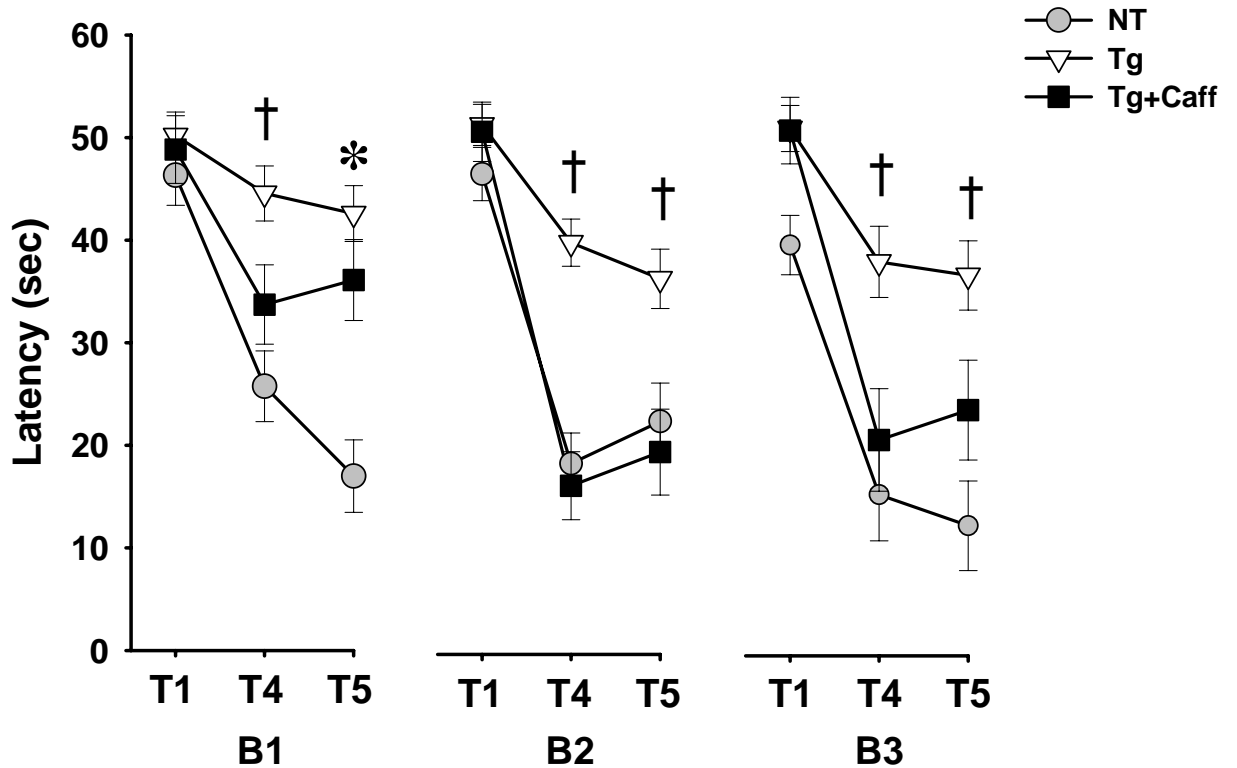


Figure 13. RAWM performance over three 3-day blocks. During the first block of testing, Tg mice had significantly slower escape latencies versus NT mice during T4 and T5 (the trials indicative of working memory) while Tg+Caff mice were not different from NT during T4. In the final two blocks of RAWM, Tg performance was significantly higher in T4 and T5 than both NT and Tg+Caff mice, the later two groups being near identical in performance. † = Tg mice significantly worse than both NT and Tg+Caff mice at $p < 0.05$ or higher level of significance. * = Tg and Tg+Caff mice significantly different from NT mice at $P < 0.0001$,

T5 compared to NT and Tg+Caff mice ($P < 0.05$ or higher levels of significance). Tg+Caff mice, by contrast, were identical in working memory performance in comparison to NT mice. A strong group by block interaction was present for T5 [$F(4,98) = 5.66$; $p < 0.0005$], which was clearly due to the much poorer T5 performance of Tg mice compared to the other two groups. The consistent impairment of untreated APP_{sw} mice in T4 and T5 of RAWM testing indicate impaired working memory in these mice, whereas the long-term administration of caffeine to APP_{sw} mice offers protection against working memory impairment.

The complete protection of working memory provided by caffeine is also manifest in overall performance across all three blocks (Fig. 14). In overall T1 performance across all three blocks, Tg mice had significantly slower escape latencies in comparison to NT mice ($P < 0.025$) while Tg+Caff mice were nearly different from NT mice ($p = 0.051$). Escape latencies in overall T4 and T5 performance showed that Tg mice were substantially impaired when compared to NT mice ($p < 0.00001$), while Tg+Caff mice were not different from NT mice and significantly better than Tg mice ($p < 0.005$). Additional support for impaired RAWM working memory in Tg mice was evident in by their increased number of overall T4 and T5 errors made in comparison to NT controls ($p < 0.0001$ for both; data not shown). This impairment was also prevented by the long-term administration of caffeine, as Tg+Caff mice performed similarly to NT mice in making significantly lower numbers of overall T4 and T5 errors compared to Tg mice ($p < 0.0001$ and $p < 0.005$, respectively). There were no group differences in swim speed present in RAWM testing, indicated by the number of seconds taken per arm choice (latency/error ratio) for T4 and T5 combined.

Overall RAWM

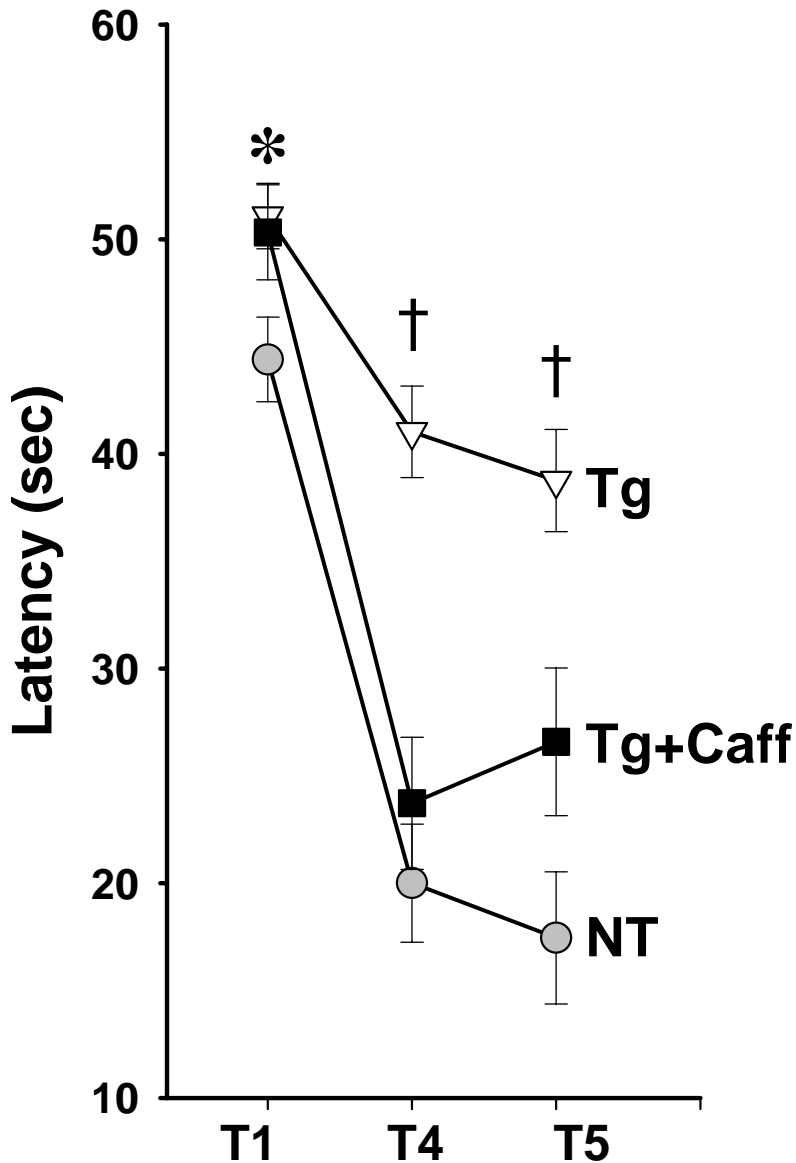


Figure 14. Overall RAWM performance. In working memory trials T4 and T5, Tg+Caff mice performed identical to NT controls and were significantly better than Tg control mice, indicating that the long-term administration of caffeine granted complete protection against working memory impairment in RAWM testing. * = Tg mice significantly higher latency than NT mice at $P < 0.025$, † = Tg mice significantly worse than both NT and Tg+Caff mice at $P < 0.005$ or higher level of significance.

Multi-metric Statistical Analysis

Factor Analysis. Factor analysis of behavioral measures was performed to determine the underlying relationships between all of these measures (Table 3). FA involving all 19 behavioral measures resulted in 13 of those measures loading on four principal factors, which accounted for 61.2% of the total variance (a measure was considered “significant” for loading on a factor if its component loading value exceeded 0.600 for that factor). All measures for RAWM, Morris maze acquisition, and platform recognition loaded heavily in factor 1, thus this factor was considered the primary cognition-based factor. The measures for activity and/or having an activity component (open field, Y-maze entries, and circular platform errors) loaded into factor 2, while circular platform latency loaded separately into factor 4 and balance beam loaded separately into factor 5. No tasks loaded into factor 3.

Correlation Analysis. Correlation analyses were performed using all 19 behavioral measures and including all mice (Tg and NT). There were no correlations between the string agility task and any of the cognitive tasks. Additionally, correlation analysis revealed that the amount of time mice spent in the open arm of the elevated plus maze correlated with an increased latency in multiple tasks (Morris maze, circular platform, platform recognition, and RAWM). This may indicate that mice with decreased anxiety are less motivated to escape certain tasks. As expected, there were numerous inter-task correlations between measures in the three water-based. For measures taken from the same task, strong intra-task correlations were also evident, particularly for RAWM and Morris maze tasks.

Table 3. Factor loadings of behavioral measures

Factor	All 19 behavioral measures
1	(32.61) RM-T4-Fin RM-T4 RM-T5-Fin RM-T5 PR-Fin PR-Avg WM-Fin WM-Avg
2	(13.97) OF CP-Err YM-Ent
3	(10.10) N/A
4	(8.247) CP-Lat
5	(6.37) BB

Numbers in bold type indicate percent of total variance explained by a given factor. Abbreviations: **BB**, balance beam; **CP-Err**, circular platform overall errors; **CP-Lat**, circular platform overall latency; **OF**, open field lines crossed; **PR-Avg**, Platform recognition overall average; **PR-Fin**, Platform recognition final day average; **RM-T4**, radial arm water maze latency overall T4; **RM-T5**, radial arm water maze latency overall T5; **RM-T4-Fin**, radial arm water maze latency last block T4; **RM-T5-Fin**, radial arm water maze latency last block T5; **WM-Avg**, water maze latency overall average; **WM-Fin**, water maze latency last day; **YM-Ent**, Y-maze entries.

Discriminant Function Analysis. DFA was utilized to determine if multiple behavioral measures could distinguish the three groups (NT, Tg, Tg+Caff) from one another (Table 4). The “direct entry” DFA method (which includes all behavioral measures) and the “stepwise forward” DFA method (which selects behavioral measures from the total number evaluated based on their contribution to the variance) were used in this analysis. Direct entry DFA, using all 19 behavioral measures, was very effective in completely discriminating between all three groups (rank order of performance was NT > Tg+Caff > Tg). Additionally, the stepwise forward DFA (using all 19 behavioral measures) was also very effective in completely discriminating between all three groups ($P < 0.0001$). For the stepwise forward DFA, five behavioral measures were retained as providing maximal discriminability: three cognitive-based measures (RAWM overall T4 latency, Morris maze retention, and circular platform overall latency) and two sensorimotor measures (Y-maze entries and elevated platform time in open arms).

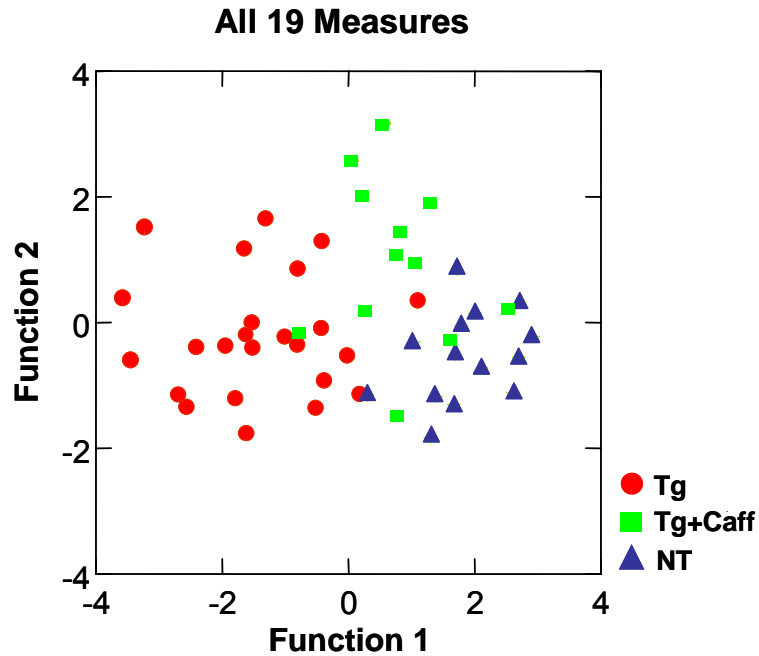
Additional DFAs were performed using only the eight cognitive-based measures that loaded on factor 1 in FA (see Table 3). Both direct entry and stepwise forward DFAs (using these eight cognitive-based measures) were able to completely discriminate between all three groups. Six measures (three from RAWM, two from platform recognition, and one from Morris maze) providing the maximal discrimination between NT, Tg, and Tg+Caff groups. Canonical plots of both the 19 and 8 measure stepwise forward DFAs are shown in Fig. 15. DFA’s involving all 19 behavioral measures was slightly better at correctly classifying individual animals into their treatment/genotypic group (84-90% correct) compared to DFA’s involving the 8 cognitive measures in factor 1 (75-79%).

Table 4. Summary of discriminant function analyses^a

Measures	Direct Entry Method		Stepwise-forward method		Measures Retained
	Significance	% Correct	Significance	% Correct	
All 19	P<0.0001	90%	P<0.0001	84%	RM-T4 WM-Ret CP-Lat YM-Ent EP-TO
Factor 1 (Eight cognitive measures)	P<0.0001	79%	P<0.0001	75%	RM-T4 RM-T5 RM-T4-Fin WM-Avg PR-Fin PR-Avg

^a P-values are from Wilks's λ . Post hoc analysis revealed complete discrimination between groups.

Canonical Scores Plots



8 Cognitive Measures from Factor 1

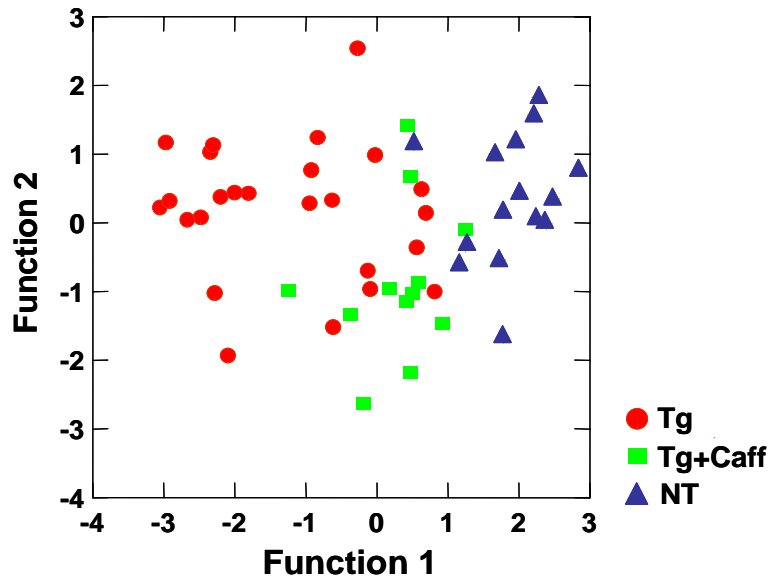


Fig. 15. Canonical score plots of stepwise-forward DFAs used to compare the “overall” cognitive performance of the three mouse groups. Each symbol represents the cognitive performance of one animal graphed from the two linear functions derived in the DFA. In both DFAs, all three groups could be completely distinguished from one another.

Neuropathologic/Neurochemical Measures: Study A

Adenosine Receptor Measures. Adenosine A₁ and A_{2A} receptor densities from striatum, hippocampus, and frontal cortex from mice in Study A were analyzed using western blotting (Fig. 16). No group differences in adenosine A₁ receptors were seen in striatum or hippocampus, but Tg and Tg+Caff mice both had significantly higher levels of A₁ receptors in the frontal cortex compared to NT mice ($p < 0.05$). Furthermore, no group differences were seen in adenosine A_{2A} receptors in striatum or frontal cortex, but Tg mice had significantly higher levels of A_{2A} receptors in the hippocampus versus NT mice ($p < 0.05$).

The mRNA levels for A₁ and A_{2A} adenosine receptors were also analyzed in the corresponding areas using RT-PCR. This evaluation revealed no significant group differences in A₁ receptor mRNA expression in striatum, hippocampus, or frontal cortex. A_{2A} receptor mRNA expression was below detectable levels in frontal cortex and hippocampus, while in the striatum no group differences were qualitatively found in A_{2A} receptor expression.

A β (1-40) and (1-42) Analysis. Shortly following completion of behavioral testing in Study A at 9 months of age (and approximately 4 months into long-term caffeine administration), A β analysis by ELISA was performed on hippocampus tissues (Fig. 17). Long-term administration of caffeine resulted in significant reductions of both soluble A β ₁₋₄₀ ($\downarrow 37\%$) and insoluble A β ₁₋₄₂ ($\downarrow 32\%$). From this data, it is clear that the long-term administration of caffeine has an impact on brain A β production and/or clearance.

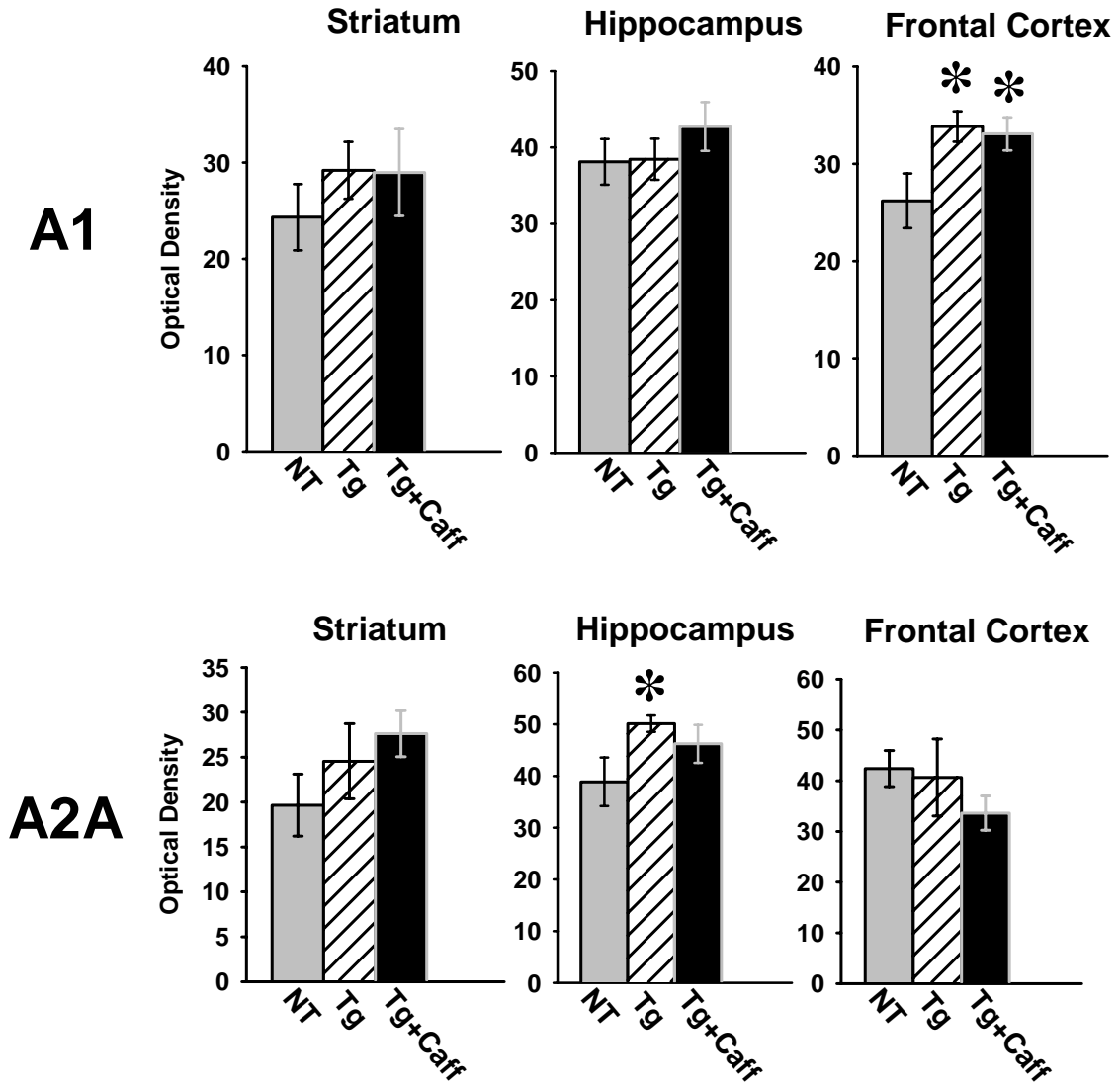


Figure 16. Adenosine A1 and A2A receptor densities in the striatum, hippocampus, and frontal cortex (Study A). The long-term administration of caffeine had no effect on A1 receptors in any areas of the brain examined, although a transgenic effect of increased A1 receptor density in the frontal cortex was observed. A further transgenic effect was seen with A2A receptor densities increased in hippocampus of Tg mice, with caffeine administration reducing this effect below significance. * = significantly different from NT at $p < 0.05$.

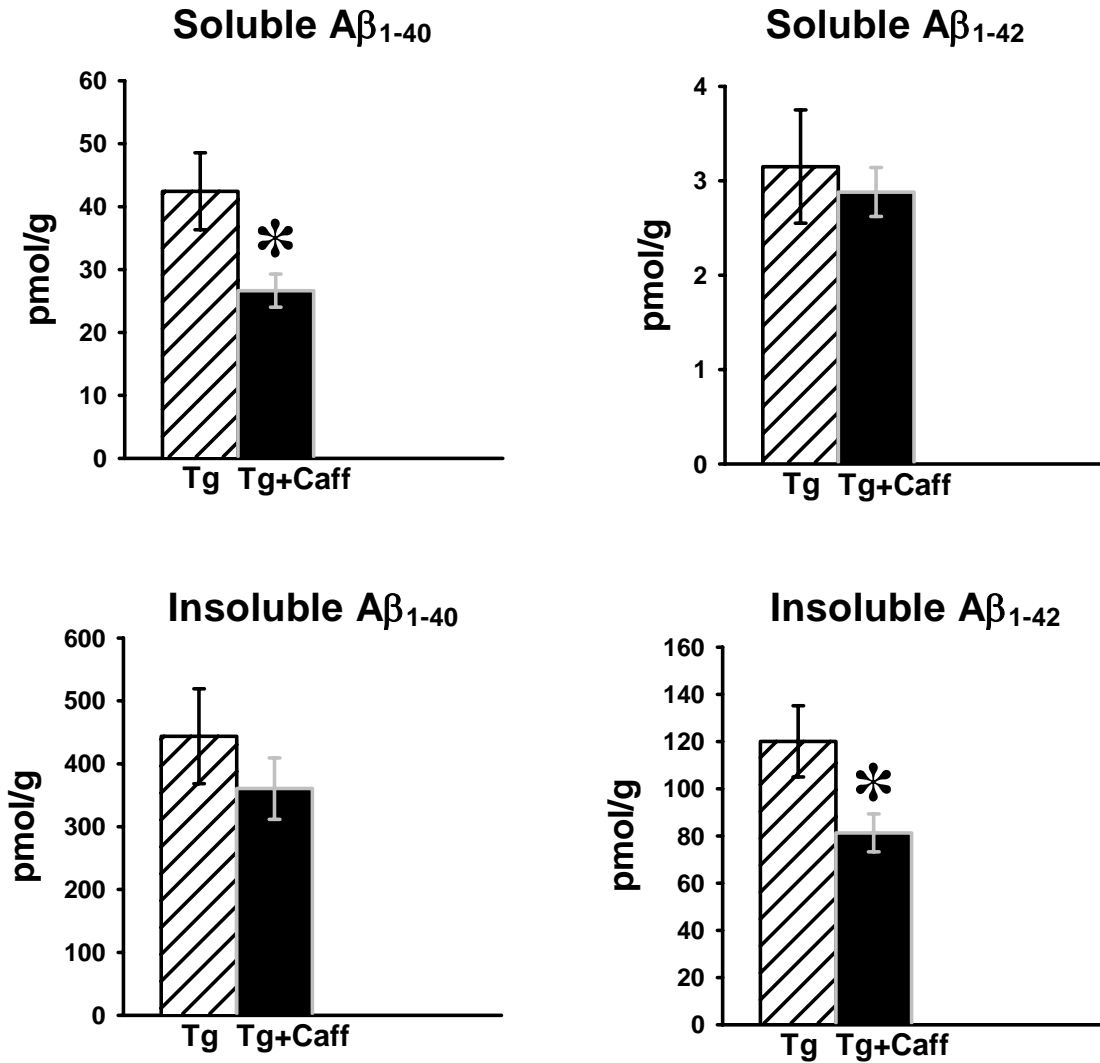


Figure 17. Quantification of soluble/insoluble Aβ₁₋₄₀ and Aβ₁₋₄₂ in hippocampus of behaviorally tested APP_{sw} mice (Study A). Caffeine-treated mice had significantly reduced soluble Aβ₁₋₄₀ and insoluble Aβ₁₋₄₂ levels when compared to untreated Tg mice. * Tg+Caff mice significantly lower versus Tg mice at p<0.05.

Comparison of β - and α - CTFs. To elucidate any changes in α - and/or β -secretase activity in combined frontal and posterior cortex tissue from behaviorally-tested mice of Study A, β -, and α -CTFs (carboxyl-terminal fragments) were analyzed by western blotting (Fig. 18). Qualitative assessment of the resultant gel revealed the same six Tg+Caff mice tested in the A β ELISA all had twice the β -CTF blot intensity of their α -CTF blot intensity (2 to 1 ratio), whereas the same six Tg control mice from the A β ELISA had more mixed blot intensities that leaned towards an even ratio between the two CTFs (1.33 to 1 ratio). Using a non-parametric Mann-Whitney test showed the difference between these group in CTF ratio was statistically significant ($p < 0.025$). This data implies that caffeine has an impact on the amyloidogenic processing of APP. In NT mice, cortical levels of β - CTF's were below the limits of detection.

Neuropathologic/Neurochemical Measures: StudyB

A β (1-40) and (1-42) Analysis. An 18-day administration of caffeine to aged 17 month old APP_{sw} mice (Study B) resulted in a significant reduction of insoluble A β ₁₋₄₂ (\downarrow 30%) in the hippocampus (Fig. 19).

β -CTF to α -CTF Ratio

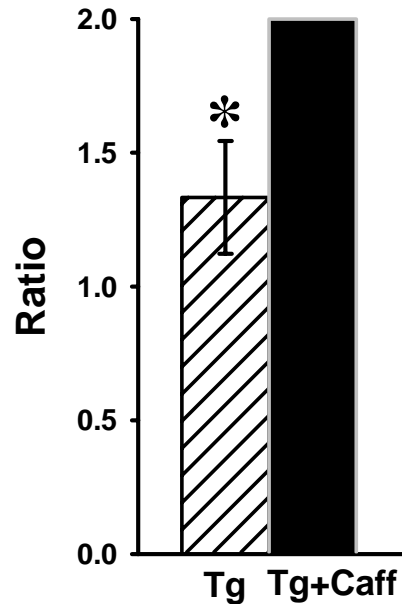


Figure 18. Comparison of β -CTF to α -CTFs ratio in APP_{sw} mice following long-term caffeine administration (Study A). Caffeine-treated mice had a significantly greater ratio compared to untreated Tg mice, consistent with a shift in amyloidogenic processing. * Tg+Caff mice significantly higher versus Tg mice at $p < 0.025$.

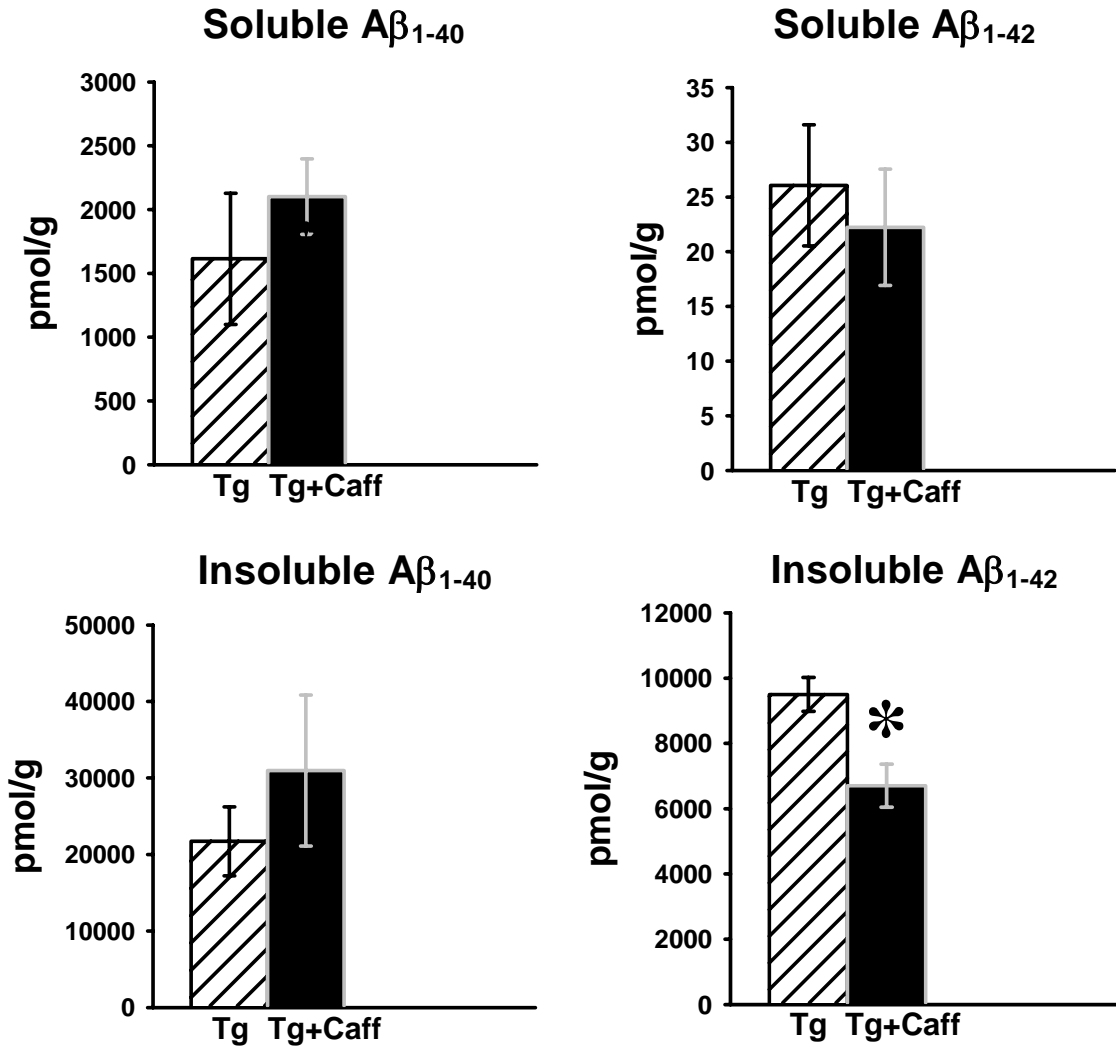


Figure 19. Quantification of soluble/insoluble Aβ₁₋₄₀ and ₁₋₄₂ in hippocampus of aged APPsw mice (Study B). Caffeine-treated mice had significantly reduced insoluble Aβ₁₋₄₂ levels compared to untreated Tg mice. * Tg+Caff mice significantly lower versus Tg mice at p<0.05.

Determination of γ -secretase Activity. To more fully determine if a reduction in γ -secretase activity is involved in the reduction of amyloidogenic processing after caffeine administration, combined frontal and posterior cortex tissue from the mice in Study B were used to determine enzymatic activity of the γ -secretase class of proteases using a fluorometric reaction. There were no significant group differences between Tg+Caff and Tg mice (Fig. 20). However, γ -secretase activity in Tg+Caff mice was comparable to NT mice, with both of these groups having slightly reduced γ -secretase activity (\downarrow 10%) when compared to untreated Tg mice. It is possible that endogenous mouse secretase activity, unaffected by caffeine treatment, may be present and masking any actual reductions in harmful γ -secretase activity that may have been provided by caffeine treatment. Regardless, even slight reductions in γ -secretase activity might have beneficial effects on amyloidogenic processing, as evident by caffeine's ability to reduce hippocampal A β levels in these same animals.

SAM Quantification. To determine if long-term administration of caffeine has an impact on SAM (*S*-adenosyl-methionine) levels, combined frontal and posterior cortex tissue from the mice in Study B were used in a SAM fluorescence assay (*Mediomics, LLC*). There was no significant group difference between the Tg+Caff and Tg mice (Fig. 21), yet SAM concentrations were 49% higher in the Tg+Caff mice (7.89 μ M) than the Tg mice (5.30 μ M). These results suggest that this analysis should be repeated, but next time using a greater number of samples and a more precise technique in the micromolar ranger.

Gamma Secretase Activity

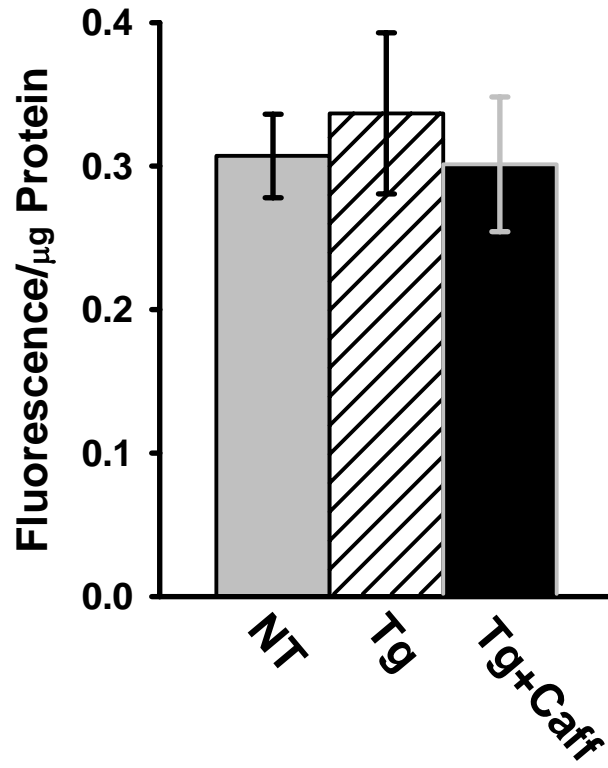


Figure 20. Gamma- Secretase activity in cerebral cortex of APP_{sw} mice following caffeine administration (Study B). Caffeine treatment did not significantly affect γ -secretase activity in our assay. Although it is possible that endogenous mouse secretase activity, unaffected by caffeine treatment, may be present and masking any actual reductions in harmful γ -secretase activity.

SAM Concentration

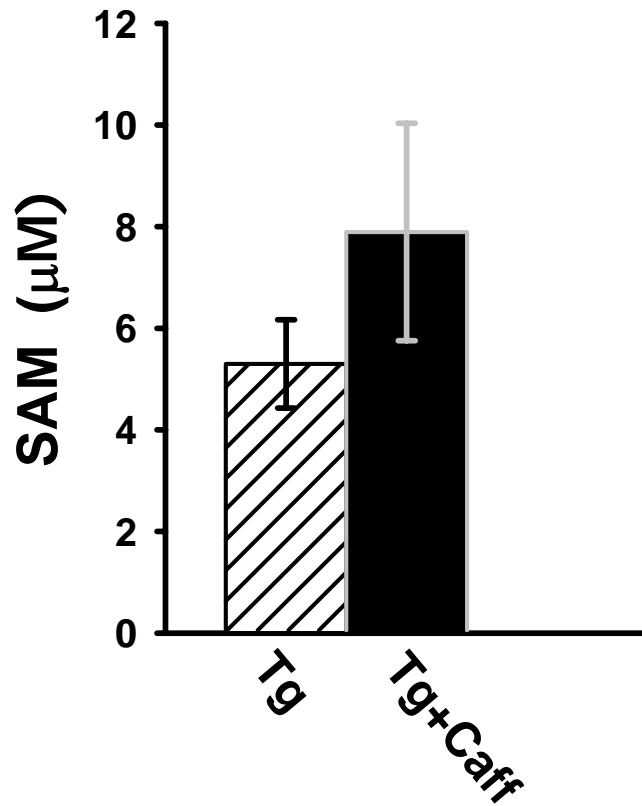


Figure 21. SAM (*S*-adenosyl-methionine) levels in cerebral cortex of APP_{sw} mice following caffeine administration (Study B). There were no significant group differences in SAM concentrations following the caffeine treatment.

Extracellular Adenosine Analysis. The left brain hemispheres from the aged APP_{sw} mice used in Study B were analyzed for adenosine levels by mass spectrometry utilizing an ion-trap Finnegan LC-MS (Fig. 22). Interestingly, Tg mice had significantly reduced extracellular brain adenosine levels (↓27%) compared to age-matched NT (p<0.005). By contrast, Tg+Caff mice had extracellular brain adenosine levels that were similar to NT mice and significantly higher than Tg mice (p<0.05 for Tg vs. Tg+Caff comparison). These results suggest Tg mice have pathologically lowered extracellular adenosine levels and caffeine administration restores those levels to normal.

Correlation Analyses involving neurochemical/neuropathologic measures.

Correlation analyses were performed between all 19 behavioral measures and the four measures of A β (e.g., soluble and insoluble A β ₁₋₄₀, A β ₁₋₄₂) for all Tg mice. Other than significant correlations between circular platform impairment and both soluble and insoluble A β ₁₋₄₂, no significant correlations were found between the various forms of quantified A β and any of the other behavioral tasks. As expected from the data presented in Figures 16 and 20 involving aged Tg mice, a correlation was found between elevated adenosine levels and decreased levels of insoluble A β ₁₋₄₂ (r=0.856; p=0.03) within the hippocampus.

Extracellular Brain Adenosine

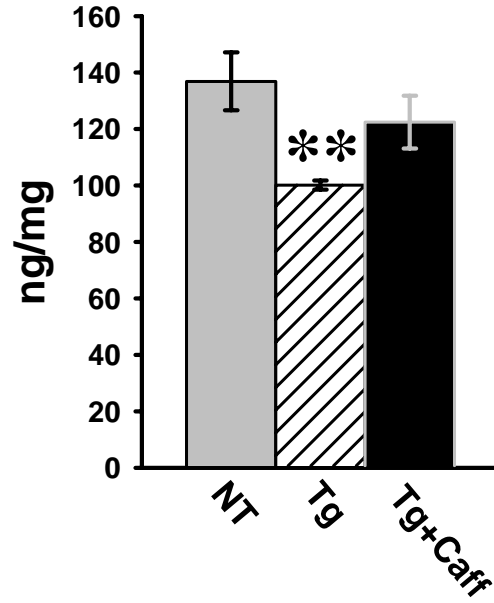


Figure 22. Quantification of extracellular brain adenosine levels in Aged Mice (Study B). The administration of caffeine resulted in a significant elevation of extracellular adenosine levels in Tg+Caff mice to near that of NT mice, while untreated Tg mice had significantly reduced levels. ** = significantly lower than NT ($p < 0.005$) and Tg+Caff mice ($p < 0.05$).

VII. Discussion

General Summary

In the present study, we determined behavioral and pathological changes in AD transgenic mice mediated by the long-term administration of caffeine to APP_{sw} transgenic mice. The 4-month, daily administration of approximately 1.5 mg of caffeine to each mouse, equivalent to about five cups of coffee in humans, resulted in global protection against cognitive impairment. This behavioral protection was associated with reductions in both soluble and insoluble forms of A β in hippocampus, supporting other reports that cognitive impairment is A β dependent. Furthermore, an 18-day administration of caffeine to aged APP_{sw} mice also led to reductions in A β , and normalized the otherwise decreased extracellular brain adenosine levels in untreated APP_{sw} control mice. Importantly, results from this study establish a direct link between long-term caffeine usage and the SAM/SAH cycle, which when potentially disrupted in AD, has crucial implications in APP processing and specifically gamma-secretase activity.

The complete cognitive protection and reductions in AD-like pathology found in this study, together with the recent epidemiological study indicating a reduced risk of Alzheimer's disease from moderate caffeine use (Maia and Mendonca, 2002), provide a compelling argument for future use of caffeine-based treatments in clinical trials to protect against AD. Given the already widespread use and acceptance of caffeine and the lack of serious side effects associated with long-term intake of caffeine in moderate

amounts, it would be advisable to include a moderate, sustained daily caffeine intake throughout life as a preventative against AD.

Behavioral Measures. Caffeine administration protected Tg mice against cognitive impairment across tasks testing multiple cognitive domains (reference learning/memory, working memory, recognition/identification, strategy switching). The global protection afforded by chronic caffeine administration was not only evident in every task wherein impairment was present in Tg mice, but also inclusive of all cognitive tasks, as discriminant function analysis clearly showed. These wide-ranged cognitive benefits of caffeine administration did not involve significant side-effects on sensorimotor function that impacted cognitive performance because there were no correlations between sensorimotor function and cognitive performance. A discussion of results from each task, in the order performed, is presented below.

No differences in Y-maze alternations were seen between the three groups, thus it is likely that the Y-maze task is not strict enough to detect any obvious abnormalities in basic mnemonic processing in 8-9 month old APP_{sw} mice as previous studies have shown (Holcomb et al., 1999; King et al., 2002). Therefore, with no transgenic impairment present, the long-term caffeine administration to transgenic mice was unable to have any effect in this task.

Results from the acquisitional phase of Morris water maze revealed Tg mice had significant impairment in the fourth and fifth blocks, indicating that these mice have impaired spatial learning, as earlier studies have found (Hsiao et al., 1996; Westerman et al., 2002; Arendash et al., 2004). Further impairment was seen in Tg mice in the memory retention phase of this task, as these mice failed to show any preference for the quadrant

formerly containing the submerged escape platform. In contrast, Tg mice given caffeine had significantly quicker escape latencies during the acquisition phase of testing. Additionally, Tg+Caff mice showed an exclusive preference for the quadrant formerly containing the submerged escape platform, as indicated by their annulus crossings and time spent in this particular quadrant. These results indicate the long-term administration of caffeine protected APP_{sw} mice against spatial/reference learning and memory impairment.

Tg mice showed additional impairment in the circular platform task, a behavioral task that also relies on spatial/reference learning and memory. In previous studies, Pompl et al. (1999) reported seven month old APP_{sw} mice were impaired in circular platform performance when the escape hole's location was changed (e.g., reversal learning). In the present study, Tg mice were worse across all eight days of testing when compared to NT mice in the circular platform task. By contrast, Tg+Caff mice performed identically to NT mice and substantially better than Tg controls in this task. Additionally, Tg mice made significantly more errors than the other two groups on the last day of testing, further highlighting the cognitive impairment that the Tg group alone had and the protective affect of caffeine against this impairment. It is noteworthy that the circular platform impairments evident in the present study's APP_{sw} mice were present without changing the escape whole's location, as was the case for Pompl et al. (1999).

If performed after the Morris maze task, the platform recognition task relies heavily on the ability of mice to switch from the cued (spatial) strategy used in Morris water maze to a search/recognition strategy. Difficulties doing this are associated with higher escape latencies. Previous behavioral characterizations reported APP_{sw} mice of

similar age were impaired in this task (Hsiao et al., 1996; King et al., 2002; Arendash et al., 2004). Tg mice showed an overall impairment in platform recognition testing compared to NT controls, while Tg mice given caffeine performed equivalent to NT mice overall. Moreover, on the last day of testing in this study, Tg mice had significantly higher escape latencies when compared to both NT and Tg+Caff mice. Thus, chronic caffeine administration resulted in protection of strategy switching abilities in Tg mice.

As the most sensitive of all the cognitive-based behavioral tasks in our test battery, the RAWM task is proficient in elucidating working memory impairment in transgenic mice. An earlier study reported 15 month old APP_{sw} mice were impaired in RAWM testing (Morgan et al., 2000), and Arendash et al. (2004) found recently that this impairment is evident as early as 6.5 months of age. Consistent with both of these prior studies, 8-9 month old Tg mice in the present study were impaired overall in both T4 and T5 of RAWM (the trials indicative of working memory), as evident by significantly higher escape latencies than NT mice. As with all other cognitive tasks wherein the Tg group was impaired, Tg+Caff mice were protected against RAWM working memory impairment in performing significantly better than Tg controls and no different from NT mice. The similar performance between NT and Tg+Caff mice in this sensitive task indicates that long-term caffeine treatment grants powerful protection against working memory impairment in these Alzheimer's transgenic mice.

Consistent with earlier characterizations of this APP_{sw} mouse model (Holcomb et al., 1999; King et al., 2002; Arendash et al., 2004; Leighty et al., 2004), no differences in overall sensorimotor performance that might indicate a gross motor dysfunction were seen in either Tg or Tg+Caff mice compared to NT controls. The absence of differences

in the elevated plus maze between Tg and Tg+Caff mice shows that the long-term administration of caffeine does not result in any anxiety-based side effects that could explain the profound differences in cognitive performance between these two groups. These results are consistent with an earlier study reporting the lack of anxiolytic properties associated with long-term caffeine administration to rodents (Bhattacharya et al., 1997).

In factor analysis, the loading of RAWM, platform recognition, and Morris water maze measures into factor 1 led this factor to be dubbed the primary cognitive-based factor. Furthermore, both the direct entry and the step-wise forward methods of discriminant function analysis were able to completely discriminate between all three groups when using all 19 behavioral measures, as well as only the cognitively-based measures in factor 1. This indicates that the long-term administration of caffeine resulted in an overall protection of cognitive function across multiple behavioral measures, although the performance of NT mice across those multiple behavioral measures could still be distinguished from that of Tg+Caff mice. Thus, multiple cognitive domains were protected by caffeine treatment in Tg mice.

The consistently poor performances of untreated APP_{sw} transgenic mice in four of the five cognitive-based tasks used in the present study indicates that these particular mice exhibit widespread cognitive deficiencies that negatively impact spatial memory/learning, working memory, and recognition/identification in a manner similar to that of early to moderate Alzheimer's disease in humans. These impairments currently reported are consistent with an earlier behavioral characterization of APP_{sw} mice involving the same transgenic colony (Arendash et al., 2004). Even more significant, the

long-term administration of caffeine to Tg mice, presumably started before these mice become cognitively impaired, resulted in a behavioral phenotype similar to that of NT mice. These results indicate caffeine's potentially beneficial influences on the cognitive-destabilizing processes apparent in Alzheimer's disease are worth investigating in a clinical manner.

Previous studies investigating the behavioral effects of long-term caffeine administration to normal (wild type) rodents form a consensus that this treatment has no effects on cognition (Von Lubitz et al., 1993; Molinengo et al., 1994), although none of these studies lasted beyond 15 days. Therefore, it is sufficient to assume that the behavioral improvements seen in the caffeine-treated mice from the present study are a result of behavioral protection against over-expression of the mutated APP gene in APP_{sw} mice, rather than a behavioral improvement resulting directly from caffeine's immediate effects on cognition. Furthermore, the present study aimed to duplicate moderate dietary caffeine use in humans by placing mice on a truly long-term diet of caffeine administration lasting for four months and continuing throughout behavioral testing, thus establishing the mice are fully tolerant to caffeine and avoiding the potentially harmful effects of caffeine withdrawal. By ensuring the mice consumed a caffeine dose equivalent to approximately five cups of coffee in humans, the present study is the first of its kind to closely mimic the nature of human caffeine consumption that was found to be associated with a reduced risk of Alzheimer's disease in a retrospective study (Maia and Mendonca, 2002).

Neurochemical/Neuropathological Measures. Long-term administration of caffeine resulted in no significant effects on A₁ adenosine receptor densities in the

striatum, hippocampus, or frontal cortex of 9 month old, behaviorally-tested APP_{sw} mice. Untreated APP_{sw} mice did have significantly increased A_{2A} receptors in the hippocampus, a finding that may indicate increased glial activation in this area as A_{2A} receptors are generally only associated with glial or vascular cells when found within the hippocampus (Fiebach et al., 1996). Interestingly, caffeine administration did reduce these elevated hippocampal A_{2A} receptor levels to a level that was not statistically different than NT mice, although the reduction was not great enough to result in a significant difference between Tg and Tg+Caff mice. No differences in A₁ or A_{2A} mRNA expression were seen following long-term caffeine administration in any of the groups in any of the brain areas examined, as other labs have reported in normal, wild type mice (Johansson et al., 1993; Shi and Daly, 1999).

The long-term administration of caffeine resulted in significantly less soluble A β ₁₋₄₀ and insoluble A β ₁₋₄₂ in the hippocampi of caffeine-treated APP_{sw} mice when compared to the age-matched APP_{sw} mice used in this study. Earlier characterizations of the APP_{sw} mice have indicated their behavioral impairment was A β dependent, as age-related increases in amyloid levels (particularly the soluble isoforms) were found in the hippocampus and/or associate cortices that coincided with deterioration in cognitive tasks (Chen et al., 2000; Westerman et al., 2002). In the present study, reduced amyloid burden thus appears to be a primary cause for the lack of impairment in cognition-based tasks that the caffeine-treated APP_{sw} mice exhibited. It is also noteworthy that the 18-day administration of caffeine to 17.5 month old APP_{sw} mice also resulted in a significant reduction of insoluble A β ₁₋₄₂ in the hippocampus. This implies that caffeine administration has an effect on amyloid levels after they have reached substantial

amounts in aged APP_{sw} mice, indicating long-term caffeine intake may have a treatment role as well as a preventative role in Alzheimer's disease.

The reductions in soluble A β ₁₋₄₀ and insoluble A β ₁₋₄₂ following caffeine treatment are likely associated with changes in amyloidogenic processing, as caffeine is too small molecularly to have an effect on binding to and clearing the much larger A β species. In cerebral cortical tissues from behaviorally-tested Tg mice, the increased ratio between β - and α -CTFs mediated by caffeine treatment thus suggests that caffeine has a direct impact on the secretases involved in amyloidogenic processing. Increases in β -CTFs could potentially be construed as a harmful precursor to A β generation that might result from increases in β -secretase or decreases in α -secretase activity (as the β -CTF results from β -secretase cleavage of APP and is the intermediate precursor that when cleaved by γ -secretase cleavage yields A β). Yet in this case we theorize the increase in β -CTFs is a result of decreased γ -secretase activity. Less γ -mediated cleavage of β -CTFs in the caffeine-treated Tg mice would explain why they had a greater amount of β -CTF present relative to α -CTF and would also explain the decreases in A β found in these mice.

When γ -secretase activity was assayed directly from cortical tissues of aged Tg mice after 18 days of caffeine administration, no statistically significant difference in γ -secretase activity was seen between Tg and Tg+Caff mice. However, there is the possibility that a larger decrease in γ -secretase activity was masked by the presence of other endogenous mouse proteins that are unaffected by caffeine treatment yet share γ -secretase's preferred substrate used in our assay.

In the present study, aged APP_{sw} mice had significantly reduced brain levels of extracellular adenosine, while the long-term (18 day) administration of caffeine restored adenosine levels in aged APP_{sw} mice to those of NT mice. Although mice were given *ad libum* access to caffeine-treated water, it can generally be assumed that mice consume the most water during nighttime hours and therefore plasma caffeine levels were likely greatest during the night. In this study, mice were sacrificed at mid-morning, indicating extracellular brain adenosine levels were significantly elevated hours after peak plasma levels of caffeine have fallen. In a related finding, Conlay et al. (1997) found caffeine administration to rats resulted in elevated plasma adenosine levels eight hours later. Although it is likely caffeine transiently raises adenosine levels after increasing cellular metabolism while increasing vasoconstriction in the brain, this effect should diminish as caffeine is metabolized and its antagonism of adenosine receptors removed. The longer duration of increased adenosine levels (i.e. eight hours after caffeine administration) thus may be a result of increased S-adenosyl-homocysteine (SAH) hydrolysis, resulting in the freeing of intracellular adenosine.

This potential link between caffeine administration and increased SAH hydrolysis suggests that caffeine may have an impact on the impaired SAM/SAH cycle in Alzheimer's disease (see Figure 2), which has been shown through *in vitro* studies to affect PS1 and BACE expression (Fuso et al., 2005). Decreases in SAM (S-adenosyl-methionine) concentrations have been reported in AD patients (Morrison et al., 1996), while SAH levels are found to be increased in AD patients (Kennedy et al., 2004). In the present study, we found caffeine treatment raised SAM levels by 49% compared to untreated Tg mice. Although this increase was not statistically significant, possibly due

to either small group sizes (n=3) or euthanasia at a time well after maximal drinking (e.g., caffeine intake), the data suggest caffeine raises SAM levels, leading to decreased expression of PS1 and BACE and subsequent decreased production of A β (see Figure 3).

Proposed Mechanism of Caffeine-Mediated Cognitive Improvement

The leading theory in Alzheimer's research is the amyloid hypothesis, which proposes that an increased production of β -amyloid is responsible for a cascade of NFT formation, oxidative damage, neuroinflammation, and neurodegeneration/neuronal dysfunction. Supporting this theory, transgenic mice overexpressing a hAPP gene with amyloidogenic mutations over-express mutant APP, resulting in production of A β and ensuing cognitive impairment. In the present study, long-term caffeine treatment reduced A β and protected the mice from cognitive impairment.

A previous study investigating the potential neuroprotective effects of caffeine was limited to an *in vitro* investigation that failed to account for caffeine-mediated physiological changes aside from the pharmacological blockade of adenosine receptors (Dall'Igna et al., 2003). These investigators reported that A_{2A} receptor blockade by caffeine in cultured rat cerebellum cells resulted in neuroprotection against β -amyloid toxicity. Past literature suggests, however, that A_{2A} receptors are limited to insignificant numbers in cerebellum cells (Fredholm et al., 1999; Fisone et al., 2004). It is also known that cerebellum cells are not normally subjected to high levels of β -amyloid until possibly the very late stages of advanced Alzheimer's disease. If caffeine-mediated blockade of A_{2A} receptors was neuroprotective, then this would also dictate that caffeine must be present in the bloodstream at all times. The epidemiological study by Maia and Mendonca (2002) reported that a caffeine intake of 199 mg/day (approximately two cups

of coffee) reduced the risk of Alzheimer's disease, indicating that a sustained presence of caffeine is not required for caffeine's protective effects on cognitive function. In the present study, the nature of the long-term caffeine treatment was designed to closely mimic the normal consumption of caffeine in a typical caffeine user. With this in mind, the *in vivo* effects of long-term caffeine intake on the cognitive function and pathological features of APP_{sw} mice could be examined through the present controlled study, which eliminated other potential variables over a protracted longitudinal treatment format.

Caffeine is derived from a biosynthetic pathway in tea leaves that begins with the precursor S-adenosyl-methionine (SAM) (Koshiishi et al., 2001). SAM is a major methyl donor in the brain and is involved in the methylation status of various genes including PS1 and BACE (Fuso et al., 2005). Under normal circumstances, SAM is converted to SAH by catechol-O-methyltransferase (COMT) while donating its methyl group and SAH is rapidly hydrolyzed to adenosine and homocysteine. When homocysteine is present in higher concentrations, such as AD, the equilibrium reaction proceeds in the opposite direction and favors the formation of SAH from adenosine and homocysteine. Elevations in SAH could potentially block the methylation reaction leading to increased expression of PS1 and BACE (Scarpa et al., 2003), and would be evident by decreases in extracellular adenosine as it is synthesized into intracellular SAH.

Elevated plasma levels of homocysteine are considered a strong independent risk factor for AD (Seshadri et al., 2002), and it was recently reported that AD patients over the age of 60 consumed significantly less dietary vitamin B6 and folate than controls (Mizrahi et al., 2003). Vitamin B6 and folate are both required for the recycling of homocysteine back to methionine, and homocysteine accumulates when these compounds

are present in insufficient quantities (Fuso et al., 2005). Dietary influences such as these, as well as other genetic or physical alterations that might affect homocysteine metabolism, result in accumulation of SAH and the subsequent state of hypomethylation proposed in the genes implicated in AD (Scarpa et al., 2003). It is therefore relevant to address the link between caffeine, SAM, and SAH in Alzheimer's disease.

It is currently unknown how caffeine might raise SAM levels as this is the first study to suggest that a caffeine-induced elevation in SAM occurs. It is possible caffeine is directly metabolized back to SAM once absorbed into the body, as caffeine is a downstream product of SAM biosynthesis in tea leaves. Another proposed theory relies on caffeine down-regulating astrocytic metabolism by antagonizing A_{2A} receptors in these cells, which contain the majority of COMT present in the brain. The potential inactivation of COMT in this manner would lead to a decrease in transformation of SAM to SAH, promoting the methylation status of PS1 and BACE. On this note, COMT inhibitors have been used to treat depression (a symptom of Alzheimer's disease) and are reported to decrease the L-dopa induced increases in homocysteine that accompany Parkinson's disease patients (Miller et al., 1997). It was also recently found that direct activation of COMT stimulates homocysteine synthesis in astrocytes, which in turn export homocysteine to neighboring neurons (Huang et al., 2005). A caffeine-mediated inhibition of COMT-induced homocysteine synthesis would thus have beneficial implications in a variety of diseases in addition to AD.

Clinical Implications of Caffeine Administration Study Findings and Potential Future Investigations

In and of itself, the near-complete protection against cognitive impairment granted by the long-term administration of caffeine to APP_{sw} mice warrants further studies into the relationship between caffeine and Alzheimer's disease. Furthermore, the substantial decreases in A β following caffeine treatment provide evidence that caffeine does have a positive effect (perhaps indirectly) on amyloidogenic processing that results in behavioral improvements. The proposed link between a caffeine-induced change in the SAM/SAH cycle and the effect this relationship potentially has on SAM methylation of BACE and PS1 activity would be novel in providing a link between a dietary influence and the genetics of Alzheimer's disease. In addition to this proposed "indirect" mechanism for caffeine affecting amyloidogenic pathways, it is possible that "direct" effects of caffeine on neuronal amyloidogenic pathways are also present. *In vitro* neuronal culture studies could determine if such direct affects are, indeed, part of the beneficial mechanisms through which caffeine reduces brain A β levels and protects cognitive function.

The suggested diminishment of PS1 expression following caffeine treatment has potentially powerful repercussions. First, decreased PS1 expression following long-term caffeine use would result in decreases in the pathological amyloidogenic processing found in Alzheimer's disease as suggested, but this decrease in expression would also not result in a complete loss of function of the PS1 gene, sidestepping the potential harmful effects that often accompany PS1 inhibitors. Secondly, the 18-day treatment of caffeine used in study B of this investigation also resulted in a substantial decrease in A β as well,

indicating that caffeine administration may serve as an effective treatment against those already diagnosed with AD. Lastly, the use of caffeine is already widely accepted by the global community and is cost effective. It has also been established that moderate use of caffeine is unaccompanied by harmful side effects as long as caffeine intake is maintained on a daily basis. Thus, it is a safe, naturally-occurring nutraceutical agent that could have significant prophylactic and therapeutic value against AD, whether taken alone or in combination with other AD therapeutics.

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