The Effect of Rho Kinase Inhibitors on Alzheimer's Disease

by

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ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease that affects 5.4 million Americans. AD leads to memory loss, changes in behavior, and death. The key hallmarks of the disease are amyloid plaques and tau tangles, consisting of amyloid-β oligomers and hyperphosphorylated tau, respectively.

Rho-associated, coiled-coil-containing protein kinase (ROCK) is an enzyme that plays important roles in neuronal cells including mediating actin organization and dendritic spine morphogenesis. The ROCK inhibitor Fasudil has been shown to increase learning and working memory in aged rats, but another ROCK inhibitor, Y27632, was shown to impair learning and memory. I am interested in exploring how these, and other ROCK inhibitors, may be acting mechanistically to result in very different outcomes in treated animals.

Preliminary research on thirteen different ROCK inhibitors provides evidence that while Fasudil and a novel ROCK inhibitor, T343, decrease tau phosphorylation in vitro, Y27632 increases tau phosphorylation at a low dose and decreases at a high dose. Meanwhile, novel ROCK inhibitor T299 increases tau phosphorylation at a high dosage. Further, an in vivo study using triple transgenic AD mice provides evidence that Fasudil improves reference memory and fear memory in both transgenic and wild-type mice, while Y27632 impairs reference memory in transgenic mice. Fasudil also decreases tau phosphorylation and Aβ in vivo, while Y27632 significantly increases the p-tau to total tau ratio.

DEDICATION

This dissertation is dedicated to my mom, who always supported me. My education is in her honor. This dissertation is also dedicated to my loving husband, Justin, and my son, Connor.

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CHAPTER 1

ALZHEIMER'S DISEASE AND RHO KINASE INHIBITORS

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that results in memory loss, behavioral changes, and deficits in thinking and language skills. There is no definitive test that can be used to diagnose a person with AD, and a final confirmation post-mortem is necessary to determine whether a person did suffer from AD. A physician must diagnose the disorder with a thorough medical examination that can include medical history, mental status testing, physical exams, blood tests, and brain imaging. Some of the testing required is used to rule out other dementias. Although AD is not easy to diagnose, it affects 5.4 million Americans and is the 6th leading cause of death in the United States. AD accounts for 60-80% of dementia cases, with the yearly socioeconomic cost of AD estimated at approximately $221.3 billion in 2015 (Gaugler, James, Johnson, Scholz, & Weuve, 2016).

In the decade between 2000 and 2010, the top five causes of death in the United States showed decreases in mortality rates due to increased awareness of early diagnoses, better therapeutic options, and preventative care (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). Meanwhile, AD, ranked as the 6th leading cause of death, showed a 68% increase in mortality rates due longevity of people in the United States, coupled with continued lack of early diagnosis, preventative options, and therapeutics.

The first clinical signs of AD include deficits in episodic and spatial memory, followed by deficits in working memory, and in late stages of the disease, long-term

memory. The behavioral hallmark of AD also includes the inability for patients to form new memories.

Clinically, AD can be divided into two groups, early onset and late onset. Development of AD before the age of 60 is often considered early onset, whereas after 60 years of age is considered late onset. Early onset AD has been characterized as familial, with autosomal dominant mutations in amyloid precursor protein (APP), presinilin 1 (PS1), and presinilin 2 (PS2). Despite this, the vast majority of AD cases are late onset, and develop sporadically without a clear genetic component. The primary risk factor for AD is aging, with approximately 20% of people between 75-84 years of age and 50% of people 85 years and older suffering from the disorder. Additional risk factors include, family history of AD, carrying the apolipoprotein E (APOE)-e4 gene (Naj et al., 2014), and traumatic brain injury (Sennik, Schweizer, Fischer, & Munoz, 2016).

Current treatment options for AD include only five drugs—four cholinesterase inhibitors and Memantine (Szeto & Lewis, 2016). Cholinesterase inhibitors prevent the breakdown of acetylcholine, which is a key chemical messenger for learning and memory. Cholinesterase inhibitors are prescribed to patients with mild to moderate AD (Birks, 2006). However, the evidence suggests that these drugs do not slow the progression of dementia over time. Memantine is an N-mehtyl-D-aspartate receptor antagonist, and regulates glutamate activity, which is also involved in learning and memory. Memantine is prescribed in moderate to severe stages of AD, and there has been some evidence suggesting that Memantine may have neuroprotective potential (Danysz & Parsons, 2003). It can also be used in conjunction with a cholinesterase inhibitor. These drugs, however, are only symptomatic treatments, and do not affect the pathophysiology

underlying AD. Furthermore, they do not alter the course of the disease, and the relief they provide is relatively short-lived.

As the "baby-boomer" generation ages and enters the average age at which late onset AD is clinically determined to be the cause of memory problems, the number of people who are susceptible to AD will increase dramatically. This year, the first baby boomer will turn 65 years old. The Alzheimer's Association predicts an increase from 5.4 million to 10 million Americans with AD by 2030. Without adequate early biomarkers and treatments for AD, the cost of AD will also skyrocket.

Instead of the traditional approach in treating AD, in which therapy begins after patients develop memory problems and cognitive deficits, preventative measures may lead to better outcomes, ultimately lowering the prevalence of the disease. Neurobiologists, with the support of the FDA, are working toward the identification of early biomarkers for AD and subsequent development of preventative therapeutics, including tools to decrease amyloid plaque formation and neurofibrillary tangle (NFT) formation, as well as tools to enhance declining cognitive function in AD patients (Anand, Gill, & Mahdi, 2013; Tayeb, Yang, Price, & Tarazi, 2012)

Neuropathology

AD is characterized by atrophy of the cortex, including enlarged ventricles and gyral shrinkage, along with the presence of two hallmark biomarkers, intracellular neurofibrillary tangles composed of hyperphosphorylated microtubule-associated protein tau, and extracellular plaques composed of amyloid-ß (Aß). The accumulation of these misfolded proteins results in oxidative and inflammatory stress, leading to both energy

failure and synaptic dysfunction. Furthermore, proper diagnosis of AD typically requires post-mortem histological confirmations of both plaques and tangles. Thus, it is noted that both the pathology of tau and Aß likely play a key role in the pathogenesis of the disease.

Aß

AD was first diagnosed by Alois Alzheimer in 1906, when he observed amyloid plaques in a woman with progressive dementia (Cipriani, Dolciotti, Picchi, & Bonuccelli, 2011). Amyloid plaques are composed mostly of Aβ peptides, which consist of 36 to 43 amino acids derived from the amyloid precursor protein (APP). APP, in turn, is encoded by the APP gene located on chromosome 21 (Yoshikai, Sasaki, Doh-ura, Furuya, & Sakaki, 1991). The protein APP is a type 1 transmembrane protein consisting of 695-770 amino acids. Aß is produced as a monomer but aggregates to form multimeric complexes. The severity of cognitive symptoms in AD does not correlate with the amount of Aß in the brain, but instead, is correlated with the levels of oligomers in the brain (Takahashi, Nagao, & Gouras, 2017). There is no conclusive evidence regarding the normal functionality of Aβ, or whether the total loss of Aβ would cause detrimental effects—in animal studies, physiological function does not seem to be affected (Phinney et al., 1999); however, they may be involved in the activation of kinase enzymes, serve as protection against oxidative stress, or play a role as a transcription factor, among other possibilities.

APP is processed into Aß through a multi-step process. First, full-length APP is cleaved by either α- or β-secretase, to produce two large soluble fragments, sAPPα or sAPPß, and two membrane-anchored C-terminal fragments, CTFα or CTFß, respectively. The C-terminal fragments are then processed by γ-secretase to generate either P3 peptide

or Aß peptides, along with the APP intracellular domain fragment (Zhang, Thompson, Zhang, & Xu, 2011). Aß plaques are composed mostly of $Aß_{40}$ and $Aß_{42}$, predominantly with the latter, which is the isoform of Aß with an extended C-terminal, and is insoluble, while $Aß_{40}$ is relatively soluble. That said, $Aß_{40}$ has been shown to significantly increase in concentration in the AD brain (Näslund et al., 1994) as well.

The Aß cascade hypothesis proposed by Hardy and Selkoe suggests that the accumulation of extracellular Aß aggregates, or senile plaques as an outcome of those aggregates, catalyzes the cascade of neurotoxic events that lead to neurodegeneration in AD. These neurotoxic events include the facilitation of tau phosphorylation, disruption of proteasome and mitochondria function, dysregulation of calcium homeostasis, impairment of synaptic function, inhibition of long-term potentiation, and neuronal death.

Tau

Tau is a microtubule-associated protein that is essential for microtubule assembly and stability (Drechsel, Hyman, Cobb, & Kirschner, 1992; Lindwall & Cole, 1984; Trinczek, Biernat, Baumann, Mandelkow, & Mandelkow, 1995). Tau is highly expressed in neurons, with low levels of tau expression also found in astrocytes and oligodendrocytes (Binder, Frankfurter, & Rebhun, 1985; LoPresti et al., 1995). Microtubule Associated Protein Tau (MAPT) is a gene that encodes for six different isoforms of tau found in the adult human brain through alternative mRNA splicing. MAPT consists of 16 exons on chromosome 17q21, and transcription is differentially expressed through the nervous system based on the stage of neuronal maturation and type. While mutations in the MAPT gene are not linked to AD, studies have shown a

correlation between MAPT haplotype variants and AD risk (Myers et al., 2005; Randall et al., 2009).

Tau function is highly regulated by post-translational modifications, including the addition and removal of phosphate groups through the activity of several phosphates and kinases. There are 79 potential serine (Ser) and threonine (Thr) residues in the longest tau isoform that are able to act as phosphate acceptor sites. Phosphorylation at approximately 30 of these sites has been confirmed in normal tau proteins. Tau phosphorylation is developmentally regulated and the degree of phosphorylation in each of the isoforms increases with age. While some phosphorylation sites associated with AD are found in the healthy brain as well, there are also many phosphorylation sites that are found only in the AD brain.

An increase in tau phosphorylation decreases the binding of microtubules. Hyperphosphorylated tau is insoluble, and is unable to dephosphorylate or bind microtubules. Hyperphosphorylated tau self-associates into paired-helical filament, which congregate to create neurofibrillary tangles. Intermediate aggregates of abnormal tau molecules are cytotoxic and impair cognition. The levels of phosphorylated and total tau in cerebrospinal fluid inversely correlate with scores on cognitive exams and could serve as a biomarker for pathology progression.

Dual Pathway Hypothesis

The dual pathway hypothesis supports the view that Aß plays a large role in the pathogenesis of AD; however, it is apparent that Aß is not the sole driving factor of AD, therefore, warranting further investigation in uncovering additional components driving

the pathology. For example, in a phase 1 study immunizing AD patients against Aß, post mortem results confirmed a decrease in both amyloid and plaques; however, there was no change in survival rates, or in latency and severity of end stage dementia (Holmes et al., 2008; Weller & Nicoll, 2003). Thus, the dual pathway hypothesis suggests that upstream factors drive both Aß and tau pathology. That said, treating downstream effectors of either of these pathologies would not be an effective treatment strategy.

Many studies to date have been targeting the investigation of either amyloid pathology, or tau pathology. Targeting tau pathology takes several forms, including the prevention of the phosphorylation of tau, prevention of the misfolding of tau, prevention of the aggregation of tau, and tau immunotherapy. However, it may be more beneficial to target both amyloid and tau pathologies simultaneously.

Rho-associated protein kinase

RhoA is a small GTPase that works through Rho-associated protein kinase (ROCK) to mediate actin organization and cellular migration. ROCK has two isoforms, ROCK I and ROCK II. While both isoforms are similar in both amino acid sequence and kinase domains, they differ in expression. ROCK I is found mostly in non-neuronal tissues such as the stomach, liver, spleen, kidneys, and testis. Meanwhile, ROCK II is found mostly in the brain and muscle tissues.

ROCK Inhibitors: Fasudil and Y27632

There are two main methods for treatment of disease: small, chemically manufactured molecules (SMOLs) and monoclonal antibodies. SMOLs constitute the

majority of pharmaceuticals. The major advantage that SMOLs have over antibodies is that SMOLs are absorbed when administered orally, although this is not always the case.

Fasudil is a SMOL ROCK inhibitor of both ROCK I and ROCK II isoforms, to varying degrees (Table 1). Fasudil has been widely used in Japan since 1995 as a treatment for cerebral vasospasms occurring after a subarachnoid hemorrhage, and has since also become clinically available in China (Feng, Lograsso, Defert, & Li, 2016). ROCK increases angiotensin-converting-enzyme (ACE) activity, which then increases the conversion of angiotensin I to angiotensin II, a peptide hormone that increases blood pressure. Fasudil decreases the amount of ACE and thereby angiotensin II, leading to a decrease in blood pressure and improves cerebral vasospasms. The ROCK pathway has been under investigation as playing a key role in several different diseases, including neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis. There is evidence that shows Fasudil treatment in a Parkinson's mouse model has protective effect on the mice, both in neurons and learning and memory behavior (Tönges et al., 2012).

Fasudil has been demonstrated to have neuroprotective effects on both murine models and humans. Studies have also reported a protective effect against injury to neurons after cerebral ischemia (S. Satoh et al., 1996; S. I. Satoh et al., 2001; Toshima, Satoh, Ikegaki, & Asano, 2000) and hypoxia-reoxygenation in mouse models (Ding et al., 2010; Jing D, Qin-Ying L, Xin W, Chang-Hai S, Chuan-Zhen L, 2010). Furthermore, Fasudil treatment in humans within 48 hours of acute ischemic stroke has been shown to have significant improvement in clinical outcomes. In addition, Fasudil has shown to

have a neuroprotective effect via suppression of an inflammatory response in autoimmune encephalomyelitis.

Y27632, another ROCK inhibitor, inhibits both ROCK I and ROCK II by competing with ATP by binding to the catalytic site of the kinase. Inhibition of ROCK is selective, and Y27632 does not significantly inhibit other kinases such as PKC, MLCK, and cAMP-dependent protein kinases. Y27632 is cell permeable (Ishizaki et al., 2000) via a carrier-mediated facilitated diffusion into the cell. Y27632 has also been shown to affect brain structures when administered via drinking water (Abouhamed et al., 2009) or intraperitoneal injections (Inan & Büyükafşar, 2008).

ROCK and AD

The KIBRA gene was associated with variation in normal episodic memory performance in adults (Papassotiropoulos et al., 2006) via a 500K single nucleotide polymorphism genome-wide associate study. There has also been evidence suggesting a genetic link between KIBRA variation and Alzheimer's disease (Corneveaux et al., 2010). KIBRA is a substrate for PKCζ (Büther, Plaas, Barnekow, & Kremerskothen, 2004) and has been shown to interact with Dendrin, a postsynaptic cytoskeleton modulatory molecule. The ROCK pathway is upstream of PKCζ (Kampfer et al., 2001; Scott, Arioka, & Jacobs, 2007; Uberall et al., 1999; Van Kolen & Slegers, 2006), and could therefore have an effect on cognition in both the healthy and AD populations as well. Along with this, the inhibition of ROCK can potentially be an effective therapeutic for AD through the prevention of tau hyperphosphorylation, and amyloid plaques, or through generally improved cognition.

An early case study reported the effects of Fasudil treatment on cerebral vasospasm patients (Kamei, Oishi, & Takasu, 1996), in which two patients with cerebrovascular dementia exhibiting wandering symptoms and forgetfulness were treated with Fasudil for 8 weeks at 30 mg/kg or 60 mg/kg. Results showed that patient memory was slightly improved and their wandering symptoms disappeared during Fasudil treatment, reappearing a few days following treatment.

Fasudil has also been reported to decrease learning and memory deficits and attenuate neuropathology in rats injected with streptozotocin in order to produce synaptic dysfunction similar to that found in the early stages of AD (Hou, Zhou, Yang, Du, & Li, 2012). Fasudil-treated rats not only showed improvement on the Morris water maze, but they also had increased expression levels of SYP, which plays a key role in synapse number and plasticity. Fasudil treatment also attenuated the increased phosphorylation of both LIMK and cofilin due to the streptozotocin injection. Furthermore, Fasudil has been reported to improve cognition and attenuate neuropathology in rats injected with $Aß_{42}$ into the left lateral ventricle (Song, Chen, Wang, Gao, & Zhu, 2013). Rats treated with Fasudil improved on the Morris water maze and had decreased neuronal injury in the hippocampus.

Fasudil has been tested as a cognitive enhancer in aged rats. Wild-type aged rats were treated with Fasudil and tested on the Morris water maze. The results showed that the Fasudil-treated rats had increased learning and memory scores on the maze in comparison to vehicle-treated rats (Huentelman et al., 2009).

ROCK inhibitor Y27632 has been tested on ischemic and wild-type rats as a cognitive enhancer (Castro-Alvarez, Gutierrez-Vargas, Darnaudéry, & Cardona-Gómez,

2011). Y27632 treatment in ischemic rats led to an improvement in learning and memory scores; however, in normal rats, Y27632 treatment led to a decrease in learning and memory scores.

Inhibition of ROCK has been shown to in turn inhibit cyclin dependent kinase 5 (Cdk5), a regulator of cell differentiation and morphology that has been well established as an important kinase in the pathology of AD (Cho et al., 2013; Chu, Li, & Praticò, 2013; Flaherty, Soria, Tomasiewicz, & Wood, 2000; Shukla, Skuntz, & Pant, 2012). Aberrant activation of Cdk5 leads to tau hyperphosphorylation and NFT deposition. ROCK may affect AD risk through its effect on Cdk5 (Castro-Alvarez et al., 2011), implying that ROCK inhibition would inhibit Cdk5, leading to tau phosphorylation inhibition and decreased risk of AD.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to decrease $Aß_{42}$ *in vitro.* While some studies have suggested that this is due to the effects NSAIDs have on cyclooxegenase, others have shown that NSAIDs decrease $Aß_{42}$ in the absence of cyclooxegenase activity. One hypothesis is that NSAIDs affect $Aß_{42}$ levels through the Ras family of proteins, including ROCK. Inhibition of Rho has been shown to decrease $Aß_{42}$ concentrations *in vitro* (Zhou et al., 2003). Further, it showed that application of ROCK inhibitor Y27632 reduced $Aß_{42}$ in a concentration-dependent manner *in vitro*, and lowered cortical $Aß_{42}$ in Parkinson's disease APP mice injected with Y27632.

ROCK inhibits myosin light chain (MLC) phosphatase, which acts on acto-myosin interactions and on MLC (Kawano et al., 1999), both of which have been associated with synaptic plasticity (Hayashi et al., 1996; Lamprecht, Farb, & LeDoux, 2002; Lamprecht & LeDoux, 2004). ROCK inhibition in a non-diseased brain, therefore,

would deregulate MLC phosphatase, decreasing both MLC and acto-myosin interactions, diminishing the capacity for synaptic plasticity and learning. However, in an excitotoxic state, typically seen as a constant in the AD brain, MLC has been shown to be overactive, causing disruptions in synapses and decreasing learning. A ROCK inhibitor used in that state could decrease MLC, and stabilize synapses to increase cognition.

ROCK has also been found to increase Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Akt activity (Wu et al., 2012), thereby reducing the phosphorylation of Glycogen synthase kinase-3 beta (GSK-3β). Due to GSK-3β inhibition through phosphorylation has been previously shown to decrease p-tau in the AD brain (Flaherty et al., 2000; Hamano, Yen, Gendron, Ko, & Kuriyama, 2012), and ROCK inhibition has been shown to maintain phosphorylation of GSK-3β (Castro-Alvarez et al., 2011), ROCK inhibition should therefore also reduce p-tau. Consequently, decreased levels of p-tau would lead to decreased NFT.

However, ROCK phosphorylates LIM kinase (LIMK) (Maekawa, 1999), which in turn phosphorylates cofilin and is crucial in F-actin stabilization (Munsie & Truant, 2012; N. Khan, 2012; Y. Wang et al., 2013), both of which are important factors in learning and memory. LIMK has also been associated with CREB phosphorylation and the subsequent neurogenic factor-induced neuronal differentiation in CNS hippocampal progenitor cells - a critical task for memory formation (Blum, Moore, Adams, & Dash, 1999). This pathway could prove deleterious for learning and memory when a ROCK inhibitor is applied, as synaptic plasticity and neuronal differentiation may decrease. However, during excitotoxic events, overactivation of ROCK causes high cytoskeletal reorganization, leading to irreversible neuronal disconnection and cell death (Esposito et

al., 2013; Salińska, Danysz, & Łazarewicz, 2005). ROCK inhibitors could reverse the effects of excitotoxicity, a prevalent factor in AD.

Modeling AD

in vitro

When selecting an *in vitro* methods of modeling AD, there are a multitude of options that include 3D culture, organotypic brain slice cultures, induced pluripotent stem cells, and cells that have been transfected to overproduce APP or tau proteins.

For an initial screening of drugs with a hypothesis of decreased phosphorylation of tau, however, 3D culture, organotypic brain slices, and induced pluripotent stem cells are not efficient or cost-effective. Using a cell line that is transfected to overproduce tau is the most efficient option to test whether drug treatment would improve tauopathy. The cell line selected was the H4 neuroglioma cells overexpressing four repeat tau (4R0N), or H4-tau cells. H4-tau cells are transfected to overproduce tau, and are a standard in the AD field as a model for tauopathy in AD. It is necessary to use this cell line to produce sufficient levels of phosphorylated tau for significant detection.

in vivo

There are many *in vivo* models for AD, made up for the most part by mouse models. There are both transgenic and non-transgenic mouse models of AD. Non-transgenic mouse models are mice that have direct infusions of Aβ or tau into different areas of the brain, depending upon the model. Transgenic mouse models are more commonly used as models of AD. These include a multitude of single transgenic mice as well as multi-transgenic mice.

Single transgenic models

There are several transgenic models with APP mutations. Some have mutations at the β-secretase cleavage site (Tg2576(Swe)), some have mutations at the gamma-secretase cleavage site (PDAPP(Indiana)), and some have mutations within the Aβ sequence (TgAPParc, APPDutch). These models have Aβ plaques, but no neurofibrillary tangles. They do have synaptic and memory deficits that generally precede the plaques. There are also higher levels of diffuse amyloid deposits compared to wild-type.

Multi-transgenic models

APP/PS1 mice are a bigenic AD mouse model that were produced using the APP Swedish and PSEN1 L166P mutations, both under the control of the Thy1 promoter. The expression of human APP is 3-fold higher than the endogenous APP in this mouse model. Aβ deposits begin at 6 weeks of age in the cortex. By 4 months of age, Aβ deposits are also found in the hippocampus. While some phosphorylated tau processes have been found around plaques in this mouse model, there have been no mature tangles. APP/PS1 mice tested in the Morris water maze have been shown to have diminished spatial learning and memory at 7 months of age, and at 8 months of age, the mice have shown diminished learning in a food-rewarded spatial maze task.

APPSwe-Tau mice are a bigenic AD mouse model that were produced by crossing Tg2576 mice with JNPL3 mice. These mice develop significant levels of plaque pathology and neurofibrillary tangles respectively. The mutations used in the progenitor mice are APP Swedish and MAPT P301L. Plaques develop gradually starting at approximately 6 to 7 months of age, with none found at 5 months. By 9 months of age,

plaques are scattered throughout the brain including the cortex, hippocampus, and amygdala. There is a significantly higher number of plaques at 12 months of age than at 9 months of age, indicating a continued increase of plaques over time. Meanwhile, some neurofibrillary tangles are found as early as 3 months in the spinal cord and pons. However, the tangles are more consistently found by 6 months of age. Female mice had an increase in tangles in the olfactory cortex, entorhinal cortex, and amygdala by 6 months of age as well.

Triple transgenic AD (3xTg-AD) mice are widely regarded as a reliable model of AD. These mice express exogenous mutations in APP and tau, as well as an endogenous familial mutation in presinilin associated with familial AD, making it a desirable model for phenotyping AD. The mutations are APP Swedish, MAPT P301L, and PSEN1 M146V. To produce these transgenic mice, single cell embryos of mice with the knock-in of the PSEN1 mutation were injected with the human transgenes for the APP and MAPT mutations. The transgenes are integrated at a single locus under the control of the Thy1.2 promoter.

The overexpression of these transgenes produce progressive AD plaque and tangle pathology. Aß deposition increases over time, and intracellular deposits can be seen as early as 4 months of age in the neocortical region. Extracellular deposits can be detected by 6 months of age in the frontal cortex and CA1 pyramidal neurons, and this becomes more extensive as the mice age. By 12 months of age, Aß deposits spread to other hippocampal regions as well as other cortical regions. Tau hyperphosphorylation starts later, beginning with tau being phosphorylated at Ser202, Thr205, and Thr231 sites at 12 months. Paired helical filaments and tangles can also be detected in the

hippocampus at approximately 12 months of age. By 18 months of age, late stage phosphorylation sites, Ser396 and Ser404 are also hyperphosphorylated. Tau pathology spreads from the hippocampus, especially within the CA1 pyramidal neurons, to the cortical structures over this course of time. 3xTg-AD mice were also shown to manifest cognitive impairment beginning at 4 months of age (Billings, Oddo, Green, McGaugh, & LaFerla, 2005; Oddo et al., 2003).

The present study utilized 3xTg-AD mice and their wild-type complement, C57BL/6. Only female mice were used due to the higher variability in phenotype found in male 3xTg-AD mice.

Learning and Memory Behavior Tests

A variety of cognitive behavioral tests are available to assess learning and memory in mice. Because AD pathology originates and is most severe in the hippocampus, hippocampal-dependent learning and memory tests are the most relevant in studying the efficacy of a treatment. The three behavioral tasks most commonly used to assess learning and memory in mice modelling AD are the Morris water maze, the radial arm water maze, and the fear conditioning test (Puzzo, Lee, Palmeri, Calabrese, & Arancio, 2014).

Morris Water Maze

The Morris water maze is a learning and memory task created by Dr. Richard Morris in 1981 (Morris, 1981). Briefly, the Morris water maze is a spatial learning task that uses a large circular tub filled with opaque water. A small platform is placed in one

quadrant of the tub, and is not moved from that spot. The mouse is placed in one of the non-platform quadrants and recorded as she swims for up to 60 seconds, with recording ending early if she finds the platform. There are extra-maze cues that the mouse can use to find the platform. Data is accumulated for each trial, and can be analyzed in several ways. The amount of time spent searching for the platform and the distance swam searching for the platform can both be analyzed for each trial, to determine whether the time and distance decrease with each trial. If the mouse is learning, they should decrease. These variables can also be analyzed for only the first trial of each day. This is called overnight retention, and can be informative for whether the mouse has retained the information learned from the previous day. This can be considered long-term memory. Finally, on the last day of the task, the platform is removed and the mouse is recorded swimming for 60 seconds for a probe trial. The amount of time spent in the platform quadrant and the opposite quadrant are measured, along with the number of times the mouse crosses the space in which the platform was previously. The more time spent in the platform quadrant, and the higher number of platform crossings is considered evidence that the mouse has retained spatial memory. Longer time spent in the opposite quadrant is considered evidence that the mouse has impaired spatial memory.

The Morris water maze is widely used due to the lack of food deprivation or electric shock as motivators. The task uses the animal's dislike of water as a motivator instead. However, there are some weaknesses. Some mice will swim in circles, remaining close to the edge of the tub, increasing the amount of time spent "looking for the platform", when in fact the mouse is not doing so. Some of the variance in performance scores in the Morris water maze may be due to differences in thigmotaxis, the tendency

for animals to stay close to walls, rather than to differences in spatial memory. Thigmotaxis is seen significantly more in female rodents (Devan, Tobin, Dunn, & Magalis, 2016). Mice are more affected by not only thigmotaxis (Gerlai & Clayton, 1999), but also passivity, or the tendency to float (Vorhees & Williams, 2006; Whishaw & Tomie, 1997), in comparison to rats. Some mice also will float passively in the water until "rescued" by the experimenter—this variability was decreased by decreasing the water temperature to increase aversion to the environment (Lipp & Wolfer, 1998) and when that failed, issuing a loud sound when a mouse floated for longer than five seconds without movement. While this is not a perfect task measuring spatial memory, it is widely used and informative in testing hippocampal-dependent spatial learning.

Radial Arm Water Maze

The radial arm water maze is a learning and memory task created by Drs. Olton and Samuelson in 1976 (Olton & Samuelson, 1976). Reference memory and working memory are both assessed during this task, and can be measured by keeping track of two separate types of incidents. Working memory is a short-term memory process that allows for temporarily holding information to guide behavior (Buresova, Bures, Oitzl, & Zahalka, 1985). Briefly, the task consists of an eight-arm maze radiating from a circular central area. One arm is the "goal arm" and includes a small platform at the end. Extra-maze cues are placed for the mouse to use to find the platform. The mouse is placed in the maze from any of the non-goal arms and the time the mouse takes to find the platform, as well as the number of times the mouse enters each of the incorrect arms is counted. The total number of times a mouse enters incorrect arms prior to reaching the

platform, or to the end of the timed trial, is used to measure reference memory. The number of times a mouse re-enters incorrect arms prior to reaching the platform, or the end of the timed trial, is used to measure working memory. If a mouse re-enters the incorrect arm many times even though the platform was not there during previous attempts, the mouse is considered to have poor working memory. The radial arm water maze has been used to test working memory in a variety of AD mouse models, and has been shown to detect impairment in mutant tau mice (Arendash et al., 2006; Golub et al., 2008; Rees et al., 2005) and in mutant APP mice (Volianskis, Køstner, Mølgaard, Hass, & Jensen, 2010; Xiong et al., 2011).

Fear Conditioning

While the Morris water maze and the radial arm water maze are both spatial memory oriented tasks, fear conditioning tests whether an animal learns to predict aversive events. The amygdala is largely involved in the emotional formation and storage of memories, such as those involving fear (LeDoux, 2003); however, learning the context in which the fear inducing stimulus occurred largely involves the hippocampus (Phillips & LeDoux, 1992). Briefly, a mouse is placed in a novel chamber for three minutes and toward the end of the task, a light turns on and a shock is delivered to the mouse. The mouse is returned to her home cage, until the next day when she is placed back into the chamber. The number of times the mouse freezes, as well as the length of each freeze, is measured and analyzed. The sympathetic response to fear in rodents has been demonstrated as a defensive behavior represented by freezing. Freezing is a total absence of movement outside of breathing (Blanchard & Blanchard, 1969). Significantly

decreased amount of freezing behavior in comparison to the control is considered an impaired fear response, while increased freezing behavior is considered an improvement in fear response and memory. Patients with mild to moderate AD have exhibited impaired conditioned fear responses, and mouse models of AD have also exhibited impairment that worsens with age.

Aims of Research

I. *in vitro* drug selection: To better understand the effects of ROCK inhibitors on tau phosphorylation, thirteen different ROCK inhibitors were used to treat H4-tau cells at an LD-10 dosage over a 96-hour time course. Eight of these drugs were novel ROCK inhibitors, developed at TGen.

   A. Phosphorylated tau (S396) and total tau levels were measured at each time point for each treatment. The four final drug candidatees, Fasudil, Y27632, T299, and T343 were tested again at a higher dosage (LD-50) to validate results.

   B. RNA was collected from cells treated by the four drugs at the LD-50 dosage. Next Generation Sequencing was performed to test for significant transcriptional changes in line with difference in the drug effects on the ratio of phosphorylated to total tau.

II. *in vivo* drug testing: While Fasudil and Y27632 have previously been tested on learning and memory in rodent models, they have never been tested on triple transgenic Alzheimer's disease (3xTg-AD) mice, and alongside one another. It is beneficial to test both drugs on the same model, and in the

same manner, to better parse out whether the differences in their effects are due to the different models or due to the drug mechanisms.

A. ROCK inhibitors were administered via drinking water to both 3xTg-AD and wild type mice. Vehicle treated animals were used as controls. Treatment began at 3 months of age, 1 month prior to literature based reports of pathology and significant cognitive deficits. Learning tasks were administered at 10 months, 12 months, and 15 months, critical time points for changes in pathology and behavior in 3xTg-AD mice. The total study period was 12 months.

B. Neuropathology was conducted in post-mortem brain tissue following behavioral testing. Western blots were performed on protein extracted from the hippocampus to test for changes in APP and APP cleavage sites, total tau, and phosphorylated tau. Immunohistochemistry was performed to visualize differences in phosphorylated tau and $A\beta_{42}$, which are indicative of tangle and plaque formation, in the hippocampus and the cortex. Finally, ELISAs were utilized to test for $A\beta_{40}$ and $A\beta_{42}$ in both soluble and insoluble fractions in the hippocampus.

CHAPTER 2

THE EFFECT OF ROCK INHIBITORS ON TAU PHOSPOHORYLATION: AN IN VITRO STUDY

Abstract

Rho kinase (ROCK) is an enzyme that plays an important role in neuronal cells. The ROCK inhibitor Fasudil has been shown to increase learning and working memory in aged rats, while another ROCK inhibitor Y27632 was shown to impair learning and memory in normal rats. These observations suggest different mechanisms of action underlying these ROCK inhibitors, albeit their structural differences.

Thirteen different ROCK inhibitors, five of which are commercially available and eight of which were newly designed and synthesized for this study, were used to treat human neuroglioma cell overexpression of 4-repeat tau (H4-tau) across a 96-hour time course, which was broken down into 8, 12-hour intervals: 12, 24, 36, 48, 60, 72, 84, and 96 hours. The ratio of Serine 396 phosphorylated tau (p-tau) to total tau was measured using ELISA at each time point. All drug treatments were compared against the corresponding time point for vehicle-treated cells. Fasudil was the only commercially available drug to decrease the p-tau to total tau ratio. Of note, Y27632 did not decrease this ratio. In addition, several of the novel ROCK inhibitors significantly decreased the p-tau to total tau ratio. Of these, T343 had the greatest significance. T299, another newly designed ROCK inhibitor, displayed no change in the p-tau to total tau ratio despite its similarity to T343 in ROCK-I and ROCK-II inhibition. With an increased dosage, Fasudil, T343, and Y27632 were found to decrease the p-tau to total tau ratio, while T299 was found

to increase the ratio. These findings detail several drugs with a shared target with differential effects on tau.

Phosphorylation of tau at Serine 396 decreases tau mobility and the ability of tau to bind microtubules, contributing the tauopathy of Alzheimer's disease. These results present a unique opportunity to utilize these molecules to dissect associated changes in p-tau to total tau ratio, and tailor drugs to more effectively target p-tau. The differential effects of ROCK inhibitors on the p-tau to total tau ratio, as well as on learning and memory on healthy animals, are compelling. Further research is necessary to parse out whether the effects of Fasudil on learning and memory are mediated through changes in p-tau to total tau expression, or through other on- or off-target effects.

Experimental Procedures

Drugs

Originally, 13 different ROCK inhibitors were screened. 8 of the 13 drugs were novel inhibitors designed at the Translational Genomics Research Institute and the rest were commercially available ROCK inhibitors. The drugs and their structures, molecular weights, and enzymatic inhibition of ROCK I and ROCK II are available in Table 1.

The top 4 candidates selected based on the first round of *in vitro* experiments were Fasudil, Y27632, T343, and T299. Fasudil and Y27632 are commercially available ROCK inhibitors, while T343 and T299 are novel ROCK inhibitors.

Cell Culture

H4-tau cells were used for this study. H4-tau cells are a standard use in the AD field as a model for tauopathy in AD. It is necessary to use this cell line to produce sufficient levels of tau and phosphorylated tau for detection of significant changes in those levels. Cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% geneticin (Invitrogen), and 25% HEPES (ThermoFisher). Cells were split at 80% confluency every two weeks until they were plated at $8x10^4$ cells per well in 6-well plates (surface area = $9cm^2$).

Protein extraction

Cells were lysed using 150ul of RIPA buffer and 10 ul Halt Protease Inhibitor Cocktail (Thermo Scientific). The plate was incubated on ice for one hour. Lysate was harvested into 1.5mL centrifuge tubes, and kept on ice. Each sample was then sonicated briefly at amp=1. Lysate was spun in the centrifuge at 10,000g for 15 minutes at 4C and supernatant was collected and transferred to a fresh tube, to be used in ELISAs.

Determining the LD-10 value

Viability assays were performed using a 96-well plate paradigm, with metabolic activity measured using alamar Blue (Invitrogen). 10% alamar Blue was added directly to H4-tau cells in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% Geneticin (Invitrogen), and 25% HEPES. This assay works based on the ability of metabolically active cells to convert alamar Blue reagent into a fluorescent signal, proportional to innate metabolic activity. The LD-50 value was determined using curve fitting statistics, and the LD-10 value was determined from there.

Performing a 96-hour time course

Each drug was applied to H4-tau cells in a 6-well plate. Cells were plated at $8x10^4$ cells per well in normal growth media as described in 2.2. After 24 hours, normal growth media was removed and growth media supplemented with drug at the LD-10 value was added. Eight wells were used for each drug, providing a well for each time point per drug. Every 24 hours, media was removed and the cells were washed with 1X PBS before new LD-10 dosed media was added. Every 12 hours, cells treated with each drug were lysed using RIPA buffer and protein was collected. There were three biological replicates for each drug and time point. Time points were as follows: 12 hr, 24 hr, 36 hr, 48 hr, 60 hr, 72 hr, 84 hr, 96 hr.

Enzyme-Linked Immuno Sorbent Assays (ELISAs)

The concentration of protein from cells treated with each drug at each time point was measured using a Bicinchoninic acid (BCA) kit (Pierce). Pre-coated 96-well ELISAs (Life Technologies) were used to measure the S396 (a form of p-tau found in higher concentrations in AD patients) and total tau concentration for each sample. 25000 pg/ul of protein was used for each well in the ELISA, and samples were run in triplicate.

Validation in H4-tau cells

The two drugs that had the most significant impact on the p-tau to total tau ratio (Fasudil and T343) were tested again to validate the results. In addition, two drugs that did not affect the p-tau to total tau ratio were re-tested. Y27632 was of interest due to previous

research stating that treatment using the drug decreased learning and memory in sham animals, which was contradictory to our previous results. T299 was of interest because it had a similar ROCK inhibition profile with T343, yet produced different results.

Three changes were made in the protocol. First, 4 biological replicates were used per drug treatment time point. Second, two time points were added previous to the 12-hour time point: 0 hours and 6 hours. Finally, the drugs were applied at their LD-50 dosages to ensure that the two negative drugs did not have an unnoticeable effect at the LD-10 dosage.

RNA collection and sequencing

The four drugs of interest selected during the protein stage (Fasudil, T343, Y27632, and T299) were used to treat H4-tau cells over a 36-hour time course at their LD-50 dosages. RNA was collected to be sequenced at the following time point: 0 hr, 3, hr, 6 hr, 12 hr, 24 hr, 36 hr. RNA was collected using Trizol and eluted to 20ul using RNAse free water. Concentrations of RNA were determined using Ribogreen (Invitrogen), and quality was assessed using 2100 Bioanalyzer (Agilent Technologies). The TruSeq RNA Sample Preparation Kit (Illumina Inc) was used to prepare samples for sequencing, and the final libraries were validated on the 2100 Bioanalyzer and quantified using qPCR (7900HT, Applied Biosystems). Libraries were sequenced by 100bp paired-end sequencing on a HiSeq2000 (Illumina Inc).

Calculation

To analyze results from ELISAs, two-way ANOVAs with factors 1) drug vs vehicle and 2) time, were used to determine significance for each drug across the time course.

Pairwise t-tests were performed in order to determine which of the drug treatments caused a significantly different p-tau to total tau ratio in comparison to vehicle treatment.

Analysis of RNA results were completed as follows: Illumina BCL files were converted and demultiplexed (bcl2fastq 2.170. Fastq files were trimmed of adapter sequences (Cutadapt 1.8.3) and aligned to the human reference genome GRCh37 (STAR 2.5.0). Sequencing and RNA quality control reports were generated (FastQC 0.11.4 and Qualimap 2.1.3). Aligned reads were summarized as gene counts (featureCounts 1.4.4). Pairwise differential expression was conducted between groups with the R package DESeq2 (v1.10.1).

Results

Enzymatic Activity

The enzymatic activity for Fasudil, Y27632, T343, and T299 are available in Table 1. Each number is the IC-50 value, or the amount of drug necessary to decrease ROCK I or ROCK II to 50% of the original amount.

Protein

ELISA results from protein collected from the cells were analyzed using a two-way ANOVA, for the ratio of ptau to total tau with variables of treatment and time. Treatment, time, and the interaction of treatment and time were found to be significant ($p = 2.2 \times 10^{-16}$, 1.8×10^{-8}, and 0.0014 respectively).

Of the 13 tested drugs, Fasudil ($p = 0.0003$; Fig. 4), T303 ($p = 0.02$), and T343 ($p = 0.01$; Fig. 6) were the only drugs that displayed a significant change in the p-tau to total

tau ratio (Fig. 1-3) using pairwise t-test. These drugs caused a significant decrease in the ratio. Surprisingly, Y27632 showed no significant difference across the time course (p=0.8) (Fig. 5). T299, a drug that has similar enzymatic activity levels for both ROCK I and II when compared to T343, also surprisingly showed no significant difference across the time course (p= 0.9) (Fig. 7).

ELISA results from H4-tau cells treated by either Fasudil, Y27632, T299, or T343 at the LD-50 were analyzed using a two-way ANOVA, for the ratio of p-tau to total tau with variables of treatment and time. Treatment, time, and interaction of treatment and time were found to be significant ($p = 5.3 \times 10^{-12}$, 9.6×10^{-6}, and 0.04 respectively). Pairwise t-test showed that the results were similar to the LD-10 dosage for Fasudil and T343, but not for Y27632 nor T299. Figure 9 shows that Y27632 decreased the p-tau to total tau ratio ($p = 0.0009$) and T299 increased the ratio ($p = 1.9 \times 10^{-7}$). Both Fasudil and T343 were validated as "positive" drugs that decreased the p-tau to total tau ratio in the cells. Fasudil was found to significantly decrease the p-tau to total tau ratio ($p = 0.001$), as was T343 ($p = 1.2 \times 10^{-7}$).

RNA

At every point of the RNA time course, Fasudil showed a significant decrease in MAPT expression (Figure 10). None of the other three drugs tested for changes in RNA expression showed such robust effects; however, there were additional expression changes that lasted through several time points (Table 2). For example, Fasudil showed a significant decrease in expression of SOX6 at timepoints 6 hours through 36 hours, and T299 showed a significant decrease in expression of ILII and ESM1 at timepoints 6 hours through 36

hours. It is also important to note that T343 showed a significant decrease in expression of GDA, which has been considered to play an important role in microtubule stabilization. All significant results for each drug treatment can be found in Table 4c.

Discussion

ROCK inhibitors did not produce the same effect on the ratio of p-tau to total tau in H4-tau cells. While a lack of significant difference in the p-tau to total tau ratio in cells treated by drugs at the LD-10 dosage was expected, as well as differences in effect size between different drug treatments, an opposite treatment effect with the treatment of T299 at the LD-50 dosage was unexpected.

Examining the IC-50, or the concentration of the drug at which the kinase response is reduced by half, all four of the drugs tested at the LD-50 dosage are similarly effective in inhibiting ROCK II. Fasudil is the least effective, with an IC-50 of 1.9uM, and T343 is the most effective with an IC-50 of 0.013uM. T299 and Y27632 have similar IC-50 for ROCK II, at 0.6uM and 0.3uM respectively. It is interesting that both Fasudil and T343 significantly decrease the p-tau to total tau ratio at the lower dosage of LD-10, even though Fasudil has the highest IC-50, indicating that it is the least efficient at inhibiting ROCK II. This cannot be explained by the IC-50 for inhibition of ROCK I, as Fasudil is again the least efficient of the four in inhibiting ROCK I at 10.7um. The other three drugs inhibit ROCK I with an IC-50 below 1uM. Finally, Y27632 seemed to have a dose-dependent molecular effect on the ratio of p-tau to total tau, with a trend toward an increased ratio with a low dose of LD-10, and a significantly decreased ratio with a high dose of LD-50. Along with the surprising increase of ratio with treatment by T299, these dose-dependent

differences beg the question of what mechanistically is occurring, possibly outside of the ROCK pathway, to cause these differences.

Next Generation Sequencing was performed on RNA from treated cells to determine which genes are controlled by the downstream targets of the ROCK inhibitors. We predicted that ROCK inhibition would create differences in gene expression in those genes, which may be related to the proteins that are known to be affected—i.e., MLC phosphatase, LIMK, PTEN, Akt, and cofilin. Results from Next Generation Sequencing also yielded interesting results, in that there were no transcriptional changes that were similar between Fasudil and T343, the two drugs that had caused the largest decrease in the p-tau to total tau ratio. This could indicate that the two drugs, while ROCK inhibitors with the same effect on p-tau, are not working through the ROCK pathway in order to produce those results. Due to the result showing that MAPT expression levels are significantly decreased over the entire time course when Fasudil is applied, it is highly likely that the change in phosphorylated tau is due to the changes in MAPT. Another gene expression change that is relevant to microtubules was GDA. GDA is a gene that has been associated with microtubule assembly, but has not been associated with AD. Application of T343 significantly decreased GDA across time points 3, 6, and 12. GDA could be the mechanism of action for changes in tau phosphorylation in cells treated by T343.

One approach that could better our understanding of the mechanisms through which these drugs are acting on the p-tau to total tau ratio is to perform kinase assays for proteins that are known to be involved in the phosphorylation of tau. This could provide information on the non-RNA molecular mechanisms through which each of the drugs is affecting, or not affecting, the ratio of p-tau to total tau. A more elegant, but costly, method would be to

use a mass proteomics approach, to determine whether there are changes in proteins outside of what we would expect as well.

CHAPTER 3

THE EFFECT OF ROCK INHIBITORS ON ALZHEIMER'S DISEASE: AN IN VIVO STUDY

Abstract

ROCK is an enzyme that plays a key role in neuronal cells. ROCK inhibitors Fasudil and Y27632 have been shown to increase learning and working memory in aged rats and rats with concussion-induced brain injury, respectively. Conversely, Y27632 has been shown to decrease learning and working memory in normal rats, exhibiting no pathology. Previous work with thirteen different ROCK inhibitors applied to human neuroglioma cells overexpressing 4-repeat tau (H4-tau) cells has shown that Fasudil and Y27632 decreases the ratio of phosphorylated tau to total tau in comparison to cells treated with vehicle. T343, a novel ROCK inhibitor, also decreased the ratio, while T299, another novel ROCK inhibitor with similar enzymatic inhibition, increased the ratio. With both behavioral and molecular data showing that ROCK inhibition has the potential for improving the outcome of Alzheimer's disease patients, we tested these drugs on triple-transgenic Alzheimer's disease mice. Fasudil, Y27632, T343, and T299 were tested on adolescent mice for sixty days to first determine whether the drugs, when mice are treated orally via drinking-water, would cross the blood-brain barrier and be present in the brain. All drugs but T299 conclusively reached the brain; however, only Fasudil and Y27632 were tested on a larger group of mice. T299 and T343 were intended to be used in a comparative study, and therefore, neither was used. The second group of mice tested were treated for 12 months beginning at 3 months of age, and tested on a series of learning and memory tests: the radial arm water maze, Morris water maze, and fear

conditioning test at 10, 12, and 15 months respectively. Fasudil treatment in wild-type mice showed a marked improvement in spatial reference memory at 10 months and fear memory at 15 months, while Y27632 treatment in AD mice showed a significant impairment in spatial reference memory at 10 months. Brains were dissected and tested for markers of Alzheimer's disease. Fasudil-treated mice showed decreases in Aß and in phosphorylated tau. Y27632-treated mice showed an increase in phosphorylated tau and Aβ. Thus, Fasudil continues to be a drug of interest in the treatment of Alzheimer's disease.

Experimental Procedures

Mice

The triple transgenic Alzheimer's disease (3xTg-AD) mice were generated as previously described (Oddo et al., 2003). Only female mice were used, as the neuropathologic variability in females is small relative to males. All mice were housed 4–5 per cage, kept on 12 hours light/dark cycle and given ad libitum access to food and water. Animal care and treatments were in accordance with the applicable regulations of the vivarium (The Institutional Animal Care and Use Committee of the Banner Sun Health Research Institute; The Institutional Animal Care and Use Committee of Arizona State Univeristy). 3xTg-AD mice were used along with C57BL6/129S mice (wild-type). In the pilot study, a total of 30 3xTg-AD mice were used. During the final study, a total of 43 3xTg-AD mice and 45 wild-type mice were used. For the course of the entire study, a total of 74 3xTg-AD mice and 45 wild-type mice were used.

During the pilot study, commercially available ROCK inhibitors Fasudil and Y27632 were used along with 2 novel ROCK inhibitors developed at TGEN—T343 and T299. However, for the final study, only Fasudil and Y27632 were used.

Pilot

3xTg-AD mice were treated with Fasudil, Y27632, T343, T299 or vehicle (25mg/L sucrose) through drinking water over the course of 60 days. Drinking water was freely provided and replaced twice weekly and in the case of empty bottles between bottle replacements. Drugs were added to the water at two dosages: 30mg/kg and 100mg/kg.

Water intake was monitored over the course of 60 days to ensure consumption levels and overall health in the mice. Mice were sacrificed after 60 days, and brain tissue was collected to test drug blood brain barrier crossing. The hippocampus was removed and fresh frozen, and prepared for HP-LC. Results from HP-LC were not completed in time for the start of the main study —however Fasudil and Y27632 have previously been shown to reach the brain in rodents at 30mg/kg; therefore these two drugs were used for the remainder of this study.

Treatment

3xTg-AD mice and wild-type mice were continuously treated with 30mg/kg Fasudil, Y27632, or vehicle (25mg/L sucrose) through drinking water starting at 3 months of age until 15 months of age. Mice were weighed weekly to ensure that treatment dosage remained accurate over time.

Behavior

Mice were tested on memory at three separate times, using three different memory tests. Mice were tested at 10, 12 and 15 months of age on the radial arm water maze, Morris water maze, and contextual fear conditioning test respectively.

Radial Arm Water Maze

The radial arm water maze, which tests spatial reference memory (Alamed et al 2006) was conducted at 10 months of age. The radial arm water maze apparatus consisted of eight identical arms, each with a slot for holding a clear plastic escape platform at the end. Each mouse had a randomly designated goal arm with the platform. On the first day, a flag was placed at the top of the platform during the first trial, and every other trial thereafter. Mice were randomly placed in each of the seven non-goal arms to start each trial. Extramaze cues are used by the mice to find the goal arm. Mice were given 60 sec to find the platform, with errors being considered as a full body entry into an incorrect arm. After 60 sec, if the mouse did not find the platform on its own, it was gently moved to the platform by the experimenter. Once on the platform, a 10 sec acclimation period to the location was given, followed by the placement in a heated cage for 20 sec. After six successive trials, mice were returned to their home cage until all experimental animals underwent their first set of six trials. Then, each mouse received another set of six successive trials. The flag was placed at the end of the goal arm during every other trial for the second set of trials as well. After each mouse received their second set of trials, the mice underwent a final set of three trials. The flag was not placed in the goal arm

during the third set of trials. On the second day, the mice underwent two sets of six trials and then a set of three trials, the same as the first day. However, on the second day, the flag was not placed in the goal arm at all.

Morris Water Maze

The Morris water maze consists of a circular plastic tank of 1.5 m in diameter filled with water and kept at 25°C. A platform (14 cm diameter) was placed 1.5 cm under the surface of the water and made invisible to mice by adding white nontoxic paint to the water. The water was maintained at 25°C throughout the duration of the testing. The tank was in a room with several extramaze visual cues, which served as reference points for mice. The location of the cues and platform were kept constant throughout the testing period. Mice were trained to find the hidden platform for 5 consecutive days, four training trials per day. Before the first trial of the first day, mice were placed on the platform for 10 seconds, after which they were placed in the water until they reached the platform for a maximum of 60 seconds. When a mouse found the platform, it was placed in a warm holding cage for 25 seconds before starting the next trial. If a mouse failed to find the platform in 60 seconds, it was gently guided to the platform location by the experimenter and allowed to stay on it for 10 seconds after which it was placed in the warm holding cage. Extreme care was taken to minimize animal stress during these procedures. Spatial memory was assessed during a probe trial, conducted 24 hours after the last training trial. During the probe trials, the platform was removed from the water and mice freely swam in the tank for 60 seconds. The entire test was recorded with a

video camera that was mounted on the ceiling. Data were obtained using specialized tracking software (Noldus Information Technology, Leesburg, VA, USA).

Fear Conditioning

The contextual fear conditioning (CFC) test was performed using an apparatus from San Diego Instruments. On day 1, mice were placed in the conditioning chamber and allowed to freely explore for 210 seconds, at which point, a foot shock was administered (0.3 mA, 2 s). After the shock, mice remained in the chamber for an additional 30 seconds. On day 2 (24 h after training), mice were placed back in the chamber for 4 minutes. Freezing, defined as the complete lack of motion, except breathing, for a minimum of 2 seconds, was recorded and analyzed by Freeze Monitor software (San Diego Instruments). The percent of freezing during each testing trial was reported.

Sacrifice

After the contextual fear conditioning test was performed, mice were sacrificed. Mice were sedated by CO2. Mice underwent cervical dislocation and heads were removed using scissors. The brain was removed and sliced sagittaly. Half of the brain was placed into 4% paraformaldehyde (PFA) for 48 hours. PFA was replaced using PBS with 0.02% sodium axide, to be used for histology. The hippocampus was removed from the other half of the brain and placed on dry ice to fresh freeze the tissue. The hippocampus was lysed and protein extraction was used to perform western blots and ELISAs.

Molecular

Protein extraction

Mice were sacrificed by CO2 asphyxiation, their brains removed and sagittally bisected. Half of the brain was drop-fixed in 4% paraformaldehyde and used for histological and immunohistochemical experiments. The hemibrain or different brain areas, such as hippocampus and cortex, were collected and stored at -80°C until use. Frozen brain areas (hemibrain without cerebellum, hippocampus or cortex) were processed as described previously (Caccamo et al., 2013). Briefly, brains were homogenized in a solution of tissue protein extraction reagent (T-PER, Pierce) containing 0.7 mg/mL of pepstatin A supplemented with a complete mini protease inhibitor tablet (Roche Applied Science) and phosphatase inhibitors (Invitrogen). The homogenized mixtures were centrifuged at 4°C for 1 hour at 100,000 g, and the resulting supernatant was stored as the soluble fraction. The pellet was re-homogenized in 70% formic acid and centrifuged as described above. The supernatant was stored as the insoluble fraction.

Western Blot

Proteins from the soluble fraction were resolved by 10% or 4-12% BisTris SDS polyacrylamide gel electrophoresis (Life Technologies) under reducing conditions and transferred to a nitrocellulose membrane. Membranes were developed as described previously (Orr et al., 2014). Briefly, membranes were incubated for 1 hour in 5% nonfat milk in TBST (0.1% Tween 20, 100 mM Tris, pH 7.5; 150 mM NaCl) and then incubated overnight in primary antibody.

The blots were rinsed in TBST for 30 minutes and then incubated in goat anti-mouse IRDye 680LT or goat anti-rabbit IRDye 800CW LI-COR secondary antibodies (1:10000) for 1 hour at room temperature. The membranes were rinsed for 30 minutes in TBST, imaged, and analyzed using the Odyssey (LI-COR, Lincoln, NE, USA). The protein levels reported were obtained as a ratio between the band intensity of the protein of interest and the band intensity of β-actin or GAPDH, used as loading control.

Western Blot Antibodies

Actin was used as a loading control. Western blots were used to measure levels of total tau, tau phosphorylated at Ser 202, full length APP, and the APP c terminal. The antibodies used to measure these levels were HT7 (Thermo Fischer), CP13 (Cell Signaling), 6E10 (BioLegend), and CT20 (Millipore) respectively.

Immunohistochemistry

Hemibrains were drop fixed in 4% paraformaldehyde in phosphate-buffered saline for 48 hours and then transferred into 0.02% sodium azide in phosphate-buffered saline until slicing; 50-μm-thick free-floating sections were subsequently obtained using a vibratome.

Sections were washed twice with TBS (100 mM Tris pH 7.5, 150 mM NaCl), 5 minutes each, followed by a 30-minute incubation in 3% H2O2 to quench endogenous peroxidase activity. Next, sections were transferred into TBS-A (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and TBS-B (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 2% bovine serum albumin) for 15 and 30 minutes, respectively, to

block nonspecific binding. Finally, the proper primary antibody was applied overnight at 4°C; antibody dilution was made in TBS-B.

Sections were washed 3 times in TBS in order to remove excess antibody, and incubated in the suitable secondary antibody for 1 hour at 25°C. Signal was enhanced by incubating sections in the avidin-biotin complex (Thermo Scientific) for 1 hour at 25°C. Sections were then washed and developed with diaminobenzidine substrate using the avidin– biotinhorseradish peroxidase system (Vector Labs). Images were obtained with a digital Zeiss camera and analyzed with ImageJ software. Quantification of staining was achieved using pixilation detection acquired by ImageJ. A threshold was set using a positive control and a standard mean gray area function, which allowed the set software to recognize positive staining and decrease error caused by background staining. Abeta42 (Millipore) was used to visualize $Aß_{42}$. AT8 (Fisher) used to visualize tau phosphorylated at Thr205/Ser202.

Enzyme-linked immunosorbent assay

ELISAs were performed using Invitrogen's prepared $Aß_{40}$ and $Aß_{42}$ plates. ELISAs were performed on both soluble and insoluble fractions of protein extracted from the hippocampus.

Statistical Analyses

All data were analyzed using R. Data were analyzed using one- or two-way ANOVA followed by Bonferroni's post hoc analysis or Dunnet's multiple comparison

tests when applicable. Selective experiments were analyzed using Student's t-test, as specified in the results section.

Results

Pilot

Over the course of 60 days, none of the treated mice suffered adverse effects, other than weight loss in the Y27632 treated mice. These mice were not drinking the treated water in the beginning of the study. After sucrose was added to each treatment including Y27632, the mice began drinking the treated water, and regained weight and health. While the Y27632-treated mice did not receive the same amount of drug during the beginning of the pilot, this was resolved by the time the main study began with a new set of mice. All groups of mice drank similar amounts of water after the sucrose was added. No mice died before the end of the 60 days.

Behavior: Radial arm water maze

Total Errors

Fasudil-treated wild-type mice committed fewer total errors than vehicle-treated mice ($p = 0.01$) on Day 2 (Figure 12). Fasudil-treated 3xTg-AD mice did not have a significantly different number of total errors than vehicle-treated mice ($p = 0.22$) on Day 2 (Figure 11). Y27632-treated wild-type mice did not have a significantly different number of total errors than vehicle-treated mice ($p = 0.35$) on Day 2 (Figure 14). Y27632-treated 3xTg-AD mice committed more total errors than vehicle treated mice ($p = 0.016$) on Day 2 (Figure 13).

Pairwise t-test were used to determine whether there were significant differences between treatment groups during each individual trial—the only significant differences were found between Y27632-treated and vehicle-treated 3xTg-AD mice during trials 18 ($p = 0.00045$) and 29 ($p = 0.0028$).

There were no significant differences between groups on Trial 16, which measures overnight retention (Figure 19).

Re-entries

The number of re-entries into incorrect arms was not significantly different between any drug-treated and vehicle-treated groups on Day 2 (Figures 14-17). However, pairwise t-test was used to determine whether there were significant differences between groups at each individual trial, and some differences were found. Fasudil-treated 3xTg-AD mice had significantly fewer re-entries during trial 21 ($p = 0.0042$) in comparison to vehicle-treated mice, and Y27632-treated 3xTg-AD mice had significantly more re-entries during trials 18 ($p = 0.00014$), 23 ($p = 0.026$), and 29 ($p = 0.022$).

Time

Across all points, 3xTg-AD mice treated with Y27632 took significantly longer than vehicle-treated 3xTg-AD mice ($p=0.012$) to reach the platform. Fasudil treated 3xTg-AD mice did not take a significantly different amount of time to reach the platform in comparison to vehicle-treated mice ($p=0.53$). Fasudil treated wild-type mice performed significantly better than vehicle-treated wild-type mice ($p<0.001$). Y27632-treated wild-

type mice did not perform significantly differently in comparison to vehicle-treated mice (p=0.59).

On the first trial of the second day, 3xTg-AD mice treated with Y27632 performed significantly worse than untreated wild-type (p=0.02). While the untreated 3xTg-AD mice were not significantly different from the untreated wild-type mice, this shows that the Y27632 treatment decreased performance further than pathology alone.

Behavior: Morris Water maze

On Days 1 and 2, there were no significant differences in distance swam on the MWM between treatment groups. On Day 3, Fasudil-treated 3xTg-AD mice trended toward a decreased distance swam (p = 0.069) and Y27632-treated 3xTg-AD mice had a significantly decreased distance swam (p = 0.04) in comparison to vehicle-treated mice (Figure 20). On day 4, Y27632-treated wild-type mice swam a significantly shorter distance than vehicle-treated mice (p = 0.005, Figure 21).

Probe trial

The number of times the Fasudil-treated 3xTg-AD mice (M = 1.75) entered the platform area was not significantly different from vehicle-treated mice (M = 1.1; t = 1.017, df = 19.678, p = 0.3). The number of times Y27632-treated 3xTg-AD mice (M = 1) entered the platform area was not significantly different from vehicle-treated mice (t = -0.187, df = 15.265, p = 0.9). The number of times Fasudil-treated wild-type mice (M = 2.1) entered the platform area was not significantly different compared to vehicle-treated mice (M = 3.8; t = 2.055, df = 19.052, p = 0.054). The number of times Y27632-treated

wild-type mice (M = 3.5) entered the platform area was not significantly different from untreated wild-type mice (t = 0.279, df = 26.799, p = 0.8) (Figure 24).

The amount of time, within the 60 second timeframe allotted to each mouse, that each mouse spent in each of the quadrants was also measured. The time spent in the quadrant the platform was previously located, as well as the time spent in the quadrant opposite the platform quadrant, was analyzed. Neither the Fasudil-treated nor Y27632-treated 3xTg-AD mice spent a significantly different amount of time in the platform quadrant nor in the opposite quadrant, in comparison to the vehicle-treated 3xTg-AD mice (Figure 25, 27). The same null results between drug-treated and vehicle-treated mice were found in the wild-type mice (Figure 26, 27).

Overnight retention

There was no significant difference in the distance swam on the final trial of each day in comparison to the distance swam on the first trial of the next day (i.e. the final trial of Day 2 in comparison to the first trial of Day 3), for any treatment group for either 3xTg-AD mice (Figure 22) or wild-type mice (Figure 23).

Behavior: Fear Conditioning

Fasudil-treated 3xTg-AD mice showed significantly increased freezing levels when compared to vehicle-treated 3xTg-AD mice (t(393.82) = 3.23, p = 0.0013) and vehicle-treated wild-type mice (t(441.07) = 3.23, p = 0.02) on the second day (Figure 28, 29). Y27632-treated and vehicle-treated 3xTg-AD mice did not have a significantly different freeze duration (t(172.52) = 0.41, p = 0.68) on the second day (Figure 30, 31).

Western Blot

The antibody 6E10 was used to measure total APP. The expression of 6E10 was significantly decreased in Fasudil treated mice compared to vehicle treated animals ($t(5.35) = 4.63$, $p = 0.0048$). 6E10 expression was not significantly different in Y27632 treated mice in comparison to vehicle treated mice (Figure 37). CP13 was used to measure tau phosphorylated at Ser202, one of the abnormal phosphorylation sites for AD patients. HT7 was used to measure total tau. CT20 was used to measure APP C-terminal fragments. CT20 (Figure 38), CP13 (Figure 40), and HT7 (Figure 39) expression was not changed in either Fasudil- nor Y27632- treated groups in comparison to vehicle-treated mice. All samples showed similar actin expression levels.

Further analysis was necessary for the ratio of ptau to total tau. The ratio of CP13 expression and HT7 expression was used to determine the ratio of ptau to total tau in the 3xTg-AD mice. Fasudil treatment elicited no changes in the ratio; however Y27632-treated mice had a significantly higher ratio than vehicle-treated mice ($t(6.0774) = -2.4837$, $p = 0.047$) (Figure 41).

ELISA

Treatment with Fasudil in the 3xTg-AD mice showed a significant decrease in Aß-40 in both soluble ($t(45.5) = 2.3$, $p = 0.03$) (Figure 44) and non-soluble ($t(40.91) = 2.8$, $p = 0.008$) (Figure 42) layers compared to vehicle treatment. Aß-42 levels were not significant different in either layer (Figure 43, 45). Treatment with Y27632 in the 3xTg-AD mice did not have a significant effect on Aß-40 in either layer, or Aß-42 in the non-

soluble layer. However, in the soluble layer, Y27632 treatment significantly increased the level of Aß-42 ($t(42.1) = -3$, $p = 0.005$).

Immunohistochemistry

In the hippocampus, Fasudil-treated mice (M = 65700.36) did not have significantly different levels of $A\beta_{42}$ staining in comparison to vehicle-treated mice (M = 82259.94), although there was a trend toward decreased staining ($p = 0.055$). Y27632-treated mice (M = 70657.13) did not have significantly different levels of $A\beta_{42}$ (Figure 46). In the cortex, neither drug treatment (Fasudil M = 48387.68; Y27632 M = 35736.6) produced a significantly different level of $A\beta_{42}$ in comparison to vehicle treatment (M = 45953.82; Fasudil: $t = -0.202$, $df = 9.245$, $p = 0.844$; Y27632: $t = 1.254$, $df = 7.505$, $p = 0.247$) (Figure 48).

Fasudil-treated mice (M = 14673.07) had significantly decreased AT8 staining than vehicle-treated mice (M = 23187.98; $t = 4.354$, $df = 4.758$, $p = 0.0082$). Y27632-treated mice (M=21400.11) and vehicle-treated mice did not have a significantly different amount of AT8 staining ($t = 0.778$, $df = 5.552$, $p = 0.4683$) (Figure 50).

Discussion

In the radial arm water maze, the number of errors is indicative of working memory, while the amount of time to reach the platform is indicative of reference memory. Working memory and reference memory are two of many types of memory affected in AD. Working memory is the mental processing system that is used to hold and manipulate transitory information to guide behavior. In the radial arm water maze, after a

mouse makes an error, working memory would be used to avoid making that error again. The total number of errors is used to measure working memory. If a mouse continually makes the same errors, it is likely that the mouse is not holding and manipulating the information about the location of the platform. Our findings show that neither of the treatments made a significant difference in the number of re-entries into incorrect arms, for either 3xTg-AD nor wild-type mice in comparison to their respective vehicle-treated controls. This indicates that working memory is not affected by Fasudil or Y27632 treatment at 10 months of age at this dosage.

Reference memory is learned knowledge that can be used throughout the behavioral task, and is correlative to human semantic memory. Semantic memory is a type of long-term memory that is considered common knowledge. While the location of the platform is not considered common knowledge, reference memory is as similar as we are able to measure in mice. The time that the mouse takes to reach the platform is measured to analyze reference memory because if a mouse remembers that the platform is in the direction of a specific extra maze cue, the mouse has consolidated the information from prior trials. The ability to remember this information should decrease the amount of time that it takes the mouse to reach the platform. Our findings indicate that Fasudil treatment in wild-type, but not 3xTg-AD mice, improves reference memory at 10 months of age at this dosage. However, Y27632 treatment in 3xTg-AD, but not wild-type mice, impairs reference memory at 10 months of age at this dosage.

Y27632 also caused 3xTg-AD mice to perform worse on reference memory in overnight retention trials. Overnight retention is a better indicator of long-term memory due to the longer period of time between trials. Y27632-treated mice may be utilizing

working memory to reach the platform at a faster time in subsequent trials during the second day.

The Morris water maze tests for reference memory as well. The Morris water maze was used in order to create a battery of tests to better understand the effects of the drugs on memory, and because the Morris water maze is a classic task used to test memory in AD rodent models. There was a lack of significant results, both for Fasudil-treated and Y27632-treated mice in comparison to vehicle-treated mice, in both the AD model as well as the wild-type mice. These results suggest that neither treatment had an effect on memory at 12 months of age and with the dosage used. However, as we did see an effect on spatial reference memory at 10 months of age with the use of the radial arm maze, it is possible that the lack of effect is due to practice effects, or due to a difference in sensitivity between the tasks. Also, only on Day 3, Y27632 treatment in 3xTg-AD mice decreased overall swim distance significantly, and Fasudil treatment in 3xTg-AD mice showed a trend toward decreased swim distance. While these results are not seen on other days, it is possible that treatment could have an effect with higher dosage, or that a task with a higher sensitivity to different effects would have shown a significant improvement. Finally, if mice were not previously tested on spatial reference memory, it is possible that a lack of practice effect may have produced significant results at 12 months of age. Future work could include groups that are tested only on at one of the three time points, in order to minimize these effects, and could use the same task for each of the groups, and therefore each time point.

Overnight retention in the Morris water maze task was measured by comparing the final trial of one day to the first trial of the next day, for each day. There were no

significant differences in vehicle-treated 3xTg-AD mice, indicating that we were not able to show that overnight retention was impaired. Therefore, while neither Fasudil nor Y27632 treatment in 3xTg-AD mice led to an impairment in overnight retention, we are unable to determine whether this was due to the drug or due to an issue with the behavioral task. However, while there was no significant difference between both Fasudil- or Y27632-treated and vehicle-treated 3xTg-AD mice in time spent in the platform area during the probe trial, there seems to be a trend toward increased time in the platform area in comparison to the vehicle-treated mice, signifying that both treatments may be improving reference memory slightly.

Western blot results for Fasudil-treated mice show no difference in the expression of p-tau, or the ratio of p-tau to total tau. Meanwhile, immunohistochemistry results show a significant decrease in the expression of p-tau. This is likely due to differences in antibody—the western blot antibody measured tau phosphorylated at Ser202, while the immunohistochemistry antibody measured tau phosphorylated at both Ser202 and Thr205. Different antibodies were used due to their efficiency in the particular molecular tests they were used for. Y27632 treatment resulted in an increase in p-tau, as seen in the western blot, but not with immunohistochemistry. Thus, it seems that Fasudil and Y27632 treatment may decrease tau phosphorylation at Thr205, as Y27632's effect on Ser202 was muted. Increased phosphorylation at both sites are associated with an increase in abnormal tau processing and paired helical filaments, in the pathogenesis of AD as well as in other tauopathies.

While no memory tasks were conducted at 4 months of age, the first point of significant cognitive deficits, it would have been informative to do so, and should be

tested in the future. The improvements seen in fear memory in the 3xTg-AD mice may have been due to a shift in decreased cognition, rather than in an overall improvement in cognition. The treatment with Fasudil may have slowed down the progression of the disease, rather than halting it, as we did not see improvements in spatial memory at 10 or 12 months of age. Improvements in pathology were also noted in both tau phosphorylation and levels of $A\beta_{40}$; however, this could also be due to a shift in AD pathology. Future work could further parse this out by adding sacrifice time points at 9 months of age (significant amyloid pathology) and 12 months of age (significant early tau pathology).

In conjunction with the results from the *in vitro* study, the results indicate that Fasudil decreases phosphorylation of tau. Y27632 treatment *in vitro* is also consistent with the results produced *in vivo*. While *in vitro* results for a high dosage of Y27632 resulted in a decrease in the ratio of p-tau to total tau, a low dosage resulted in no significant difference, but with a trend toward an increased ratio. Thus, we hypothesized that there may be a dose-dependent difference in the molecular mechanisms of the drug, and its effect on p-tau. The dosage used for the *in vivo* study was on the low end of the dosages previously used in mice receiving drug orally. It is possible that the same mechanisms that caused a trend toward increased p-tau to total tau ratio *in vitro* also caused a significant increase in the p-tau to total tau ratio *in vivo*.

CHAPTER 4

CONCLUDING REMARKS

AD is the most common subset of dementia, affecting an estimated 5.4 million Americans in 2015 (Gaugler et al., 2016). An estimated 236 billion dollars was spent on AD in 2016. Because patients in the final stages of AD are bedbound and incapable of caring for themselves, they require around-the-clock care, creating a large need for caregivers. This can mean high costs both financially and in time spent away from work for family members of those with AD. While some risk factors, such as carrying the APOE-e4 allele, have been identified, there have been no identifiable early biomarkers of the disease in non-familial AD. Further, no effective treatment options for AD are available, with the only therapeutics currently available alleviating symptoms for short periods with no effect on the pathology of the disease. It is critical to find early biomarkers and to develop a reliable and effective therapeutic to prevent and treat AD. This report identifies the current state of biomarker research as well as the potential for ROCK inhibitor Fasudil as an AD treatment that affects the neuropathology of AD.

Our early results are particularly interesting due to previous studies that have shown conflicting outcomes in learning and memory through the use of different ROCK inhibitors. While Fasudil has been shown to improve learning and memory in aged rats, for example, Y27632 has been shown to lead to decreased scores on learning and memory tests in normal rats. However, Y27632 has also been shown to increase learning and memory in rats that have ischemia. Our study adds to complex understanding of how these drugs affect cognition and elucidates underlying molecular pathways in learning and memory.

While the H4-tau cells used for this study are convenient for studying the effect of drugs on p-tau and total tau levels due to elevated levels of tau protein, the cells do not molecularly or functionally express similarly to AD neurons. Not only do the cells lack an overexpression of Aβ, the tau phosphorylation can also be called into question. First, the cells overexpress 4-repeat tau, which is a non-physiological condition. Second, the overexpression of tau can create the possibility of a cytoplasmic pooling of tau not bound to microtubules. This could lead to normally protected phospho-epitopes becoming exposed and hyperphosphorylated. Further investigation is necessary, not only to determine the cause of the difference in ROCK inhibitor treatments on the p-tau to total tau ratio, but also to ensure that these differences and changes are seen in AD neurons as well. Future *in vitro* work will include molecular and morphology studies using cells more similar to AD neurons than H4-tau cells, such as primary neuronal cultures from 3xTg-AD mice.

While others have studied the effects of Fasudil or Y27632 on tau or amyloid pathology, or learning and memory behavior, ours was the first to study two different ROCK inhibitors on an animal model, and to examine the drugs' effects on both tau and amyloid pathology. This is the first study to create a comprehensive overview of the different effects of Fasudil and Y27632 in an AD mouse model. This study was also one of the first studies to test possible preventative therapeutics for AD with a chronic time course, beginning prior to both pathology and significant cognitive deficits, and continuing treatment and testing until old age. This study also used drinking water to treat mice orally, rather than through injections or other methods that may not be as accessible to patients with AD, if the treatment were to succeed. These methods are important, as acute high

dosage treatments injected into the bloodstream are not the treatment method that will likely be used for AD patients; yet many studies use this paradigm to test treatments *in vivo*.

Non-steroidal anti-inflammatory drugs have been shown to decrease Aß, and it has been proposed that the pathway through which this occurs is the downregulation of the Rho-GTPase and downstream effectors, ROCK I and ROCK II. Application of ROCK inhibitor SR3677 *in vitro* has shown a marked decrease in Aß production and levels (Herskowitz et al., 2013). Fasudil treatment in rats injected with $Aß_{42}$ improved learning and memory and reduced neuronal loss and injury (Song et al., 2013). The current study confirmed these findings—hippocampal tissue from Fasudil-treated mice had significantly decreased $Aβ_{40}$ levels, as well as significantly reduced expression of APP, in comparison to vehicle-treated mice.

Recent research has shown that ROCK I and ROCK II proteins are increased in AD patients as well as patients with mild cognitive impairments (Henderson et al., 2016; Herskowitz et al., 2013). However, our work also shows that there was no difference in the transcription of either ROCK I or ROCK II in AD patient samples in comparison to non-AD samples (Figure 49). In conjunction, this supports the theory by Henderson et al., that the changes in ROCK protein levels are due to the increase in Aß oligomers. Depletion of ROCK I *in vitro* was found to decrease levels of APP, sAPPα, and $Aß_{40}$. ROCK I knock-down mice were also found to have decreased levels of $Aß_{40}$ (Henderson et al., 2016). However, other research shows an increase in $Aß_{40}$ *in vitro* with the depletion of ROCK I (Herskowitz et al., 2013). Our research does not use full depletion of ROCK I or ROCK II, but does show a decrease in both $Aß_{40}$ in the hippocampus in Fasudil-treated 3xTg-AD

mice, confirming the results of Henderson et. al, but also shows an increase in $A\beta_{42}$ with Y27632 treatment.

Our work *in vitro* showed a significant decrease in the ratio of p-tau to total tau using Fasudil. We hypothesized that this would translate to a decrease in p-tau and the p-tau to total tau ratio *in vivo* in animals treated with Fasudil, as well as to an improvement in learning and memory results through behavioral testing. However, in our present study, while Fasudil significantly reduced tau phosphorylated at residues Ser202 and Thr205, Fasudil treatment did not significantly improve learning and memory on all behavioral tasks. Fasudil did, however, improve spatial references memory in wild-type mice, and improved contextual fear memory in both 3xTg-AD and wild-type mice. It is possible that an increased dosage would further improve cognition.

Meanwhile, *in vitro* work showed that Y27632 decreased the p-tau to total tau ratio, but only with a higher dosage, and we hypothesized that this would translate to p-tau levels that were lower than vehicle-treated mice but higher than Fasudil-treated mice, and that there may be no change in learning and memory results. However, Y27632-treated mice exhibited a higher ratio of p-tau to total tau than vehicle-treated mice. The results from behavioral testing showed no changes in learning and memory, save for a worsening in the radial arm water maze at 10 months. These results could be due to the use of a low dosage of Y27632, which was shown to trend toward an increased p-tau to total tau ratio *in vitro*. A comprehensive summary of behavioral and molecular results for the *in vivo* study is available in Table 3.

It is possible that the dosage of Fasudil in the present study was not strong enough to have a significant effect on learning and memory, but was strong enough to elicit changes

in tissue pathology, and to show a significant difference in learning only at the end-stages of the disease. Fear conditioning is also considered a very sensitive test, and may have been more capable of indicating an improvement than less sensitive tests. Further work should be considered to increase the dosage safely, to test whether Fasudil is able to decrease learning and memory issues in AD mice.

This study attempted to discern whether Fasudil or Y27632 could be used as a preventative therapeutic, so the mice were treated beginning prior to the pathology onset. We are not able to determine whether the changes in tau pathology or total APP were due to consistent and early intervention, or due to the drug being administered at all. It would be interesting to test whether the same results can be obtained by treating mice with higher dosages after pathology onset, and for shorter duration. Further, in this study, drug was administered via drinking water. Since this study began, there have been improvements in technology of osmotic infusion pumps, such that the longest study using a pump has been 7 months, with replacement every 4 weeks. Future work could use these infusion pumps to better regulate and control the amount of drug administered to mice, to ensure consistency in treatment.

Dosage is not the only variable that could have an effect on the cognitive and pathological outcome of treatment. Timing may also play a key role. While we began treatment at 3 months of age in order to treat preventatively, this may not have been early enough to use as a preventative therapeutic. 3xTg-AD mice have significant cognitive deficits beginning at 4 months of age. This means that there could already be some cognitive impairment prior to 4 months of age, even if it is not significant. If this is the case, our preventative treatment may be too late. In humans, dementia onset occurs two

decades after the first neuritic plaque; in 3xTg-AD, cognitive impairments precede pathology. Thus, it is difficult to time the treatment in such a way that mimics the treatment paradigm we aim for. However, beginning treatment earlier, such as 1 month of age, may allow us to begin treatment significantly prior to the onset of cognitive impairment.

Here, we provide results from a preventative therapeutic paradigm, using two different ROCK inhibitors to treat 3xTg-AD mice and wild-type mice. Fasudil treatment resulted in significantly decreased amyloid and tau AD pathology, two hallmarks of the disease. Fasudil treatment also resulted in improved spatial reference memory at 10 months of age and contextual fear memory at 15 months of age in wild-type mice, and improved contextual fear memory in 3xTg-AD mice. Y27632 treatment, on the other hand, resulted in increased amyloid and tau AD pathology load, along with spatial reference memory impairment at 10 months of age in 3xTg-AD mice. While results from Fasudil treatment are promising, some changes in dosage and timing, along with future research on other ROCK inhibitors, will further provide clarity regarding the mechanisms through which these drugs may work to prevent and treat AD.

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APPENDIX A

TABLES

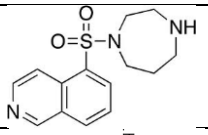
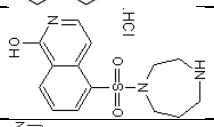
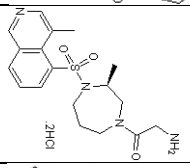
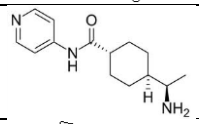
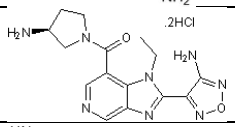
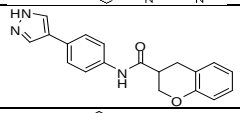
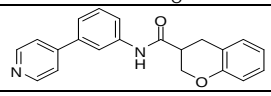
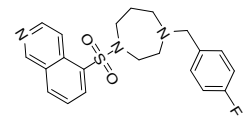
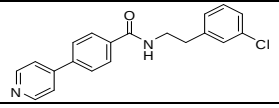
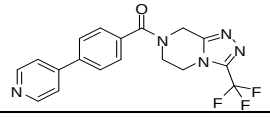
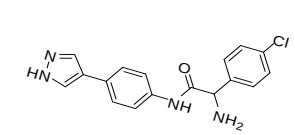
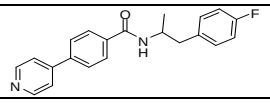
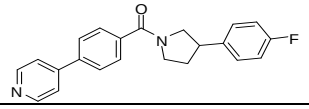
Drug	Structure	Molecular weight	ROCK I IC-50 (uM)	ROCK II IC-50 (uM)
Fasudil			10.7	1.9
HA1100			0.9	1.9
H1152			0.002	0.012
Y27632			0.14	0.3
SB77207B			0.006	0.006
T299		319.36	1.675	0.115
T303		330.38	2.955	0.665
T306		399.48	6.845	3.735
T343		336.81	0.027	0.013
T347		373.33	Not Available	Not Available
T349		326.78	Not Available	Not Available
T350		334.39	Not Available	Not Available
T355		346.4	Not Available	Not Available

Table 1. Structures, molecular weights, and kinase inhibition profiles, as available, for each of the ROCK inhibitors that was used during the study. All T- drugs are novel ROCK inhibitors synthesized at TGen.

Commercially available drugs	p-value	Novel drugs	p-value
Fasudil	0.000043	T303	0.007
H1152		T343	0.003
Y27632	0.75	T299	0.9
HA1100	0.6	T306	0.2
Harmine	0.1	T347	0.5
SB77	0.6	T349	0.8
		T350	0.09

Table 2. The difference between the p-tau to total tau ratio of H4-tau cells treated with each drug at LD-10 dosage in comparison to H4-tau cells treated with vehicle over a 96 hour time course. The p-value is determined via a pairwise t-test.

	Fasudil	Y27632	T343	T299
3, 6, 12, 24, 36 hr	MAPT			
6, 12 24, 36 hr	SOX6			IL11, ESM1
12, 24, 36 hr	IGFBP3, TMEM100, PTGER4			SPRY2, SLC16A6, IL24, IHHBA
3, 6, 12 hr			AHRr, SERPINB2, TFAP2C, GDA	
3, 6, 12, 24 hr			CPY1B1	
6, 12, 36 hr		ANKRD1, CPA4		

Table 3. Significant changes in expression levels in H4-tau cells after treatment with ROCK inhibitors Fasudil, Y27632, T343, or T299. Time points included in the study are 3, 6, 12, 24, and 36 hours.

	Fasudil – 3xTg	Y27632 – 3xTg	Fasudil – Wild-type	Y27632 – Wild-type
Radial arm water maze (10 months)	No change	Reference memory impaired	Reference memory improved	No change
Morris water maze (12 months)	No change	No change	No change	No change

Fear Conditioning (15 months)	Increased freezing	No change	Increased freezing	No change
p-tau	No change – WB Significantly better – IHC	No change	N/A	N/A
Aβ	Significantly better – ELISA No change – IHC	Significantly worse, soluble $A\beta_{42}$ only – ELISA No change – IHC	N/A	N/A

Table 4. The summary of results for the *in vivo* experiments. Overall, Fasudil treatment improved learning and memory results in wild-type mice. Fasudil treatment did not lead to changes in learning and memory in 3xTg-AD mice until the final behavioral experiment. Y27632 treatment did not cause a change in learning and memory in either 3xTg-AD mice nor in wild-type mice. Fasudil treatment decreased both p-tau and Aβ, although results vary based on testing method. Y27632 treatment did not change p-tau or Aβ levels, except for soluble $A\beta_{42}$ levels, which were decreased.

APPENDIX B

FIGURES

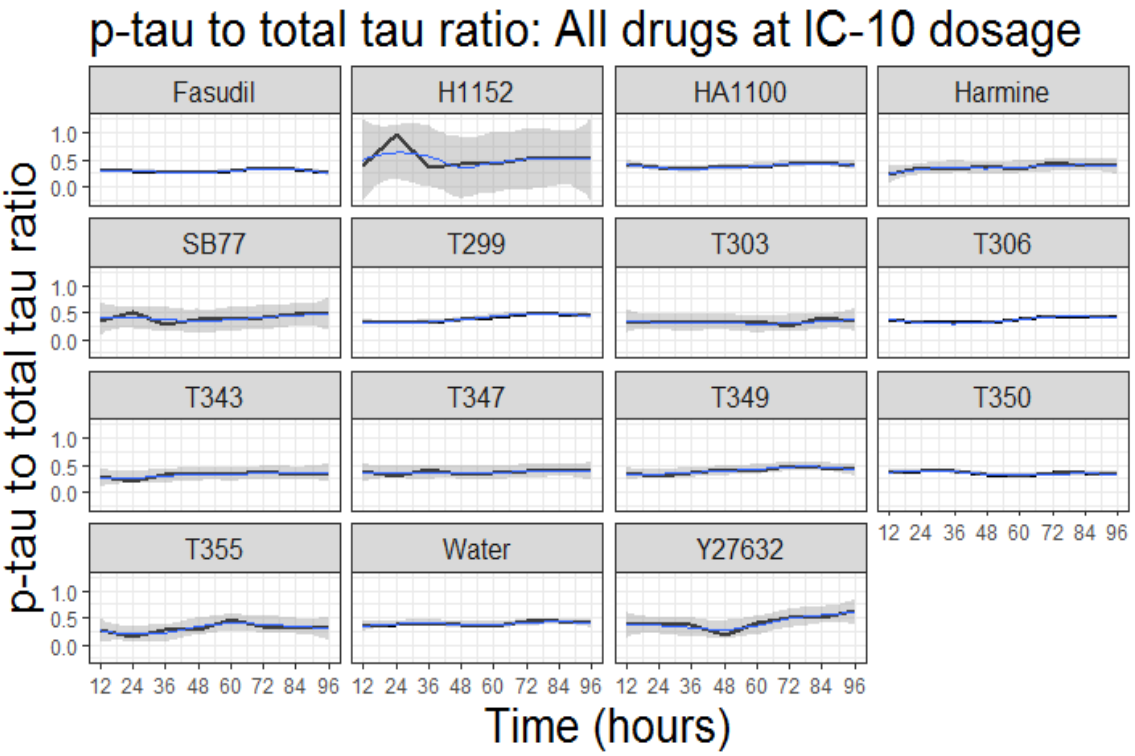


Figure 1. The ratio of p-tau to total tau for each drug, and water, treatment across all time-points. The water treatment led to a fairly stable ratio nearing 0.5, which is similar to many of the drug treatments. Notably, Fasudil and T343 remain closer to a ratio of 0.4.

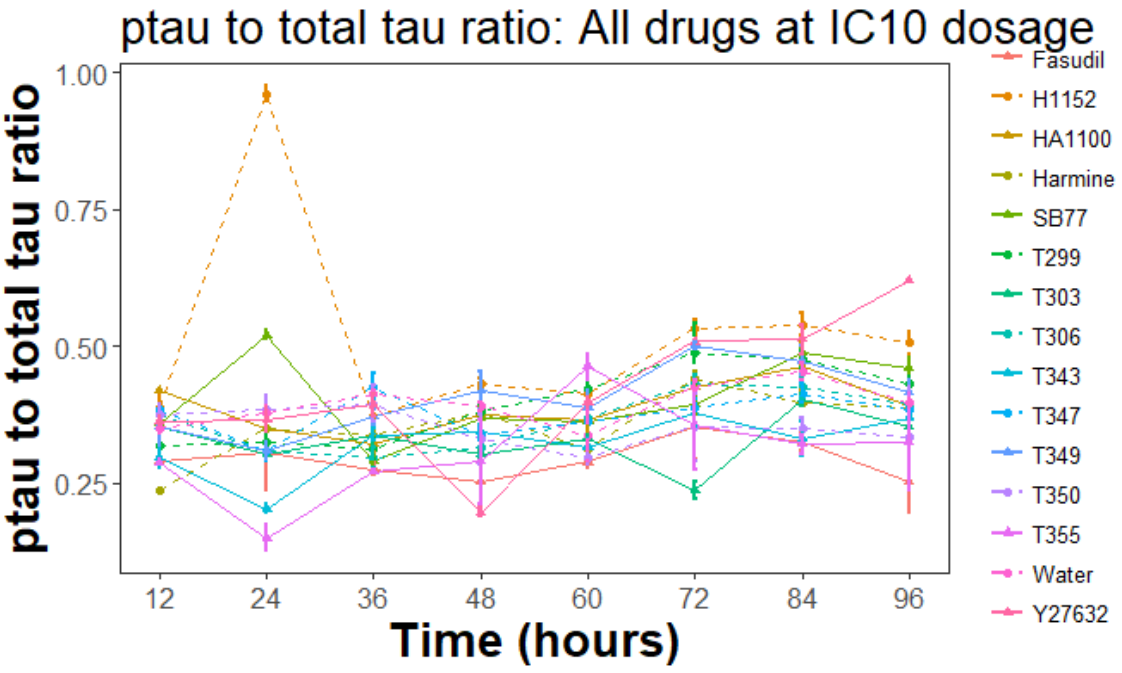


Figure 2

The p-tau to total tau ratio for all drugs at the LD-10 dosage. Most drug treatment did not lead to significant differences in the p-tau to total tau ratio. However, Fasudil, T303, and T343 treatments resulted in a significantly lower p-tau to total tau ratio.

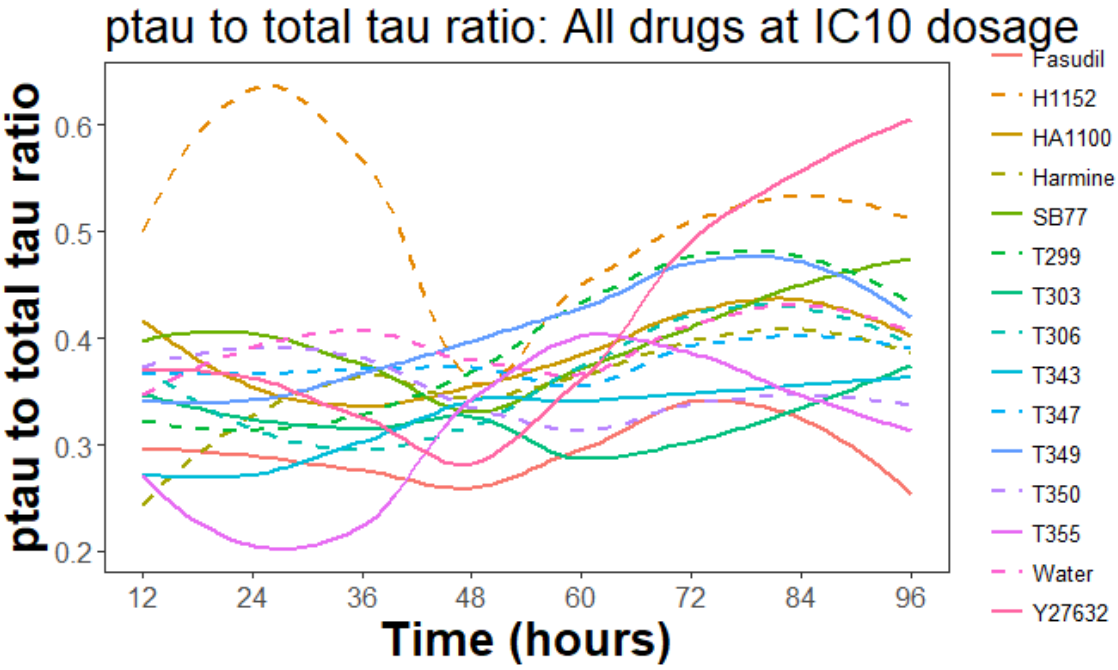


Figure 3.
The ptau to total tau ratio for all drugs at the LD-10 dosage, using loess plotting to show the trendlines for the ratio. The lines for Fasudil and T343 treatment remain lower than the line for water treatment.

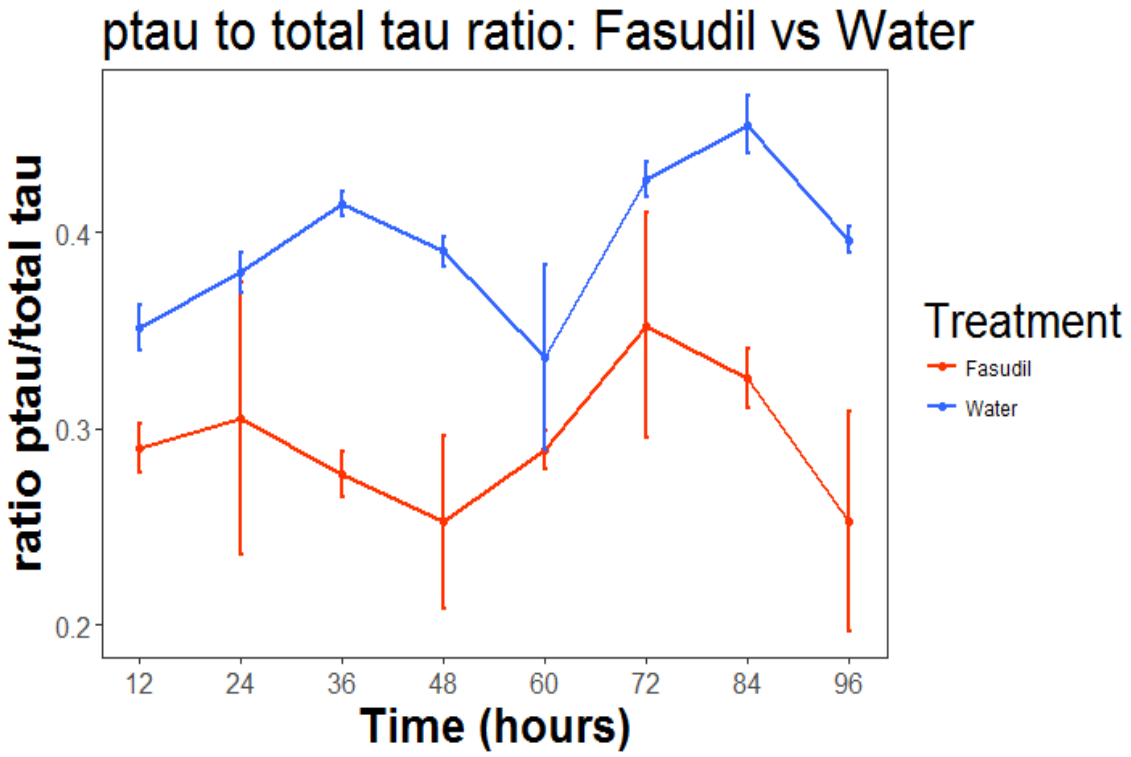


Figure 4.
Fasudil-treated H4-tau cells had a significantly reduced p-tau to total tau ratio at a low dosage in comparison to water-treated cells.

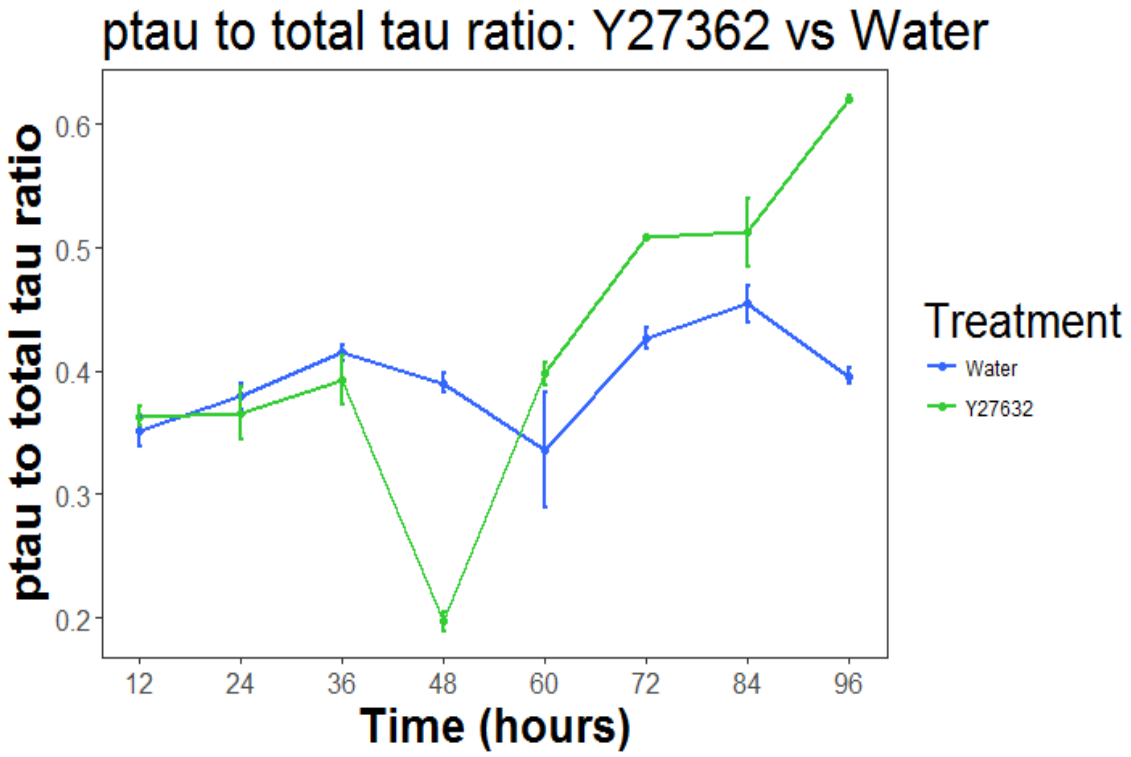


Figure 5.
Y27632-treated H4-tau cells did not have a significant effect on the p-tau to total tau ratio at a low dosage, in comparison to water-treated cells.

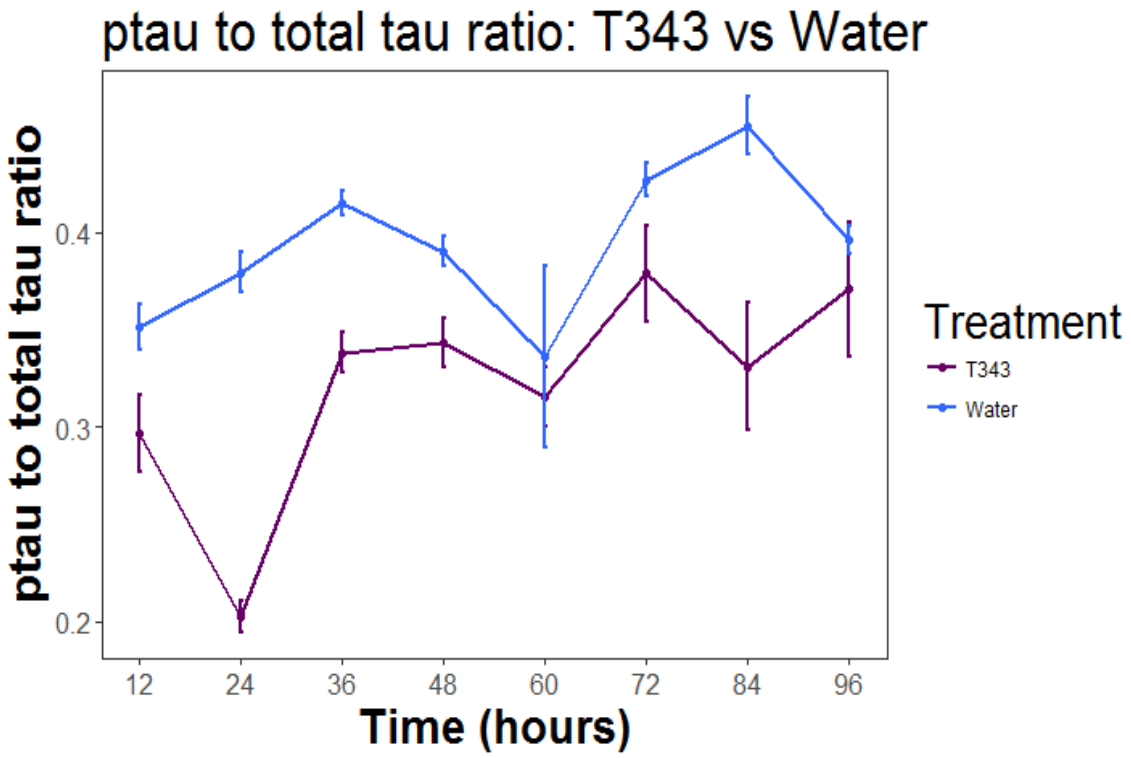


Figure 6.
T343-treated H4-tau cells had a significantly reduced p-tau to total tau ratio at a low dosage, in comparison to water-treated cells.

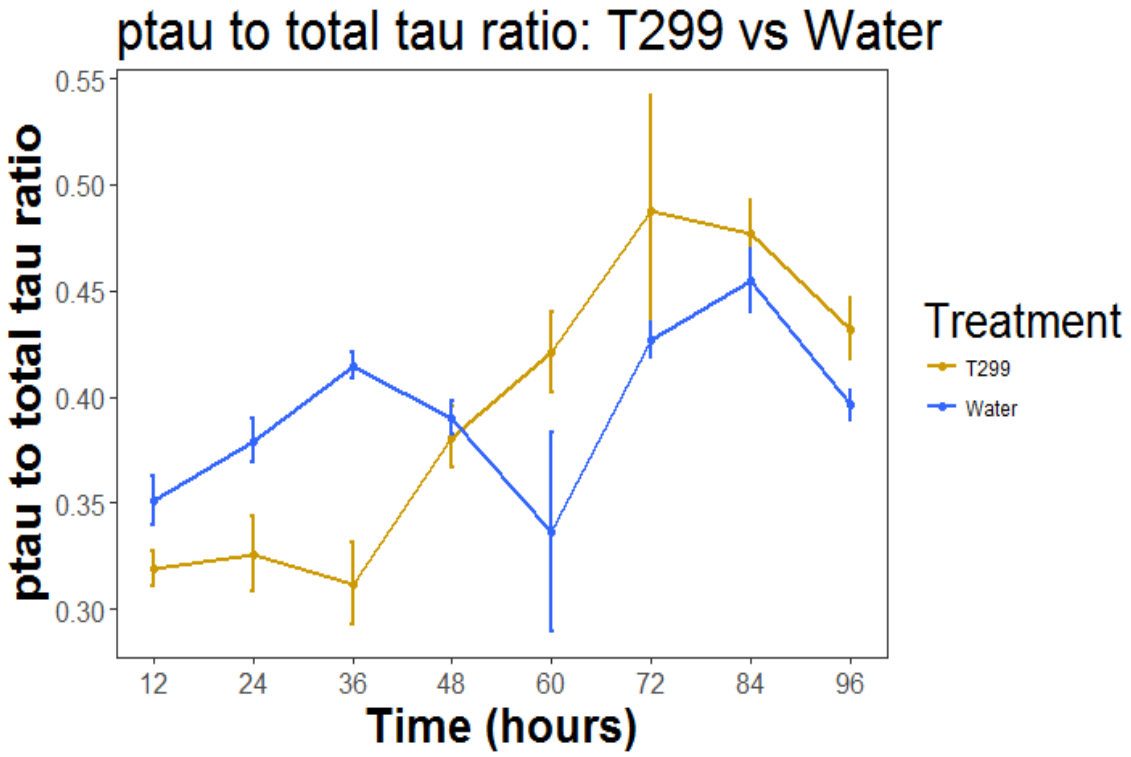


Figure 7.
T299-treated H4-tau cells did not have a significantly different ratio of p-tau to total tau at a low dosage, in comparison to water-treated cells.

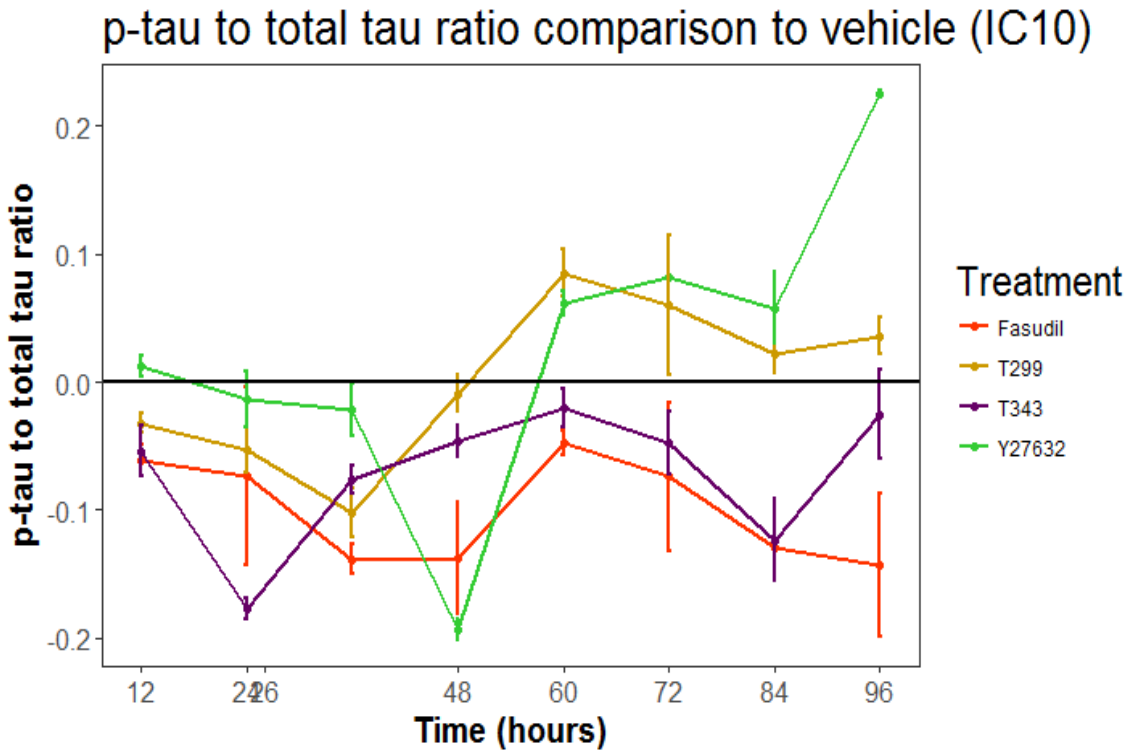


Figure 8.
The ratio of p-tau to total tau after treatment by Fasudil, Y27632, T343, and T299 at a low dosage are shown in comparison to treatment by water. Fasudil and T343 treatment decrease the ratio, while Y27632 and T299 do not cause a significant difference over the time course. However, Y27632 treatment leads to a significant increase at the final time point.

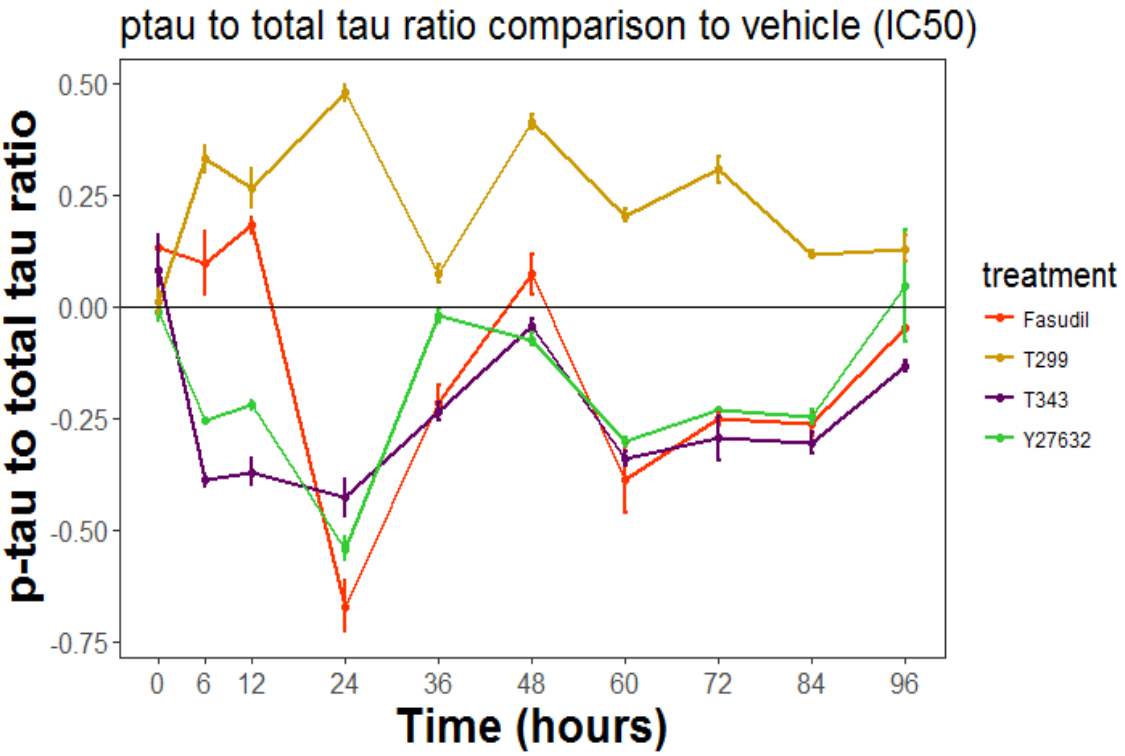


Figure 9
The ratio of p-tau to total tau after treatment by Fasudil, Y27632, T343, and T299 at a high dosage are shown in comparison to treatment by water. Treatment by Fasudil, Y27632, and T343 all lead to a significant decrease in the ratio across the time course, while Y27632 leads to a significant increase.

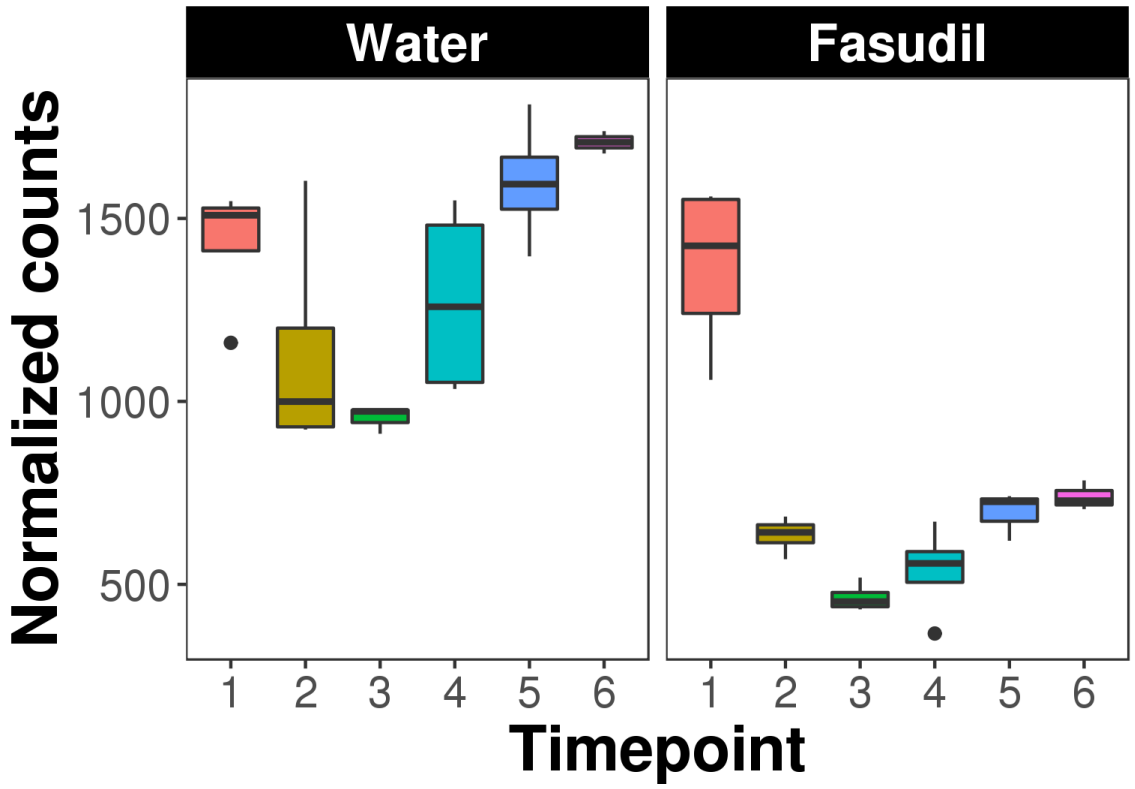


Figure 10.
High dosage Fasudil treatment in H4-tau cells led to a significant decrease in the expression levels of MAPT mRNA in comparison to water treatment over the entire time course. No other drug treatment, or DMSO treatment, led to a significant difference in MAPT mRNA at any time point.

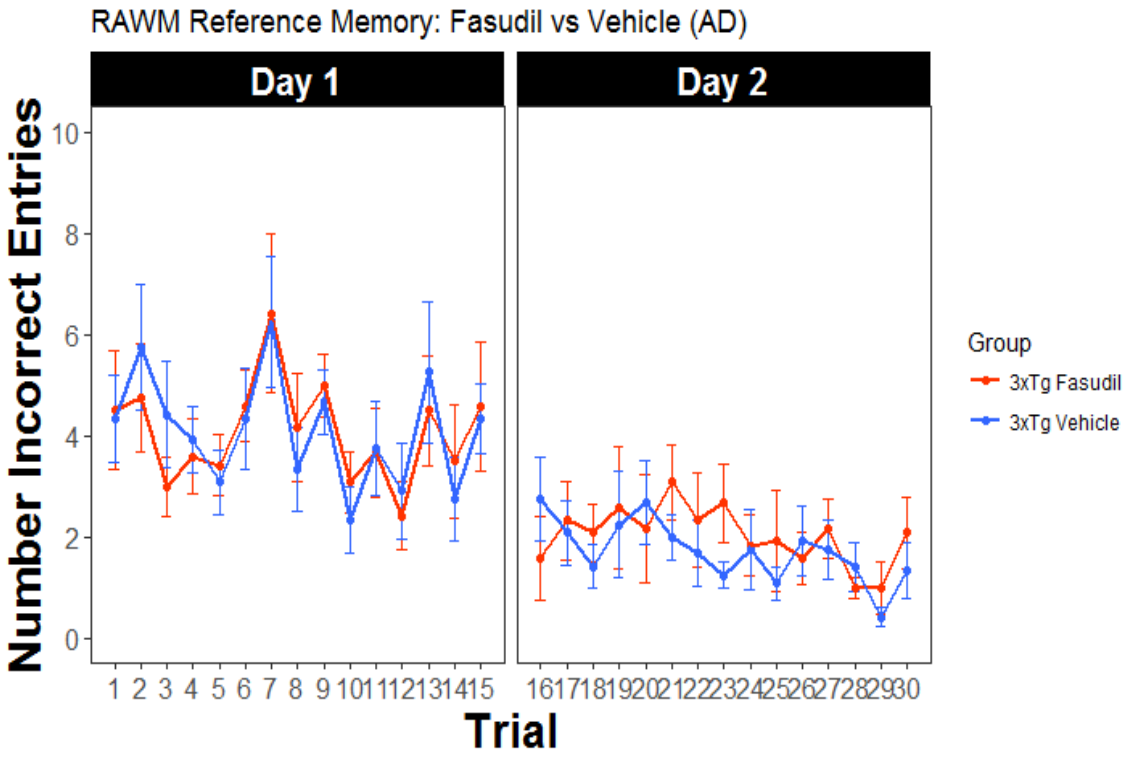


Figure 11
Fasudil treatment in 3xTg-AD mice did not have a significant effect on the number of total incorrect entries over the course of Day 2, indicating that Fasudil does not lead to changes in reference memory at 10 months of age for 3xTg-AD mice.

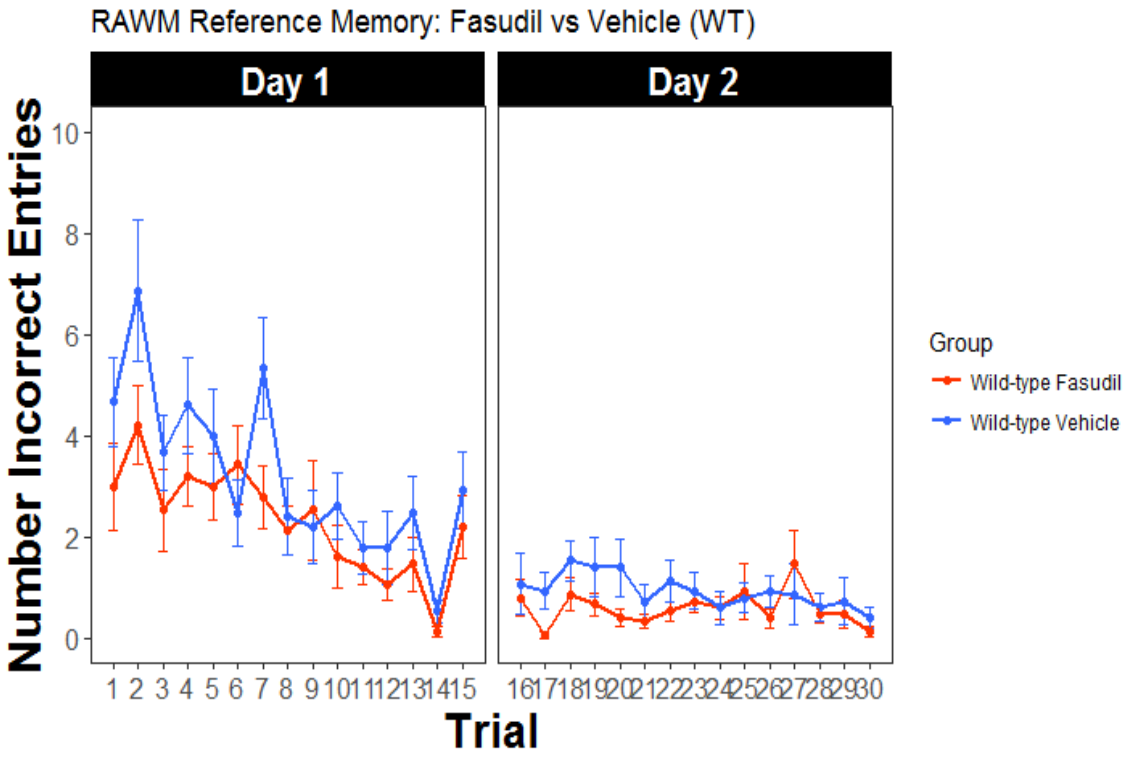


Figure 12
Fasudil treatment in wild-type mice led to a significant decrease in the number of total incorrect entries over the course of Day 2, indicating that Fasudil improves reference memory in wild-type mice at 10 months of age.

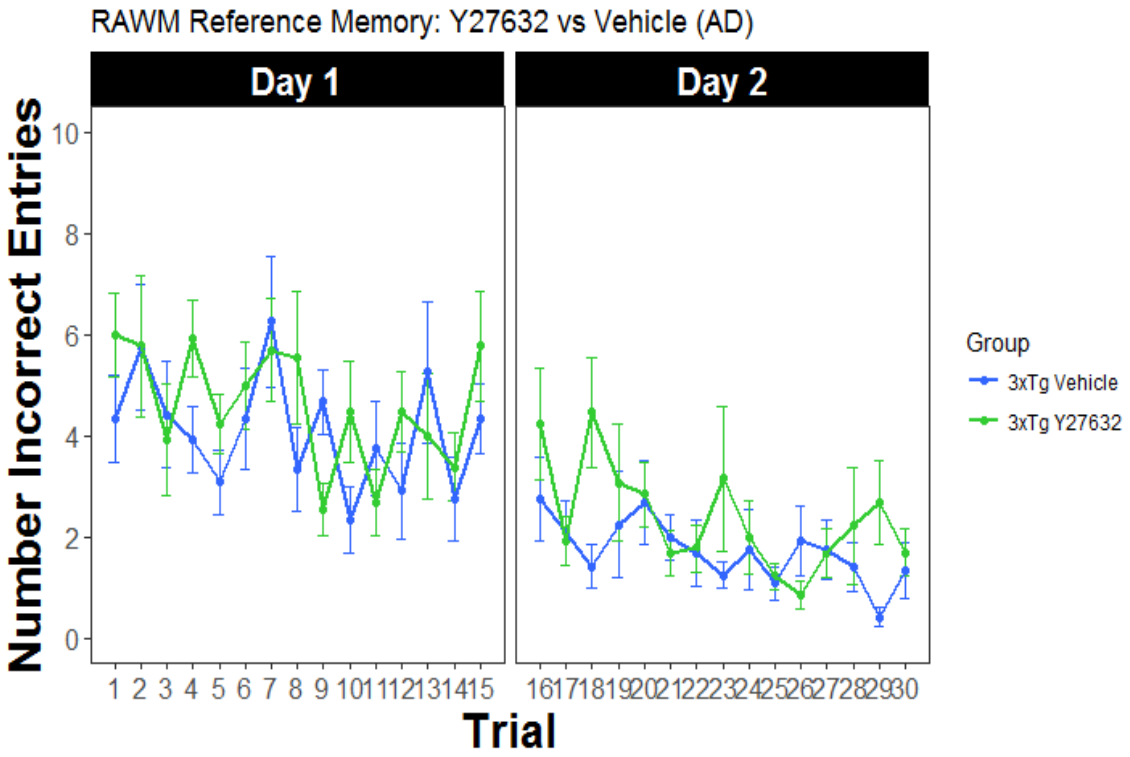


Figure 13
Y27632 treatment in 3xTg-AD mice led to a significant increase in total number of incorrect entries across Day 2, indicating that Y27632 impairs reference memory at 10 months of age in 3xTg-AD mice. There were also significant differences during two specific trials on Day 2: trial 18 and 29.

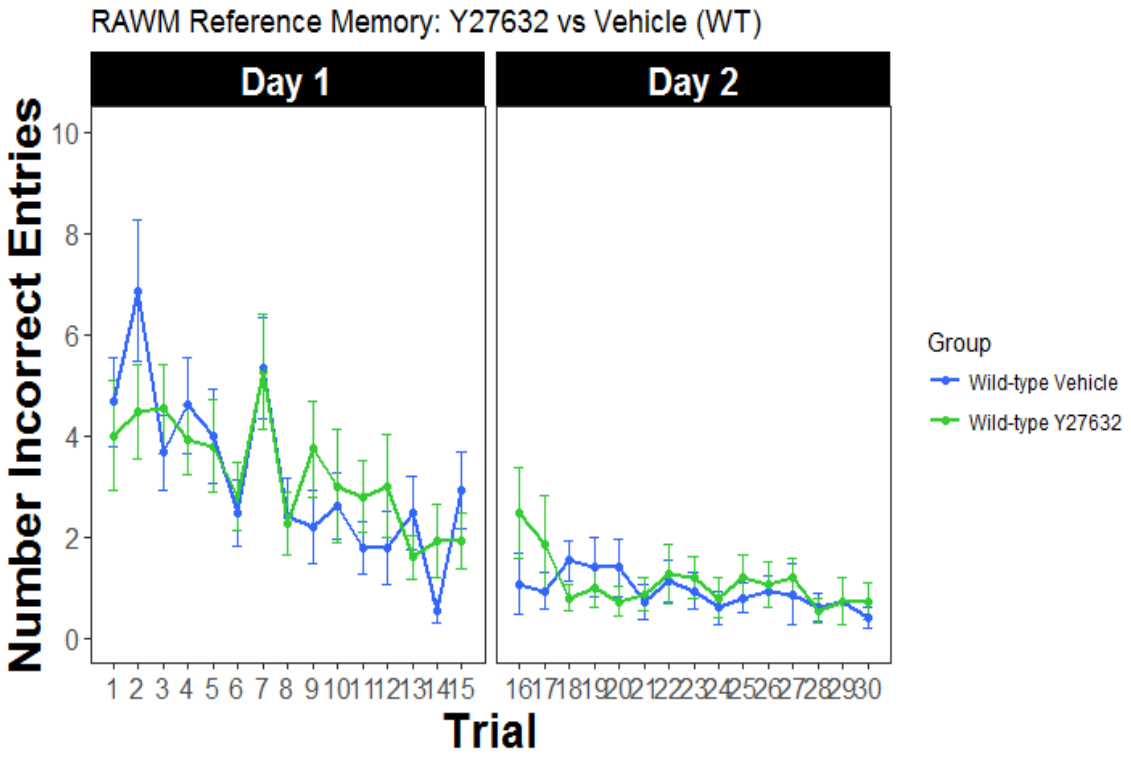


Figure 14
Y27632 treatment in wild-type mice led to no significant difference in the total number of incorrect entries on Day 2, indicating that Y27632 leads to no changes in reference memory at 10 months of age in wild-type mice.

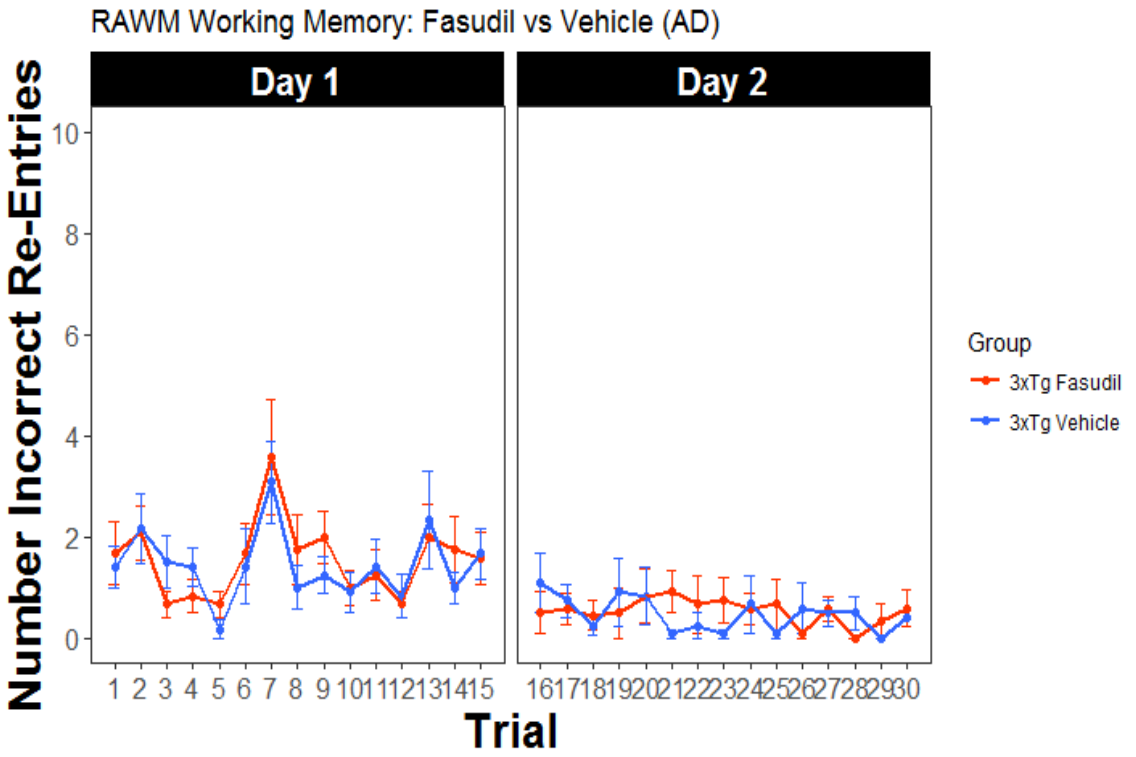


Figure 15
Fasudil treatment in 3xTg-AD mice did not lead to a significant difference in the number of re-entries into the incorrect arm over the course of Day 2, indicating that Fasudil does not have an effect on working memory at 10 months of age in 3xTg-AD mice. However, there was a significant decrease in the number of incorrect entries during one trial (Trial 21), indicating that perhaps there is a trend toward an improvement.

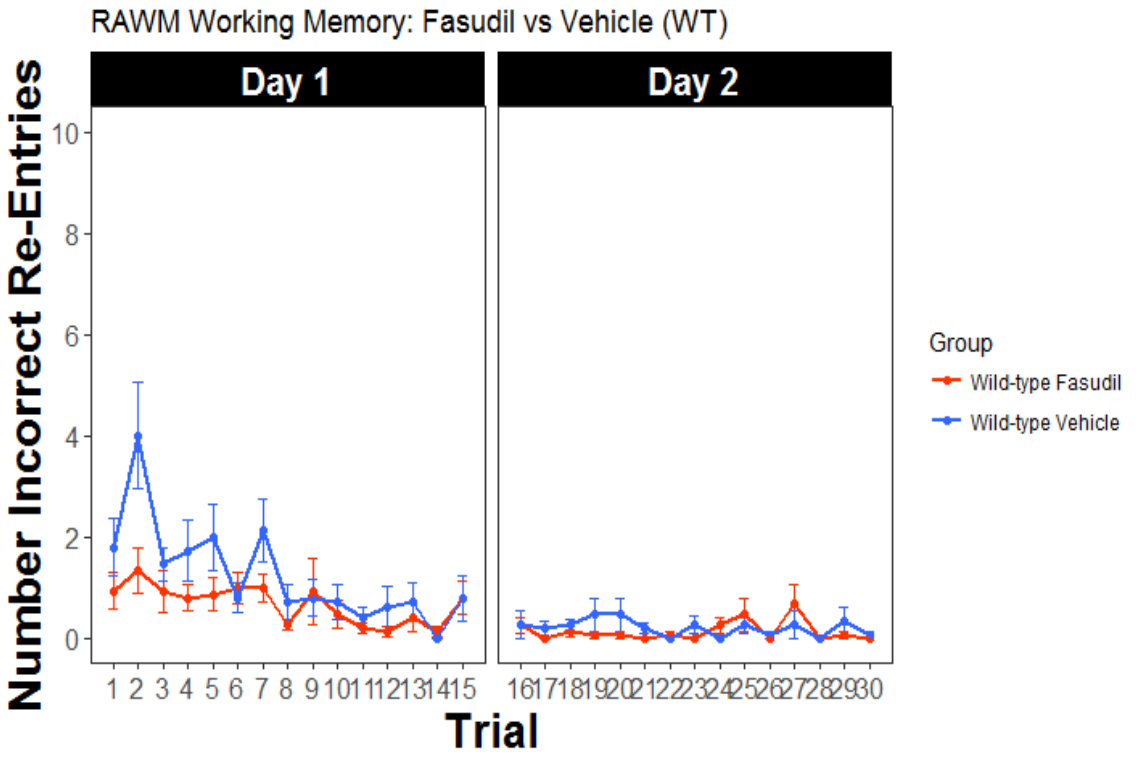


Figure 16
Fasudil treatment in wild-type mice did not lead to a significant difference in the number of re-entries into the incorrect arm over the course of Day 2, indicating that Fasudil does not influence working memory at 10 months of age in wild-type mice.

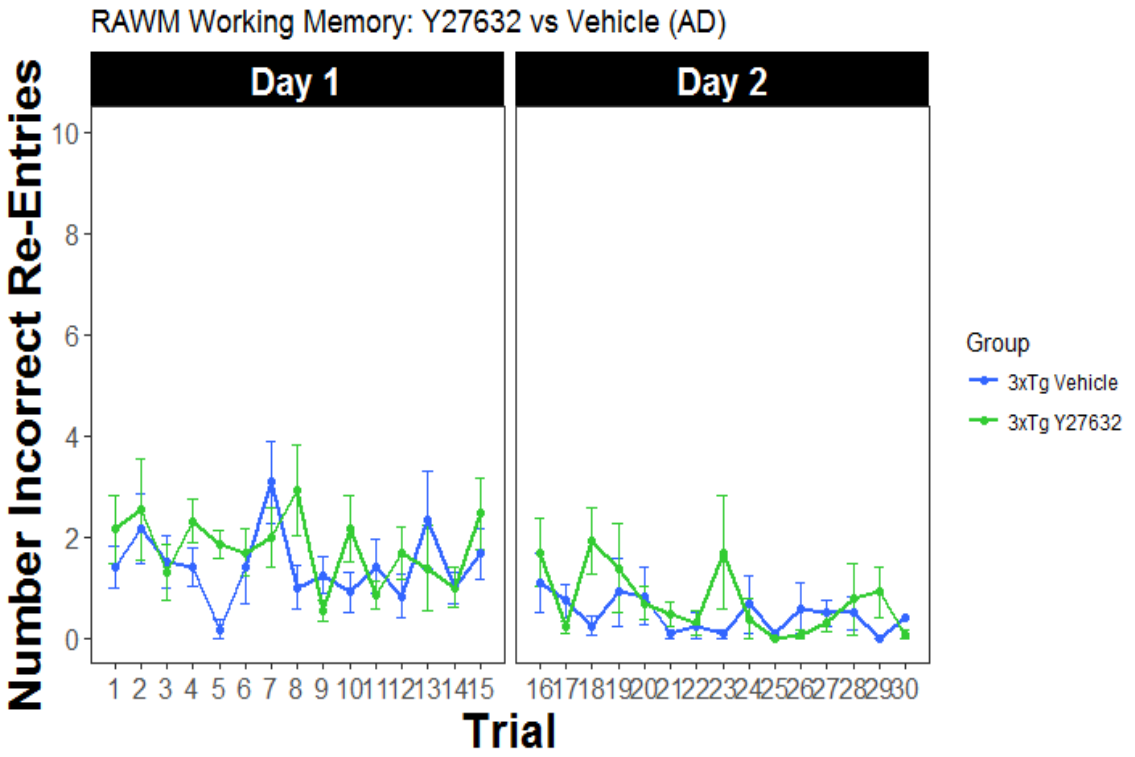


Figure 17
Y27632 treatment in 3xTg-AD mice did not lead to a significant difference in the number of re-entries into an incorrect arm over the course of Day 2, indicating that Y27632 does not influence working memory at 10 months of age in 3xTg-AD mice. However, Y27632 significantly increased the number of incorrect re-entries during three trials (18, 22, and 29) indicating that there may be a trend toward an impairment in working memory.

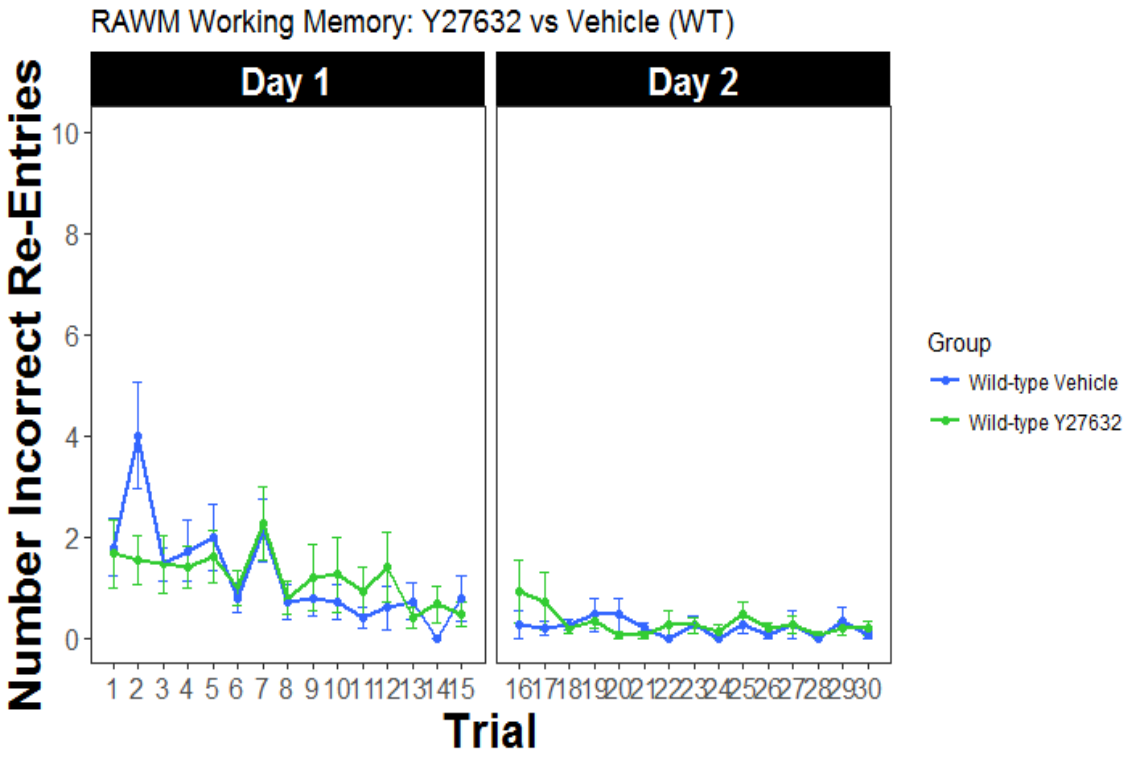


Figure 18
Y27632 treatment in wild-type mice led to no significant differences in the number of re-entries into incorrect arms during Day 2, indicating that Y267632 does not affect working memory at 10 months of age in wild-type mice.

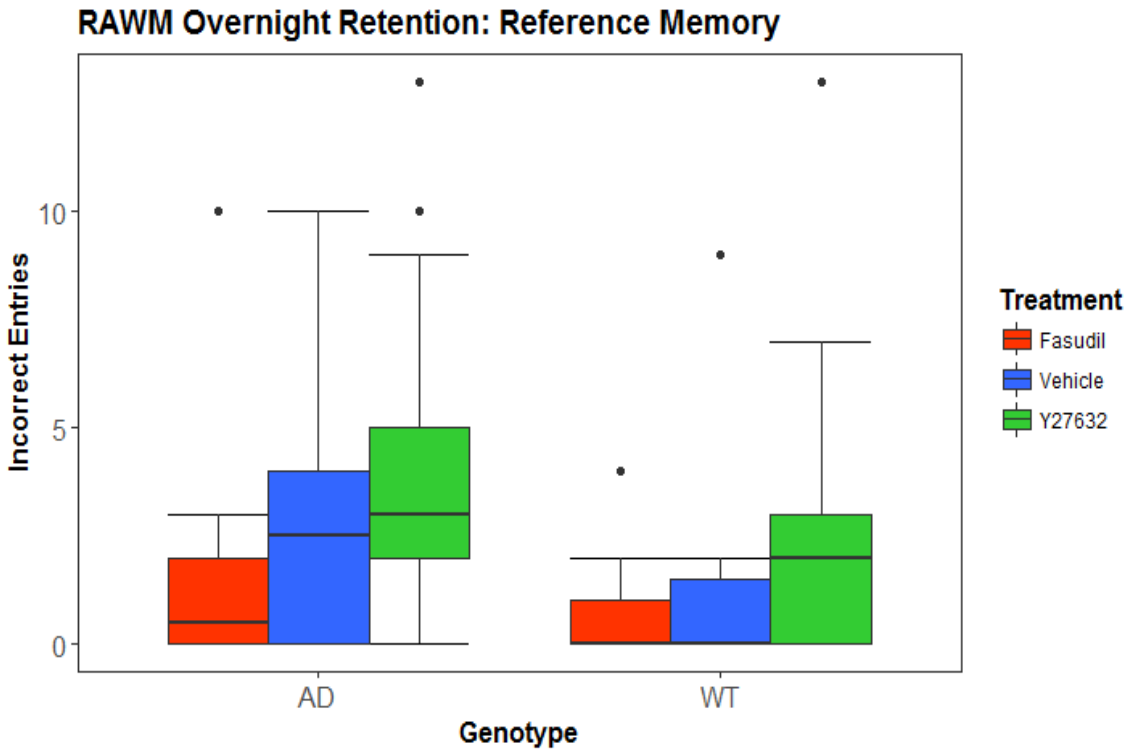


Figure 19. 3xTg-AD mice show no overnight retention impairment.
There is no significant difference in overnight retention for any groups, except for Y27632-treated 3xTg-AD mice and vehicle-treated wild-type mice, indicating that treatment does not affect overnight retention at 10 months of age.

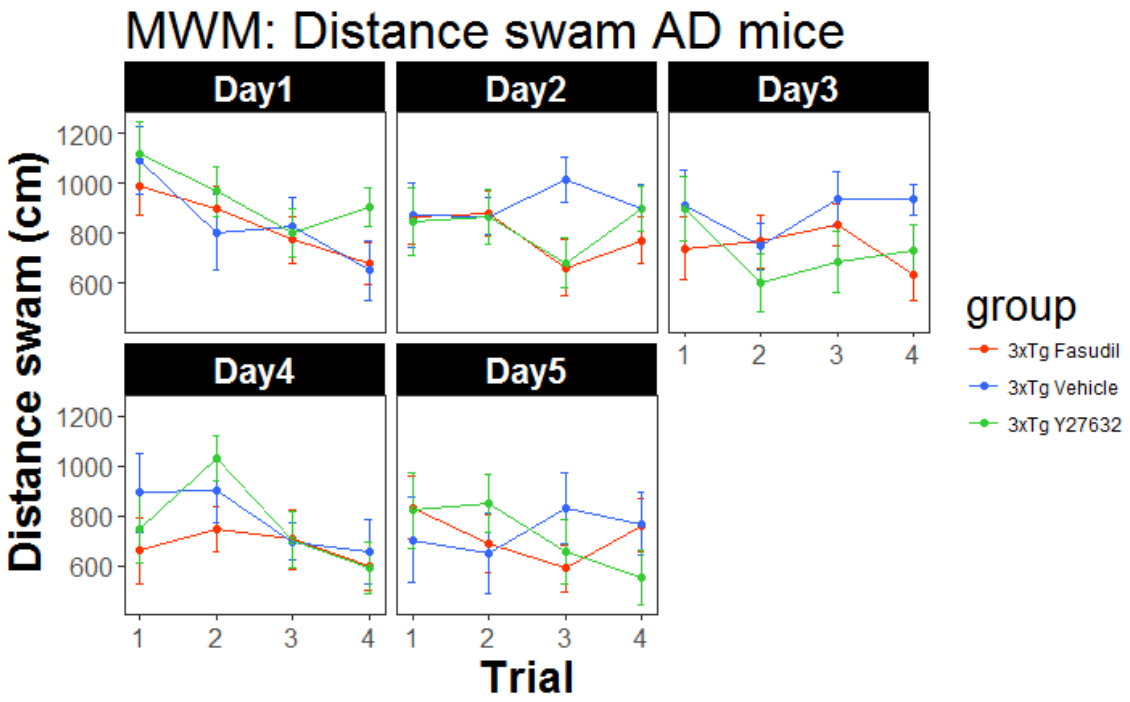


Figure 20. Treatment does not affect total swim distance in 3xTg-AD mice.
On Day 3, Y27632-treated 3xTg-AD mice swam a significantly shorter distance than vehicle-treated mice before reaching the platform, and Fasudil-treated mice trended toward a shorter distance, indicating that these drugs may have some effect on reference memory at 12 months of age. There were no other significant differences on the other days, indicating that this effect is not robust.

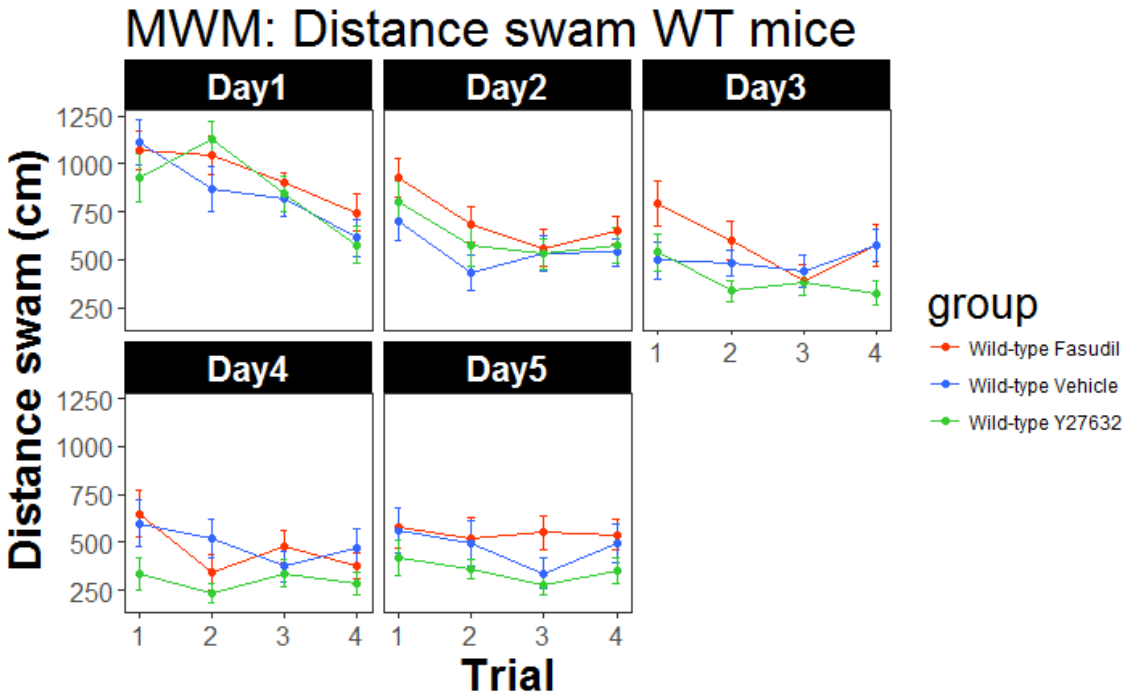


Figure 21. Treatment does not affect total swim distance in wild-type mice.
On Day 4, Y27632-treated mice swam a significantly shorter distance than vehicle-treated mice before reaching the platform, indicating that Y27632 may have some effet on reference memory at 12 months of age. There were no other significant differences on the other days, indicating that this effect is not robust.

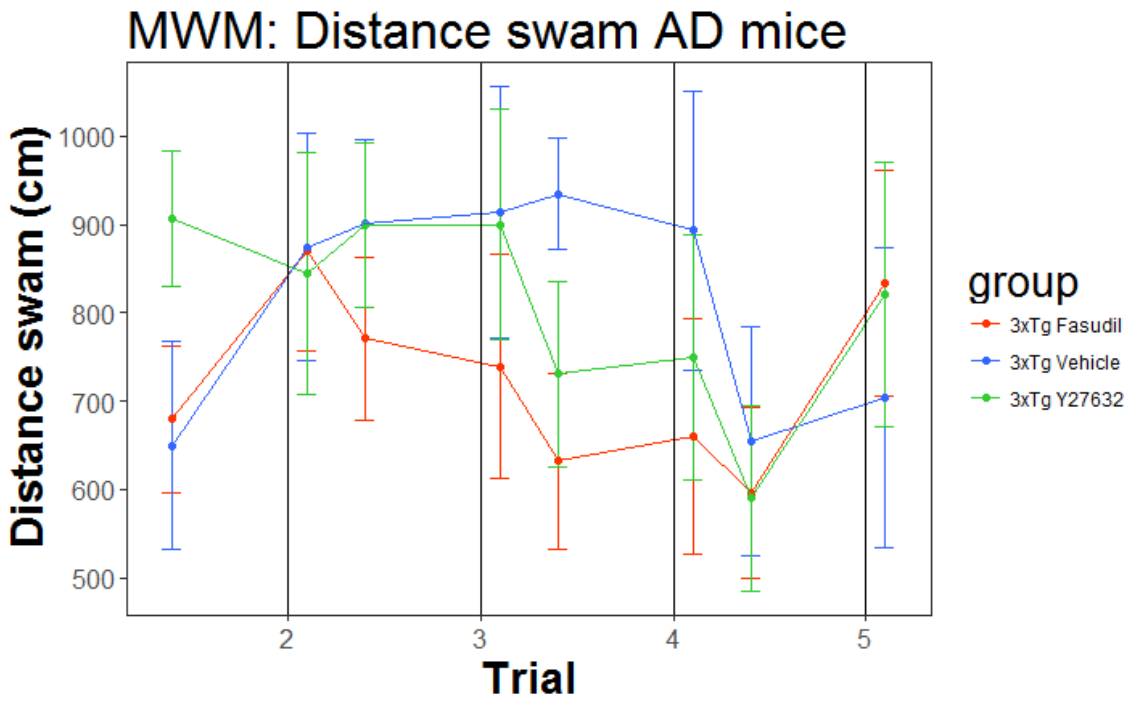


Figure 22. 3xTg-AD mice show no overnight retention impairment.
The distance swam by each of the 3xTg-AD treatment groups for the final trial and first trial of each day shows that none of the groups displayed significant differences in overnight retention on any day.

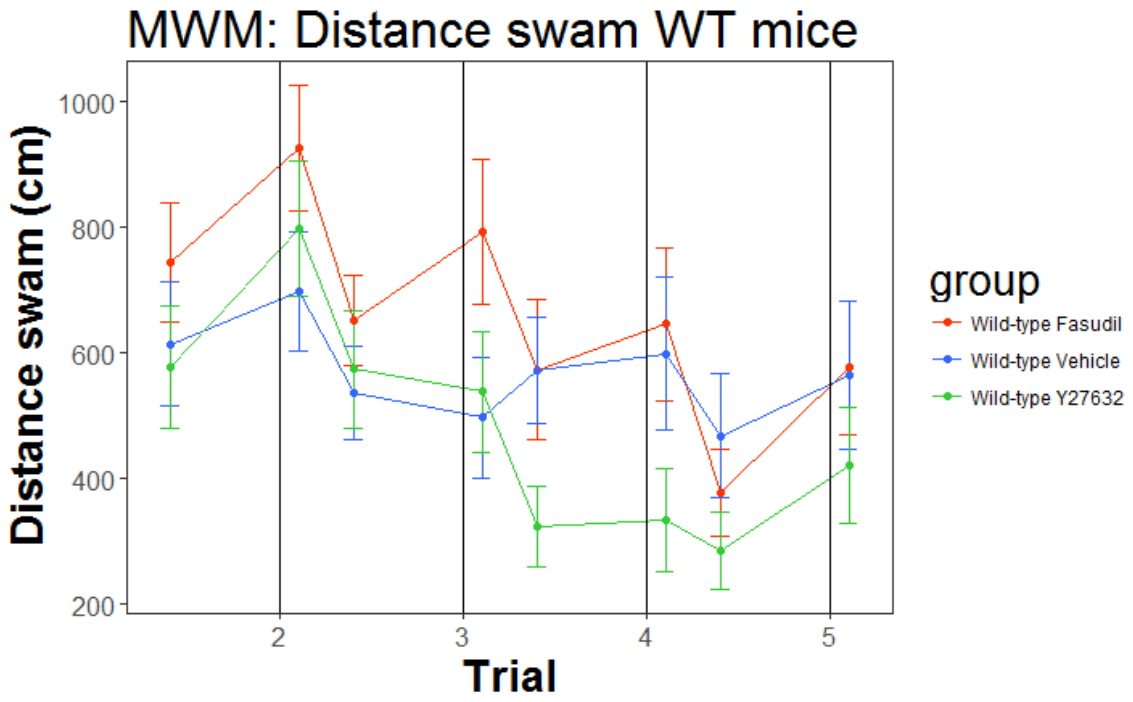


Figure 23. Wild-type mice show no overnight retention impairment.
The distance swam by each of the wild-type treatment groups for the final trial and first trial of each day shows that none of the groups displayed significant differences in overnight retention on any day.

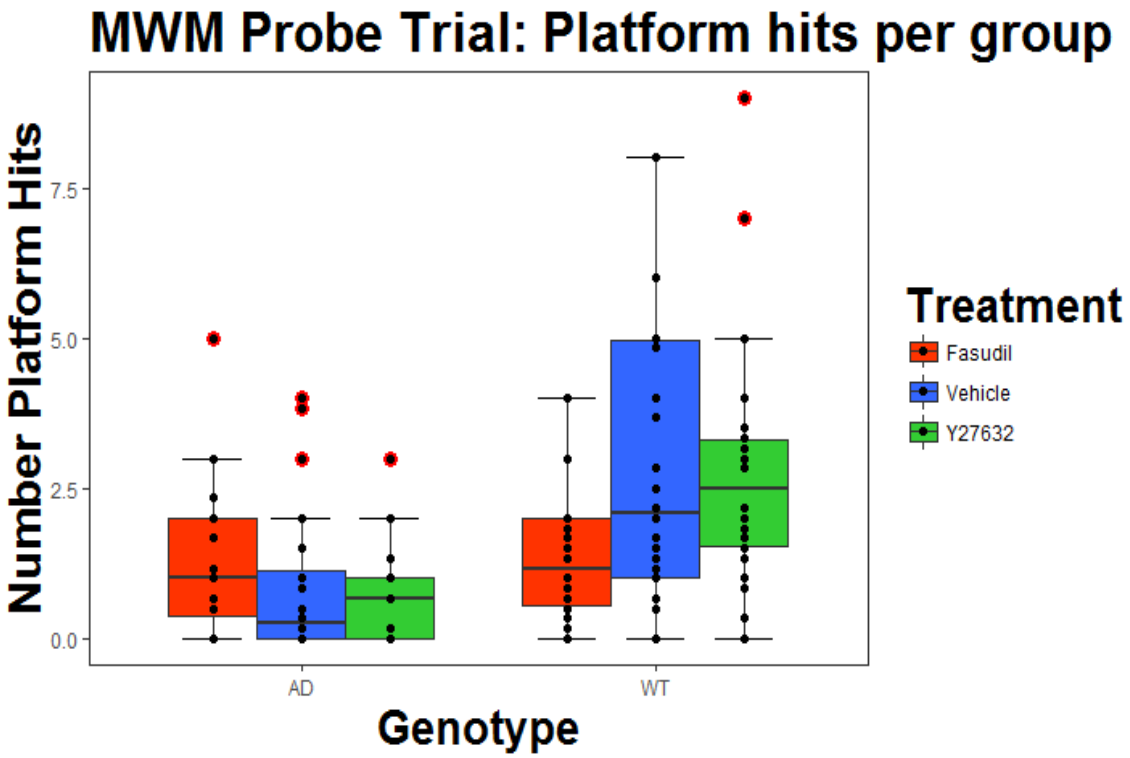


Figure 24. Treatment does not improve number of platform hits during probe trial.
During the probe trial, treatment by either Fasudil or Y27632 does not change the number of times 3xTg-AD mice or wild-type mice pass over the area the platform was in previous trials. 3xTg-AD mice pass over the platform space significantly less than wild-type mice.

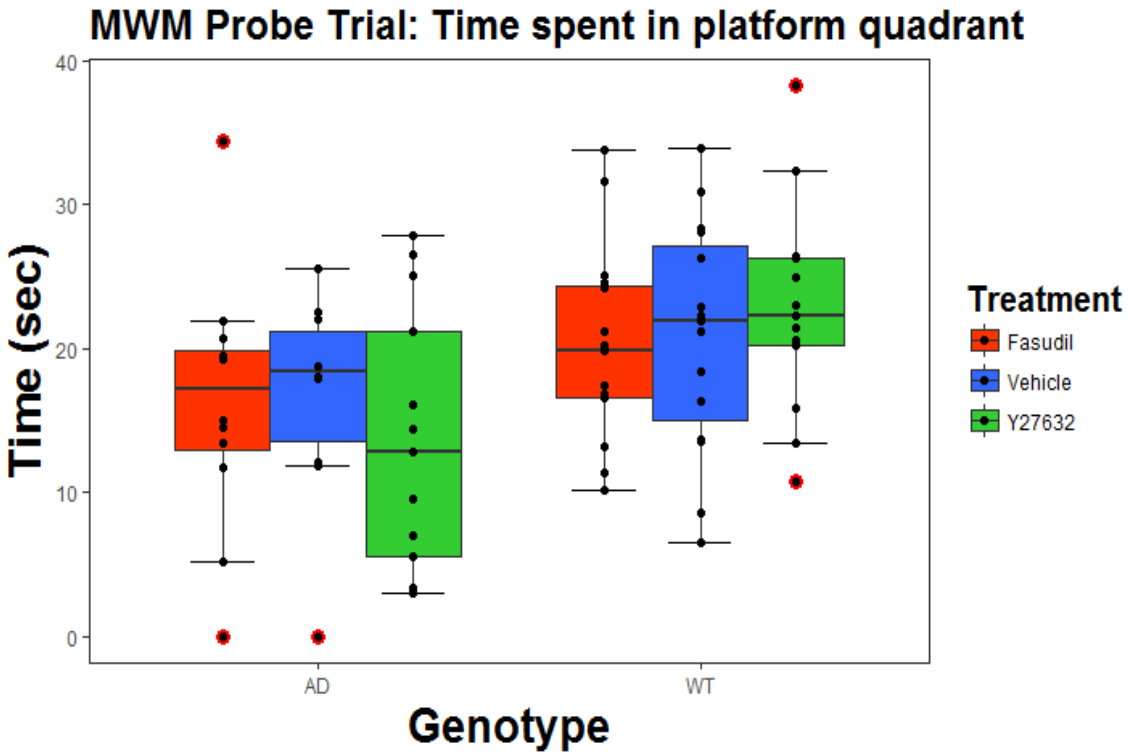


Figure 25. Time in platform quadrant not affected by group or treatment.
There is no significant difference in the amount of time, within a 60 second timeframe, during which any group of mice spent in the platform quadrant.

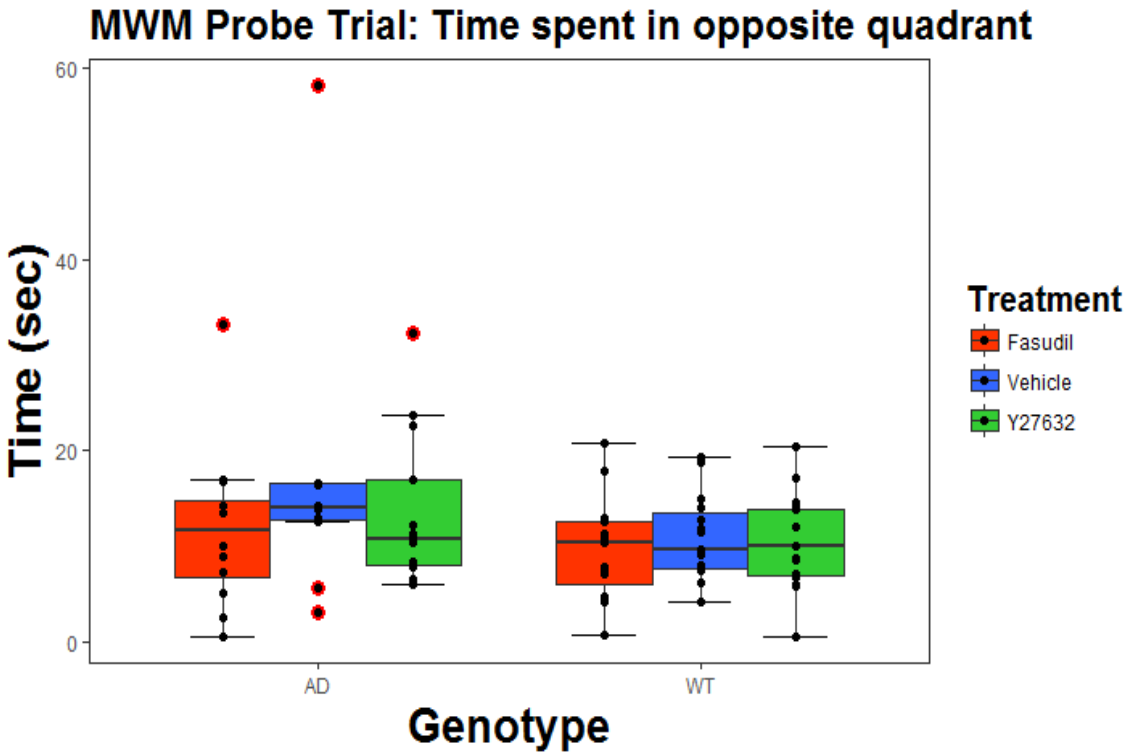


Figure 26. Time in opposite quadrant not affected by group or treatment.
There is no significant difference in the amount of time, within a 60 second timeframe, during which any group of mice spent in the quadrant opposite the platform quadrant.

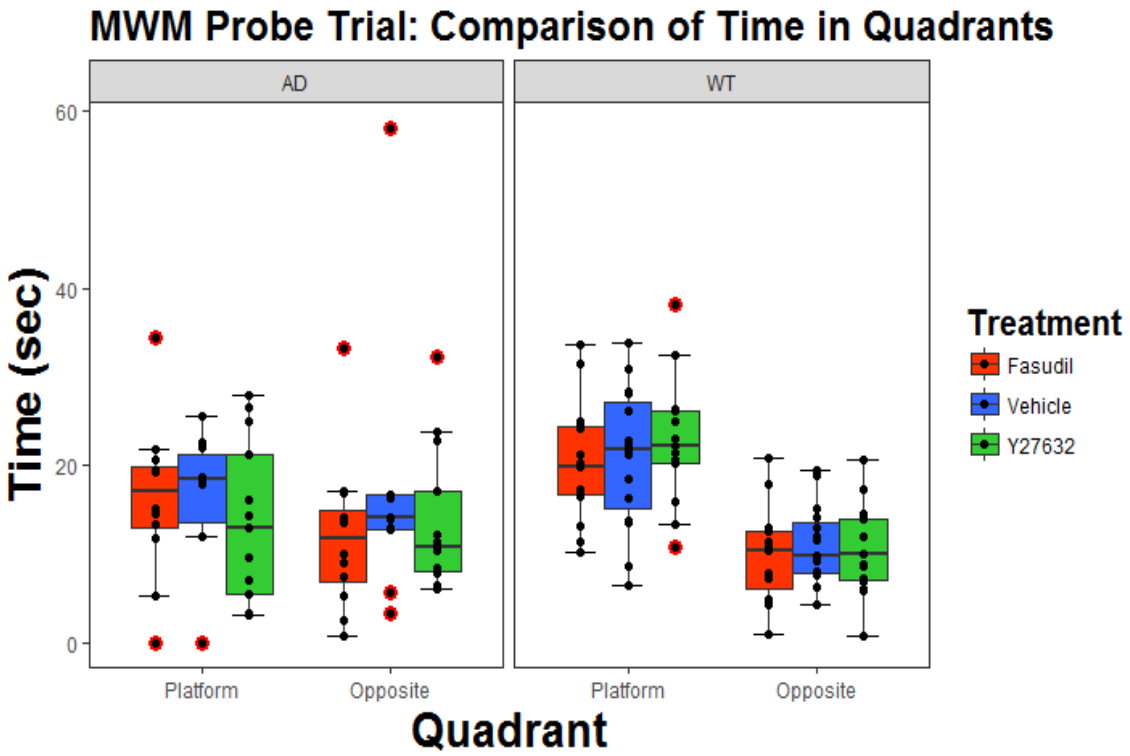


Figure 27. Ratio of time spent in opposite vs platform quadrant not affected by group or treatment.
The average amount of time spent in the platform quadrant in comparison to the opposite quadrant. In wild type vehicle-treated mice, there is a more comparable difference between the amount of time. The 3xTg-AD mice do not have as comparable a difference between the amount of time in the platform vs opposite quadrant. However, this is not a significant ratio.

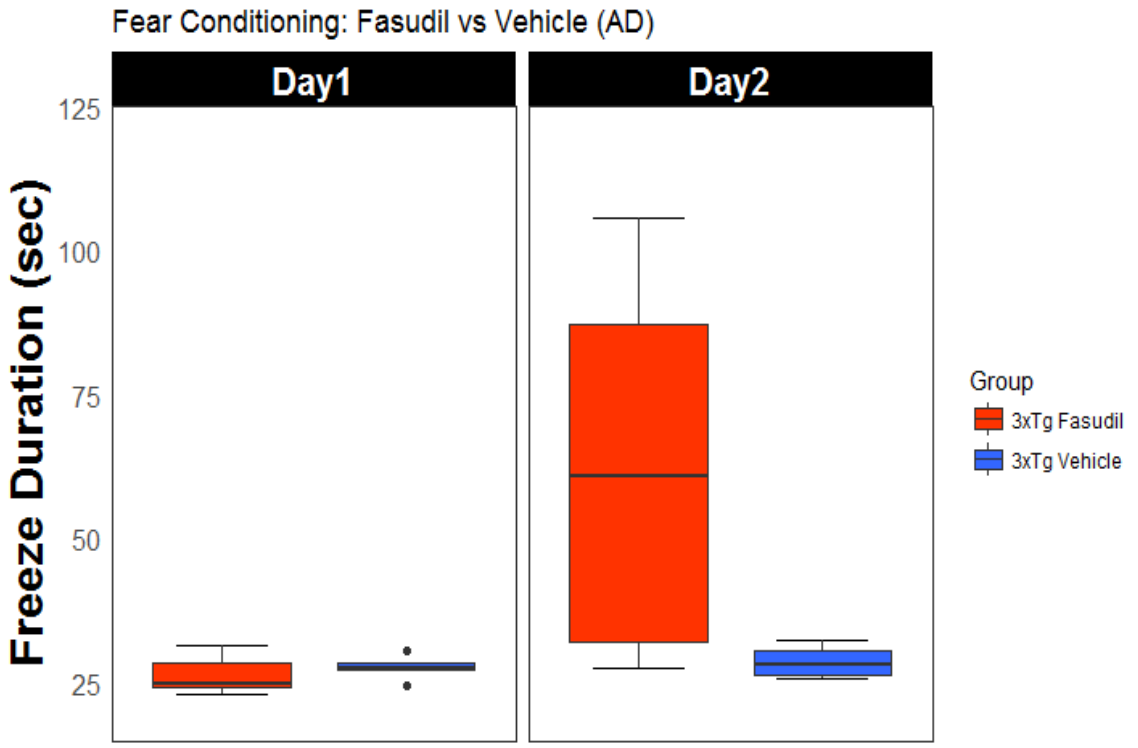


Figure 28. Fasudil treatment improves memory in 3xTg-AD mice at 15 months. Fasudil-treated 3xTg-AD mice freeze for a longer time per freeze event in comparison to vehicle-treated 3xTg-AD mice, indicating that Fasudil treatment increases memory at 15 months of age in 3xTg-AD mice.

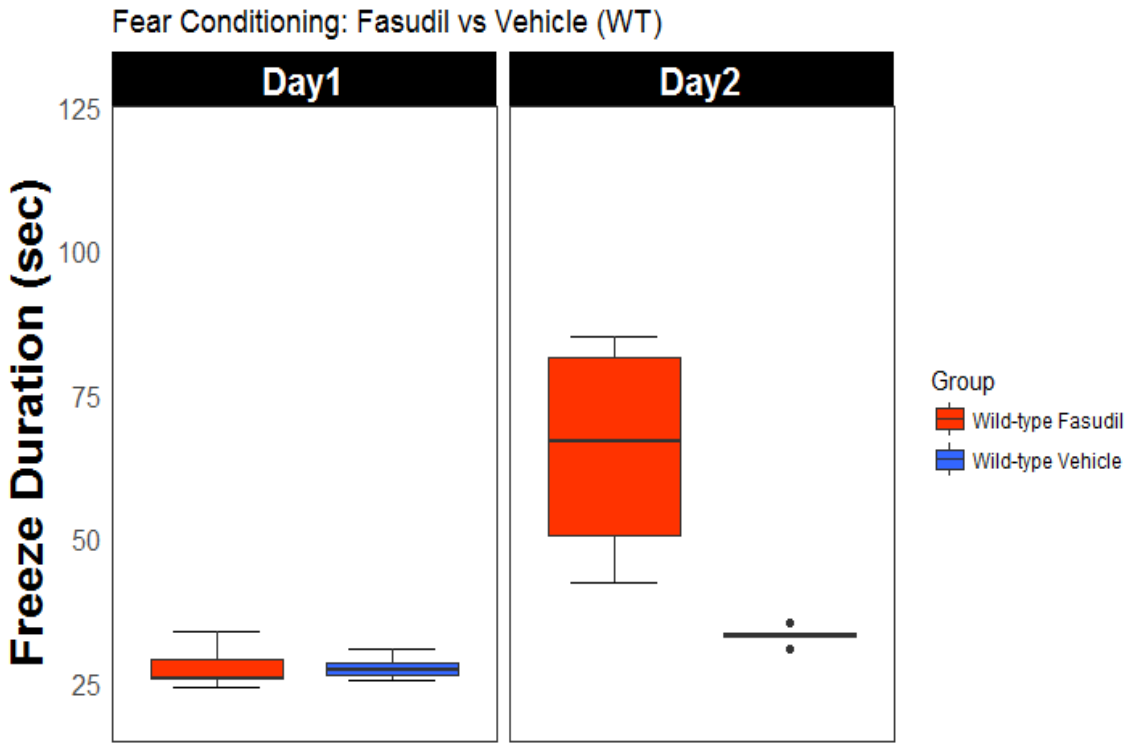


Figure 29. Fasudil treatment improves memory in wild-type mice at 15 months. Fasudil-treated wild-type mice freeze for a longer time per freeze event in comparison to vehicle-treated wild-type mice, indicating that Fasudil treatment increases memory at 15 months of age in wild-type mice.

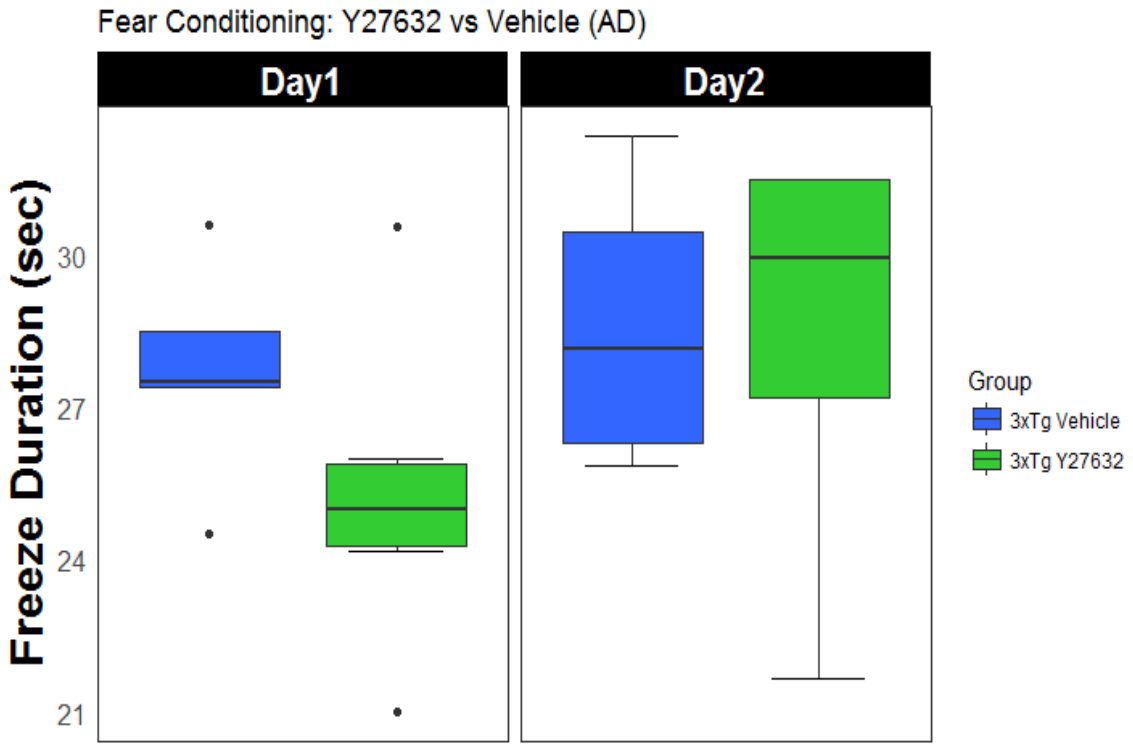


Figure 30. Y27632 treatment does not affect memory in 3xTg-AD mice at 15 months.
Y27632-treated 3xTg-AD mice do not have a significantly different freeze duration in comparison to vehicle-treated mice, indicating no effect on memory at 15 months of age.

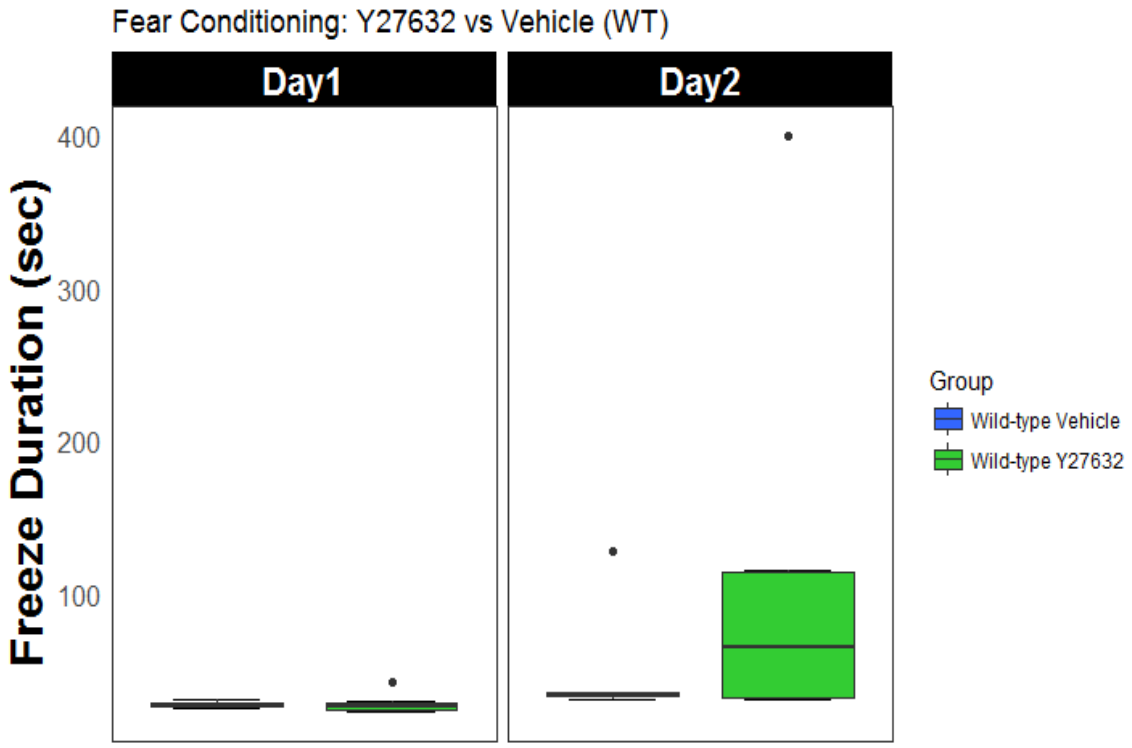


Figure 31. Y27632 treatment does not affect memory in wild-type mice at 15 months.
Y27632-treated wild-type mice do not have a significantly different freeze duration in comparison to vehicle-treated mice, indicating that Y27632 does not have an effect on memory at 15 months of age.

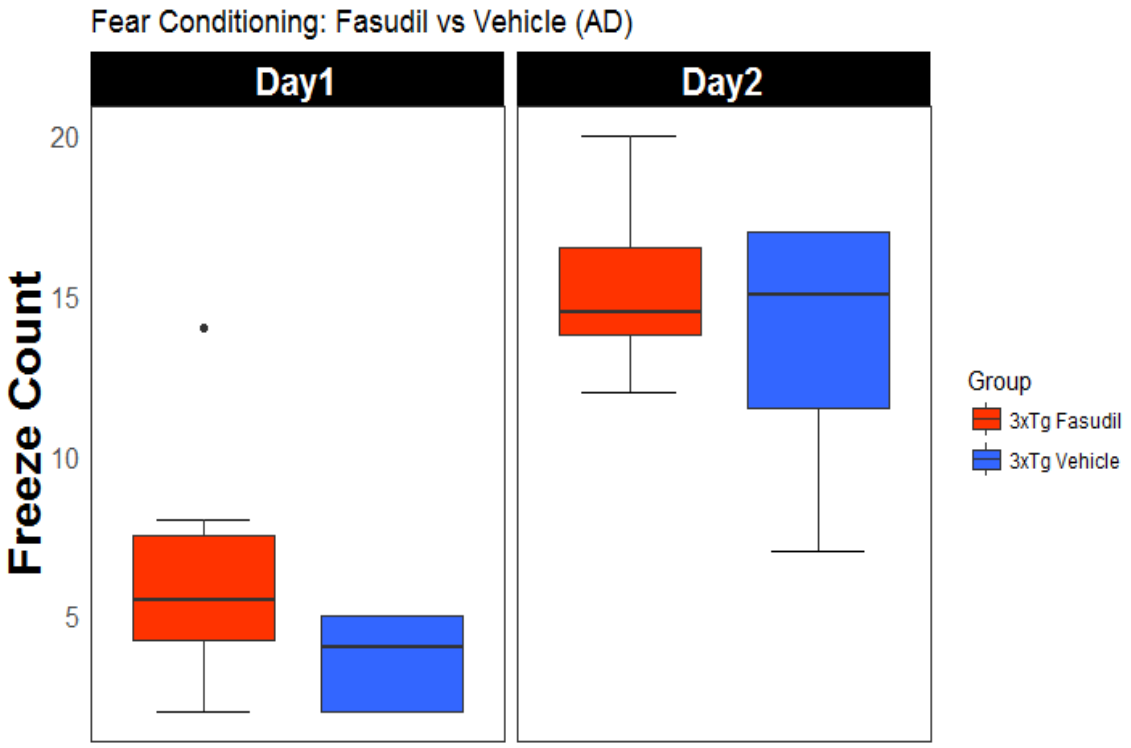


Figure 32. No difference in freeze count with Fasudil treatment in 3xTg-AD mice. There is no significant difference in the number of times Fasudil-treated and vehicle-treated 3xTg-AD mice froze on Day 2.

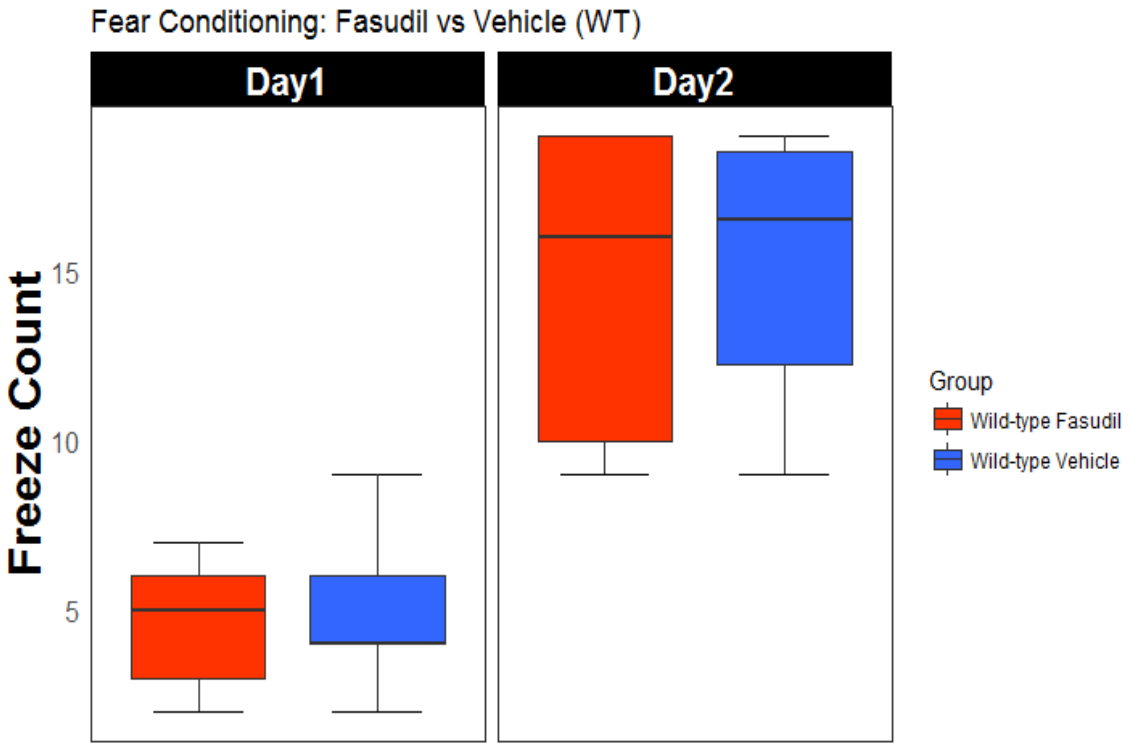


Figure 33. No difference in freeze count with Fasudil treatment in wild-type mice. There is no significant difference in the number of times Fasudil-treated and vehicle-treated wild-type mice froze on Day 2.

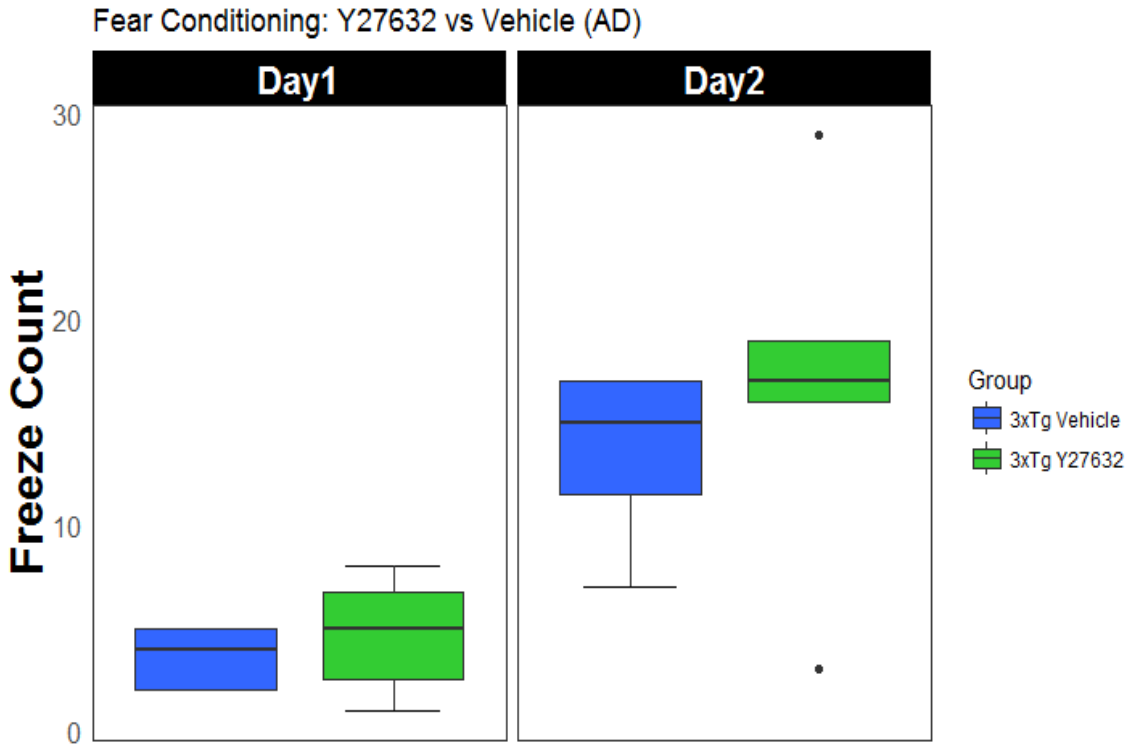


Figure 34. No difference in freeze count with Y27632 treatment in 3xTg-AD mice. There is no significant difference in the number of times Y27632-treated and vehicle-treated 3xTg-AD mice froze on Day 2.

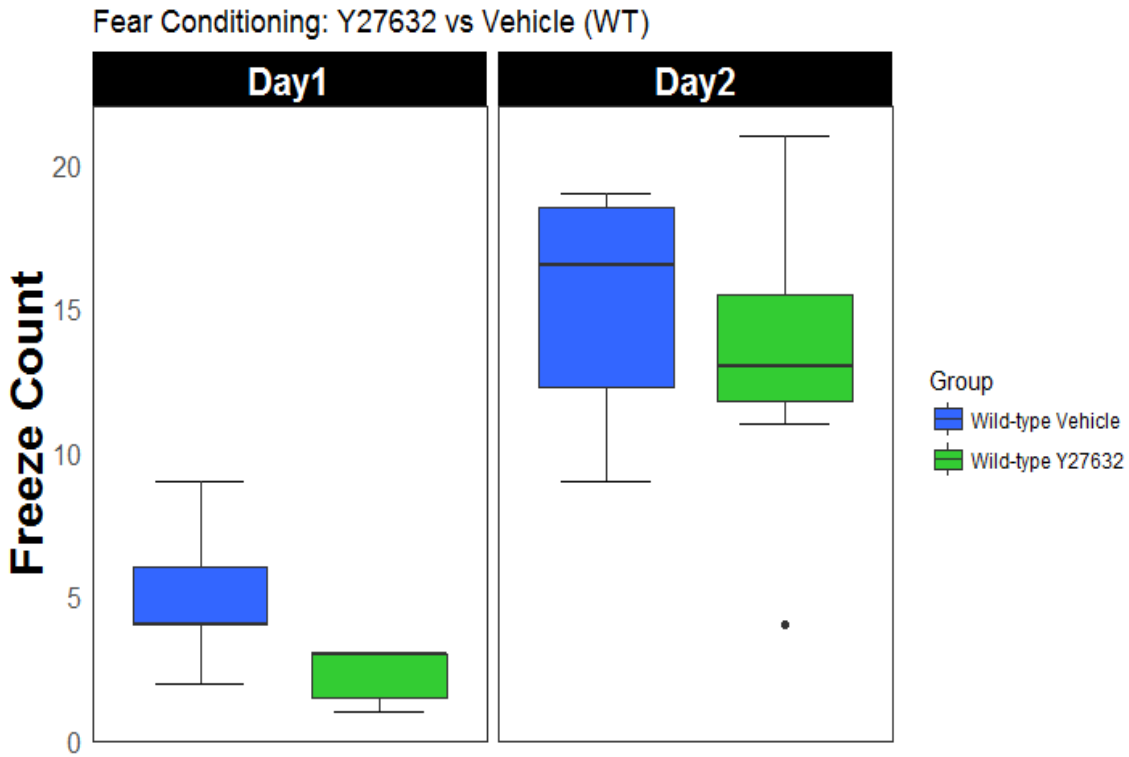


Figure 35. No difference in freeze count with Y27632 treatment in wild-type mice. There is no significant difference in the number of times Y27632-treated and vehicle-treated wild-type mice froze on Day 2.

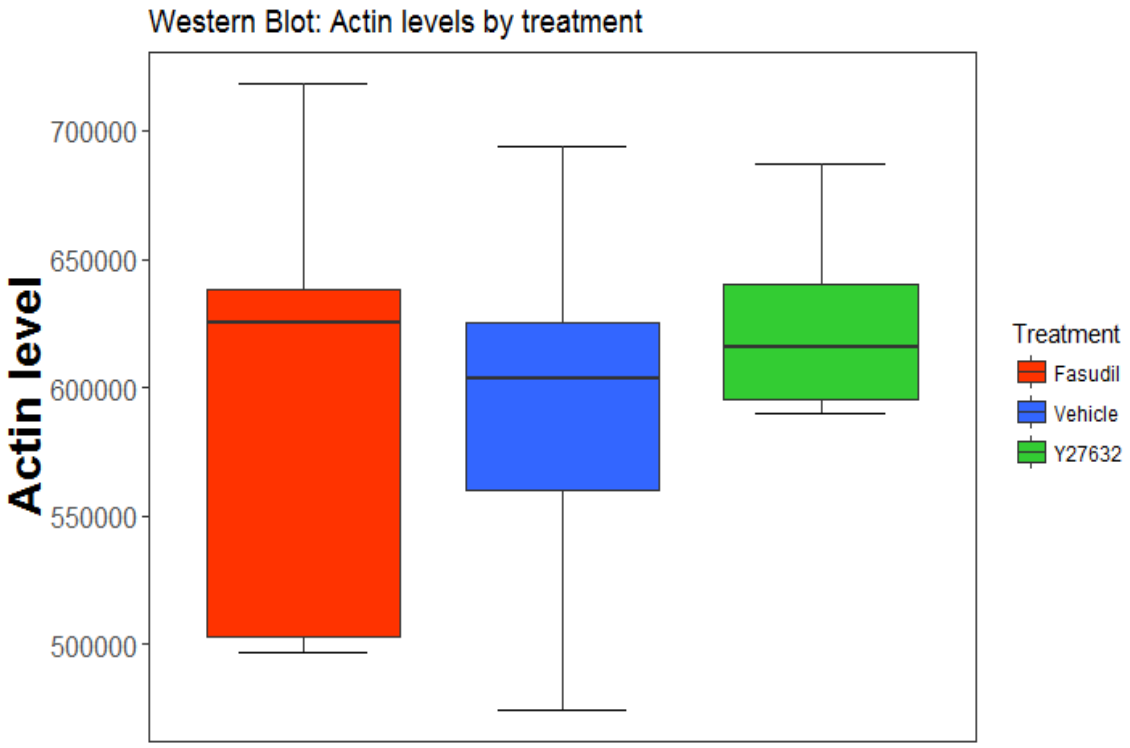


Figure 36. Western blot wells loaded equally.
Actin levels are not significantly different between groups, indicating that significant differences with other antibodies are not an artifact of unequal loading.

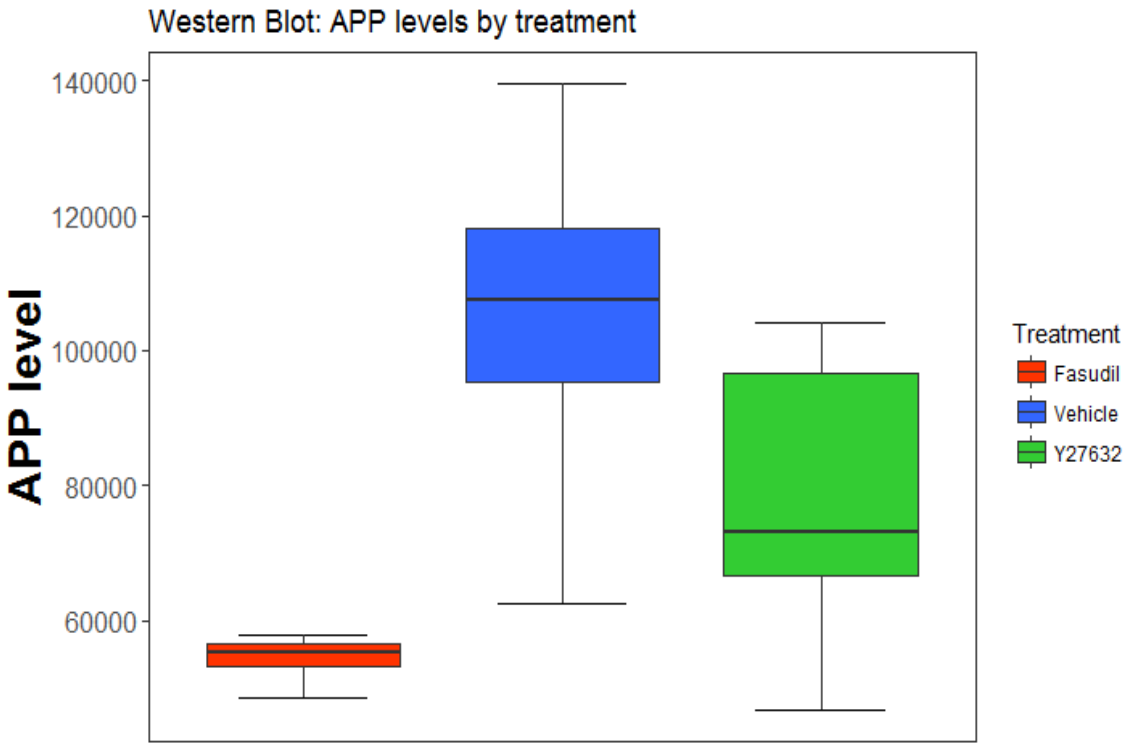


Figure 37. Fasudil decreases APP in 3xTg-AD mouse hippocampus.
Fasudil-treated, but not Y27632-treated, 3xTg-AD mice have a significantly decreases level of APP in the hippocampus.

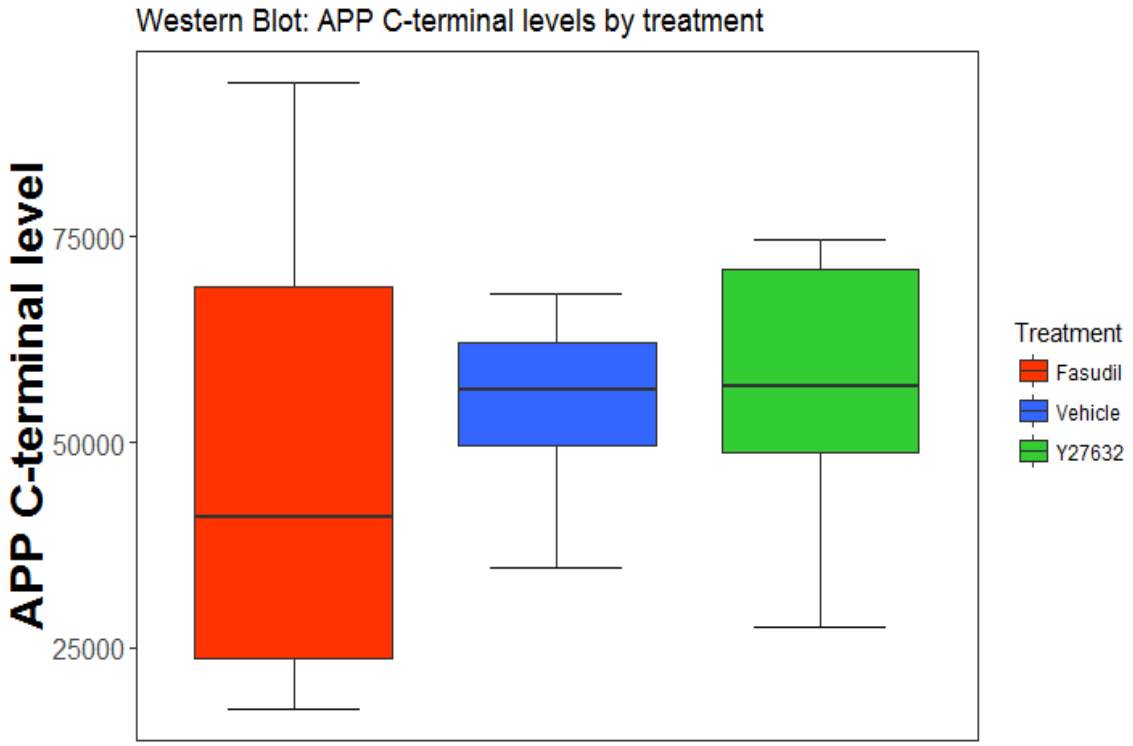


Figure 38. APP C-terminal levels not affected by treatment.
Neither Fasudil-treated nor Y27632-treated 3xTg-AD mice had a significant difference in APP C-terminal fragments in the hippocampus.

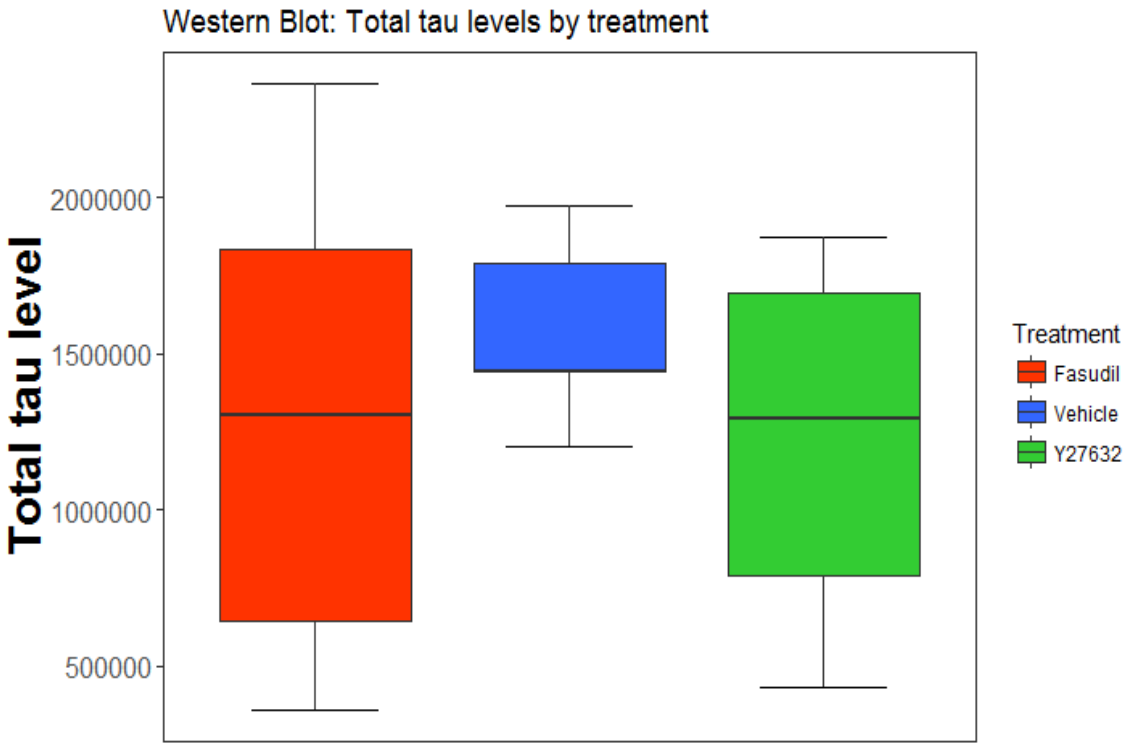


Figure 39. Total tau levels not affected by treatment.
Neither Fasudil nor Y27632 treatment significantly changed the level of total tau in the 3xTg-AD hippocampus.

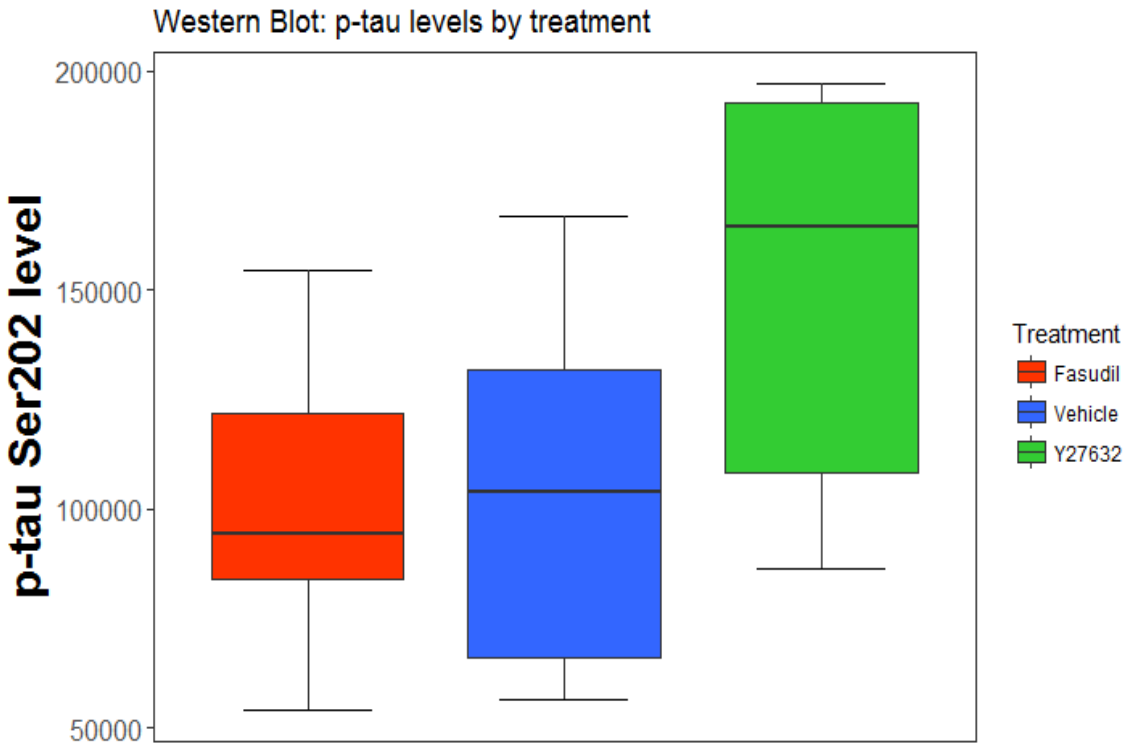


Figure 40. p-tau levels not affected by treatment.
Neither Fasudil nor Y27632 treatment significantly changed the level of p-tau at the Ser202 residue in the 3xTg-AD hippocampus.

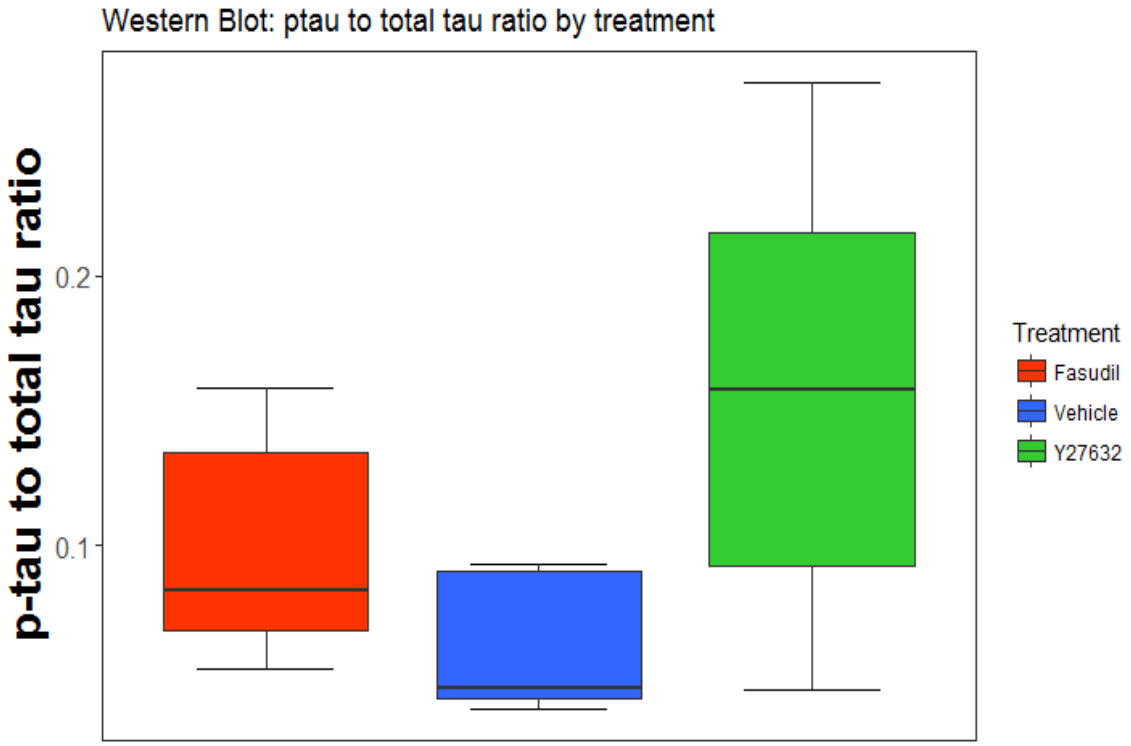


Figure 41. Y27632 treatment increases p-tau to total tau ratio.
Y27632 treatment led to a significant increase in the p-tau (Ser202) to total tau ratio in comparison to vehicle-treated 3xTg-AD mice, indicating an increase in pathology. There was no significant difference between Fasudil-treated and vehicle-treated mice.

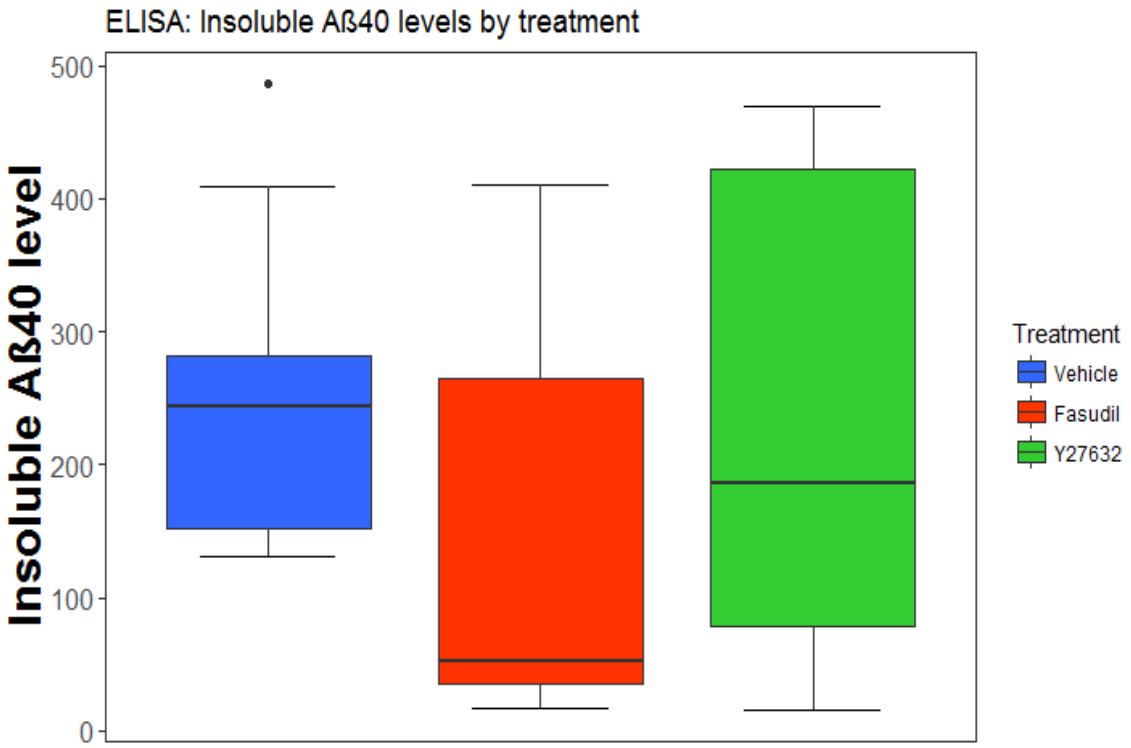


Figure 42. Fasudil treatment decreases insoluble Aβ$_{40}$.
Fasudil-treated mice, but not Y27632-treated mice, had a significantly decreased level of insoluble Aβ$_{40}$ in comparison to vehicle-treated mice, indicating that Fasudil improves pathology.

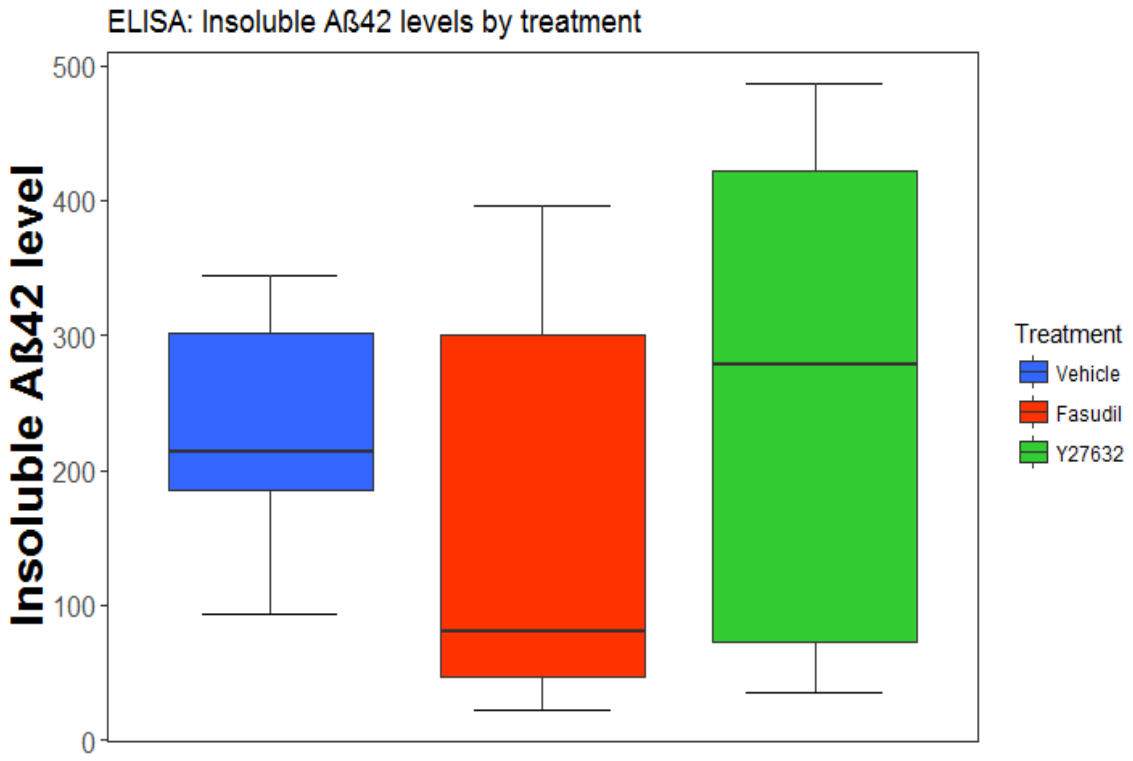


Figure 43. Insoluble Aβ$_{42}$ levels not affected by treatment.
There is no significant difference in levels of insoluble Aβ$_{42}$ between Fasudil- or Y27632-treated mice and vehicle-treated mice.

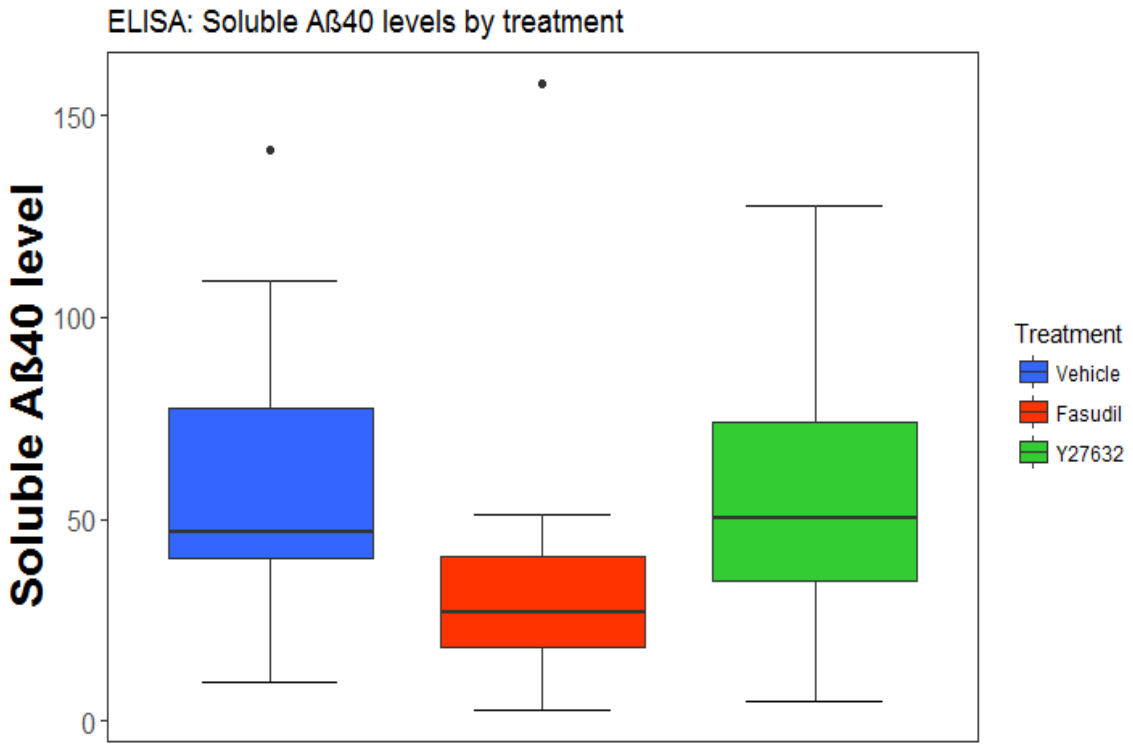


Figure 44. Fasudil treatment decreases soluble Aβ40.
Fasudil-treated mice, but not Y27632-treated mice, had a significantly decreased level of soluble $A\beta_{40}$ in comparison to vehicle-treated mice, indicating that Fasudil improves pathology.

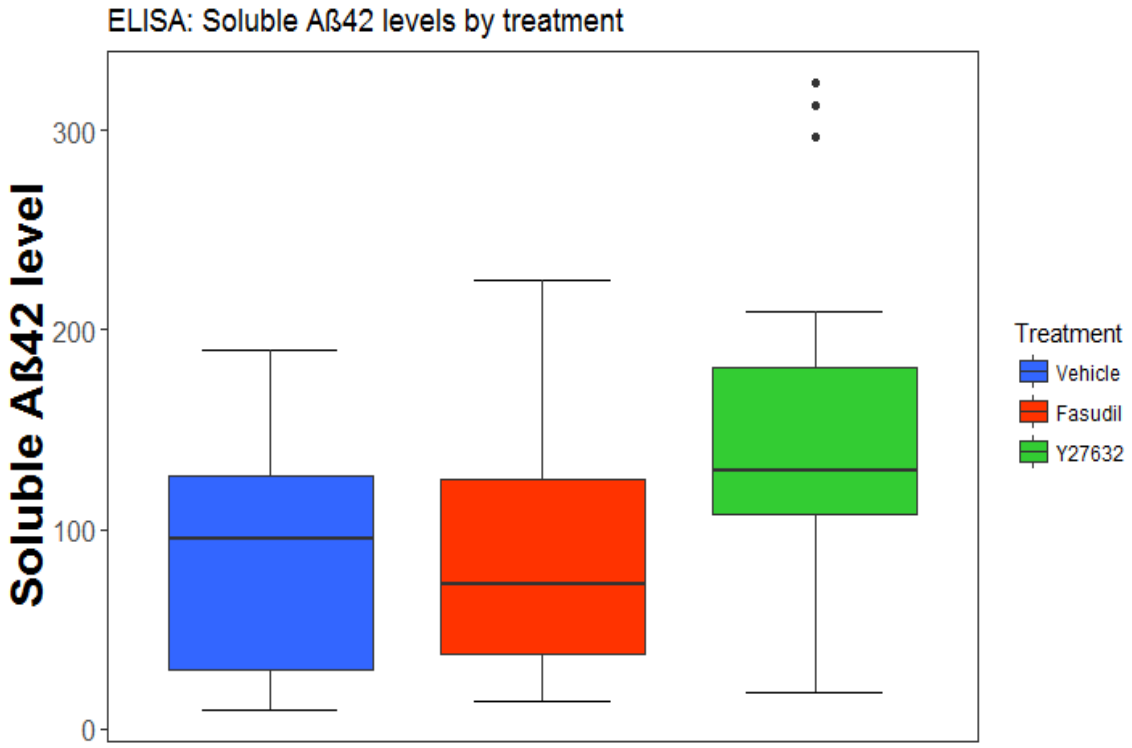


Figure 45. Y27632 treatment increases soluble Aβ42.
Y27632 treatment significantly increases the level of soluble $A\beta_{42}$ in comparison to vehicle treatment, indicating that Y27632 increases pathology. Fasudil treatment did not cause a significant change in soluble $A\beta_{42}$.

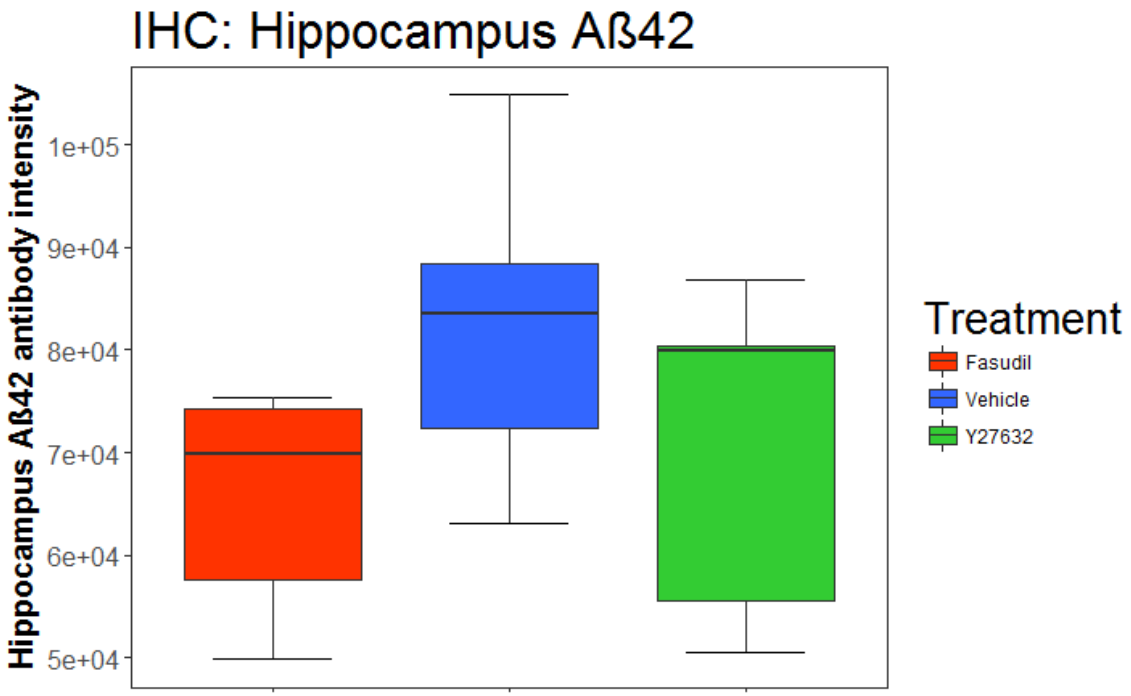


Figure 46. Hippocampal Aβ$_{42}$ not affected by treatment
While neither Fasudil nor Y27632 treatment significantly change the level of Aβ$_{42}$ seen in the hippocampus with IHC, Fasudil treatment trends toward a decrease with p = 0.055, indicating that there may be some improvement in the pathology.

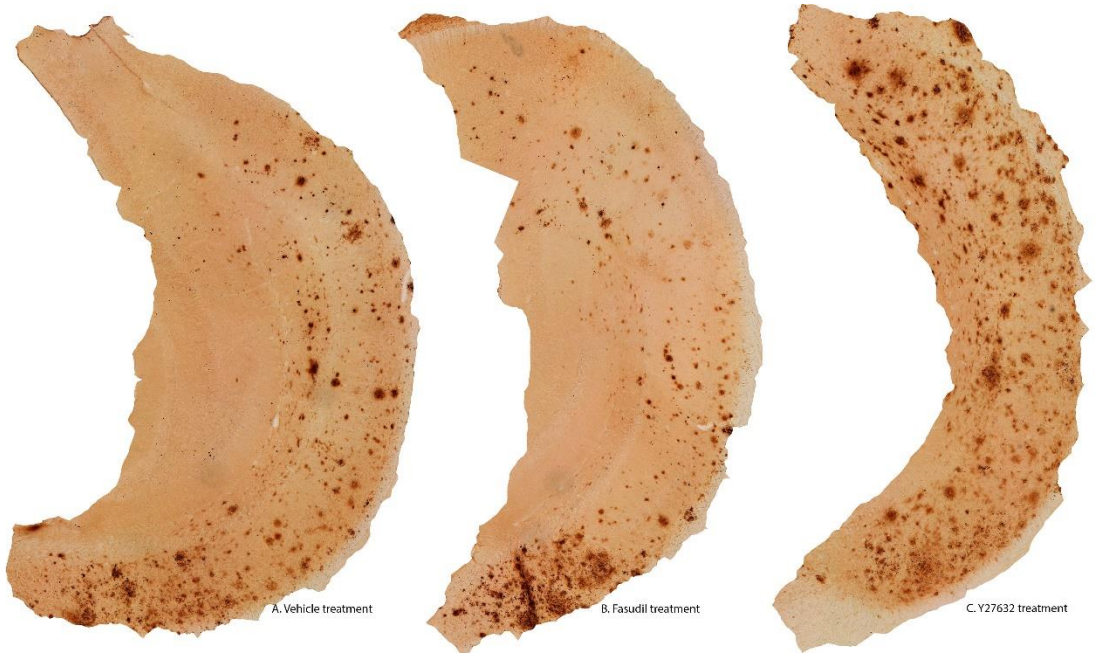


Figure 47. Representative images of IHC staining of hippocampal Aβ$_{42}$.
Each image represents its treatment group, and is the image with the median amount of staining, as represented by the box plot in Figure 46. Aβ in hippocampus of (a) vehicle-treated mouse, (b) Fasudil-treated mouse, and (c) Y27632-treated mouse.

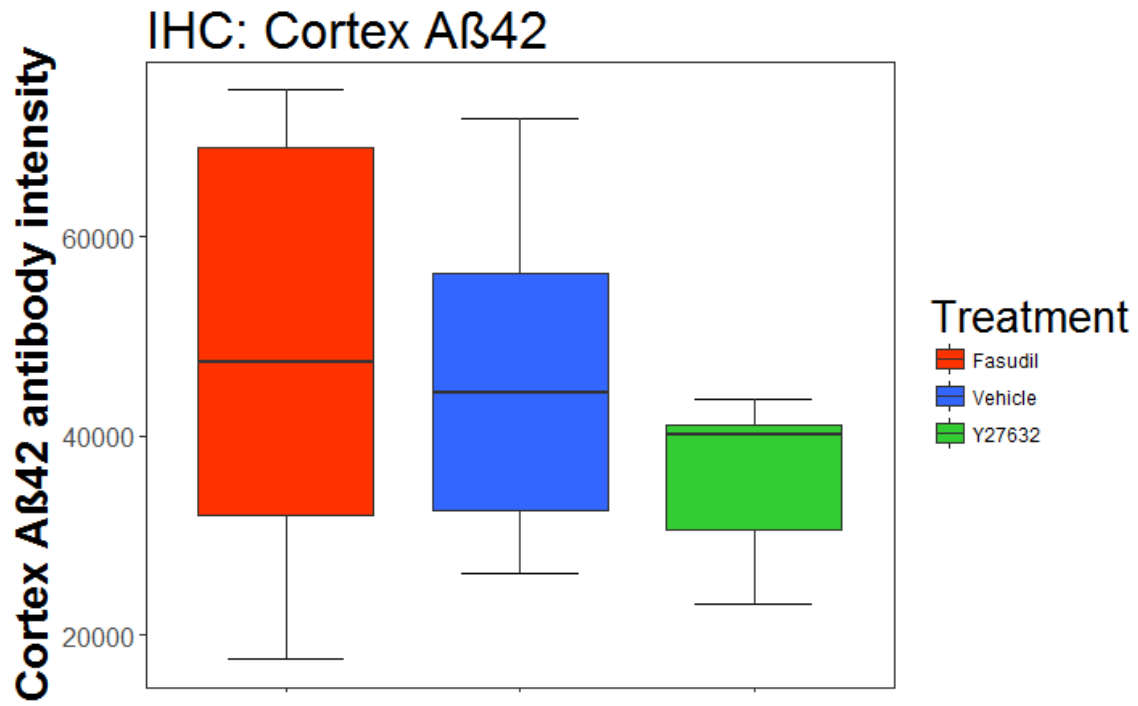


Figure 48. Cortex Aβ42 levels not affected by treatment.
There is no significant difference in the level of Aβ42 staining in the cortex between mice treated with Fasudil or Y27632 in comparison to mice treated with vehicle.

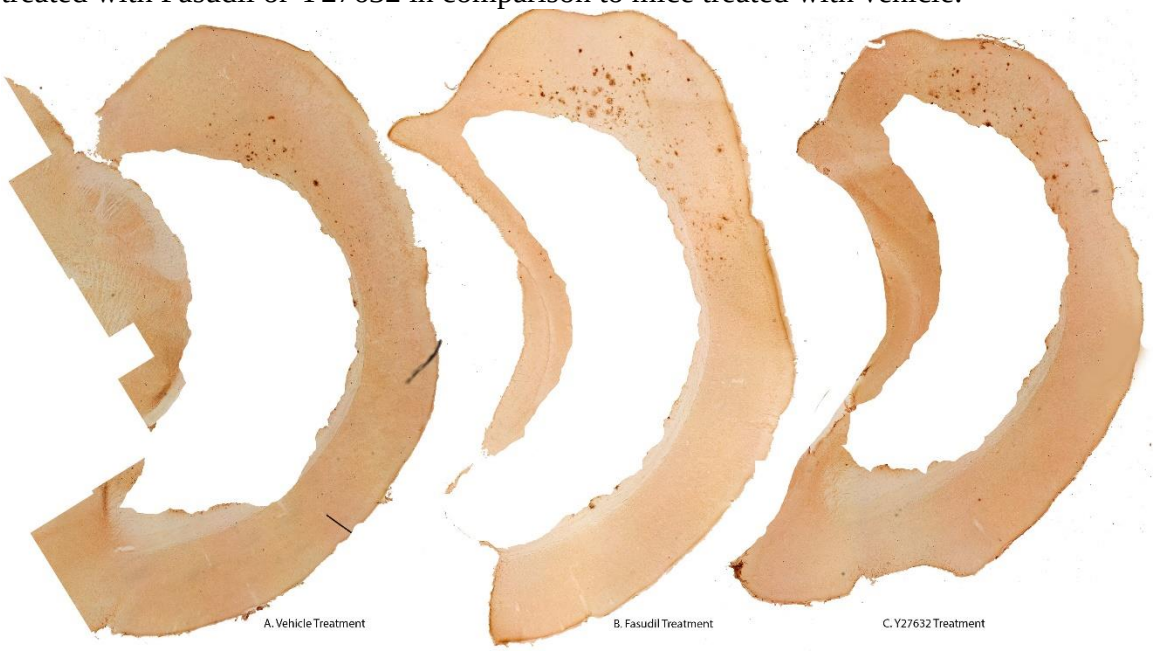


Figure 49. Representative images of IHC staining of cortex Aβ42.
Representative staining images from IHC of cortex Aβ42. Each image represents its treatment group, and is the image with the median amount of staining, as represented by the box plot in Figure 46. Aβ in cortex of (a) vehicle-treated mouse, (b) Fasudil-treated mouse, and (c) Y27632-treated mouse.

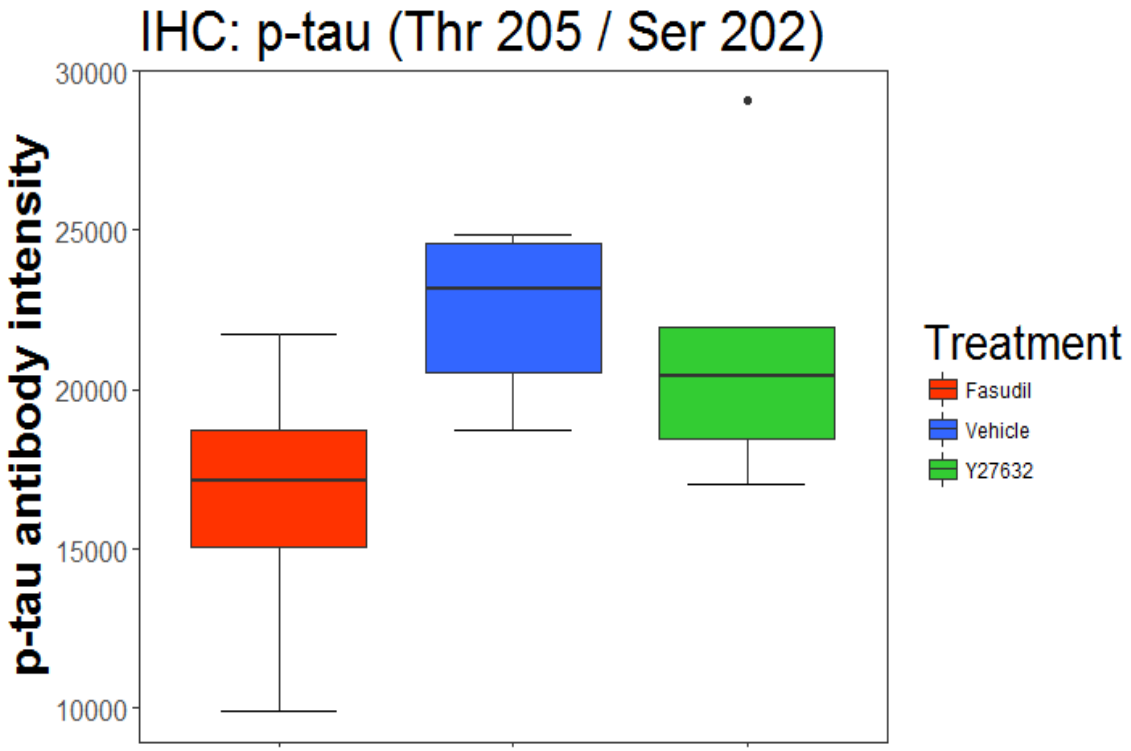


Figure 50. Fasudil decreases p-tau levels (Thr205/Ser202).
Fasudil treatment leads to a significant decrease staining using AT8, and antibody that stains for tau phosphorylated at residues Thr205 and Ser202. Y27632 treatment does not alter AT8 staining in comparison to vehicle treatment.

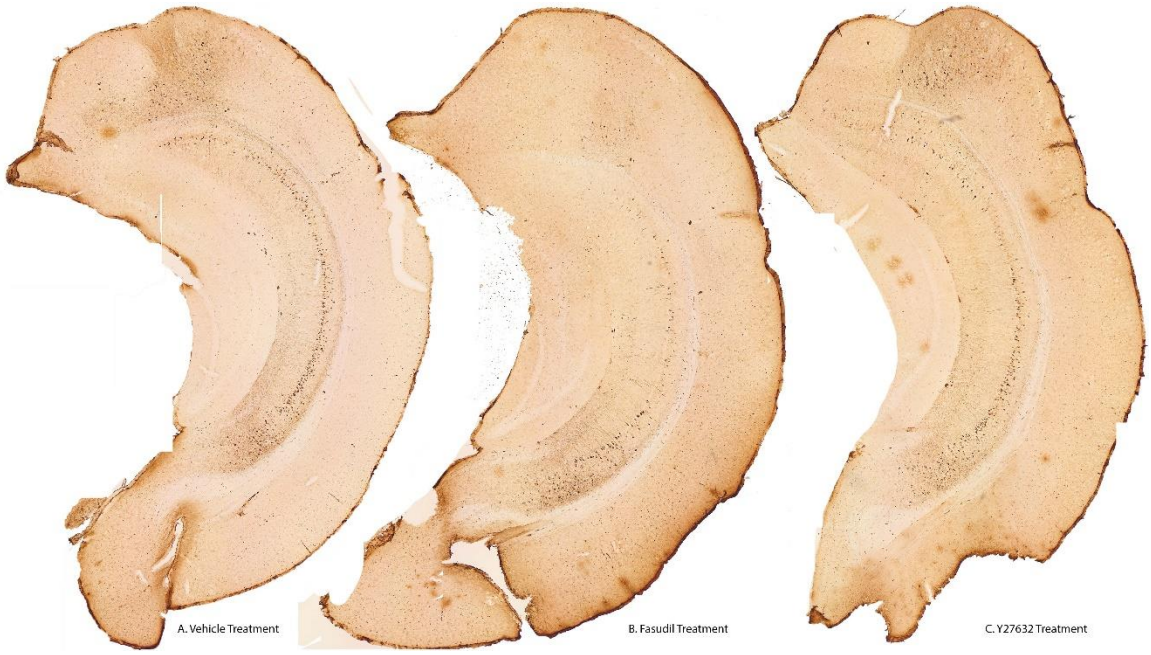


Figure 51. Representative images of IHC staining of p-tau.
Representative staining images from IHC of phosphorylated tau (Ser202/Thr205). Each image represents its treatment group, and is the image with the median amount of staining, as represented by the box plot in Figure 48. Phosphorylated tau in the brain of a (a) vehicle-treated mouse, (b) Fasudil-treated mouse, and (c) Y27632-treated mouse.

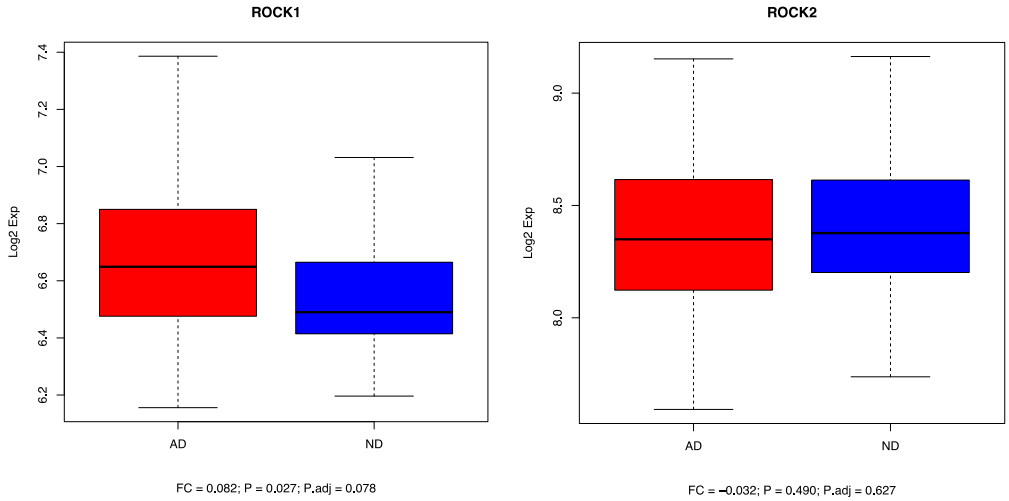


Figure 52. ROCK I and ROCK II expression levels do not differ between human AD and non-AD brain samples.
ROCK I and ROCK II expression levels in human samples. Expression levels were compared between AD patients and non-diseased (ND) samples. Results are from whole expression profiling analysis performed with HumanHT-12_v4_BeadChip (Illumina) in 97 AD vs 98 ND medial temporal gyrus samples. Data were normalized and analyzed with R package LUMI and p-values for differential expression analysis were corrected for multiple comparisons with FDR method. ROCK II shows two results due to two different probes in the chip. These were analyzed at the probe level as suggested by authors of R package LUMI.

APPENDIX C

LIST OF ABBREVIATIONS

AD – Alzheimer's disease
APP – Amyloid precursor protein
PS1 – presinilin 1
PS2 – presinilin 2
Apolipoprotein E – APOE
NFT – neurofibrillary tangle
Aβ – amyloid-β
CTF – C-terminal fragments
MAPT – Microtubule Associated Protein Tau
Ser – Serine
Thr – Threonine
ROCK – Rho-associated protein kinase
SMOL – Small, chemically manufactured molecules
ACE – Angiotensin-converting-enzyme
CDK5 – Cyclin dependent kinase 5
NSAID – Nonsteroidal anti-inflammatory drug
MLC – Myosin light chain
PTEN – Phosphotase and tensin homolog deleted on chromosome 10
GSK-3β – Glycogen synthase kinase-3 β
LIMK – LIM kinase
H4-tau – H4 neuroglioma cells overexpressing four repeat tau (4R0N)
3xTg-AD – Triple transgenic AD
BCA – Bicinchroninic acid
IHC – Immunohistochemistry
ELISA – Enzyme-linked immunosorbent assay

APPENDIX D

IACUC APPROVAL

ASU PROTOCOL NUMBER: 15-1435R RFC 17 PROTOCOL TITLE: DISSECTING THE MECHANISMS OF COGNITIVE DEFICITS IN ALZHEIMER'S DISEASE
PRINCIPAL INVESTIGATOR: SALVATORE ODDO
DATE OF ACTION: 9/20/2016

APPENDIX E

NUCLEIC ACID RISK FACTORS AND BIOMARKERS

CURRENT AND FUTURE USE IN ALZHEIMER'S DISEASE CLINICAL TRIALS

A REVIEW ARTICLE PUBLISHED IN PERSONALIZED MEDICINE (2015) 12(5), 475-82.

MARI N TURK AND MATTHEW J HUENTELMAN

Introduction:

Alzheimer's disease (AD) is a progressive neurological disorder and the most common form of dementia in the United States. AD is also the sixth leading cause of death in the United States. At this time, diagnosis only occurs after memory loss symptoms have become moderate to severe. Treatment must occur before these symptoms begin, as neuropathology begins and progresses for an estimated 15-20 years before this point. The AD field needs both new treatment options, coupled with early diagnostic tools, such as blood or cerebrospinal fluid biomarkers and neuroimaging assessments. This review aims to briefly explore new biomarkers and genetic risk factors and delve deeply into the future of nucleic acid-based diagnostic medicine for AD.

Biomarkers:

The National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disease and Related Disorders Associations discussed the classifications and methods used for diagnostic testing of AD in 1984, and the guidelines have not been updated since then(McKhann et al., 1984). Diagnoses use neuropathological patterns and clinical testing methods in order to determine whether the patient falls under classifications of "possible", "probable" or "definite" AD.

Biomarkers are necessary to better classify and diagnose AD prior to disease onset. Present biomarkers are associated with AD post-onset, and are not capable of providing information prior to symptomatic pathology. However, those biomarkers are still useful for classification purposes for patients and their families in order to provide the framework for appropriate care of the patient, as well as for research purposes, allowing patients and their

families to enroll in AD-specific studies. In short, the biomarkers that exist today were designed to classify individuals into healthy versus diseased classes and were not necessarily developed to determine risk in an otherwise healthy individual. Movement toward this type of biomarker is important for longevity as treatment starting at the current diagnostic mark is not able to return patient brain function to baseline levels.

Biomarkers must be predictable and should ideally be simple to supervise and inspect over the course of both prognosis and disease. While protein biomarkers have been attractive and highly studied for AD, and there have been a variety of promising possibilities, these potential biomarkers are complex due to the posttranslational modifications, sequence variations, and complications with development of high affinity and high sensitivity assay methods. Additionally, the protein biomarkers discovered thus far have also only been associated with AD after the disease onset, and thus would not be effective biomarkers for preventative medicine (Esteras et al., 2013; Harari et al., 2014; Ruiz et al., 2013; C. Wang et al., 2014).

Nucleic acids – DNA and RNA – have significant potential as effective biomarkers for AD and other neurological disorders. They have the benefits of being stable and are able to be amplified during assay allow the signal to be increased for even the lowest expressed molecules (something that cannot be currently done for rare protein species). Additionally, the maturation of so-called next generation sequencing approaches has significantly improved our ability to assay nucleic acids in an entirely un-biased digital fashion, therefore, measurements are much more reproducible in other laboratory environments and have a dramatically expanded dynamic range when compared to microarray-based measurements of nucleic acids.

Nucleic acid biomarkers come in two main classes, those that are personalized and represent heritable risk factors (e.g. the DNA variants that were passed on to you by your mother or father in your germline genome) or those that represent markers of a change in physiology or development of a pathophysiology (e.g. circulating RNAs that may be released from the brain in response to the progression of AD-relevant pathology or site-specific DNA methylation changes).

Genetic risk factors:

Genome wide association studies (GWAS) have been used to identify susceptibility loci for AD under the common disease-common variant hypothesis for approximately the last decade. Most studies on late onset Alzheimer's disease (LOAD) have been performed on non-Hispanic white people of European ancestry. The International Genomics of Alzheimer's Project combined all non-Hispanic white data sets, leading to 12 additional susceptibility loci and 13 candidate loci being found through this study.

The Apolipoprotein E gene (APOE) has been established as a susceptibility gene for LOAD, with an increased risk of 12-fold for ε4 homozygotes(Chen et al., 2007; Reiman et al., 2007). This makes up approximately 25% of the estimated heritability of ~80% for LOAD. While a large portion of the heritability risk is accounted for by APOE, this still leaves room for other genetic factors that could modify the risk of LOAD(Seshadri et al., 2010). Over 1200 papers have claimed or refuted an association between AD and a variety of putative risk genes, with often contradictory results. Many of these studies are not replicated, likely due to small genetic effects and low powered data sets. However, 20 other susceptibility loci have been identified with replicable results including SORL1, CLU,

PICALM, ABCA7 and BIN1(Harold et al., 2009; Hollingworth et al., 2011; J.-C. Lambert et al., 2009; J. C. Lambert et al., 2013; Naj et al., 2011). Many of these loci have been associated with immune function, and are likely to be found in other disease states, and while they may be risk factors functioning as decreased immune function or overactive immune function leading to glial overactivity and increased neurodegeneration, they may not be useful for AD risk assessment. However, some of the susceptibility loci identified continue to be of interest to the AD community due to their function. SORL1 and ABCA7, for example are involved in APP processing and trafficking(Andersen et al., 2005, 2006; Mack, Townsend, Beljanski, & Tew, 2007; Spoelgen et al., 2006; Yin, Yu, & Tan, 2014). Single nucleotide polymorphism (SNP) variants of PICALM have been highly associated with AD, but not with other neurodegenerative disease. Overexpression of normal PICALM increases the internalization of APP and the formation of Aß42 and amyloid plaques(Xiao et al., 2012). CLU expression has been shown to be upregulated in AD patient brains. CLU mutations have also been associated with LOAD, as well as with APOE allele ε4(Lidstro, 1998; McGeer, Kawamata, & Walker, 1992). BIN1 mutations may be a risk factor for LOAD through the trafficking of APP(Carrasquillo et al., 2011). Presently, it is possible to screen an individual's germline genome and quantitate their heritable risk for developing AD, however, even with the best models we can likely only explain half of their overall genetic risk, therefore, more work is needed to fully characterize the remaining factors in our genome that are linked to altered AD risk.

Oxidation levels of RNA may be used as a biomarker of AD, although it is decidedly non-specific. DNA is oxidized and repaired under non-disease states; however, extensive RNA oxidation may be a mechanism for disease development. RNA oxidation

results in ribosomal stalling and dysfunction, and can be identified through those means(Poulsen et al., 2012). However, studies have not yet parsed out the differences in RNA oxidation that may occur between different neurodegenerative diseases and other diseases as well, therefore the question remains regarding the specificity of such a marker for AD in particular.

Another route through which mRNA can be studied in the context of AD is through RNA editing. Adenosine to inosine (A to I) RNA editing occurs within precursor mRNA, and is prevalent in the human brain. It occurs at both the protein-coding and noncoding regions of the RNA(Bass, 2002; Valente & Nishikura, 2005). A to I RNA editing has been shown to differ between controls and patients with amyotrophic lateral sclerosis, providing a possible mechanism of action for neuronal death(Kwak & Kawahara, 2005). Few studies have examined RNA editing in AD patients specifically, although one has found a decrease in RNA editing in the prefrontal cortex of AD patients(Akbarian, Smith, & Jones, 1995) and another has found differences in RNA editing between AD patient hippocampus and control hippocampus(Gaisler-Salomon, Kravitz, & Feiler, 2014). The usefulness of this approach as a biomarker tool remains to be seen, as much of the work to date has been conducted on autopsy derived material.

Profiling of blood-based buffy coat derived RNA has been used to examine multiple types of human disease with limited success. The same case can be made for AD. We are not certain as to why this approach has yielded limited success as a biomarker, however one could hypothesize that the gene expression patterns present within circulating nucleated cells is not significantly altered enough – especially in the early stages of neurological disease – to result in a biomarker with appreciable sensitivity and specificity.

A modern example of a blood-based signature can be found in the AclarusDx™ assay(Fehlbaum-Beurdeley et al., 2012). This approach examines 136 known genes involved in multiple types of biological processes with the hope of assigning an individual to either an AD case or control group. The assay is reported to have a sensitivity of 81% and a specificity of 67%, however, this biomarker panel is focused on aiding in the diagnosis of AD once already suspected by a clinician – it is not designed to determine AD risk before symptom onset.

microRNAs (miRNA) have been of high interest to AD and other neurological disease researchers for several years due to their demonstrable stability outside of the cell and identification of them packaged in extracellular microvesicles. miRNAs are non-coding RNA containing approximately 22 nucleotides and are recognized as critical regulators of gene expression. Notably, individual miRNA species can target several different transcripts, thereby providing a mechanism of action that could perturb entire signaling pathways through the altered expression of one miRNA. miRNA have been found in extracellular fluids such as plasma, serum, urine, and saliva, while changes in extracellular miRNA have been observed and studied in the instance of cancer, cardiovascular disease, and injury(Hanke et al., 2010; Mitchell et al., 2008; Park et al., 2009; Zubakov et al., 2010). Similarly, this deregulation of miRNA has also been associated with degenerative diseases such as AD(Cogswell et al., 2008; Geekiyanage, Jicha, Nelson, & Chan, 2012; Lukiw, 2007). Some hypothesize that extracellular miRNA are found in healthy individuals through the circulating blood cells, but that in illness and injury the affected tissue(s) releases miRNA as well. Thus, the miRNA released by the affected tissue, such as the brain in AD, could serve as an effective biomarker.

The brain has been found to have the highest levels of tissue specific miRNA(Babak, Zhang, Morris, Blencowe, & Hughes, 2005). miRNA extracted from brain tissue that have been found to be dysregulated in AD cases include miR-9, miR-20a, and miR-132(Hébert et al., 2009; Hébert, Sergeant, & Buée, 2012; Makeyev, Zhang, Carrasco, & Maniatis, 2007). However, brain tissue biomarkers are not useful in clinical diagnoses, and extracellular miRNA biomarkers are necessary. Extracellular miRNA that originated in the brain can be found in the CSF, blood, saliva, and urine(Baraniskin et al., 2012; Hunter et al., 2008; Ogawa, Taketomi, Murakami, Tsujimoto, & Yanoshita, 2013; Patel et al., 2011; Qi, Wang, Katayama, Sen, & Liu, 2013). Extracellular miRNA can be an effective biomarker because they are protected from degradation by binding to RNA binding proteins, or are contained in membrane derived microvesicles such as exosomes(Arroyo et al., 2011; Mitchell et al., 2008; Vickers & Remaley, 2012). While circulating miRNA have been shown to be useful biomarkers in cancer studies, using them for AD may not be as fruitful because the mechanism and the level at which brain specific miRNA pass through the blood brain barrier is unknown. However, it is known that highly abundant brain specific miRNA are found in the CSF, blood, and urine. Therefore, there is a possibility that circulating miRNA will be an effective biomarker for AD. A notable shortcoming to these efforts may lay in the current inability to identify and purify microvesicles that are released from the brain – distinguishing those microvesicles from others derived from non-disease relevant tissues would theoretically aid in the increase of disease biomarker signal relative to the healthy tissue noise.

CSF is perhaps the most relevant, albeit invasive to obtain, source of biomarkers for AD and other neurodegenerative diseases, as it is produced in close proximately and

surrounds the organ of interest. The most commonly used platform to measure miRNA levels in CSF is qRT-PCR; however, more recently, next generation sequencing has been utilized and optimized for miRNA quantification. qRT-PCR has allowed for the discovery of 60 miRNA that are significantly altered in the CSF of patients with AD, as compared to healthy elderly control CSF. These miRNA were associated with changes in the immune system, however, and are therefore likely linked to general neurodegeneration rather than to AD specifically. Other studies have also attempted to find differential expression of miRNA in CSF between AD patients and controls; however, the upregulation and deregulation of the miRNA found in each study were not replicated in subsequent studies(Alexandrov et al., 2012; Cogswell et al., 2008; Lehmann et al., 2012; Lukiw, Alexandrov, Zhao, Hill, & Bhattacharjee, 2012). These contradicting results could be due to methodological differences in quantification approach—the studies used either microarrays, qPCR, or target candidate miRNA approaches.

Next generation sequencing is a potent technique for quantifying differential expression of miRNA between AD patients and controls. Brain specific miRNA dilutes in the circulatory system, leading to a greater signal to noise ratio when compared to quantification of miRNA in the brain tissue(Cheng, Quek, Sun, Bellingham, & Hill, 2013). While blood biomarkers would be a minimally invasive and simple diagnostic test, few studies have profiled miRNA differences in AD patients compared to controls. However, these studies have found a number of brain specific miRNA down regulated in the blood of AD patients, as well as miRNA that were upregulated in patients with early AD symptoms(Geekiyanage et al., 2012). Further studies could include exosome separation

from the blood in order to find differential expression specific to exosomes, as has been done with plasma and serum.

An attractive minimally invasive and very cost effective clinical diagnostic biospecimen is urine. Circulating extracellular RNA is released into the urine through renal epithelial cells bound to RNA binding proteins or packaged into microvesicles. RNA in urine has been used for diagnosis in bladder or prostate cancers(Hanke et al., 2010); however, the same techniques may not be effective for neurodegenerative diseases such as AD because the cells in urine are highly concentrated from hematologic, renal, or urothelial origin. Cell-free urine may be useful; however, separating the cells from the urine may also remove miRNA from tissues outside the excretory system. Exosomes may be the most effective solution for miRNA studies from urine samples.

Epigenetics

Another possible risk factor involves the transient, reversible changes to chromatin, and the modifications these changes can have on gene expression and transcription activity. These changes are classified as epigenetics, and include chromatin remodeling, histone modification, and DNA methylation, among other DNA alterations. Epigenetics could have an effect on the risk of developing AD. Genetic risk factors can play a large role in the disease; however, even human monozygotic twins differ in developing AD(Brickell et al., 2007). Environmental risk factors must have a significant effect on the disease and epigenetics allows environmental factors to influence genetic expression without altering DNA sequence. Epigenetic changes repress or silence the expression of specific genes, or release that repression, enhancing gene expression.

DNA methylation has been investigated in post-mortem brain tissue in both AD mouse models and human AD patients(Brohede, Rinde, Winblad, & Graff, 2010; Cadena-Del-Castillo et al., 2014; Chouliaras et al., 2013; Coppieters & Dragunow, 2011; Mastroeni, McKee, Grover, Rogers, & Coleman, 2009; Siegmund et al., 2007). Genetic risk may partially explain the DNA methylation differences found in these studies; however, GWAS studies have not found differences in genes linked to DNA methylation between controls and AD patients. There has been evidence for environmental factors such as diet having an effect on DNA methylation. For example, a study showed that folate deficiency in late life is associated with an increased percentage of AD, and that vitamin B treatment in AD patients with low baseline folate levels benefit the patients(Hinterberger & Fischer, 2013). Because these epigenetic changes would not be available until later in life, they may not be as useful as a biomarker for preventative therapeutics; however, they could be used as a risk assessment tool for making lifestyle changes in the older population.

While twin studies would be an excellent method to parse out the genetic and environmental effects of AD, especially with regards to epigenetics, it is difficult to find pairs of monozygotic twins within which at least one twin has AD. One pair of monozygotic twins that was discordant for AD was studied for methylation differences in the temporal neo-cortex and the superior frontal gyrus. In the AD twin, both regions had significantly decreased global methylation levels. However, the twins had no differences in methylation in the cerebellum, which is largely unaffected by AD(Mastroeni et al., 2009). This study demonstrated that epigenetics may play a role in the development of AD.

One study used the triple transgenic model of AD (3xTg-AD) in order to assess methylation changes in both an early age (pre-AD) and a late age (post-AD) AD model and

confirm differences within the two categories. Due to the difficulty in finding more monozygotic twin pairs discordant for AD, an animal study was necessary to confirm the results. The study both confirmed the decrease in methylation and added that there were markers for demethylation as the mice aged. They also found that young 3xTg-AD mice had increased levels of demethylation that matched aged wild type mice, indicating early aging occurring in mice with AD(Cadena-Del-Castillo et al., 2014). This study demonstrates that epigenetic changes that could be risk factors for AD may occur earlier in life, and therefore may be risk factors to consider at an early age, or could even be used as biomarkers.

DNA methylation, as a risk factor, is an interesting and exciting line of study as it also has potential as a biomarker. Statistical methods for analyzing DNA methylation as a biomarker have been produced(Alonzo & Siegmund, 2007) and biomarkers through DNA methylation have been implicated and are currently being pursued in different cancer studies(Kan, Liou, & Wang, 2014; Winham et al., 2014).

Future Perspective:

In the next five years, AD treatment is likely to go through a significant metamorphosis because of increasing assessment of "prevention" trials. If successful, these trials will guide the use of effective and safe medications in individuals who do not yet demonstrate dementia but are at elevated risk for the disease. This strategic approach to AD, and hopefully other progressive degenerative neurological diseases, is important not only to the specific field of AD therapeutics but also for the potential treatment of other slow onset disorders. It is very likely that the therapeutics, when administered earlier in the course of the disease than ever before, will demonstrate a positive effect.

Unfortunately, how early pending AD is diagnosed or how early personalized risk is reliably assessed remains a problem in society. This raises issues and is important because it is unlikely that the best preventative medicines will come without side effects and/or significant cost to the patient. Therefore, at least in the beginning stages of the prevention era, decisions will need to be made regarding the individuals who utilize prevention therapeutics versus those who do not. Imaging- and genomics-based biomarkers will likely contribute significantly to fill this void. The use of amyloid imaging has largely shown to be beneficial in the diagnosis of AD and it can also help assess risk in presymptomatic individuals. Many biotechnology and pharmaceutical companies are also working on developing tau-based imaging agents. However, imaging tests are not cost effective, they are data intensive, and expose the patient to some risk associated with the agent itself. Although nucleic acid biomarkers, as reviewed here, are still in their infancy, the field is advancing rapidly, with novel approaches and focus on extracellular RNA measurements in easy to obtain biofluids (like saliva and urine) and the use of next

generation sequencing, to both quantitate and sequence the RNA/DNA analyses. We believe it is likely that a blood-based nucleic acid focused biomarker test for AD will mature in the coming five years, with urine and/or saliva tests developing soon after. It is likely that these tests will need troubleshooting once developed, but they will act as an initial screen in determining if an individual had a tau or amyloid imaging test prior to starting prevention therapy. In short, we foresee the approval of at least one prevention therapeutic in the coming decade and the likely use of combined biomarker approaches – imaging and nucleic acid – to determine if an individual should or should not be prescribed AD alleviating medicine.

Lastly, we recognize that innovative approaches are needed in conducting human studies. Many of the trials in the AD field are small and too focused to provide a complete picture of the potential patient or even healthy human being. To address this, several groups, ours included, have resorted to recruiting and studying individuals using the internet. Our study, MindCrowd (at mindcrowd.org) seeks to recruit one million healthy volunteers to take a ten-minute episodic memory test. To date, over 30,000 people have completed the study, providing us answers to over 20 demographic questions. We have had at least one participant from over 150 countries in the world. Through this study, we are not only able to examine the most common demographics and their influence on our cognitive task, but we can also examine combinations of factors, including rare events such as the co-occurrence of early onset hypertension and a first degree relative with AD, allowing us to tease out how they might work together to influence cognition. Importantly, these large surveys enable us to identify those individuals who are significant outliers (think of a 60 year old who scores perfectly on our episodic memory task). These test takers represent

less than 0.5% of their respective age group and therefore may harbor novel genetic changes that enhance their cognition or protect them from some aging effects. Moreover, large internet-based studies may be the only way to identify these rare genetic changes of large effect.

APPENDIX F

CURRICULUM VITAE

Education

- **Arizona State University**
  - Ph.D. in Neuroscience, 2011-Present [Projected Graduation: May 2017]
  - B.S. in Psychology, 2007-2011
    - Barrett Honors College: 2007-2011
    - Academic Dean's Honor's List: 2009-2011

Research Experience

8/2011-Present	Pre-doctoral Research Associate: Translational Genomic Research Institute. (Mentor: Dr. Matt Huentelman)
1/2010-5/2011	Undergraduate Research Assistant: School of Life Science, Arizona State University. (Mentor: Dr. Janet Neisewander)
4/2011	Undergraduate Thesis Defense: Novel cues surprisingly drive reward-seeking behavior as effectively as reward-related cues.

Publications

Bastle RM*, Kufahl PR*, **Turk MN**, Weber SM, Pentkowski NS, Thiel KJ, Neisewander JL. Novel Cues Reinstate Cocaine-Seeking Behavior and Induce Fos Protein Expression as Effectively as Conditioned Cues. Neuropsychopharmacology. Epub 2012 April 25. PubMed PMID: 22534624

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Dean DC 3rd, Jerksey BA, Chen K, Protas H, Thiyyagura P, Roontiva A, O'Muircheartaigh J, Dirks H, Waskiewicz N, Lehman K, Siniard AL, **Turk MN**, Hua X, Madsen SK, Thompson PM, Fleisher AS, Huentelman MJ, Deoni SC, Reiman EM. Brain differences in infants at differential genetic risk for late-onset Alzheimer disease: a cross-sectional imaging study. JAMA Neurol. 2014 Jan. PubMed PMID: 24276092

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1. Bastle RM, Weber SM, **Turk MN**, Sanabria F, Cheung THC, Neisewander JL. Contextual and discrete cue contributions to renewed cocaine-seeking behavior. 40th Annual Meeting of the Society for Neuroscience. November 2010.

2. **Turk MN**, Bastle RM, Neisewander JL. Novel cues surprisingly drive reward-seeking behavior as effectively as reward-related cues. Celebrating Honors Symposium of Research and Creative Projects. April 2011.
3. Bastle RM, Kufahl PR, **Turk MN**, Pentkowski NS, Thiel KJ, Weber SM, Neisewander JL. Novel and conditioned stimuli reinstate extinguished reward-seeking behavior and induce similar patterns of fos expression. 41st Annual Meeting of the Society for Neuroscience. November 2011.
4. Hoang LT, Richards A, Allen AN, Biwer LA, **Turk MN**, Alexander GE, Hale TM, Mitchell KD, Huentelman MJ, Barnes CA. Cognitive consequences of the gradual induction of hypertension in middle age using Cyp1a1-Ren2 transgenic rats. 41st Annual Meeting of the Society for Neuroscience. November 2011.
5. **Turk MN,** Huentelman MJ. Longitudinal biomarker characterization in a viral vector induced model of Alzheimer's disease in the rat. 2012 Arizona Alzheimer's Consortium Conference. May 2012.
6. Corneveaux JJ, Myers AJ, Allen AN, **Turk M**, Hardy J, Chen K, Reiman EM, Huentelman MJ. Common genetic variation within BACE2 is associated with altered Alzheimer's disease risk in APOE ε4 non-carriers. 2012 Arizona Alzheimer's Consortium Conference. May 2012.
7. Swaminathan S, Huentelman MJ, Corneveaux JJ, Myers AJ, Faber KM, Foroud T, Mayeux R, Shen L, Kim S, **Turk M**, Hardy J, Reiman EM, Saykin AJ. Replication and meta-analysis of the CHRFAM7A copy number variant in a cohort of clinically characterized and neuropathologically verified individuals. Alzheimer's Association International Conference (AAIC) 2012. July 2012.
8. Fitzhugh MC, Totenhagen JW, Yoshimaru ES, Richards A, Hoang LT, Allen AN, **Turk M**, Krate J, Biwer LA, Hale TM, Chen K, Moeller JR, Coleman PD, Mitchell KD, Huentelman MJ, Barnes CA, Trouard TP, Alexander GE. Regional brain network of MRI gray matter with gradual induction of hypertension in the Cyp1a1-Ren2 transgenic rat. 42nd Annual Meeting of the Society for Neuroscience. October 2012.
9. **Turk MN,** Mennenga SE, Siniard A, Corneveaux JJ, Hewitt L, Tsang C, Caselli J, Braden BB, Bimonte-Nelson HA, Huentelman MJ. The PKCβ selective inhibitor, Enzastaurin, impairs learning and memory without changes to hippocampal transcription. 42nd Annual Meeting of the Society for Neuroscience. October 2012.
10. Siniard AL, Corneveaux JJ, **Turk M**, Allen A, Chawla M, Reiman R, Rose H, Barnes CA, Huentelman MJ. Activity regulated transcript identification in the hippocampus and the genetic association with AD risk. Arizona Alzheimer's Consortium (AACC) 2013. May 2013.
11. **Turk MN**, Adams MD, Wang T, Dunckley T, Huentelman MJ. ROCK inhibitor development for cognitive enhancement and blockade of tau phosphorylation. 43rd Annual Meeting of the Scoeity for Neuroscience. November 2013.

12. Corneveaux J, Schrauwen I, Siniard A, Peden J, **Turk M**, De Both M, Richholt R, Mueller M, Langbaum J, Reiman EM, Caselli RJ, Coleman P, Barnes C, Glisky E, Ryan L, Huentelman MJ. An internet-based study of over 25,000 individuals demonstrates significant effects of age, gender, education, and Alzheimer's disease family history in paired associate learning. Arizona Alzheimer's Consortium (AACC) 2014. June 2014.

13. Schrauwen I, Corneveaux J, Siniard A, Peden J, **Turk M**, De Both M, Richholt R, Mueller M, Langbaum J, Reiman EM, Caselli RJ, Coleman P, Barnes C, Glisky E, Ryan L, Huentelman MJ. Web-based paired associates testing of over 25,000 individuals demonstrates significant main effects of chronological age, gender, education, and Alzheimer's disease family history on performance. Arizona Alzheimer's Consortium (AACC) 2014. June 2014.

14. Siniard A, Schrauwen I, Corneveaux J, Peden J, **Turk M**, De Both M, Richholt R, Mueller M, Langbaum J, Reiman EM, Caselli RJ, Coleman P, Barnes C, Glisky E, Ryan L, Huentelman MJ. The influence of demographic and disease risk factors on paired associates learning in an internet recruited cohort of 25,000 individuals. Arizona Alzheimer's Consortium (AACC) 2014. June 2014.

15. **Turk MN**, Adams MD, Wang T, Dunckley T, Huentelman MJ. ROCK inhibitor development for cognitive enhancement and blockade of tau phosphorylation. Arizona Alzheimer's Consortium (AACC) 2014. June 2014.

Review Article

Turk MN, Huentelman MJ. Nucleic Acid Risk Factors and Biomarkers: Current and Future Use in Alzheimer's Disease Clinical Trials. Personalized Medicine. Issue #3 2015.

Invited Talks

Turk MN, Adams MD, Wang T, Dunckley T, Pirrotte P, Oddo S, Huentelman MJ. ROCK inhibitors for modulation of tau phosphorylation: An opportunity to target Alzheimer's disease pathology and enhance memory. Nanosymposium. Society for Neuroscience 2015.

Turk MN, Huentelman MJ. The Birds and Bees of Alzheimer's Disease: Novel Approaches to Studying at 100 Year-Old Puzzle. Hot Topics Speaker Selection—one of six selected abstracts. TGen Scientific Retreat 2013.

NOTE:
* Equal contribution by these authors

Awards and Grants

2013 Tuition Award: Summer Institute in Statistical Genomics (University of Washington)

2013	Travel Award: Summer Institute in Statistical Genomics (University of Washington)
2012	Conference Travel Award (ASU)
2011-2012	Doctoral Enrichment Fellowship (ASU)
2011	Conference Travel Award (ASU)

Community Service

2014-2015	Phoenix Metro St. Baldrick's Day: President
2013	Next Generation Innovator Speaker Series: Speaker
2013	Mesa Academy for Advanced Studies Science Fair: Judge
2011-2012	Hospice of the Valley: Volunteer Caregiver and Japanese Translator
2012	Arizona Junior Science and Humanities Symposium: Judge
2012	Mini-Brain Fair
2012	Larry C. Kennedy School Science Fair: Judge
2011	Next Generation Innovator Speaker Series: Speaker

Institutional Service

2011-Present	ASU, Graduate and Professional Student Association: Peer Reviewer
2011-Present	Ask A Biologist: Volunteer
2013-2015	Graduate Assoc. of Interdisciplinary Neuroscience: Student Representative

Mentored Students

2015-2016	Prakriti Shukla, Undergraduate Student	Volunteer Intern
2015	Satya Sette, Undergraduate Student	Volunteer Intern
2012	Erika Kollitz, High School Student	Basis Senior Project
2012	Matthew Adams, Medical Student	Volunteer Intern