The Dissertation Committee for Jordan B Jahrling
Certifies that this is the approved version of the following dissertation

PPARγ-pERK interaction restores memory consolidation in an Alzheimer's disease model

Committee:

Kelly T. Dineley, PhD
Supervisor

Larry Denner, PhD

Kathryn Cunningham, PhD

Fernanda Laezza, MD, PhD

Dean P Edwards, PhD

Dean, Graduate School

PPARγ-pERK interaction restores memory consolidation in an Alzheimer's disease model

by

Jordan B Jahrling, B.Sc

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Dedication

This work is dedicated to my wife, Dr. Christina Jahrling, my father, Dr. Peter B Jahrling, and my mother, Daria Baldovin-Jahrling. Thank you all for your love and support.

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PPARγ-pERK interaction restores memory consolidation in an Alzheimer's disease model

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Abstract

Cognitive impairment is a quintessential feature of Alzheimer's disease (AD) and AD mouse models. We and others have previously reported the peroxisome-proliferator activated receptor gamma (PPARγ) agonist rosiglitazone improves hippocampus-dependent cognitive deficits in some AD patients and ameliorates deficits in the Tg2576 mouse model for AD amyloidosis. Since extracellular signal-regulated protein kinase mitogen-activated protein kinase (ERK MAPK) is required for many forms of learning and memory that are affected in AD, and since both PPARγ and ERK are key mediators of insulin signaling, we tested the hypothesis that RSG-mediated cognitive improvement induces a hippocampal PPARγ pattern of gene and protein expression that converges with the ERK MAPK signaling axis in Tg2576 AD mice. In the hippocampal PPARγ transcriptome, we found significant overlap between peroxisome proliferator response element-containing PPARγ target genes and ERK-regulated, cAMP response element-containing target genes. Within the Tg2576 dentate gyrus proteome, RSG induced proteins with structural, energy, biosynthesis and plasticity functions. Several of these

proteins are known to be important for cognitive function and are also regulated by ERK MAPK. Given that RSG-mediated cognitive enhancement induced convergence of the PPARγ signaling axis and the ERK cascade, we next tested whether PPARγ and ERK associated in protein complexes that subserve cognitive enhancement through PPARγ agonism. Co-immunoprecipitation revealed that PPARγ and active ERK (pERK) associated in Tg2576 hippocampal extracts *in vivo,* and that PPARγ agonism facilitated recruitment of PPARγ to pERK during memory consolidation. Furthermore, the amount of PPARγ recruited to pERK correlated with cognitive reserve in humans with AD and in Tg2576. Thus, PPARγ represents a signaling system that is not crucial for normal cognition yet can intercede to restore neural networks compromised by AD. Our findings implicate a previously unidentified PPARγ-pERK complex that provides a molecular mechanism for the convergence of these pathways during cognitive enhancement, thereby offering new targets for therapeutic development in AD.

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CHAPTER 1
INTRODUCTION

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common of the neurodegenerative diseases and the sixth leading cause of death amongst the aged population. Concern grows to find disease modifying interventions since AD is predicted to affect 1 in 85 people globally by 2050 (Brookmeyer et al., 2007). Along with this explosion in disease prevalence, social and economic costs will increase accordingly. In the United States alone, AD currently affects approximately 5 million people and in developed countries, AD is one of the most costly diseases to society (Bonin-Guillaume et al., 2005). In 2013, Alzheimer's will cost the United States $203 billion, and this figure is expected to rise to $1.2 trillion by 2050 (Alzheimer's Association, 2013). Current treatments serve only to temporarily alleviate symptoms and, as yet, do not successfully modify the disease process. On average, the life expectancy following diagnosis of AD is approximately seven years (Molsa et al., 1986), while fewer than three percent of individuals live more than fourteen years after diagnosis (Molsa et al., 1995). By some estimates, a hypothetical intervention that successfully delayed the onset of AD by just five years would result in a 57% reduction of patients with AD and reduce projected Medicare costs by nearly $300 billion (Sperling et al., 2011). AD is the only cause of death among the top 10 in America without a way to prevent it, cure it or even slow its progression, and deaths from Alzheimer's increased 68 percent between 2000 and 2010, whereas deaths from other major diseases, including the number one cause of death, heart disease, decreased.

1.2 AD Pathogenesis

While AD develops differently in each individual, there are common symptoms associated with the disease. Consensus now suggests that AD pathology begins years or even decades before cognitive symptoms develop (Morris, 2005); however, the majority of AD

subjects do not realize anything is wrong until they manifest physical symptoms. In this regard, sufferers most commonly exhibit deficits in hippocampus-dependent episodic memory, such as difficulty remembering recent events or conversations (Scheff et al., 2006) or the inability to learn new information (Backman et al., 2004). These initial presentations are often mistakenly attributed to normal aging or stress (Waldemar et al., 2007); however, they actually qualify as mild cognitive impairment (MCI), defined as cognitive decline greater than expected (given the individual's age and education level) that does not interfere with daily life (Gauthier et al., 2006). As the disease-state advances, symptoms can include irritability and mood swings, language problems, confusion, aggression, and long-term memory problems (Waldemar et al., 2007).

Despite being characterized over a century ago, the cause of AD has not been definitively determined. Currently, there are three main hypotheses regarding the onset of Alzheimer's pathology: 1) the cholinergic hypothesis, 2) the tau hypothesis, and 3) the amyloid cascade hypothesis. The cholinergic hypothesis is the oldest of the three and proposes that the degeneration of cholinergic neurons in the basal forebrain, combined with the associated loss of cholinergic neurotransmission in the cerebral cortex, is responsible for the cognitive decline observed in AD (Bartus et al., 1982). This concept was investigated after the observation that patients who had received the anticholinergic anesthetic scopolamine exhibited memory problems similar to those seen in early stages of AD (Drachman and Leavitt, 1974) and other reports that post-mortem AD brain samples exhibited 75% loss of cholinergic neurons in the basal forebrain (Whitehouse et al., 1982). However, it is noteworthy that individuals with inherited olivopontocerebellar atrophy lack cognitive deficits despite having diminished choline acetyltransferase activity similar to that observed in AD (Kish et al., 1989), and medications that treat acetylcholine deficiencies have proven ineffective at preventing disease stage progression from MCI to AD (Raschetti et al., 2007) and only mildly effective at treating the symptoms of disease, and therefore do not represent a cure (Desai and Grossberg, 2005; Birks, 2006). As a

result, it is now believed that while cholinergic transmission is important to memory, the loss of these neurons is a downstream effect of AD pathology and thus support for the cholinergic hypothesis has waned in recent years.

One of the hallmark pathological features of AD at autopsy is the presence of intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. Thus, the tau hypothesis contends that the hyperphosphorylation and subsequent aggregation of tau protein to form these neurofibrillary tangles within nerve cell bodies initiates the Alzheimer's disease-state (Mudher and Lovestone, 2002). The resulting destruction of the neuron's transport system is believed to result in impaired intracellular communication and ultimately cell death (Chun and Johnson, 2007). However, tau aggregates are a general marker of neuronal death, while episodic memory deficits appear long before overt neurodegeneration in both human AD and animal models of the disease (Mucke et al., 2000; Cheng et al., 2007), suggesting that tau pathology is unlikely to be the primary initiator of the disease state and more likely a secondary consequence.

The amyloid cascade hypothesis is the most widely accepted theory regarding AD pathogenesis. Briefly, this theory posits that the accumulation of beta amyloid (Aβ) due to aberrant cleavage of the amyloid precursor protein (APP) triggers the disease state (Hardy and Allsop, 1991; Selkoe, 1991; Hardy and Higgins, 1992); further detail on this process is described in section 1.3 below. Post-mortem analysis of AD brains typically demonstrates widespread deposits of Aβ protein in the form of fibrils; however, plaque counts or total Aβ load do not always correlate with clinical impairment, and in fact, intracellular hyperphosphorylated tau, neuronal loss, and synaptic depletion all exhibit stronger correlations with impairment when examined at autopsy (Sperling et al., 2011). That said, more recent research has implicated the soluble Aβ oligomers, which preclude fibril formation, as the toxic species in AD (Podlisny et al., 1995; Lesne et al., 2006; Benilova et al., 2012; Gilbert, 2013). Tg2576 AD mice expressing a

56kDa Aβ species exhibit substantial disruptions to memory (Lesne et al., 2006), and injection of this oligomeric species into young rats caused similar impairment (Lesne et al., 2006; Oddo et al., 2006; Cheng et al., 2007). Further support for the amyloid cascade theory stems from cognitive deficits and synaptic dysfunction observed in 1) transgenic animal models over-expressing human APP (Dineley et al., 2002b; Dineley et al., 2010; Rodriguez-Rivera et al., 2011) 2) wild type rats injected with Aβ (Walsh et al., 2002; Selkoe, 2008) and 3) human carriers of familial genetic mutations in APP that result in increased amyloid burden and AD symptomology (Cruts et al., 2012).

Additionally, soluble Aβ oligomers have been shown to inhibit hippocampal long term potentiation (LTP) both *in vitro* and *in vivo* (Chapman et al., 1999; Walsh et al., 2002) bolstering the argument that increased amyloid burden leads to the onset of AD pathology. Interestingly, Aβ accumulation is associated with downstream pathology including abnormal tau, neural dysfunction, glial activation, and neuronal loss and atrophy. Also, tau knock-down in cultured hippocampal neurons has been shown to decrease the cytoskeletal disruption induced by Aβ oligomers (Panda et al., 1995) and hippocampal slices from tau knockout mice are resistant to Aβ oligomer-induced LTP inhibition (Shipton et al., 2011). Thus, it is unclear whether aggregation of Aβ on its own is sufficient to initiate the AD pathological cascade (Sperling et al., 2011) or if tau and Aβ oligomers cooperate to initiate the disease state. Still, the majority of AD mouse models include some form of Aβ dysregulation in an attempt to recreate the physical symptoms of the disease.

1.3 Risk factors for the development of AD

Cases of AD are categorized as either familial (FAD) or sporadic (SAD), with the overwhelming majority (95%) of cases qualifying as the latter. Early-onset AD describes individuals who develop AD before the age of 65, and this is generally attributed to familial gene mutations. Deterministic Alzheimer's variations have been found in only a few hundred

extended families worldwide. Symptoms nearly always develop before age 60, and may appear as early as the individual's 30s or 40s. The known risk genes for the development of AD that have been identified include autosomal dominant mutations to amyloid precursor protein (APP), presenilin 1 (PS-1) and presenilin 2 (PS-2). APP was originally identified from the amyloid plaques that are a hallmark of AD pathology. These plaques are made up of beta-amyloid protein (Aβ) which is coded by chromosome 21 and synthesized by APP. All individuals synthesize APP, although the native biological role of this protein remains unknown. There is some evidence that APP is involved in both the formation and repair of synapses as APP expression is up-regulated in both cultured neurons (Priller et al., 2006) and P19 embryonic cells (Hung et al., 1992) during differentiation and in mouse embryonic stem cells following neural injury (Bibel et al., 2004). In this regard, APP is also recognized as a substrate of the Notch signaling cascade which regulates the expression of genes involved in the development and function of neurons (Selkoe and Kopan, 2003); thus, APP in the brain is considered to be both neurotrophic and neuroprotective in normal physiology (Turner et al., 2003; Priller et al., 2006).

Under normal, non-amyloidogenic conditions, APP is cleaved by α-secretase within the Aβ peptide sequence and the soluble extracellular fragment is released and undergoes proteasomal degradation. However, missense mutations in APP can result in the increased activity of β- and γ-secretase, resulting in the over-production of Aβ peptides of 40-43 amino acids (**Figure 1.1**). Mutations near the β-secretase site generally increase overall Aβ levels, while those around the γ-secretase site specifically increase Aβ42 (Citron et al., 1992; Cai et al., 1993; Haass et al., 1994). The Aβ peptide fragment can misfold and aggregate into oligomers

Figure 1.1

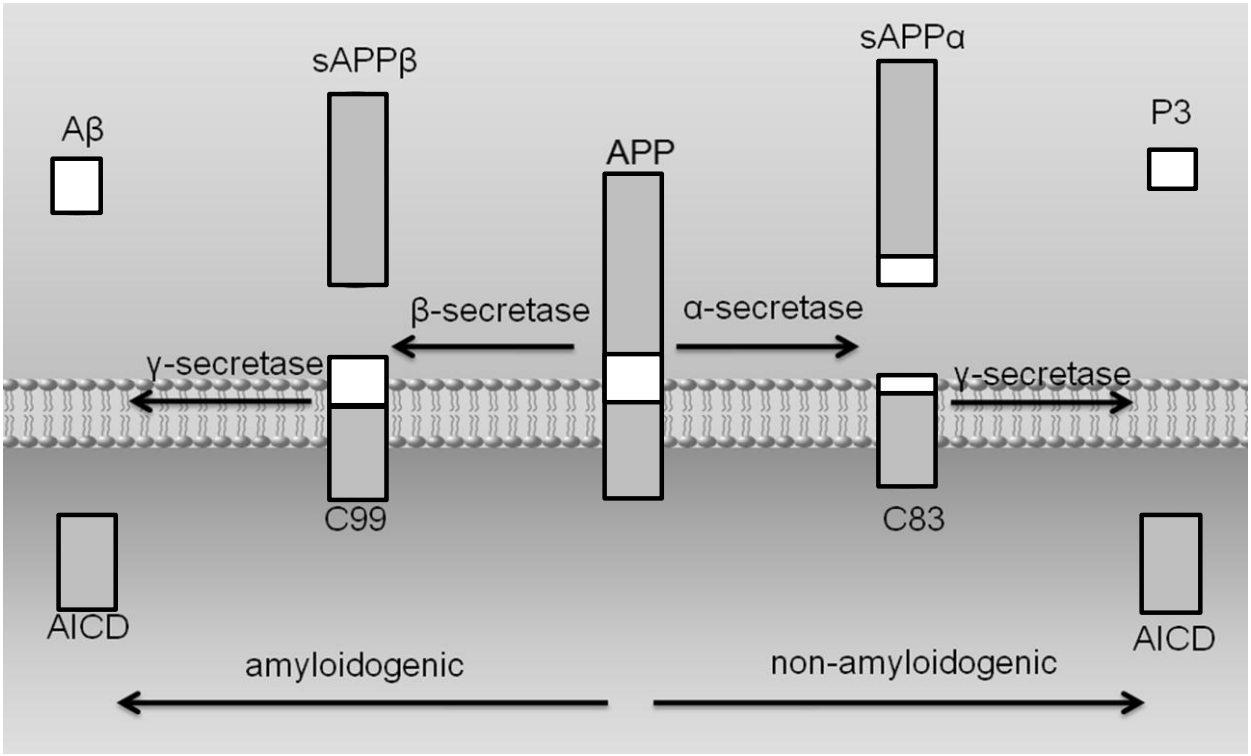

Figure 1.1 Cleavage of the Amyloid Precursor Protein. Under normal, non-amyloidogenic conditions, APP is cleaved in the middle of the transmembrane domain by α-secretase, releasing the non-toxic extracellular domain APPα which is subsequently degraded by proteasomes. Mutations that increase the activity of β- and γ-secretase result in increased production and release of the Aβ42 fragment. Aggregation of Aβ42 in soluble oligomers is believed to be responsible for the onset of AD pathology and large fibrillar aggregates of this protein are commonly observed in the brains of AD subjects at autopsy.

and ultimately large fibrils, and it is generally accepted that Aβ42 is the most "toxic" species, as it is more likely to aggregate in the brain than the shorter Aβ40 peptide, and thus its formation is highly correlated with AD (Tandon et al., 2000; Tomiyama et al., 2008). Today, over 30 APP missense mutations have been identified, the majority of which are pathogenic and result in early-onset AD (Cruts et al., 2012). Interestingly, individuals with Down's syndrome, who carry an extra copy of chromosome 21 (and therefore exhibit increased Aβ production), almost universally exhibit AD neuropathology by their 40s (Lott and Head, 2005; Nistor et al., 2007),

further supporting that Aβ overload contributes to the symptoms of AD. Patients with APP mutations generally develop disease symptoms from 40 to 60 years of age and these account for a small proportion of early-onset FAD (Alzheimer's Association 2013).

The majority of early-onset FAD cases are caused by mutations in PS-1, while a relatively small percentage of cases result from mutations in PS-2 (Alonso Vilatela et al., 2012). PS-1 is an important component of the γ-secretase complex, providing stability and enhancing complex activity. More than 180 mutations have been identified in PS-1 (Cruts et al., 2012), and many of them result in an increased production of Aβ42. In contrast, PS-2 mutations result in a significantly later age of onset (45-88 years) and the mechanism by which PS-2 enhances Aβ generation is unclear. Some research indicates PS-2 mutations increase γ-secretase activity (Citron et al., 1997), while others suggest that PS-2 actually increases β-secretase activity via ROS-mediated activation of extracellular signal-regulated kinase (ERK) (Park et al., 2012).

Still, FAD only accounts for approximately 5% of all AD cases, whereas the overwhelming majority of AD cases are sporadic (SAD). The risk of developing SAD is dependent on a number of inherent factors. The greatest known risk factor for AD is aging, as most individuals who have the disease are age 65 or older (Brookmeyer et al., 1998) and the rate of development of AD doubles roughly every five years from this point, peaking at a nearly 50 percent population prevalence by age 85 (Morris, 1999). Notably, the rate of disease in women is 1.5 to 3 times as high as that in men, as estrogen deficiency in the brain (generally brought on by menopause) has been linked to AD (Gandy and Duff, 2000; Henderson, 2009). Unlike FAD, the genetic risk factors associated with SAD are not deterministic. The largest known genetic risk factor for SAD is inheritance of Apolipoprotein epsilon-4 (APOE-4), one of the 3 forms of the APOE gene (Sadigh-Eteghad et al., 2012). Individuals who carry two copies of the e-4 allele have a higher risk than those who carry only one copy, and both groups have a higher risk than those who carry only the e-2 or e-3 forms (Slooter et al., 1998). While the exact

reason for increased risk is unclear, it is known that APOE enhances proteolytic clearance of Aβ and that the e-4 variant is less efficient at catalyzing this reaction than the e-2 or e-3 forms (Jiang et al., 2008b). It is noteworthy that being an APOE-4 carrier does not guarantee development of SAD, and lacking APOE-4 is not preventative, suggesting that factors other than Aβ burden may have significant impact on disease symptomology.

In addition to genetic risk factors, there are a number of lifestyle factors that affect the risk for SAD. Head trauma and traumatic brain injury (TBI) such as that resulting from car accidents, sports collisions, and blast-radius injuries have been linked to an increased risk for developing SAD. This is particularly true when the trauma is repetitive or involves a loss of consciousness. The link between TBI and the risk for AD originated from studies of boxers suffering from pugilistic dementia who demonstrated AD-like pathology and deposition of Aβ plaques (Roberts et al., 1990), and histopathological studies of severe TBI patients that exhibited significant Aβ deposition (Roberts et al., 1994; Gentleman et al., 1997; Ikonomovic et al., 2004). Other studies have also demonstrated dysregulated levels of CSF Aβ42, an accepted biomarker of AD, following TBI (Raby et al., 1998; Olsson et al., 2004). Biomarkers and their relationship to AD staging will be discussed in section 1.6.

On the other hand, a number of lifestyle factors including education, physical activity, and social activity have each been noted to have an inverse correlation with the incidence of AD, suggesting that keeping the mind and body active can offer protection from Alzheimer's-mediated insult (Szekely et al., 2007; Wilson et al., 2007). This idea is supported by the fact that conditions associated with poor physical fitness that can damage the heart or blood vessels, including high blood pressure, heart disease, stroke, high cholesterol (Szekely et al., 2007), or other vascular problems such as the gluco-regulatory abnormalities common to type 2 diabetes mellitus (T2DM) (Arvanitakis et al., 2004; Patterson et al., 2008; Craft, 2009; Sperling et al., 2011) increase the risk for developing AD, and other research that shows the plaques and

tangles associated with AD are more likely to cause memory impairment in the presence of vascular damage (Lee, 1994; Sparks et al., 1994; Sparks et al., 1995).

1.4 Insulin resistance in AD

Of particular interest is the increased risk of AD in sufferers of T2DM, a chronic metabolic disorder characterized by peripheral insulin resistance leading to elevated blood glucose levels and hyperinsulinemia. Epidemiological studies consistently link type 2 diabetes, as well as intermediate stages of insulin resistance, with increased risk of developing AD (Ott et al., 1999; Luchsinger et al., 2001; Watson and Craft, 2003; Arvanitakis et al., 2004; Luchsinger et al., 2004; Xu et al., 2004; Rivera et al., 2005; Akomolafe et al., 2006; Schrijvers et al., 2010) with suffers of T2DM having up to a 65% increased risk of developing AD (Arvanitakis et al., 2004). Furthermore, clinical evidence demonstrates decreased central insulin present in the AD brain (Craft et al., 1998; Talbot et al., 2012) as well as dysregulated glucose metabolism and insulin resistance in AD patients (Craft et al., 1999). Even non-diabetic individuals have an increased risk for AD if they exhibit hyperinsulinemia (Ott et al., 1999; Luchsinger et al., 2004), thereby suggesting that impaired insulin signaling is directly linked to AD pathological development.

The link between these two disease states is not surprising; the role of insulin in brain metabolism and memory is well characterized. Insulin receptors are widely distributed in brain regions known to be involved in memory function, including high concentrations at synapses in the hippocampus and amygdala, and moderate expression in cortex and cerebellum (Werther et al., 1987). Insulin readily crosses the blood-brain barrier in order to regulate glucose utilization (Woods et al., 2003) and this process regulates neuronal survival, energy metabolism, and neuronal plasticity (Wallum et al., 1987; Wickelgren, 1998; Park et al., 2000; Craft et al., 2003; Craft and Watson, 2004; Watson et al., 2006). Furthermore, acute insulin administered

intravenously improves memory in both humans and rodents (Park et al., 2000; Craft et al., 2003) and disruption of CNS insulin receptors leads to cognitive deficits in adult wild type rats (Lannert and Hoyer, 1998; Steen et al., 2005). Thus, it is clear that impinging upon the insulin signaling axis can affect learning and memory.

Given that insulin signaling is impaired in AD and that enhanced insulin signaling is linked to improvement in memory, many studies have examined the feasibility of therapeutically targeting receptors known to improve insulin signaling, and have subsequently evaluated the efficacy of compounds that activate these receptors in combating AD pathology. Although previous large-scale clinical trials failed to show efficacy of insulin sensitizer therapy in AD, their downfall was possibly due to testing in advanced stage disease, similar to many other failed AD drug candidates (Becker and Greig, 2013). This is supported by outcomes from recent clinical trials on patients with mild cognitive impairment (MCI) in that insulin sensitizers as well as intranasal insulin provided significant cognitive benefit (Stockhorst et al., 2004; Watson et al., 2005; Risner et al., 2006; Reger et al., 2008; Sato et al., 2011; Craft et al., 2012). Indeed, our own research indicates a significant therapeutic benefit of the insulin sensitizing thiazolidinedione (TZD) drug class in ameliorating AD-like cognitive dysfunction during preclinical stages; this data will be discussed in chapter 3. Thus, stimulation of the insulin signaling axis leads to the expression of genes that have the capacity to modulate memory in MCI and early AD (Watson and Craft, 2004).

1.5 Diagnostic criteria for AD

One of the primary challenges to the development of Alzheimer's therapeutics has been selecting an appropriate window for intervention. Until recently, research efforts attempted to reverse damage resulting from substantial neurodegeneration and synaptic loss at late stages of the disease (Becker and Greig, 2013), a strategy that most experts now agree was doomed

to failure. Studies of Aβ-modifying therapies in transgenic mouse models of AD have been found largely ineffective once neuronal degeneration has begun; as such, the current general consensus is that AD pathology begins years and possibly decades before the manifestation of clinical symptoms (Morris, 2005), and that the appropriate time for therapeutic intervention is likely during the pre-clinical phase that precedes overt neurodegeneration and memory impairment (Sperling et al., 2011). This has proven challenging as well, though, as it requires diagnosis of AD pathology years before a noticeable impairment develops. Therefore, recent focus by the National Institute on Aging (NIA) and the Alzheimer's Association has turned to identifying biomarkers and epidemiological factors that best predict an individual's risk for transitioning from "normal" cognition to MCI and ultimately AD (Sperling et al., 2011).

Biomarker identification

While a number of biomarkers have been considered, a direct link between a particular biomarker and subsequent disease state has not yet been firmly established. Still, several potential proteins are being investigated at early stages of disease in order to determine whether they are related to the severity or likelihood of future cognitive decline. The CSF biomarkers total tau (T-tau), hyperphosphorylated tau (P-tau) and Aβ42 are being investigated as they reflect the core pathologic features of AD – neuronal loss, intracellular neurofibrillary tangles, and extracellular senile plaques – respectively (Rosen et al., 2013). T-tau indicates the extent and intensity of neuroaxonal degeneration, P-tau may correlate with tangle pathology, and CSF Aβ42 inversely correlates with plaque pathology (Blennow et al., 2010), as it is believed that most of the Aβ42 in these individuals is accumulated in plaques. Therefore, low CSF Aβ42 levels are associated with a higher risk for AD pathology.

Preclinical studies on patients with familial AD considered those who were mutation carriers and those who were not, and found that mutation carriers had significant elevation of CSF T-tau and plasma Aβ42 15 years before symptom onset, and CSF Aβ42 was significantly

reduced 10 years before symptom onset (Rosen et al., 2013). Similarly, individuals with low CSF Aβ42 exhibit much higher rates of brain atrophy over a one year period than those with higher levels (Schott et al., 2010). Even in healthy, cognitively normal adults, decreased levels of CSF Aβ42 and increased levels of CSF P-tau were associated with worse performance on cognitive tests (Stomrud et al., 2010), indicating a link between P-tau, Aβ, and normal cognition. CSF levels of T-tau are nearly 300% higher in AD patients than control subjects; however, this is not a specific biomarker for AD as it indicates generalized neurodegeneration and is also increased in sufferers of head trauma, stroke, and Creutzfelt-Jakob disease (Hesse et al., 2001; Wang et al., 2006a; Zetterberg et al., 2006). Conversely, P-tau in the CSF of living subjects has been shown to correlate with neurofibrillary tangles and hyperphosphorylated tau during post-mortem analysis (Buerger et al., 2006; Tapiola et al., 2009), suggesting that P-tau may be a more specific marker of AD pathology (Hampel et al., 2010).

While a number of correlations have been established, a substantial portion of non-AD subjects exhibit similar biomarker profiles to confirmed AD patients but never progress to dementia (Schoonenboom et al., 2012). Thus, while CSF T-tau, P-tau, and Aβ42 may provide some insight into the potential for future cognitive decline, their correlation is not definitive and new biomarkers are still needed. Researchers remain optimistic regarding the potential for blood biomarkers, as testing in blood is far less invasive than drawing CSF. However, the concentration of brain-derived proteins in significantly lower in blood than the CSF because of the blood-brain barrier, and the clearance mechanisms employed in the blood versus those in the CSF differ substantially (Hesse et al., 2001; Randall et al., 2013). Furthermore, no correlation has been established between CSF and blood levels of the currently studied biomarkers (Handoko et al., 2013). Thus, challenging detection methods and the potential for inaccurate assessment relegate blood biomarkers for AD as a future hope and not a current reality.

In addition to fluid biomarkers, substantial research has gone into identifying structural biomarkers of AD pathology. These include increased amyloid tracer retention on positron emission tomography (PET), decreased fluorodeoxyglucose 18F (FDG) uptake with a temporoparietal pattern of hypometabolism on PET, and brain atrophy involving the medial temporal lobes, paralimbic and temporoparietal cortices on structural magnetic resonance imaging (MRI) (Rosen et al., 2013). Interestingly, combining the CSF biomarkers with structural measurements, such as assessment of cortical thickness or hippocampal volume via magnetic resonance imaging (MRI) offers much more accurate classification (Westman et al., 2012) and therefore this combined approach may provide improved diagnosis until more viable biomarkers are discovered. Finally, research is still being conducted to investigate the utility of Pittsburgh compound B (PiB), a fluorescent analog to thioflavin T that can be used to image beta amyloid on PET scans. Currently, the NIA does not consider PiB results when staging AD.

There are several caveats to consider in regards to biomarkers for AD, the first being that biomarkers are still being studied. While CSF Aβ42 and tau measures correlate with the likelihood of cognitive decline, few studies have successfully defined specific cutoff values that confer a likely progression to AD dementia. Also, because no direct relationship has been established between a particular biomarker and subsequent cognitive failure, it is conceivable that biomarker indications will conflict in individual patients (Albert et al., 2011). Biomarker studies are also potentially subject to cohort bias, as data collection from both “normal” and “impaired” elderly individuals relies heavily on volunteer subjects who are likely to be more active, both physically and socially, than the average member of the population (Sperling et al., 2011) – traits that are linked to some resistance to AD pathology (Szekely et al., 2007; Wilson et al., 2007). Similarly, biomarker studies may see a high incidence of APOE-4 carriers volunteer to participate due to subjective concerns (Sperling et al., 2011), which again may skew data collection. Finally, some biomarkers may be useful in predicting changes over brief periods (e.g.

months or 1-3 years) while others may be more useful in predicting long term changes across several years or decades (Albert et al., 2011). Thus, the effective use of biomarkers requires further study to provide more accurate and specific diagnostic criteria.

1.6 Staging of AD

Preclinical AD

Although the presently available biomarkers do not confer a definitive AD pathology, they have been utilized by the NIA to create new guidelines for the assessment and staging of AD to include several preclinical stages. The NIA broke preclinical AD into 3 stages, and following review, 2 additional preclinical stages were added for a total of 5. The progression of these stages is based upon the idea that as pathological features accumulate, the disease state progresses (Sperling et al., 2011). Stage 0 includes individuals with no pathological AD biomarkers and no signs of cognitive impairment (Jack et al., 2011). Stage 1 preclinical AD is defined by asymptomatic cerebral amyloidosis, and individuals in this stage have biomarker evidence of either low CSF Aβ42 or Aβ accumulation with elevated tracer retention on PET imaging; these biomarkers present in the absence of additional neurodegeneration or cognitive symptoms. Current evidence suggests that markers of amyloid pathology precede those for neuronal injury (Albert et al., 2011; Sperling et al., 2011); thus, stage 2 preclinical AD individuals have both amyloid positivity as described in stage 1 and evidence of synaptic dysfunction and/or early neurodegeneration. At this time, there are several validated markers of neuronal injury including 1) elevated CSF tau or phospho-tau, 2) hypometabolism assessed by FDG-PET within the posterior cingulate, precuneus, and/or temporoparietal cortices, and 3) loss of gray matter or cortical thinning in the lateral and medical parietal, posterior cingulate, and lateral temporal cortices and/or hippocampal atrophy on volumetric MRI. Fmri connectivity assays are also in development and may be employed to assess neuronal injury in the future (Sperling et al., 2011). An additional category of preclinical AD, referred to as Suspected Non-AD

Pathophysiology (SNAP), consists of subjects with evidence of abnormal biomarkers for neuronal injury (e.g. T-tau) but normal amyloid PET. These individuals do not follow the "cascade" pathology used to define the other preclinical stages, and it is currently unclear whether the neuronal damage in these individuals in caused by AD pathology or another type of dementia (Jack et al., 2011).

Finally, the last stage of preclinical AD will encompass individuals with symptoms described in stage 2 in addition to evidence of subtle cognitive decline. Notably, cognitive decline must be considered on an individual basis, as the subtle changes are only relative to one's own baseline; it is entirely feasible for an individual to perform within the "normal" range according to standard cognitive measures while still exhibiting a deficit compared to their own previous abilities. These individual differences may be due to 'cognitive reserve', a term that refers to an individual's ability to engage alternate brain networks or cognitive strategies to cope with the effects of encroaching pathology (Sperling et al., 2011). In other words, certain individuals may be able to better tolerate the effects of AD pathology by utilizing redundant pathways in the brain to accomplish a task. In this regard, it is noteworthy that there is a subset of individuals who exhibit significant AD-like pathology at autopsy who did not ever develop symptoms of the disease during their lifetime. It has been hypothesized that these individuals have high cognitive reserve and that, had they lived longer, they likely would have exhibited symptoms of AD; indeed, the percentage of cognitively "normal" amyloid positive individuals at autopsy at a given age is very similar to the percentage of individuals diagnosed with AD dementia 10 years later (Brookmeyer et al., 1998).

Cognitive reserve may be affected by a number of factors, including physical and mental activity, and may account for the inverse correlation between activity and AD incidence (Szekely et al., 2007; Wilson et al., 2007). Interestingly, individuals with higher cognitive reserve are able to delay the onset of their cognitive deterioration but seem to exhibit more rapid cognitive

decline once these compensatory mechanisms start to fail (Fotenos et al., 2008; Wilson et al., 2010). According to the NIA guidelines, individuals in stage 3 preclinical AD are on the precipice of progressing to MCI.

Mild cognitive impairment

Those subjects who advance past the preclinical stages of AD fall into the category of mild cognitive impairment (MCI). Per the NIA guidelines, the tipping point to MCI is when there is evidence of concern regarding a change in cognition relative to that individual's previous level that is more pronounced than the subtle changes seen in stage 3 preclinical AD. Patients with MCI who progress to AD dementia commonly exhibit impairment in episodic memory. Symptoms may include difficulty remembering the names of newly introduced people, forgetting material that was just read, and losing or misplacing a valuable object. Such instances could be self-reported by the patient, but would preferentially be expressed by someone who knows the patient well or even directly observed by a clinician (Albert et al., 2011). Again, in order to fall into the category of MCI, the cognitive decline exhibited must be greater than expected considering the patient's age and educational background (Albert et al., 2011), and so confirmed diagnosis of this stage relies heavily on the clinician's assessment.

Individuals can be tested with a variety of formal assays for immediate and delayed recall, such as a word-list learning test with multiple recall periods. This repeated assessment should show declining performance over time in individuals with MCI. When formal cognitive testing is available, individuals with MCI typically score 1 to 1.5 standard deviations below the mean for their age- and education-matched peers (Albert et al., 2011). In keeping with the hypothesis that AD pathology is cumulative, individuals in the MCI stage typically exhibit the biomarkers described in Stage 3 preclinical AD, including decreased CSF Aβ42 and increased CSF tau or phospho-tau. Notably, while individuals with MCI are less efficient and make more

errors in daily activities than control subjects, they are able to maintain functional independence and do not suffer any significant impairment in social or occupational situations.

AD dementia

Finally, progression to AD dementia is categorized by a significant decline in both physical and cognitive abilities. These individuals might forget recent events or their own personal history and experience changes in mood, particularly in social situations (Scheff et al., 2006). They may have difficulty performing mental arithmetic or recalling basic details about their own life, such as their address or telephone number. In late-term cases, sufferers of AD commonly become disoriented and do not know where they are or what day it is, and eventually they require assistance with daily activities such as getting dressed (Waldemar et al., 2007; Hort et al., 2010). At a cellular level, individuals in this stage have significant hippocampal and cortical atrophy and impaired synaptic transmission (Serrano-Pozo et al., 2011). Thus, as previously mentioned, intervention at this stage is unlikely to be viable as substantial cellular damage has already occurred. Ultimately, successful therapeutic strategies in AD will require early stage intervention that precludes structural changes.

1.7 Mouse models of AD

Mouse models of AD do not fully recapitulate the entire spectrum of the human disease; however, they do confer some aspects of the disease pathophysiology that are thought to play a causal role. These models have been integral to furthering our knowledge and understanding of disease pathology and have been invaluable for the effective study of potential therapeutic compounds. Due to the heightened disease prevalence and known gene mutations in familial AD, and the congruent pathology observed in both FAD and SAD, many of these models contain mutations associated with FAD in an attempt to replicate the natural disease pathology

(Bales, 2012). As such, there are a number of transgenic mouse models of AD (Gotz et al., 2004), including those that lead to aberrant accumulation of β-amyloid (Tg2576, PDAPP, presenilin conditional KO/APP, PS1/APP, CRND8, PGDF-APP_{SW}) and mutant tau for neurofibrillary tangle formation (P301S, rTg4510, 3xTg-AD).

Tg2576 provides one of the best characterized models in which to examine cognitive deficits due to aberrant accumulation of the Aβ peptide. These mice express mutant human APP695 containing the familial 'Swedish' mutation ($Lys^{670} \rightarrow Asn^{670}$, $Met^{671} \rightarrow Leu^{671}$) (Hsiao et al., 1996). Tg2576 mice exhibit progressive cognitive decline characterized by hippocampus-dependent learning and memory deficits concomitant with the dysregulation of both the ERK MAPK and insulin signaling pathways (**Figure 1.2**). Current research indicates cognitive deficits in recognition memory, associative learning and memory, and spatial navigation (Dineley et al., 2002b; Dineley et al., 2007; Escribano et al., 2009; Taglialatela et al., 2009). Additionally, Tg2576 exhibit age-dependent accumulation of Aβ and tau toxic species which are believed to be responsible for the subsequent hippocampal synaptic dysfunction (Hsiao et al., 1996; Irizarry et al., 1997a; Irizarry et al., 1997b; Chapman et al., 1999; Kawarabayashi et al., 2001; Dineley et al., 2002a; Westerman et al., 2002; Taglialatela et al., 2009).

Although Tg2576 do not experience overt neurodegeneration or synaptic loss, significant reduction of the basilar dendrites of CA1 pyramidal neurons has been observed (Perez-Cruz et al., 2011), which likely causes the loss of inhibitory interneurons and contributes to a hyper-excitable state in this region. It is noteworthy that in human AD subjects, dendritic spine loss is noted as the best pathological correlate to cognitive impairment (Cavallucci et al., 2012). Thus, while Tg2576 do not experience the massive loss of synapses common to AD, they do experience synaptic changes within the hippocampus that would preclude neurodegeneration. As such, Tg2576 best models the pathological transition from preclinical AD (0-5 months-old, MO) to MCI (5MO onward) (Albert et al., 2011; Sperling et al., 2011) and therefore its study is

well suited to the current NIA goal of identifying early biomarkers of disease and the examination of therapeutic interventions to prevent neuronal loss rather than trying to undo damage that has already occurred at a later state of disease progression.

Figure 1.2

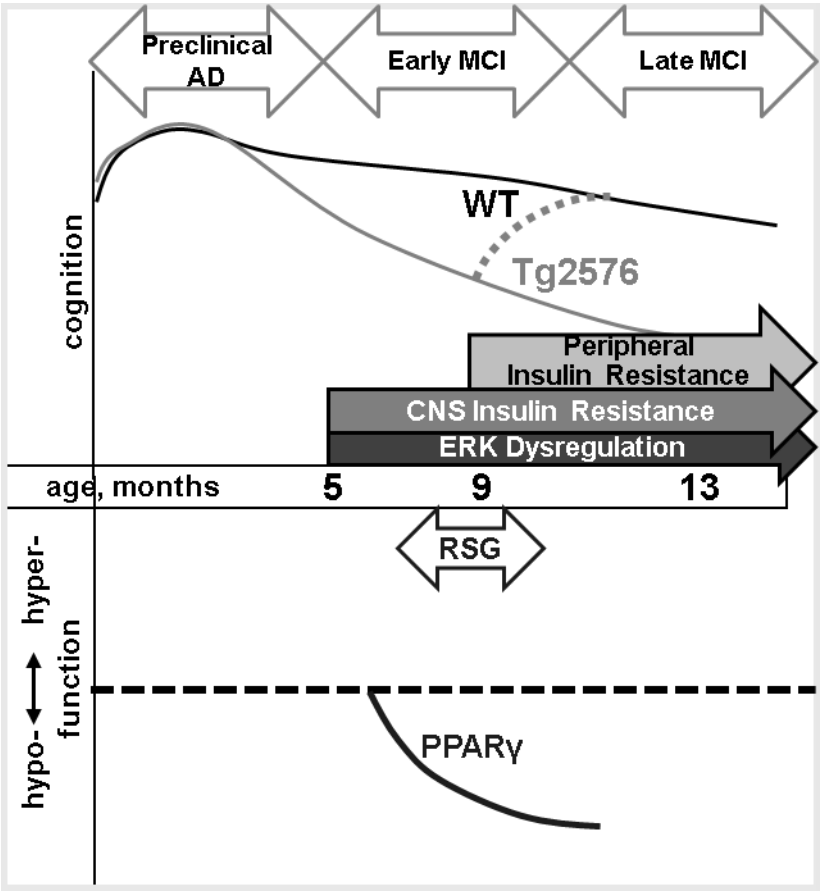

Figure 1.2 Tg2576 best models the pathological transition from preclinical AD to MCI. Tg2576 mice exhibit progressive cognitive decline concomitant with dysregulation of both the insulin and ERK MAPK signaling pathways. By 9 months of age, these mice are significantly impaired in hippocampal memory tasks and also demonstrate decreased levels of CNS PPARγ. Treatment with the PPARγ agonist rosiglitazone (RSG) from 8 to 9 months restores Tg2576 cognition to levels comparable to age-matched wild type littermates. Therefore, PPARγ may represent a viable therapeutic target during the transition from preclinical AD to MCI.

CHAPTER 2
LEARNING AND MEMORY

As episodic memory failure is one of the earliest detectable traits of AD pathology (Boeve, 2012; Cavallucci et al., 2012), it is integral that we understand the molecular processes of learning and memory if we hope to develop therapeutics that facilitate this process and/or prevent its disruption. This chapter will provide background regarding the discovery of brain regions involved in learning and memory as well as a succinct overview of the molecular mechanisms of these processes. Finally, I will highlight the role of ERK, the canonical regulatory pathway for learning.

2.1 Brain regions in L&M

Prior to the discovery that specific structures within the brain controlled different functions, it was thought that the entire brain controlled all actions. However, case studies wherein damage to certain regions of the brain resulted in specific deficits led to the examination of individual brain structures and their independent functions. One of the earliest examples of this phenomenon was the discovery by Pierre Paul Broca that damage to the posterior inferior frontal gyrus led to expressive aphasia, the loss of the ability to produce language (Dronkers et al., 2007). This ground breaking observation led researchers to question whether other mental functions, such as voluntary movement and memory, were also controlled by discrete structures in the brain.

This idea was later supported by the work of Wilder Penfield, who mapped the functions of the cerebral cortex by applying electrical stimulation to the brains of awake human patients and asking them to recount their experiences. He found that stimulation of the temporal lobes sometimes caused the subject to experience the vivid recollection of a previous experience,

leading him to suggest that the temporal lobe was critical for memory (Penfield, 1952). Further evidence for the involvement of medial temporal lobe structures in memory came from examination of surgical patients, such as the case of Patient P.B., who underwent a temporal lobectomy in two stages and only began to suffer from memory loss following removal of the medial structures of the temporal lobe (Milner et al., 1998). This idea would not be widely accepted, however, until later studies demonstrating that individuals who had surgery to treat epilepsy that involved bilateral removal of portions of the temporal lobe consistently experienced significant memory deficits.

Patient H.M. is the most well-known and comprehensively studied patient to have undergone such a procedure. He had experienced temporal lobe seizures for years as a result of an injury sustained as a child, leading his surgeon to bilaterally remove the hippocampal formation, the amygdala, and parts of the associated temporal cortex. Following surgery, H.M. suffered from severe anterograde amnesia and partial retrograde amnesia (Scoville and Milner, 1957) but maintained intact working and procedural memory; that is, he could learn new skills, but was unable to remember the actual process of having learned them. This case was highly influential as it provided insight concerning amnesia and memory as well as providing evidence that there are multiple forms of memory. These two forms would later be categorized as implicit (non-declarative) – unconscious memory for perceptual and motor skills – and explicit (declarative) – conscious recall of people, places, and objects. Mounting incidences of memory impairments resulting from temporal lobe damage, specifically to the hippocampus and amygdala, confirmed that mental functions such as memory could be controlled by discrete structures in the brain.

In order to further characterize the specific structures important in learning and memory, many researchers began to employ lesion studies in non-human primates and rodents in an attempt to re-create memory deficits. A monkey model of amnesia was ultimately developed

that allowed for the identification of the specific medial temporal lobe structures that are requisite for declarative memory; these included the dentate gyrus of the hippocampus, the subicular complex, and the entorhinal cortex, as well as the perirhinal and parrahippocampal cortices (Squire et al., 1988). Comparable structures in the mouse brain (**Fig. 2.1**) have provided a relatively inexpensive and reproducible mammalian system in which to investigate hippocampal function. The amygdala does not contribute to declarative memory, and thus the focus for the remainder of this work will focus exclusively on hippocampus-dependent learning and memory processes.

It is important to note that new memories are highly sensitive to disruption during the consolidation process, as perturbation of the hippocampal structure preferentially affects recent memories (Zola-Morgan and Squire, 1990; Kim and Fanselow, 1992; Wang et al., 2006b). An extreme example of this is retrograde amnesia, wherein an individual does not recall the events leading up to a traumatic injury due to the damage sustained and the resulting disruption of the normal consolidation process (McGaugh, 2000a; Abel and Lattal, 2001). Notably, sufferers of retrograde amnesia lose a limited window of time preceding their incident, suggesting that the consolidation process involves more permanent storage in other brain regions (Scoville and Milner, 1957; Zola-Morgan and Squire, 1990; Abel and Lattal, 2001; Wang et al., 2006b). This

Figure 2.1

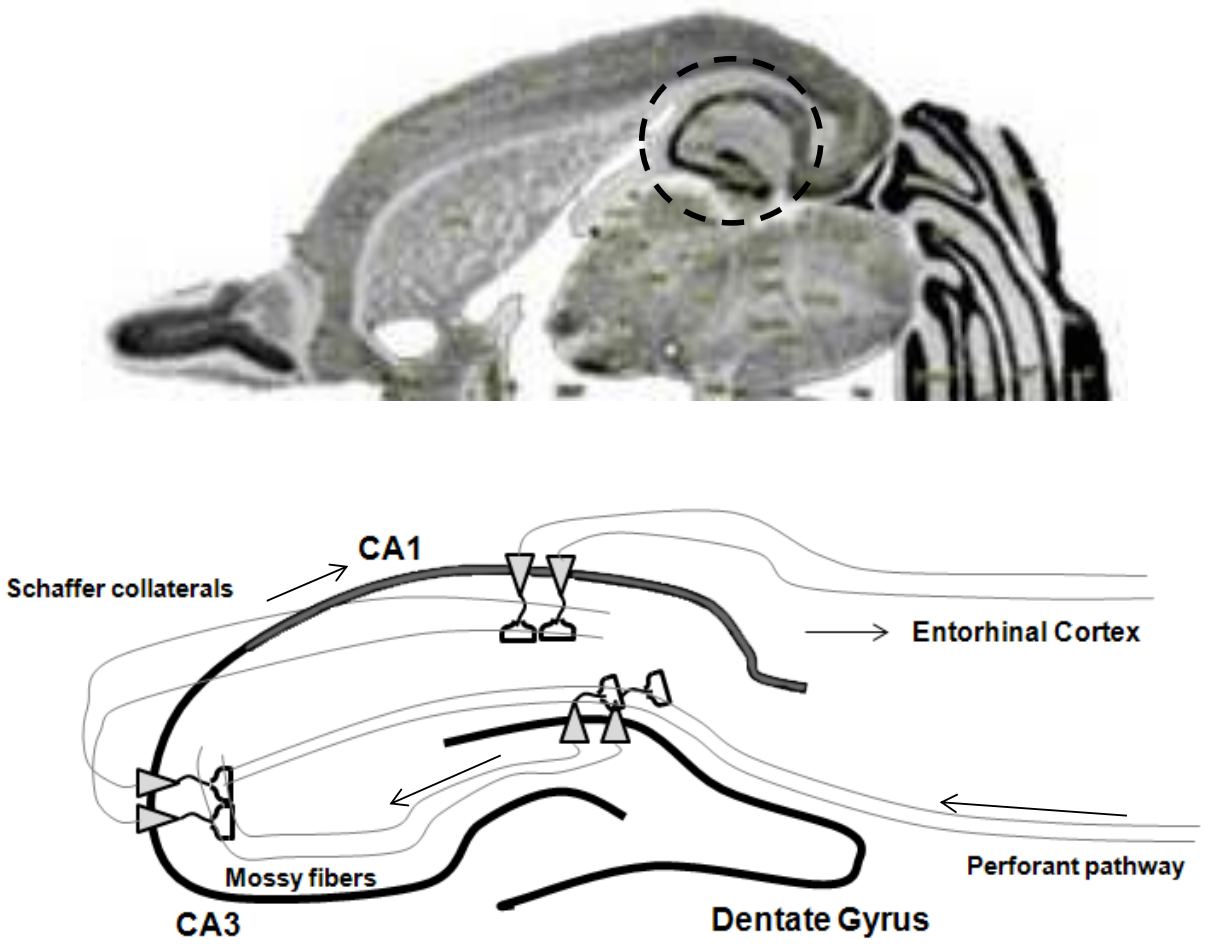

Fig. 2.1 The hippocampus in mouse brain. *Top* – a Nissl stained horizontal section of the right hemisphere from a Tg2576 negative littermate distinctly highlights the hippocampus and its subregions within the temporal lobe (circled). *Bottom* – a highly simplified schematic demonstrating the connectivity of the hippocampal subregions. Information received from the medial and lateral perforant pathways is encoded in the dentate gyrus (DG) and CA3 regions. The DG connects to CA3 via the mossy fiber pathway, and CA3 connects to CA1 via the Schaffer collaterals. Outputs from CA1 extend to the entorhinal cortex and allow for hippocampal communication with other brain structures. The hippocampus is responsible for the initial encoding of new information and is the brain structure most important for contextual memory. Bottom figure adapted from the University of Bristol Centre for Synaptic Plasticity website accessible at http://www.bristol.ac.uk/synaptic/pathways/

idea is confirmed by the fact that lesions of the hippocampus made the day after training in fear conditioning significantly impair retrieval, but do not have any discernible effect when made several weeks after training (Takehara et al., 2003). Thus, the hippocampus is important for the encoding of long term memory but is not integral for subsequent storage.

2.2 Molecular mechanisms of memory consolidation

In the years following the initial discovery that hippocampal function is integral to episodic memory, we have come a long way in our understanding of memory consolidation. We now know that the hippocampus is involved in the highest forms of memory, including declarative, episodic, and spatial, and highly developed rodent models and behavioral assays allow detailed assessment of these processes down to the molecular machinery that drives them. Initial observation of the communication between neurons yielded the concept of synaptic plasticity – the ability of synapses to strengthen or weaken over time depending on increases or decreases in their activity (Hughes, 1958).

Synaptic plasticity and memory formation are believed to be primarily mediated by the N-methyl-D-aspartate (NMDA) receptor (Snyder et al., 2005; Palop and Mucke, 2010), an ionotropic voltage- and ligand-gated glutamate receptor. The NMDA receptor only opens when it is presynaptically bound by glutamate and either glycine or d-serine (Kleckner and Dingledine, 1988; Wolosker, 2006) and the postsynaptic cell is depolarized, thereby removing its magnesium block and allowing the influx of sodium and calcium and the efflux of potassium (Liu and Zhang, 2000; Wang et al., 2006b) (**Fig. 2.2A**). Activation of the NMDA receptor produces excitatory postsynaptic potentials (EPSPs) which increase the concentration of calcium inside the cell; calcium can then act as a second messenger to activate a number of signaling pathways. In regards to memory formation, activation of the NMDA receptor is integral to the process of long term potentiation (LTP) (Malenka and Bear, 2004; Wang et al., 2006b), the cellular basis for synaptic plasticity. It is important to note that the production of EPSPs due to

neurotransmitter release is probabilistic, as this process simply requires adequate presynaptic release of glutamate. That said, glutamate release can be enhanced by repeated stimulation resulting in a high-frequency tetanus and therefore increasing the likelihood of EPSP formation (Winder et al., 1999).

LTP is the result of synchronous stimulation of two neurons that gives rise to the long-lasting enhancement of signal transmission between them. This phenomenon can be observed in many processes; perhaps one of the simplest to understand is “muscle memory”, a term used to describe the consolidation of a motor task wherein a movement that is repeated over time becomes reproducible without conscious effort (e.g. riding a bicycle or playing a scale on a piano). In the CNS, LTP is considered an integral aspect of learning and memory, as the modification of synaptic strength is believed to be the molecular basis of memory (Bliss and Collingridge, 1993). This idea was eloquently proposed by Donald Hebb in 1949 wherein he hypothesized that metabolic or connective changes occurred between synapses that were repeatedly stimulated together that served to increase the efficiency by which one stimulates the other (Hebb, 1949). This idea is often referred to as Hebbian theory and is over-simplified as the succinct statement, “cells that fire together wire together”. The phenomenon was first observed by Terje Lomo in 1966 who was studying the perforant pathway to the dentate gyrus within the rabbit hippocampus (Lomo, 2003). Lomo found that dentate gyrus EPSPs could be enhanced by delivering a high frequency stimulus to the presynaptic cells, as subsequent stimulation produced longer-lasting EPSPs in the postsynaptic cells (Bliss and Lomo, 1973). Thus, Lomo demonstrated that the synchronous stimulation of two cells resulted in an increased cellular response to future stimulation.

Although there are various forms of LTP, the best characterized is NMDA receptor-dependent LTP as observed in the CA1 region of the adult hippocampus (Malenka and Bear, 2004). The initial stage of LTP, which does not require protein synthesis, is called early form LTP or E-LTP

and this stage lasts less than 2 hours (Giovannini et al., 2001). Activation of the NMDA receptor allows calcium to enter the cell and act as a second messenger to regulate gene transcription (**Fig. 2.2B**). Calcium activates the Protein Kinase A (PKA) and Calcium/calmodulin-dependent kinase II (CaMKII) pathways, which have multiple functions in LTP. First, these kinases increase the activity of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor – the most abundant glutamate receptors in the brain that therefore mediate a majority of excitatory activity – in two ways: 1) through phosphorylation of existing AMPA receptors, which increases their activity, and 2) by mediating the addition of supplementary AMPA receptors at the postsynaptic membrane (Shi et al., 1999; Malenka and Bear, 2004). Upon glutamate binding, AMPA receptors allow the influx of sodium into the cell, further depolarizing it and facilitating the release of the magnesium block on NMDA receptors, resulting in a feed-forward mechanism to enhance synaptic transmission. In this manner, the increased efficiency and number of AMPA receptors at the synapse result in an increased response to future EPSPs and thus more efficient synaptic firing. Importantly, NMDA receptor activation of CaMKII is very specifically localized to dendritic spines and prevented from spreading to surrounding regions; thus, CaMKII modulates distinct pathways to enhance very specific synaptic transmission and facilitate a unique signal (Lee et al., 2009). In regards to AD, accumulation of Aβ reduces glutamatergic transmission and inhibits synaptic plasticity through interference with NMDA receptor endocytosis and the resulting reduction in NMDA receptor availability at synapses (Caccamo et al., 2010).

In order to effectively consolidate memory in the long term, more permanent changes are required. This phase, called late LTP (L-LTP), results from new protein synthesis and gene transcription (Frey et al., 1996; Sweatt, 1999; Giovannini et al., 2001). Persistent NMDA receptor activation leading to PKA and CaMKII activation in E-LTP leads to the synthesis of

Figure 2.2

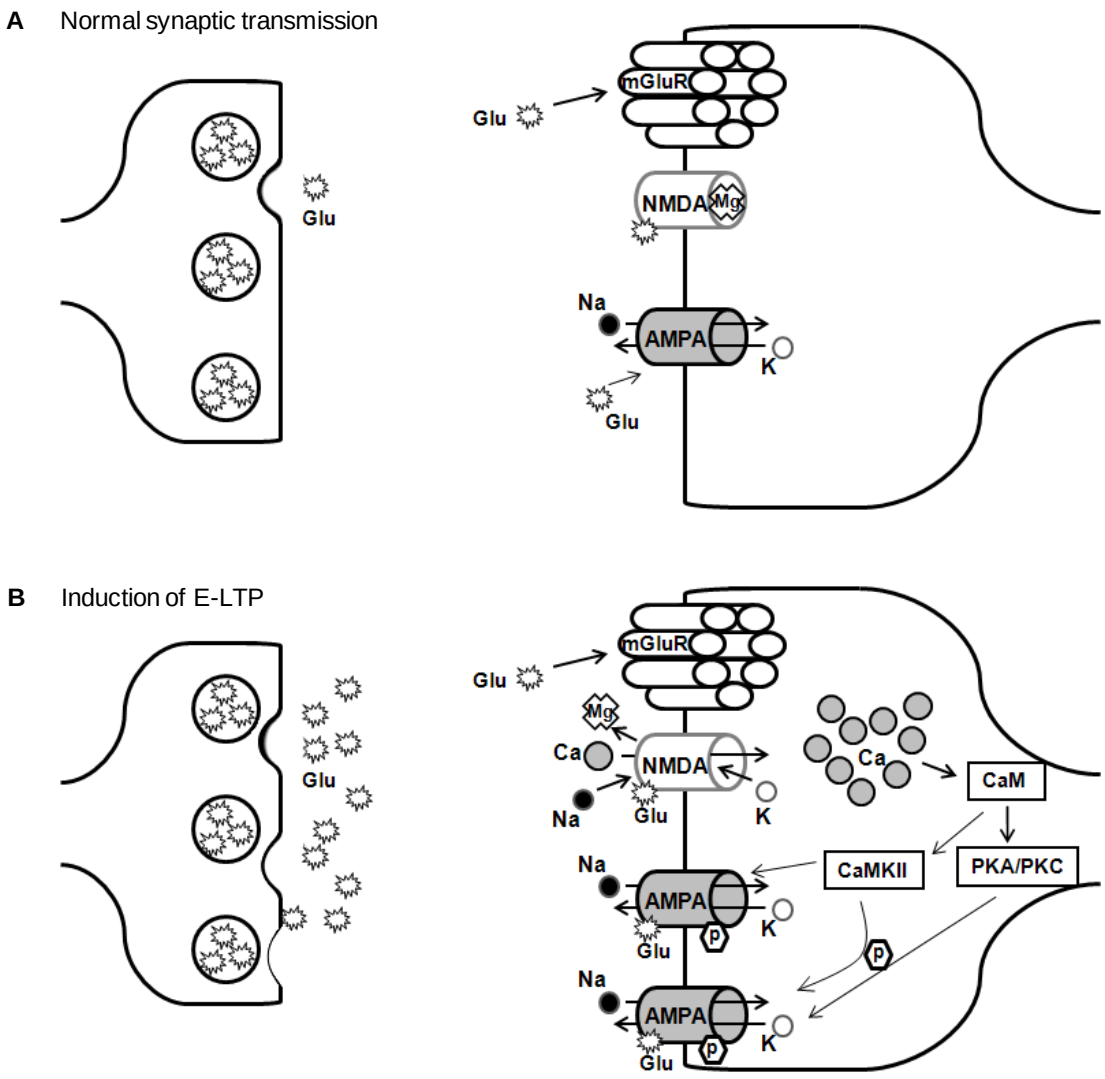

Fig. 2.2 Schematic model for synaptic transmission and induction of E-LTP. A – Cellular stimulation results in the presynaptic release of glutamate (Glu) which acts on NMDA, AMPA, and metabotropic glutamate receptors (mGluR). At resting membrane potential, the NMDA receptor is blocked by magnesium (Mg). B – During high-frequency stimulation, the postsynaptic cell is depolarized, freeing the Mg block and allowing the influx of sodium (Na) and potassium (K) through the NMDA receptor. This action further depolarizes the membrane and the NMDA receptor produces excitatory postsynaptic potentials (EPSPs) which increase the concentration of calcium (Ca) inside the cell; Ca can then act as a second messenger to activate the calcium/calmodulin (CaM) and PKA/PKC pathways. These kinases mediate both an increase in- and phosphorylation of- excitatory AMPA receptors, facilitating their activity and sensitizing the response to future synaptic transmission. This process is the molecular basis of E-LTP. More permanent consolidation during L-LTP (not shown) requires the synthesis of new proteins and gene transcription; processes stimulated by calcium and mediated by the aforementioned protein kinases. Additional abbreviations: calcium calmodulin kinase II, CaMKII; phosphorylation, p. Figure adapted from (Kandel et al., 2000).

new proteins that contribute to both structural changes, such as increased dendritic spine volume (Zhong et al., 2009), and functional changes, such as increased postsynaptic neurotransmitter sensitivity (Lynch, 2004). The combined effects of these alterations perpetuate the strengthening of the connections formed during E-LTP. Further changes in gene transcription provide an additional route to establish LTP more permanently. For example, the calcium influx mediated by NMDA receptors (and indirectly through AMPA activation) during E-LTP can lead to the activation of adenylyl cyclase which, in turn, leads to an increase in the immediate early gene cyclic adenosine monophosphate (cAMP) and the subsequent activation of protein kinase A (PKA). Along with ERK MAPK, PKA ultimately phosphorylates CREB in the nucleus and mediates the transcription of cAMP response element (CRE) – containing genes. Thus, memory formation can be broken down to a simple progression of increased calcium, kinase activation, immediate early gene activation, and finally gene transcription.

Multiple signaling systems play roles in hippocampal plasticity and memory consolidation, and there is substantial communication and transcriptional regulation between them. These pathways include the cAMP cascade, calcium/calmodulin dependent kinases, nitric oxide/Cgmp/cyclic GMP-dependent protein kinase (PKG), protein kinase C (PKC), rho/rac signaling, cell adhesion molecules, growth factor receptor tyrosine kinases, and the mitogen-activated protein kinases (MAPKs) (Sweatt, 2004). Of particular interest is the ERK MAPK cascade, which 1) plays an integral role in synaptic plasticity and memory formation across many species (Sweatt, 2004) 2) is dysregulated in early AD (Dineley et al., 2001b; Khan and Alkon, 2006) and 3) has been widely implicated in the types of memory affected in AD (Hamann et al., 2002; Hoefer et al., 2008).

2.3 ERK in learning and memory

The ERK cascade is very important to the induction and maintenance of both E-LTP and L-LTP as it converges with a number of other signaling cascades, including CaMKII, PKA, and

PKC (Kelleher et al., 2004). This signaling cascade can be activated by a number of protein kinases and receptors involved in growth and differentiation including receptor tyrosine kinases (RTK), G-protein coupled receptors (GPCR), integrins, and ion channels (Sweatt, 2004). In the canonical pathway (**Fig. 2.3**), extracellular growth factors bind to their respective RTKs leading to several phosphorylation events that activate a guanine nucleotide exchange factor (GEF) to exchange GDP bound to the small G protein Ras to GTP. Active Ras then recruits the serine/threonine kinase Raf to the membrane, where it is activated and then goes on to phosphorylate the dual specificity kinase MEK. MEK then binds to and dual phosphorylates cytoplasmic ERK, which then dimerizes and translocates to the nucleus.

Upon its activation, ERK exerts a number of downstream effects, including the phosphorylation/activation of a number of transcription factors that regulate gene expression (Winder et al., 1999; Wang et al., 2003; Sweatt, 2004) and the facilitation of protein synthesis and morphological changes (Lynch, 2004). In addition to its transcriptional regulation capabilities, ERK has been implicated as a contributing factor to learning and memory through a number of other processes. For example, ERK activation is required for PKA or PKC modulation of dendritic potassium channel function (Adams et al., 2000; Watanabe et al., 2002; Yuan et al., 2002), a process that is believed to increase membrane excitability, thereby enhancing LTP. A large body of work has confirmed that blocking ERK activation in mouse CA1 blocks LTP and even prevents increased spike bursting commonly seen in response to theta-frequency stimulation (Watabe et al., 2000; Watanabe et al., 2002) (Winder et al., 1999). Thus, ERK plays an important role in the regulation of back propagating action potentials that affect NMDA receptor activation and the basis of LTP and memory (Sweatt, 2004). More detailed analysis found that high frequency stimulation to the Schaffer collateral inputs to the hippocampal CA1 region activated only the p42 isoform of ERK (English and Sweatt, 1996), and that the p42

Figure 2.3

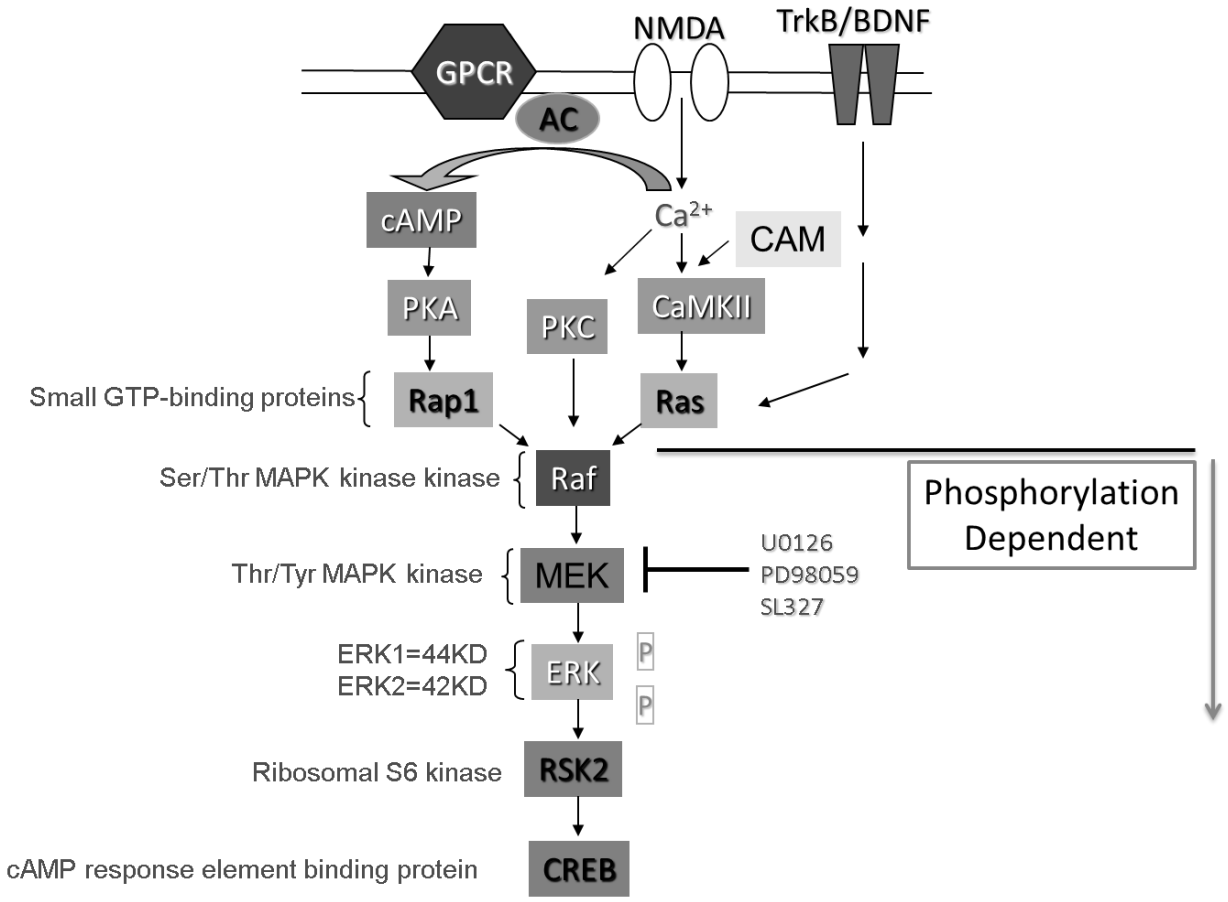

Fig 2.3 Schematic representation of the ERK MAPK signaling cascade. The ERK pathway can be stimulated by a number of protein kinases and receptors. In the canonical pathway, extracellular growth factors bind to their respective RTKs leading to several phosphorylation events that activate a guanine nucleotide exchange factor (GEF) to exchange GDP bound to the small G protein Ras to GTP. Active Ras then recruits the serine/threonine kinase Raf to the membrane, where it is activated and then goes on to phosphorylated the dual specificity kinase MEK. MEK then binds to and dual phosphorylates cytoplasmic ERK, which then dimerizes and translocates to the nucleus where it can act on downstream signaling partners such as RSK2 (aka p90RSK) and facilitate the activation of CREB and the transcription of CRE-mediated target genes. Inhibition of upstream members of this cascade (e.g. MEK) with synthetic compounds such as U0126 completely abrogates downstream activity.

isoform (heretofore referred to as ‘ERK2’) became activated in rats following training in fear conditioning (Atkins et al., 1998). ERK activation via phosphorylation can be prevented through the use of MEK inhibitors such as PD098059, SL327, or U0126; treatment with any of these compounds substantially attenuates the induction of LTP and prevents learning in animal models (Atkins et al., 1998; Selcher et al., 1999). Interestingly, studies using the NMDA antagonist MK801 found that it prevents both LTP and fear conditioning while simultaneously preventing the increased ERK activation seen following training in the FC task (Selcher et al., 1999), further demonstrating the importance of ERK activation to memory consolidation.

Aside from functional alterations, ERK has also been implicated in structural changes within the hippocampus that enhance LTP; namely the formation and stabilization of dendritic spines (Wu et al., 2001; Goldin and Segal, 2003). ERK accomplishes this regulation by promoting new protein synthesis within dendrites, a role that likely evolved from its peripheral role in preparing cells for replication (Sweatt, 2004) and one that has been shown to directly modulate LTP in hippocampal neurons (Giovannini et al., 2001).

Modulation of kinase activity is a compelling approach for therapeutic design, as dysregulated kinase activity has been implicated in a number of disease pathologies (Eldar-Finkelman and Eisenstein, 2009). However, initial efforts in this regard were unsuccessful due to the high affinity but low specificity of these compounds and the ubiquitous localization of kinases across multiple cell types and systems. As such, the resulting compounds often conferred multiple unintended and often disastrous side effects. Present research focuses on the development of small peptides which copy natural sequence motifs and may confer far more specific effects on kinase activity. The use of such peptides to regulate ERK as it relates to AD will be discussed in some capacity in chapter 5. Presently, we will focus on another method of ERK regulation. Recent evidence suggests that transcription factors may converge upon the

ERK cascade to selectively facilitate or inhibit ERK-mediated transcription (Revest et al., 2005; Song et al., 2005; Witty et al., 2012). Thus, convergence between the ERK MAPK signaling pathway and other transcriptional regulators may provide a mechanism to specifically impinge upon and restore dysregulated ERK networks in AD without affecting other signaling systems.

Figure 2.4

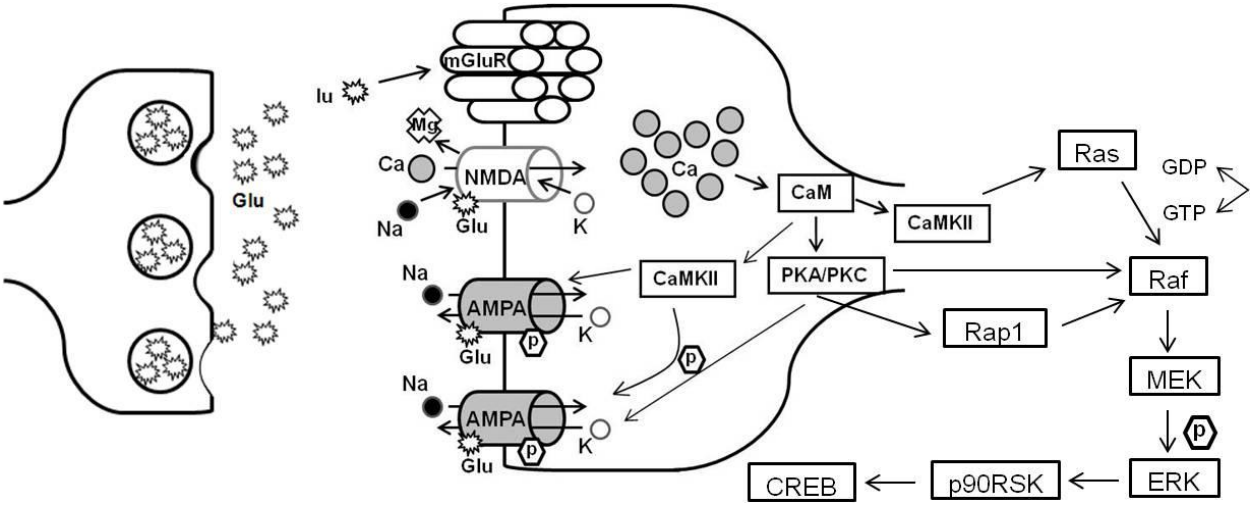

Figure 2.4 Glutamatergic transmission increases intracellular calcium and initiates the ERK cascade. During high-frequency stimulation, presynaptically released glutamate binds the NMDA receptor and the depolarized postsynaptic cell produces EPSPs which increase the concentration of calcium (Ca) inside the cell; Ca can then act as a second messenger to activate the calcium/calmodulin (CaM) and PKA/PKC pathways. In addition to their function on AMPA receptors, these kinases can initiate the GDP-GTP exchange between Ras and Raf, ultimately leading to ERK phosphorylation which then enhances the transcription of CRE-target genes in the nucleus

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CHAPTER 3
NUCLEAR RECEPTORS

3.1 Nuclear receptor structure and function

All nuclear receptors (NR) maintain a common structure that includes an N-terminal regulatory domain (A/B), a DNA binding domain I, a hinge region (D), a ligand binding domain (E), and a C-terminal domain (F) (**Fig 3.1**). NRs have the ability to directly bind to DNA at hormone response element (HRE) sequences and regulate the expression of adjacent genes; they are therefore often referred to as 'transcription factors' (Evans, 1988). This transcriptional regulation only takes place when a ligand binds the NR, inducing a conformational change and facilitating the receptor's activity. Nuclear receptors can be classified into four mechanistic classes based on their mechanism of action and subcellular distribution in the absence of ligand (Novac and Heinzel, 2004). Type I NRs are found in the cytosol and homo-dimerize. Ligand binding causes translocation to the nucleus and subsequent binding to HREs with inverted repeats. Type II NRs are bound by corepressor proteins with histone deacetylase (HDAC) activity and are typically retained in the nucleus. Ligand binding causes dissociation of the corepressor proteins and the recruitment of coactivator proteins with histone acetyl-transferase (HAT) activity as well as RNA polymerase. Type II NRs bind to DNA as hetero-dimers with the retinoid-X receptor (RXR) and bind to HREs with direct repeats. Type III NRs homo-dimerize and are very similar to Type I, with the exception that they recognize HREs with direct repeats. Finally, Type IV NRs can bind as either monomers or dimers and only recognize single sequence HREs (as opposed to the repeat sequences recognized by the other subtypes).

Figure 3.1

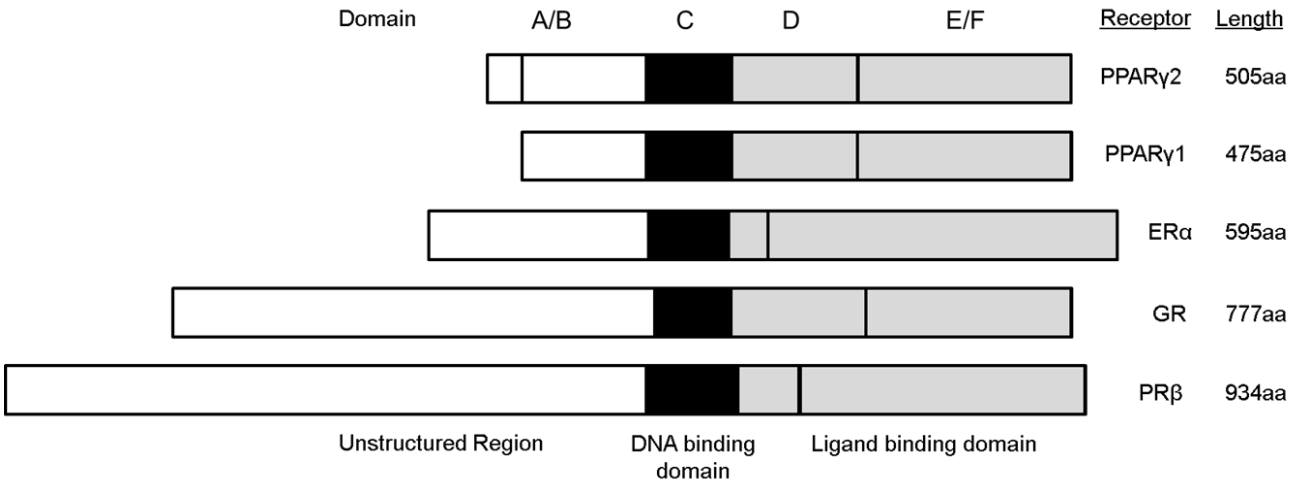


Fig 3.1 Nuclear receptors maintain a common domain structure. While the overall size of nuclear receptors varies, members of this family share a common structure that includes an N-terminal regulatory domain (A/B), a DNA binding domain I, a hinge region (D), and a C-terminal ligand binding domain (E/F). The majority of variation between these receptors is in the unstructured A/B domain. Receptors depicted have been aligned on the highly conserved DNA binding domain. Abbreviations: PPARγ2/PPARγ1, peroxisome proliferator activated receptor gamma, isoform 2/1, respectively; Erα, estrogen receptor alpha; GR, glucocorticoid receptor; PRβ, progesterone receptor beta. Total protein length is expressed in number of amino acids (aa).

A relatively high percentage (~13%) of FDA approved drugs target transcription factors due to their ability to regulate gene expression and the profound effects they can have on system function (Overington et al., 2006). Because new gene transcription is a time consuming process, a functional system effect may not be observed for several hours following ligand activation. However, study of the estrogen receptor (ER) indicates that ligand binding can have effects within minutes, suggesting that nuclear receptors may be capable of exerting non-genomic effects, although no molecular target for these effects has been definitively identified (Bjornstrom and Sjoberg, 2004; Zivadinovic et al., 2005). One hypothesis suggests that nuclear

receptors may have membrane-bound variants that are able to function through alternative signal transduction mechanisms and are therefore able to bypass gene regulation.

3.2 Nuclear receptor modulation of ERK activity

Interaction with- or impingement on- the ERK cascade is one mechanism by which nuclear receptors may be able to exert these rapid, non-genomic effects. Indeed, others have shown that multiple nuclear receptors, including the glucocorticoid receptor (GR) and estrogen receptor (ER) can directly modulate ERK activity and thereby affect memory formation. For example, acute stress causes an increase in endogenous glucocorticoids, which subsequently activate GR; this action is known to amplify the consolidation of memories associated with emotionally charged experiences (de Kloet et al., 1999; McGaugh and Roozendaal, 2002; Trollope et al., 2012), suggesting cross talk between the GR and ERK pathways. Mice subjected to contextual fear conditioning exhibit increased freezing behavior when they are exposed to exogenous glucocorticoids, and blocking ERK activation via the MEK inhibitor U0126 prevents this increase (Revest et al., 2005). Similarly, activation of GR causes increased expression levels and activity of the ERK MAPK pathway and this leads to an increase in the expression of the downstream ERK-mediated immediate early gene Egr-1 (Revest et al., 2005), which has been implicated as an important player in neuronal plasticity (Knapska and Kaczmarek, 2004). Thus, these data suggests a direct link between activation of GR and the phosphorylation/activation of ERK to cooperatively enhance Egr-1 expression and modulate memory formation (Revest et al., 2005).

Other research has found that ER activation is also linked to the modulation of learning and memory (Luine, 2008; Witty et al., 2012). Witty et al, using an aged ovariectomized rat model, examined the long-term learning and memory effects of short-term exposure to estrogens during middle age and found that exposure to estradiol enhanced future memory performance and led to increased expression of Erα in the hippocampus (Witty et al., 2012).

These effects were long lasting and did not require sustained exogenous estradiol, suggesting that increased Erα in the hippocampus directly affected activation of ERK even in the absence of ligand. This idea was confirmed by the study of ovariectomized Erα knockout mice that received viral vector delivery of Erα to the hippocampus and subsequently exhibited improved spatial learning in the hippocampus- and ERK- dependent radial arm maze task (Foster et al., 2008). Increased hippocampal Erα also facilitates increased ERK phosphorylation (Witty et al., 2012), once more suggesting that nuclear receptors can directly modulate ERK activation and thereby affect memory consolidation.

3.3 PPARγ

The nuclear receptor superfamily is a group of ligand-activated receptors and includes retinoic acid, estrogen receptor, thyroid hormone receptor, glucocorticoid receptor, and the peroxisome proliferator activated receptors (PPAR). The PPARs are Type II NRs that play a major role in energy homeostasis and are essential to cellular differentiation, development, and metabolism – specifically the regulation of fatty acid storage and glucose metabolism (Berger et al., 2000). Three PPARs have been identified: PPARα, PPARβ/δ, and PPARγ. All three forms heterodimerize with RXR and bind to the direct repeat peroxisome proliferator response element (PPRE) hexameric consensus sequence AGGTCANAGGTCA, where N is any amino acid. Interestingly, each form exhibits differential tissue distribution and function (Rosen and Spiegelman, 2001). Briefly, PPARα is widely expressed in liver, kidney, heart, skeletal muscle, and adipose tissue and primarily facilitates the oxidation and catabolism of fatty acids (Tyagi et al., 2012). PPARβ/δ has been found in skin, brain, and adipose tissue and its function is not well-characterized, although some evidence suggests it plays a role in lipid accumulation and glucose metabolism (Schmuth et al., 2004; Lee et al., 2006). PPARγ, the most well characterized form, is expressed ubiquitously and has been found in adipose tissue, kidney, heart, muscle, colon, pancreas, spleen, and brain and is integral in adipogenesis and glucose

homeostasis (Tyagi et al., 2012). In humans, two PPARγ isoforms are generated as a result of alternative splicing and promoter sites; they share identical structure with the exception of 30 additional amino acids at the N-terminus of PPARγ2. RT-PCR confirms that PPARγ1 mRNA levels are higher than those of PPARγ2 in all peripheral regions, with some tissues exclusively expressing PPARγ1 (Fajas et al., 1997). Similar analysis in our lab demonstrates a roughly 7-fold excess of PPARγ1 over PPARγ2 in the mouse CNS (Denner et al., 2012b).

Structurally, PPARγ is similar to steroid hormone receptors and like all nuclear receptors, PPARγ maintains the common structure described in section 2.1; this includes an N-terminal A/B domain that contains an AF-1 ligand-independent activation domain, a DNA binding domain containing two cysteine-rich zinc finger motifs, a hinge region where cofactors bind, and a ligand binding domain containing an AF-2 domain that serves to enhance transcription through cofactor recruitment. Ligand binding causes a conformational change that closes the AF-2 site and activates the receptor for transcription (Nolte et al., 1998; Chandra et al., 2008; Zieleniak et al., 2008), thereby allowing the PPARγ-RXR heterodimer to bind to a PPRE. The LBD also enhances DNA binding at a PPRE by stabilizing the PPARγ-RXR heterodimer through interaction with the DBD of both receptors (Chandra et al., 2008). At resting conditions, PPARγ is bound by a number of co-repressor proteins (e.g. SMRT) which prevent transcription via histone deacetylase. Ligand binding induces a conformational change in PPARγ which destabilizes the co-repressor proteins and recruits co-activator proteins with histone acetyl transferase (HAT) activity (**Figure 3.2**); this causes decondensation of chromatin and promotes the transcription of PPARγ target genes by RNA polymerase II (Zieleniak et al., 2008).

Figure 3.2

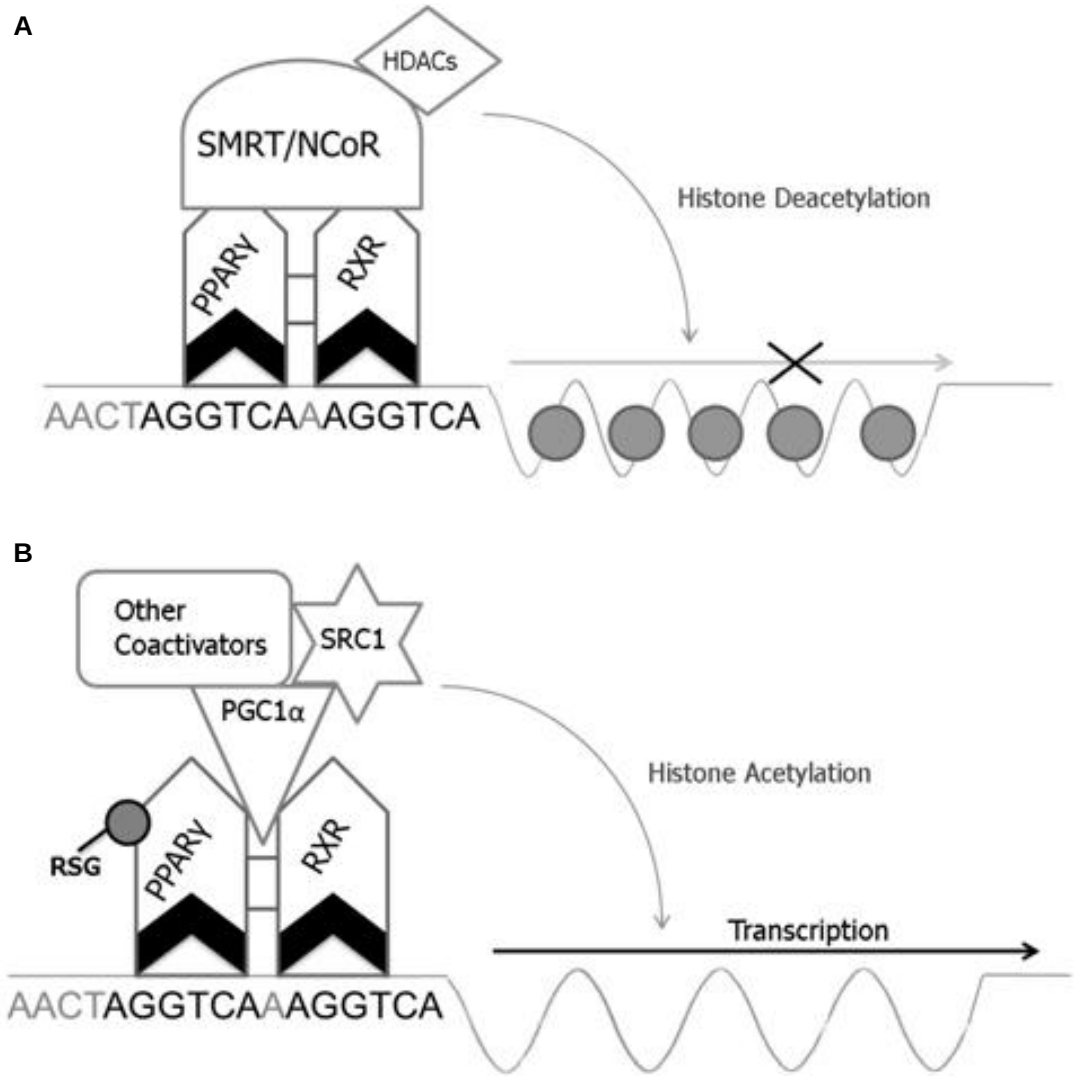


Figure 3.2 PPARγ-dependent transcription is dependent upon numerous co-regulatory proteins. PPARγ forms a heterodimer with the retinoid X receptor (RXR) and this complex recognizes the PPRE hexameric consensus sequence AGGTCANAGGTCA, where N is any amino acid. A – At resting conditions, PPARγ is bound by co-repressor histone deacetylase (HDAC) proteins which tightly pack chromatin and prevent gene transcription. B – Ligand-activation of PPARγ (here by rosiglitazone, RSG) induces a conformational change that destabilizes the co-repressor proteins and recruits co-activators with histone acetyltransferase (HAT) ability (e.g. PGC1α, SRC-1, etc), resulting in the decondensation of chromatin and the subsequent transcription of PPARγ target genes by RNA polymerase II.

3.4 PPARγ as a therapeutic target for AD

PPARs can be endogenously activated by free fatty acids and the eicosanoids, derivatives of omega-3 and omega-6 fatty acids. Pharmacologically, PPARγ can be agonized by members of the highly selective thiazolidinedione (TZD) drug class. While early research focused on the role of PPARγ in peripheral tissues (Desvergne and Wahli, 1999), a role in neuronal function emerged following immunohistological studies in the CNS that identified PPARγ expression in brain areas associated with higher cognitive function, including the cortex, basal ganglia, hypothalamus, and hippocampus (Moreno et al., 2004; Inestrosa et al., 2005; Sarruf et al., 2009). As a result, PPARγ is a therapeutic target in many disease states including inflammation, cancer, ischemia, traumatic brain injury, and T2DM. Many of these effects are attributed to inhibition of the expression of pro-inflammatory proteins (e.g. Inos, TNFα, MMP9) and attenuation of reactive oxygen species (ROS), and PPARγ agonism is generally recognized as neuroprotective (Breidert et al., 2002; Feinstein, 2003; Victor et al., 2006; Hyong et al., 2008).

Still, the effective manner in which PPARγ agonists regulate the expression of insulin-responsive genes has relegated PPARγ primarily as a therapeutic target in T2DM. Substantial research has confirmed both rosiglitazone (RSG) and pioglitazone (PIO) as effective insulin sensitizers for the treatment of T2DM (DeFronzo et al.; Hofmann et al., 1991; Strum et al., 2007). These compounds successfully activate PPARγ and facilitate the transcription of insulin-responsive genes, ultimately leading to a decrease in glucose levels without requiring an increase in pancreatic insulin output. Because insulin sensitivity is known to be impaired in AD (Craft et al., 1998; Craft et al., 1999; Ott et al., 1999; Luchsinger et al., 2001; Luchsinger et al., 2004; Talbot et al., 2012) and individuals with T2DM are seemingly predisposed to the development of AD (Watson and Craft, 2003; Arvanitakis et al., 2004; Rivera et al., 2005; Akomolafe et al., 2006), TZDs have been examined as potential therapeutics for AD pathology.

Work in neuronal cell cultures demonstrated that treatment with TZDs confers a reduction in Aβ accumulation (Camacho et al., 2004) and that TZDs inhibit expression of TNFα and interleukin-6, thereby decreasing Aβ-mediated inflammation (Combs et al., 2000). Furthermore, application of Aβ to hippocampal slice cultures has been shown to inhibit Schaffer-collateral LTP, while pre-treatment of the slices with a TZD attenuates this effect (Costello et al., 2005). As such, the synaptic dysfunction conferred by Aβ accumulation can be prevented through PPARγ agonism. Since PPARγ maintains 99% similarity and 95% identity between humans and mice (Fajas et al., 1997), transgenic mouse models have provided an excellent system in which to examine the effects of PPARγ agonism on AD pathology *in vivo*. Multiple studies have demonstrated PPARγ agonism improves cognitive performance in mouse models of AD in tasks that require intact hippocampal ERK MAPK signaling (**Fig. 3.3**) (Gemma et al., 2004; Pedersen et al., 2006; Strum et al., 2007; Landreth et al., 2008; Escribano et al., 2009; Rodriguez-Rivera et al., 2011; O'Reilly and Lynch, 2012). Furthermore, stimulation of PPARγ by either RSG or PIO has been shown to suppress expression of both β-secretase and APP, as well as promoting APP ubiquitination and subsequent degradation (Landreth et al., 2008) and PPARγ agonism can prevent β-amyloid- induced LTP deficits in hippocampal slices (Bell et al., 2004). Taken together, these data suggest a therapeutic role for PPARγ agonism to combat AD pathology.

Indeed, several small clinical trials have shown PPARγ agonism to be efficacious in a subset of subjects with early AD or pre-AD memory complaints coincident with mild to moderate insulin resistance (Strachan, 2005; Risner et al., 2006; Abbatecola et al., 2010; Watson et al., 2011). While subsequent large clinical trials using PPARγ agonists apparently failed to improve cognition in mild-to-moderate AD, it is important to note that the sponsors of these studies did not stratify their treatment outcomes based on peripheral gluco-regulatory status and thus may have overlooked a positive result in patients with concomitant insulin resistance (Becker and

Greig, 2013). TZD use for the treatment of AD has not taken off as a combined result of these negative clinical trials and the negative side effects associated with RSG treatment (e.g. adiposity, edema, increased incidence of cardiac hypertrophy). However, RSG remains a valuable tool to identify the mechanisms by which PPARγ agonism enhances cognition in individuals compromised by AD neuropathology.

Figure 3.3

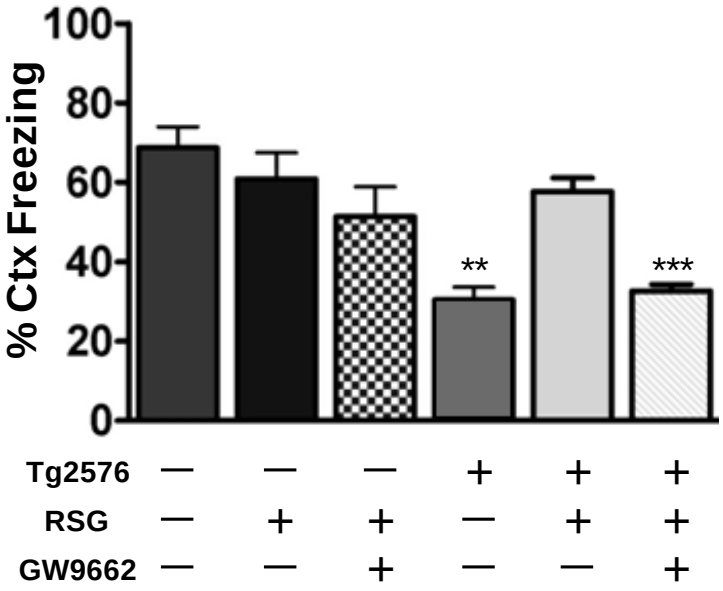


Fig. 3.3 PPARγ agonism with RSG restores 9MO Tg2576 cognitive performance to WT levels. Tg2576 and WT littermates fed either RSG or control diet for 1 month underwent 2-pair fear conditioning training and contextual testing. 9MO Tg2576 are significantly impaired in the contextual task, while RSG treatment restores performance to levels comparable to WT. RSG-treated Tg2576 given the PPARγ antagonist GW9662 do not exhibit restored cognition, indicating that the cognitive enhancing effects of RSG are mediated by PPARγ. WT mice were unaffected by either PPARγ agonism (RSG) or antagonism (GW9662). Two-way ANOVA detected a genotype effect but no treatment effect or interaction ($F(2,1,2)=0.778$ and 29.72) for genotype and treatment. Therefore, untreated Tg2576 (RSG –), vehicle-infused (GW –) Tg2576, and RSG-treated (RSG+) Tg2576 ICV infused with GW9662 (GW+) froze significantly less. Neither RSG nor GW9662 had an effect on performance of WT. Experiment by JRR.

3.5 Regulation of PPARγ

Phosphorylation – PPARγ is subject to a number of post-translational modifications that serve to regulate PPARγ-dependent gene expression in response to environmental changes. The most thoroughly studied of these is phosphorylation at serine82 and serine112 of mouse PPARγ1 and γ2, respectively. This serine falls in the midst of a conserved MAPK consensus site and can be phosphorylated by ERK in response to growth factor activation and also by both JNK1/2 and p38 in response to stress (Adams et al., 1997; Camp et al., 1999). In the periphery, this MAPK-mediated hyper-phosphorylation results in decreased transcriptional activity of PPARγ (Hu et al., 1996; Camp and Tafuri, 1997; Shao et al., 1998; Camp et al., 1999) and has been demonstrated to reduce its adipogenic functions (van Beekum et al., 2009). Phosphorylation disrupts the resting conformation of PPARγ and thereby affects ligand binding affinity, ultimately reducing the efficiency of PPARγ transcription (Shao et al., 1998). Notably, serine 82/112 can also be phosphorylated by the cyclin-dependent kinases (cdk) -7 and -9, and this modification has been found to increase PPARγ activity as it relates to adipogenesis (Compe et al., 2005; Iankova et al., 2006). Given these conflicting results, phosphorylation of PPARγ appears to have differential effects on PPARγ subcellular localization and/or transcriptional activity that may vary depending upon the cellular milieu. We previously found that phosphorylation of nuclear PPARγ is increased in Tg2576 hippocampus; RSG treatment reversed this and also led to increased PPARγ gene expression (Denner et al., 2012b), suggesting that phosphorylation inhibits PPARγ transcription in the hippocampus. Others have demonstrated that phosphorylation of Ser82 inhibits PPARγ transcription in 293T cells, although these authors also noted that occupation of the LBD of PPARγ (via agonism with RSG) reduced MAPK dependent phosphorylation while maintaining ERK activity (Camp and Tafuri, 1997). Furthermore, other reports indicate MAPK phosphorylation of ligand-bound PPARγ at Ser82 facilitates its transcriptional activity in CHO cells (Zhang et al., 1996; Prusty et al., 2002) in a

manner similar to other nuclear receptors, while still others suggest that phosphorylation tags PPARγ for proteasomal degradation (Floyd and Stephens, 2002). Interestingly, inhibition of ERK by preventing the activation of its upstream activator MEK inhibits the decay rate of PPARγ, suggesting that serine phosphorylation influences this rate (Floyd and Stephens, 2002). As phosphorylation can both facilitate and inhibit PPARγ transcription depending on the kinases involved and the cellular localization, it is difficult to make broad claims based on the phosphorylation status of this protein.

In addition to the well characterized phosphorylation site at Ser82/112, it was recently found that PPARγ2 can be phosphorylated by Cdk-5 within the LBD at Ser273 (Ahmadian et al., 2013). Cdk-5 is activated by pro-inflammatory cytokines that are elevated in obesity and phosphorylation at Ser273 alters the expression of a group of genes that are aberrantly regulated in obesity (Choi et al., 2010). Interestingly, treatment with TZDs has been shown to prevent Cdk-5-mediated phosphorylation of PPARγ (Cannon and Nedergaard, 2004), which is sensible since Ser273 phosphorylation is increased in the adipose tissue of mice fed a high fat diet (Choi et al., 2010) and TZDs have insulin sensitizing properties. See **Fig. 3.4** for a schematic of all currently known post-translational modifications to PPARγ.

SUMOylation – In addition to phosphorylation events, PPARγ is subject to post-translational modification through the covalent attachment of small ubiquitin-like modifier (SUMO) peptides at lysine 77/107 in the AF1 region and lysine 365/395 in the AF2 region. SUMOylation is involved in nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle (Hay, 2005).

Figure 3.4

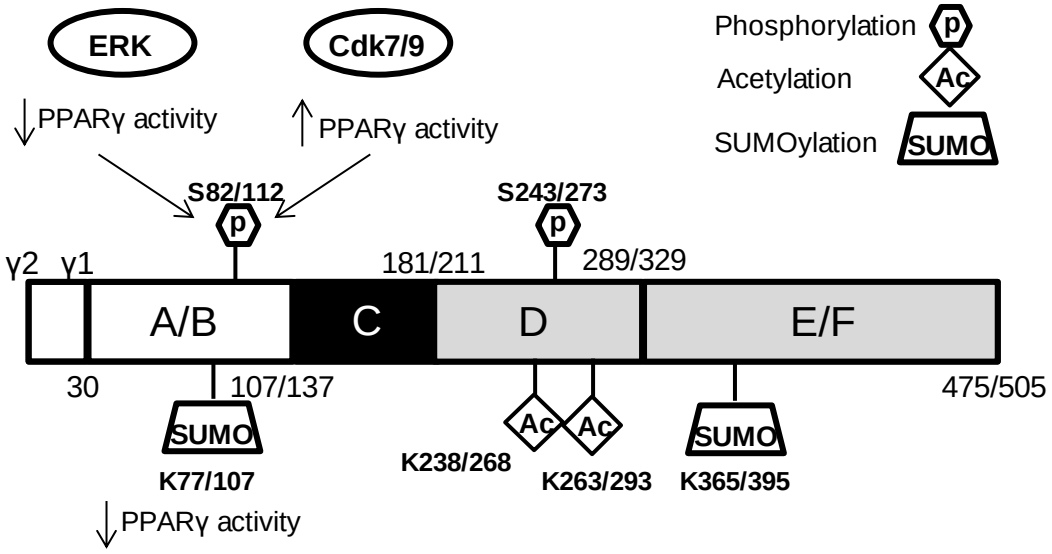


Fig. 3.4 Post translational modifications of PPARγ. ERK MAPK mediated phosphorylation at SER82/112 is generally accepted to inhibit PPARγ transcription while Cdk7/9 mediated phosphorylation at this site facilitates PPARγ transcription. PPARγ transcription can also be inhibited by SUMOylation at K77/107 and K365/395. Acetylation at K238/268 and K263/293 is associated with browning of white adipose tissue (WAT). There is evidence that the effects of these modifications may be tissue specific. Amino acids positions are expressed within PPARγ1/PPARγ2 respectively. Figure adapted from (Ahmadian et al., 2013).

Conjugation of a SUMO to lysine 77/107 represses PPARγ transcription, although the mechanism of repression has not yet been determined, but may involve promoting the specific binding of a repressor complex (van Beekum et al., 2009; Ahmadian et al., 2013). Interestingly, SUMOylation of K77/107 is linked to SER82/112 phosphorylation in that K77/107 SUMOylation is enhanced by SER82/112 phosphorylation (Yamashita et al., 2004; van Beekum et al., 2009), suggesting a feed-forward inhibitory mechanism in certain tissues. SUMOylation at lysine 365/395 has been examined in macrophages; this modification recruits PPARγ to the promoters

of inflammatory genes which prevent the clearance of co-repressor proteins and thereby inhibit PPARγ transcription (Pascual et al., 2005).

Ubiquitination – Like many proteins, PPARγ can also undergo ubiquitination, the covalent attachment of ubiquitin to lysine residues that generally label the protein for proteasomal degradation. PPARγ is no exception, as it has a relatively short half-life (t1/2=2 hours (Waite et al., 2001; Christianson et al., 2008) and is degraded by proteasomes following ubiquitination. Notably, TZDs have been found to accelerate this process (Hauser et al., 2000; Waite et al., 2001; Floyd and Stephens, 2002), suggesting that activation of the receptor leads to a quicker rate of turnover. This observation is confirmed by the fact that hypo-phosphorylated PPARγ that exhibits a high rate of transcription is degraded more rapidly than the phosphorylated form (Hauser et al., 2000; Floyd and Stephens, 2002).

Cellular compartmentalization – Recent work by Burgermeister et al. proposed that PPARγ transcription can also be regulated by MEK through subcellular compartmental redistribution (Burgermeister et al., 2007). In quiescent cells, MEK is localized to the cytosol, but translocates to the nucleus upon cellular stimulation (Yoon & Seger, 2006). However, the MEK primary sequence contains a nuclear export signal (NES) resulting in its rapid export back to the cytosol (Jaaro et al., 1997). Conversely, PPARγ is primarily localized to the nucleus in resting cells (Berger et al., 2000) and this localization facilitates PPARγ transcription of nuclear target genes (Burgermeister and Seger, 2007). In a number of cell lines (i.e. HeLa, CHO, HEK-293T, COS7) researchers found that upon mitogenic stimulation, MEK translocates to the nucleus where it can directly interact with the PPARγ AF-2 domain, and further that the resulting PPARγ-MEK complex is subsequently shuttled from the nucleus as a result of the MEK NES (Jaaro et al., 1997; Burgermeister and Seger, 2007). The result of this cellular redistribution is a reduction in PPARγ-mediated transcription of nuclear target genes and the down-regulation of PPARγ protein resulting from subsequent proteasomal degradation (Burgermeister and Seger, 2007).

One additional consequence of the nuclear export of PPARγ may be a facilitation of PPARγ interaction with cytosolic and membrane proteins; such interactions may be important in mediating the non-genomic effects of PPARγ (Losel and Wehling, 2003; Burgermeister and Seger, 2007). Also noteworthy is the fact that MEK binds PPARγ in the same region as the PPARγ co-activator SRC-1, suggesting that the binding of either protein is mutually exclusive (Burgermeister and Seger, 2007) and that the recruitment of PPARγ co-activators following ligand binding may prevent or limit MEK from exerting this shuttling effect.

CHAPTER 4

PPARγ AND ERK SIGNALING ARE LINKED

We and others have previously reported that PPARγ agonism with RSG improved hippocampus-dependent cognition in the Tg2576 AD mouse model (Pedersen et al., 2006; Rodriguez-Rivera et al., 2011). Given the precedence for nuclear receptor modulation of ERK signaling (discussed in section 3.2), we sought to provide mechanistic insight regarding how PPARγ agonism enhanced ERK-dependent gene transcription. Thus, considering that 1) RSG primarily functions as an insulin sensitizer, 2) PPARγ agonism via RSG enhances hippocampus-dependent cognition, 3) ERK is required for many forms of learning and memory affected in AD (Sweatt, 2004; Trifilieff et al., 2007; Denner et al., 2012b) and 4) both PPARγ and ERK are key mediators of insulin signaling, we investigated the hypothesis that PPARγ agonism-mediated cognitive improvement induces a hippocampal PPARγ pattern of gene and protein expression that converges with the ERK MAPK signaling axis and thereby facilitates downstream gene transcription and ultimately enhances ERK-dependent memory consolidation. Recent work has shown reciprocal PPARγ and ERK MAPK activity in several neurological disorders and cancer, suggesting a potential action for PPARγ in amelioration of memory deficits in AD (Camp and Tafuri, 1997; Kim et al., 2003; Papageorgiou et al., 2007; Schroeter et al., 2007; Rosa et al., 2008; Zhang et al., 2011). In support of this, chronic elevated Aβ leads to dysregulation of hippocampal ERK MAPK *in vitro* and *in vivo* (Dineley et al., 2001b; Bell et al., 2004; Swatton et al., 2004), while PPARγ agonism ameliorates cognitive deficits *in vivo,* possibly through suppressed expression of both β-secretase and APP and the promotion of APP ubiquitination and subsequent degradation (Landreth et al., 2008). PPARγ agonism can also prevent Aβ-induced deficits in hippocampal plasticity *in vitro* (Bell et al., 2004; Costello et al., 2005; Rodriguez-Rivera et al., 2011). Likewise, both ERK and PPARγ are dysregulated in

AD brain and certain PPARγ polymorphisms are associated with increased risk for the disease (Kitamura et al., 1999; Scacchi et al., 2007).

To investigate the molecular mechanism underlying PPARγ agonism with RSG on AD-like cognitive function, we used an extensively characterized AD mouse model, Tg2576, previously described in section 1.7; briefly, Tg2576 expresses a transgene encoding a mutant form of the human amyloid precursor protein linked to familial AD in humans (Hsiao et al., 1996). Importantly, Tg2576 mice exhibit age-dependent cognitive decline as measured in several behavioral paradigms, most notably in those requiring proper hippocampal ERK MAPK function that are similarly impaired in humans with AD (Atkins et al., 1998; Dineley et al., 2001a; Dineley et al., 2001b; Dineley et al., 2002b; Hamann et al., 2002; Hoefer et al., 2008). Therefore, the presently described study tested whether regulation of hippocampal PPARγ coincided with ERK MAPK signaling following RSG-mediated cognitive improvement. In the hippocampal PPARγ transcriptome of the Tg2576 AD animal model, we found significant overlap between peroxisome proliferator response element (PPRE)-containing PPARγ target genes and cAMP response element (CRE)-containing ERK MAPK [cAMP response element-binding protein (CREB)] target genes. This data is consistent with previous reports of glucocorticoid receptor response elements present on the promoter of Ras (Strawhecker et al., 1989), an upstream member of the ERK cascade, suggesting that transcriptional cross talk is common between members of the ERK MAPK cascade and the structurally similar nuclear receptors. Using quantitative mass spectrometry and bioinformatics on the dentate gyrus, we identified many proteins related to synaptic plasticity and memory formation that were induced concomitant with RSG-mediated cognitive rescue and activation of PPARγ and ERK2, actions reversed when hippocampal PPARγ was pharmacologically antagonized to reverse RSG-mediated cognitive improvement. We conclude that the hippocampal transcriptome and proteome induced by cognitive enhancement with RSG harness a dysregulated ERK MAPK

signal transduction pathway to overcome AD-like cognitive deficits in Tg2576 mice. Thus, PPARγ represents a signaling system that is not crucial for normal cognition yet can intercede to restore neural networks compromised by AD.

4.1 PPARγ agonism via oral RSG affects hippocampal protein expression

We initially evaluated whether oral RSG treatment increased PPARγ activity in the CNS by measuring hippocampal PPARγ binding to its PPRE. Nuclear extracts prepared from the hippocampus of Tg2576 and WT littermates showed that RSG treatment resulted in a statistically significant (30%) increase in PPARγ DNA binding in both Tg2576 and WT groups (**Fig. 4.1A**), confirming that oral RSG is blood–brain barrier permeable (Strum et al., 2007; Festuccia et al., 2008; Diano et al., 2011; Lu et al., 2011; Ryan et al., 2011) and increases steady-state DNA binding. We were unable to affect DNA binding with the PPARγ antagonist GW9662 (data not shown). Consistent with the prevailing concept that PPARγ binding to PPREs is necessary yet insufficient for regulating target gene expression, we assessed the hippocampal PPARγ transcriptome using quantitative PCR on hippocampal mRNA isolated from mice treated with or without RSG. Expression analysis from a custom array of 45 genes chosen for enrichment in PPREs, demonstrated that 34 were down-regulated in untreated Tg2576 compared with WT and 32 of those were induced by RSG treatment in Tg2576 (**Table 4.1**). For example, the PPRE-containing apolipoprotein O gene (*APO-O*) was decreased in untreated Tg2576 compared with WT, and RSG treatment reversed this (**Fig. 4.1B**). As such, untreated Tg2576 mice exhibited a 1.97-fold decrease in *APO-O* mRNA transcripts compared with WT, and RSG induced a 10.82-fold increase in this mRNA transcript in Tg2576. We next probed the hippocampal PPARγ proteome with quantitative mass spectrometry using the stable isotope ^{18}O-/^{16}O-water and LC-MS/MS method (Sadygov et al., 2010; Starkey et al., 2010). Within the

Figure 4.1

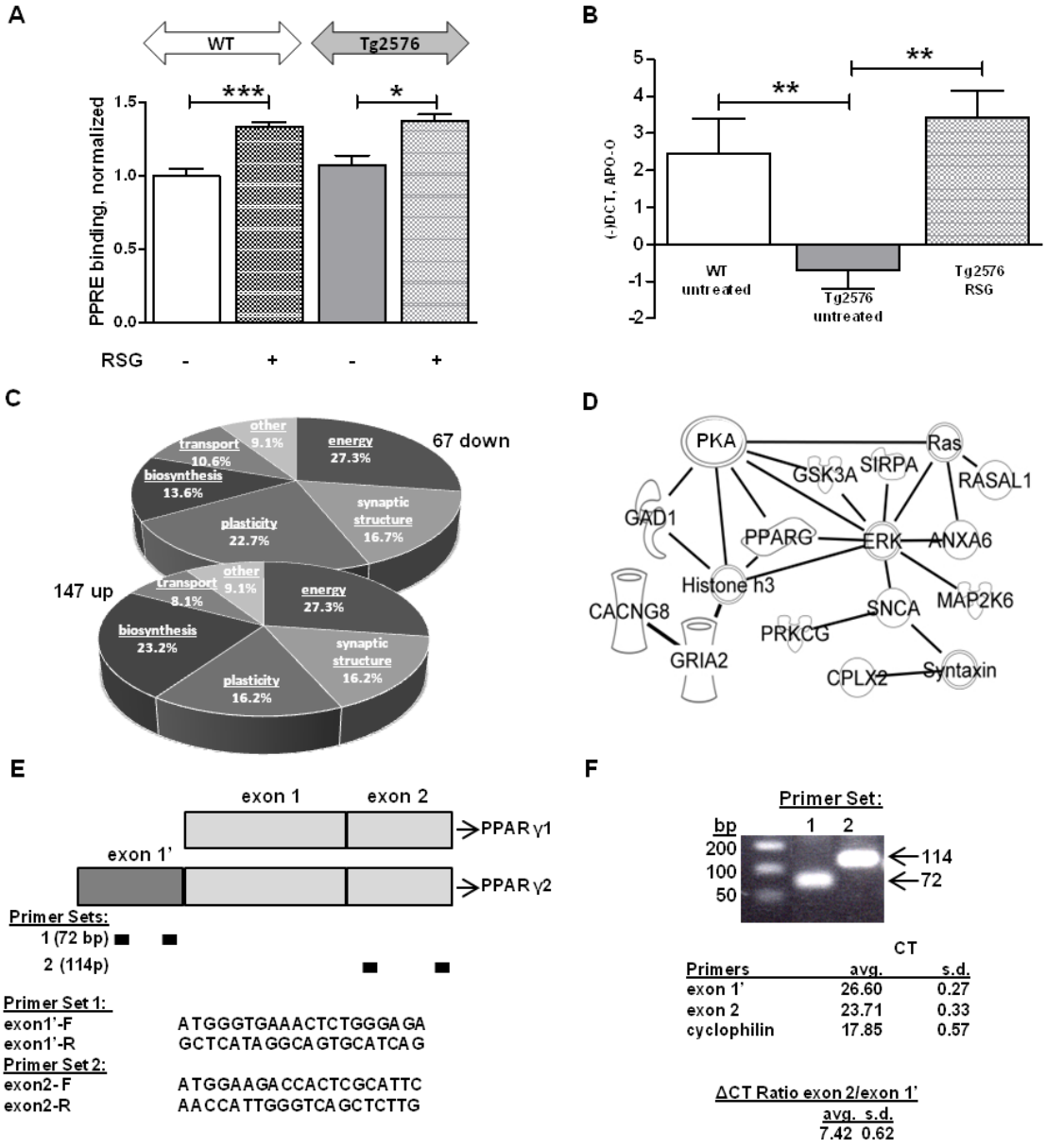


Fig. 4.1 Oral delivery of RSG impinges upon CNS PPARγ. **A**, Hippocampal PPARγ binding to its PPRE is enhanced by 1 month RSG treatment. Two-way ANOVA $F(3,31)=9.34$ for treatment; no interaction was detected. **B**, One month RSG treatment induces PPARγ target gene expression. The mRNA for the PPRE-containing *APO-O* gene is reduced in untreated Tg2576 compared with WT untreated. RSG normalizes *APO-O* expression in Tg2576. One-way ANOVA of Δ*C*T values resulted in $F(2,9)=8.6$. **C**, Quantitative mass spectrometry reveals Tg2576 hippocampal proteins altered with RSG treatment. All proteins displayed have a Benjamini-Hochberg rank sum $p \leq 0.05$. **D**, Ingenuity Pathways Analysis of synaptic plasticity proteins identified by quantitative mass spectrometry placed ERK MAPK as a central node in the protein network. ANXA6, Annexin A6; CACNG8, voltage-dependent calcium channel γ-8

subunit; CPLX2, complexin 2; GAD1, glutamate decarboxylase 1; GR1A2, glutamate receptor subunit 2; GSK3A, glycogen synthase kinase-3β; MAP2K6, dual specificity mitogen-activated protein kinase kinase; PKA, protein kinase A; PPARG, PPARγ; PRKCG, protein kinase C-γ; RASAL1, RasGAP-activating-like protein 1; SIRPA, signal-regulatory protein α; SNCA,α-synuclein (see Materials and Methods and www.ingenuity.com for a more detailed description of network statistical calculations, molecule naming, and symbol descriptions). **E**, PCR strategy to detect PPARγ1 and PPARγ2 gene transcripts in mouse hippocampus. **F**, Both PPARγ1 and PPARγ2 are detected in hippocampus by conventional PCR (gel image, top). Quantitative PCR shows PPARγ1 mRNA expression is much higher than PPARγ2 in mouse hippocampus. **p*<0.05, ***p*<0.01, ****p*<0.001. Work performed in lab of LTD.

Tg2576 dentate gyrus proteome, RSG affected proteins with structural, energy, biosynthesis, transport, and plasticity functions; in total, RSG treatment significantly up-regulated 147 proteins and down-regulated 67 proteins (**Fig. 4.1C**). For example, this approach determined that the PPARγ target gene, APO-O, exhibited 2.9-fold increased protein in RSG treated Tg2576 compared with untreated Tg2576 (Benjamini-Hochberg rank sum p=0.0015) and the ERK phosphatase PP2A was downregulated by 16% of untreated Tg2576 (Benjamini-Hochberg p= 2.54×10^{-6}).

4.2 PPARγ and ERK are both central regulators of hippocampal proteins augmented by RSG

To evaluate potential functional relationships between the Tg2576 hippocampal proteins whose expression was augmented by RSG treatment, we performed bioinformatics analysis on proteins involved in synaptic plasticity. The proteins from this category were chosen for analysis given the learning- and memory-related cognitive-enhancing properties of RSG and the importance of synaptic plasticity to memory formation (Lynch, 2004; Wang et al., 2006b). ERK MAPK emerged as a central node following Ingenuity Pathways Analysis and PPARγ itself was a target regulator of ERK MEK (mitogen-activated protein kinase kinase) in addition to glutamate decarboxylase, GSK3-α, α-synuclein, metabotropic glutamate receptor 5, and glutamate receptor 2 (**Fig. 4.1D**). The mouse PPARγ gene gives rise to two mRNAs (PPARγ1 and PPARγ2) that differ only at their 5' ends (**Fig. 4.1E**). The mouse PPARγ2 mRNA encodes

Table 4.1 PPREs and CREs in RSG-Regulated Hippocampal Genes in Tg2576

Gene Name	Protein Name	RefSeq ID	Avg.	SEM	Response Element
Actb	Actin, cytoplasmic 1	NM_007393	9.44	3.46	
ADCYAP1R1	Adenylate cyclase activating polypeptide 1 receptor 1	NM_007407	5.53	2.22	
Apba2	Amyloid beta A4 precursor protein-binding family A member 2	NM_007461	8.41	3.50	
Arpc4	Actin-related protein 2/3 complex subunit 4	NM_001170485	3.79	1.33	
Ckmt2	Creatine kinase S-type, mitochondria	NM_198415	14.23	5.05	
Csnk2a2	Casein kinase II subunit alpha' CK2α	NM_009974	18.07	8.26	
Dpysl4	Dihydropyrimidinase-related protein 4	NM_011993	16.17	4.56	
Gpr103	G protein-coupled receptor 103	NM_198192	9.05	4.59	
Gsta4	Glutathione S-transferase A4	NM_010357	5.35	1.99	
Hbb-b1	Hemoglobin subunit beta-1	NM_008220	32.82	11.44	
IL-6	Interleukin-6	NM_031168	1.45	0.41	
Kl	Klotho	NM_013823	5.81	2.24	
NARG1	N-alpha-acetyltransferase 15	NM_053089	8.17	2.43	
Ppp1ca	Serine/threonine-protein phospha-tase PP1-alpha catalytic subunit	NM_031868	10.65	3.62	
Ppp1cc	Serine/threonine-protein phospha-tase PP1-gamma catalytic subunit	NM_013636	25.13	9.46	
Prdx5	Peroxiredoxin-5, mitochondrial	NM_012021	9.04	3.91	
Rab6b	Ras-related protein Rab-6B	NM_173781	9.58	4.15	
Scd2	stearoyl-Coenzyme A desaturase 2	NM_009128	5.20	2.37	
Slc25a5	ADP/ATP translocase 2	NM_007451	7.70	2.40	
Slc35a5	solute carrier family 35, member A5	NM_028756	10.79	4.56	
Snca	α-synuclein	NM_009221	23.68	11.50	
Snph	Syntaphilin	NM_198214	4.00	0.71	
Syn1	Synapsin-1	NM_001110780	10.69	4.00	
Syp	Synaptophysin	NM_009305	5.86	1.78	
Ttr	Transthyretin	NM_013697	14.87	4.61	
TXN2	Thiredoxin 2	NM_019913	12.06	4.99	
Apoo	Apolipoprotein O	NM_026673	15.02	5.99	
ATP1A1	ATPase, Na^+/K^+ transporting, alpha 1 peptide	NM_144900	6.49	2.06	
CNN1	Calponin 1	NM_009922	18.46	8.24	
Cplx3	complexin 3	NM_146223	4.46	1.51	
Gpatch4	G patch domain containing 4	NM_001110809	11.20	5.73	
Mecr	Trans-2-enoyl-CoA reductase, mitochondrial	NM_025297	8.23	3.24	
Senp8	Sentrin-specific protease 8 (SUMO/sentrin specific peptidase 8)	NM_027838	18.83	8.42	
Slc25a22	Mitochondrial glutamate carrier 1	NM_001177576	6.52	1.62	
Zfp800	zinc finger protein 800	NM_001081678	2.79	1.67	
Ctnna1	Catenin alpha-1	NM_009818	4.43	1.31	
Mapk4	Mitogen-activated protein kinase 4	NM_172632	4.09	1.52	
Nell2	Protein kinase C-binding protein NELL2	NM_016743	7.49	2.61	
Pparg	PPAR gamma	NM_001127330	8.71	4.32	
Arpp21	cyclic AMP-regulated phosphoprotein, 21	NM_028755	14.11	5.26	
Ccdc18	Coiled-coil domain-containing protein 18	NM_028481	1.62	0.66	
Crebbp	CREB binding protein	NM_001025432	13.32	5.13	
Gpr6	G protein-coupled receptor 6	NM_199058	10.57	6.65	
NAP1L4	Nucleosome assembly protein 1- like 4	NM_008672	7.68	2.42	

Response Elements: black, PPRE + CRE; gray, PPRE only; white, CRE only; dark gray, neither. Analysis performed in lab of LTD.

An additional 30 amino acids N-terminal to the first ATG codon of PPARγ1 (Zhu et al., 1995). Our immunoblot analysis of mouse hippocampus from WT or Tg2576 mice treated with any intervention had only revealed a single band at 67 kDa. In an attempt to determine which of the two isoforms was detected by immunoblot, we performed PCR on WT mouse hippocampus using primer pairs that would selectively produce amplicons either only within the PPARγ2-specific exon 1' (primer set 1) or within exon 2 (primer set 2) that is common to both PPARγ1 and PPARγ2 (Zhu et al., 1995). We found that both mRNA forms were expressed in the hippocampus (**Fig. 4.1F,** top). However, quantitative PCR indicated that the ratio of PPARγ1 to PPARγ2 was >7 (**Fig. 4.1F**, bottom). Therefore, immunoblots most likely detected PPARγ1 protein. This was further confirmed by using a PPARγ2-specific antibody (Santa Cruz Biotechnology) to probe mouse hippocampal extracts which failed to produce a signal (data not shown).

4.3 Disinhibition of PPARγ ameliorates deficits in ERK-mediated cognition

We next determined whether there were differences between WT and Tg2576 hippocampal PPARγ, Ser84 phosphorylated PPARγ (pPPARγ), or subcellular distribution. Quantitative immunoblot analysis of hippocampal cytoplasmic fractions from sham-treated Tg2576, WT, and RSG-treated Tg2576 showed no significant differences in either total or pPPARγ (data not shown). Conversely, Tg2576 hippocampal nuclear fractions contained significantly less PPARγ than WT (**Fig. 4.2A**). Similarly, nuclear pPPARγ is decreased in untreated Tg2576 compared to WT (**Fig. 4.2B**); ERK MAPK phosphorylation of PPARγ at Ser84 is considered inhibitory by decreasing PPARγ transcriptional competency (Camp and Tafuri, 1997; Shao et al., 1998). However, because both total and phospho PPARγ are decreased in untreated Tg2576, the ratio of phospho:total PPARγ suggests a proportional increase in the ERK MAPK phosphorylated, inhibited form of PPARγ (**Fig. 4.2C**) and therefore nuclear PPARγ transcriptional competency in Tg2576 hippocampus is likely diminished.

PPARγ agonists have been shown to ameliorate several forms of cognitive deficits in Tg2576 and other AD mouse models (Pedersen et al., 2006; Escribano et al., 2009; Rodriguez-Rivera et al., 2011). We found that RSG cognitive improvement also ameliorated Tg2576 deficiencies in hippocampal nuclear PPARγ (**Fig. 4.2A,B**). These changes resulted in a decreased ratio of phospho:total PPARγ compared to untreated Tg2576 that was also statistically indistinguishable from WT (**Fig. 4.2C**). Finally, quantitative PCR analysis of hippocampal mRNA showed that PPARγ gene expression was reduced in Tg2576 compared with WT and normalized by RSG (**Fig. 4.2D**) with an 8.7-fold increase in PPARγ gene transcripts. Notably, PPARγ is not a PPRE-containing gene (**Table 4.1**), suggesting that RSG treatment has diverse effects on gene expression. This is further supported by our observation that several genes lacking identifiable PPREs were also induced by RSG treatment (**Table 4.1**). In summary, nuclear-PPARγ gene transcripts and protein are deficient in Tg2576 hippocampus and both are normalized with RSG treatment concomitant with reversal of hippocampus-dependent cognitive deficits.

4.4 Hippocampal nuclear ERK activity is enhanced by PPARγ agonism

Given the importance of ERK2 MAPK in hippocampus-dependent memory (English and Sweatt, 1996; Selcher et al., 1999), including contextual FC, we also evaluated RSG effects on hippocampal ERK2 protein, its phosphorylation (activation) status, and nuclear-cytosolic distribution. Quantitative immunoblot analysis of total-ERK2 in hippocampal nuclear and cytoplasmic fractions showed no significant differences between Tg2576 and WT animals (data not shown). Tg2576 RSG treatment, however, led to increased nuclear ERK2 activity, as noted by an increase in Thr202/Tyr204 phosphorylated ERK2 (pERK2) compared with untreated Tg2576 (**Fig. 4.2E**). No significant effects on cytosolic total or pERK2 cytoplasmic samples were found (data not shown). Thus, nuclear ERK2 activity in the hippocampus is enhanced during RSG-rescue of hippocampus-dependent cognition in Tg2576 mice. Consistent with our previous

Figure 4.2

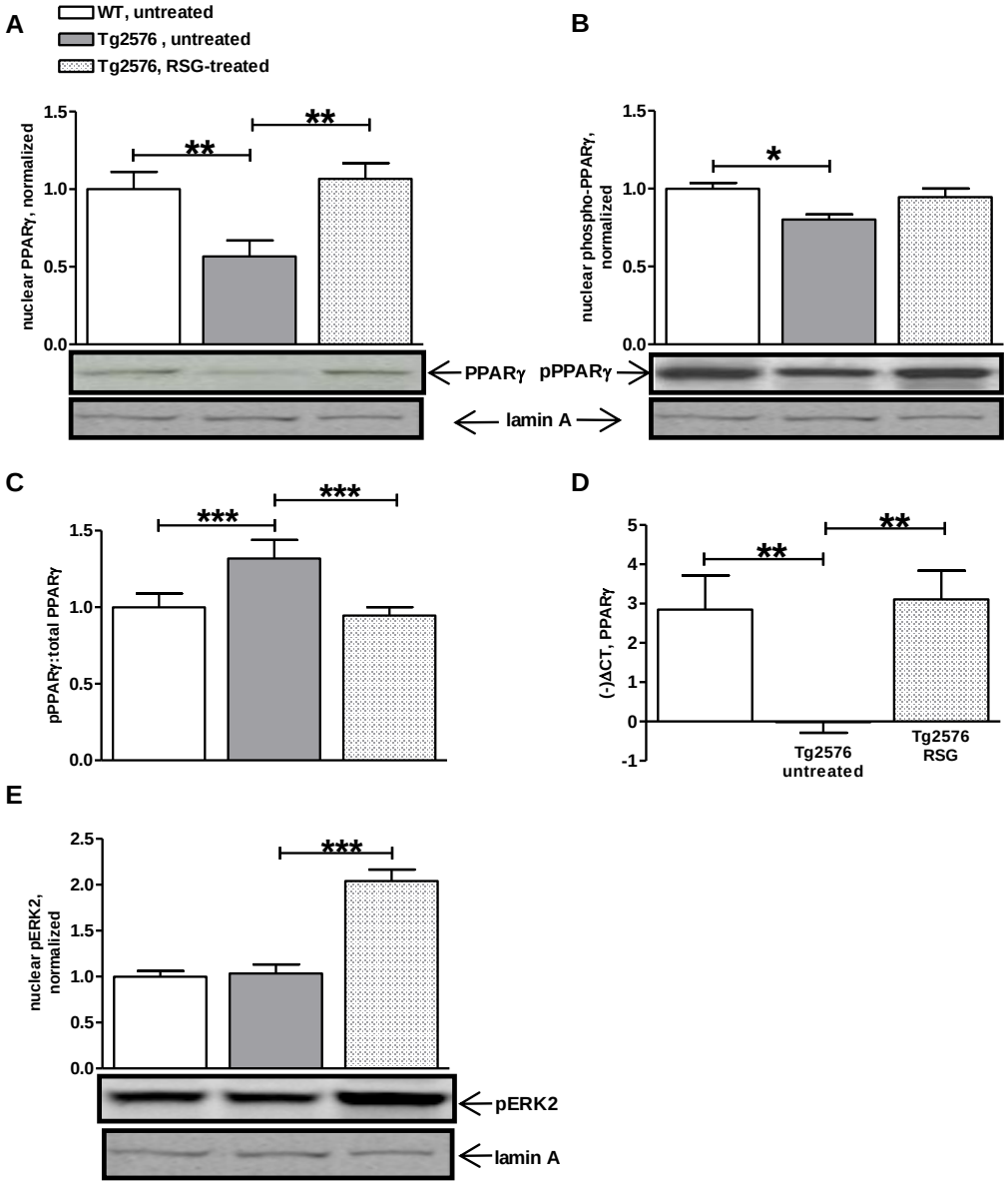


Fig. 4.2 RSG reverses deficits in nuclear PPARγ and increases nuclear ERK2 activity in hippocampus. A, Quantitative immunoblotting revealed significant downregulation of nuclear PPARγ in Tg2576 hippocampus. One-way ANOVA ($F(2,23)=7.02$; $p=0.004$). RSG treatment of Tg2576 normalized nuclear PPARγ to WT levels. **B**, Phosphorylation of nuclear PPARγ is decreased in Tg2576 and reversed with RSG treatment. One-way ANOVA, ($F(2,16)=3.2$). **C**, The nuclear pPPARγ/total PPARγ ratio is increased in untreated Tg2576 compared with wild-type, and normalized with RSG. One-way ANOVA ($F(2,16) = 19.4$). **D**, RSG increases PPARγ gene expression. Quantitative PCR showed that PPARγ mRNA was reduced in untreated Tg2576 and normalized to WT levels with RSG treatment. One-way ANOVA resulted in ($F(2,9)=8.2$). **E**, Hippocampal nuclear pERK2 levels are equivalent between untreated WT and untreated Tg2576 but increased in RSG-treated Tg2576. One-way ANOVA ($F(2,17)=37.3$) and Dunnett's *post hoc* analysis. Data reported normalized to untreated WT; mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data by JRR.

Observation that RSG has no effect on hippocampus-dependent cognition in WT littermates (Rodriguez-Rivera et al., 2011), RSG also had no effect on WT PPARγ or ERK (data not shown).

A recurring concern with TZDs is whether peripheral administration can actually affect the molecular target PPARγ in the CNS. Thus, to test whether CNS PPARγ mediates RSG cognitive improvement in 9MO Tg2576, we directly injected GW9662 (Leesnitzer et al., 2002) into the lateral ventricles of RSG-treated mice to block CNS PPARγ activity; comparable ICV administration of GW9662 has been used to establish that CNS PPARγ mediates RSG effects in animal models of energy balance and feeding behavior (Diano et al., 2011; Ryan et al., 2011). The dose employed was based on previous reports of ICV injection of GW9662 to antagonize PPARγ function in the CNS (Maeda et al., 2007; Zhang et al., 2008).

4.5 PPARγ antagonism prevents RSG-mediated cognitive rescue and restoration of both hippocampal PPARγ and ERK activity

Tg2576 and WT mice were infused with either vehicle or GW9662 4 hrs before FC training (**Fig. 4.3A**). No significant difference in behavior was detected between the groups during training, indicating that ICV injection and PPARγ manipulations do not interfere with behavior during the acquisition phase of this associative learning paradigm (**Fig. 4.3B**). Subsequent FC testing confirmed our previous findings (Rodriguez-Rivera et al., 2011) that RSG treatment ameliorates cognitive deficits in 9MO Tg2576 and that antagonism of CNS PPARγ in RSG-treated Tg2576 prevents consolidation of the hippocampus-dependent contextual FC memory. Here, neither PPARγ agonism (RSG) nor antagonism (GW9662) affected WT performance and RSG treatment restored Tg2576 freezing to WT levels, whereas untreated Tg2576 that received vehicle alone exhibited a significant deficit in this task (**Fig. 4.3C**). Notably, RSG-treated Tg2576 that received GW9662 did not experience cognitive restoration, indicating that these effects are mediated by PPARγ. It is noteworthy that neither

Figure 4.3

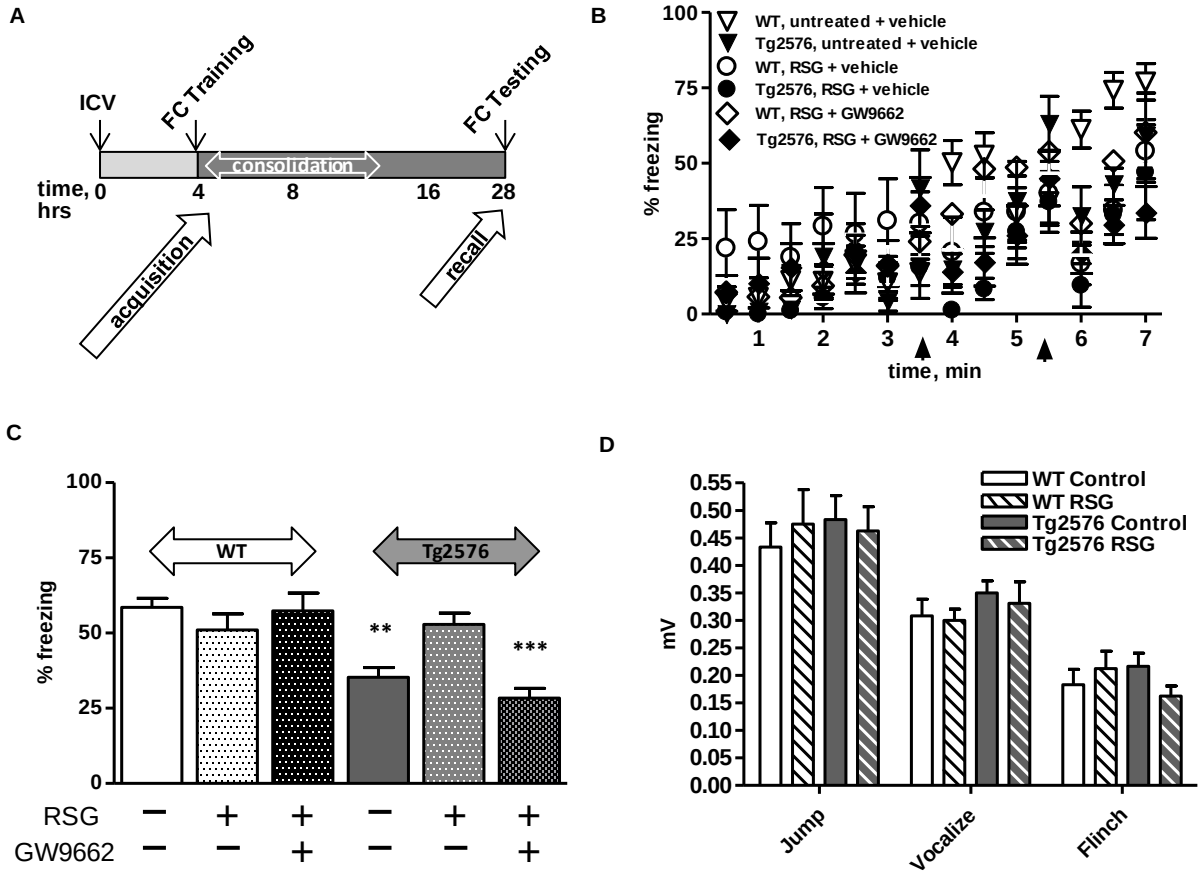


Fig. 4.3 Inhibition of CNS PPARγ blocks RSG-mediated cognitive rescue. Untreated or RSG-treated mice were infused with either vehicle or GW9662 4 h before 2-pairing FC training. **A**, Timeline for FC training and testing following ICV infusion of GW9662. ICV injection was performed 4 h before the acquisition of FC learning (FC Training). Consolidation proceeds for up to10 h following FC training. Testing for recall of FC 24 h after training tests for consolidation of FC learning. **B**, No genotype or treatment effects were detected in the 2-pairing training for FC. Repeated-measures two-way ANOVA ($F(1,1,1)=2.49$ and 2.00) for genotype and treatment, respectively; no interaction was detected. Data reported as mean percentage freezing±SEM for each 30s epoch. Vertical arrows on timeline denote the epoch within which the footshock was delivered during FC training. **C**, In the contextual test for FC, two-way ANOVA detected a genotype effect but no treatment effect or interaction ($F(2,1,2)=0.778$ and 29.72) for genotype and treatment. Therefore, untreated Tg2576 (RSG –) vehicle-infused (GW –) Tg2576 and RSG-treated (RSG+) Tg2576 ICV infused with GW9662 (GW+) froze significantly less. Neither RSG nor GW9662 had an effect on performance of WT. Data reported as mean percentage total freezing±SEM. ***$p<0.0001$ compared with RSG-vehicle groups; **$p<0.01$ compared with vehicle-infused groups. **D**, No significant genotype or treatment effect detected in 9MO WT and Tg2576, untreated or RSG-treated, with two-way ANOVA in the shock threshold test.

RSG nor RSG+GW9662 affected WT performance, as this emphasizes that PPARγ activity is not critical to hippocampus-dependent learning and memory in non-diseased mice. Additional studies in both WT and Tg2576 mice also demonstrated that RSG-treatment does not augment cued FC learning and that ICV-delivered GW9662 alone had no behavioral effect (data not shown). Furthermore, we detected no effect of genotype or treatment in an animals' threshold to flinch, vocalize, or jump in response to increasing shock intensities, indicating that 9MO Tg2576 and WT littermates exhibit equivalent sensory processing of the footshock in the FC paradigm and RSG treatment has no effect on this process (**Fig. 4.3D**). Taken together, these results suggest that RSG rescue of hippocampus-dependent cognitive deficits in Tg2576 AD mice is mediated by hippocampal PPARγ in order to compensate for a dysregulated signal transduction system that is typically necessary for this form of learning.

Since ERK MAPK is essential for hippocampus-dependent learning and memory in general, and contextual FC in particular, we hypothesized that PPARγ agonism in Tg2576 mice recruits the ERK MAPK pathway to overcome AD-like cognitive deficits in associative learning and memory. Therefore, we evaluated if PPARγ antagonism with ICV GW9662 affected hippocampal PPARγ and ERK in RSG-treated Tg2576. We sacrificed animals and collected hippocampi to evaluate GW9662 effects 4, 8, and 16 hrs following ICV-infusions; as our experimental protocol allows 4 hours of recovery time from ICV surgery, these timepoints would have correlated with 0, 4, and 12 hours post-training if these animals had been FC trained. Quantitative immunoblot revealed that ICV-injection of GW9662 had no significant effect on nuclear or cytosolic forms of total or pPPARγ at the 4 and 16 hrs timepoints compared to vehicle controls (**Fig. 4.4 A-D**). However, 8hrs after ICV-infusion of GW9662 we observed decreased nuclear PPARγ (**Fig. 4.4C**) concomitant with increased cytoplasmic PPARγ (**Fig. 4.4D**). Further, cytoplasmic pPPARγ was also increased at 8 hr (**Fig. 4.4B**). While total PPARγ decreased approximately 30% in the nucleus at this time point, analysis of the phospho:total PPARγ ratio

Figure 4.4

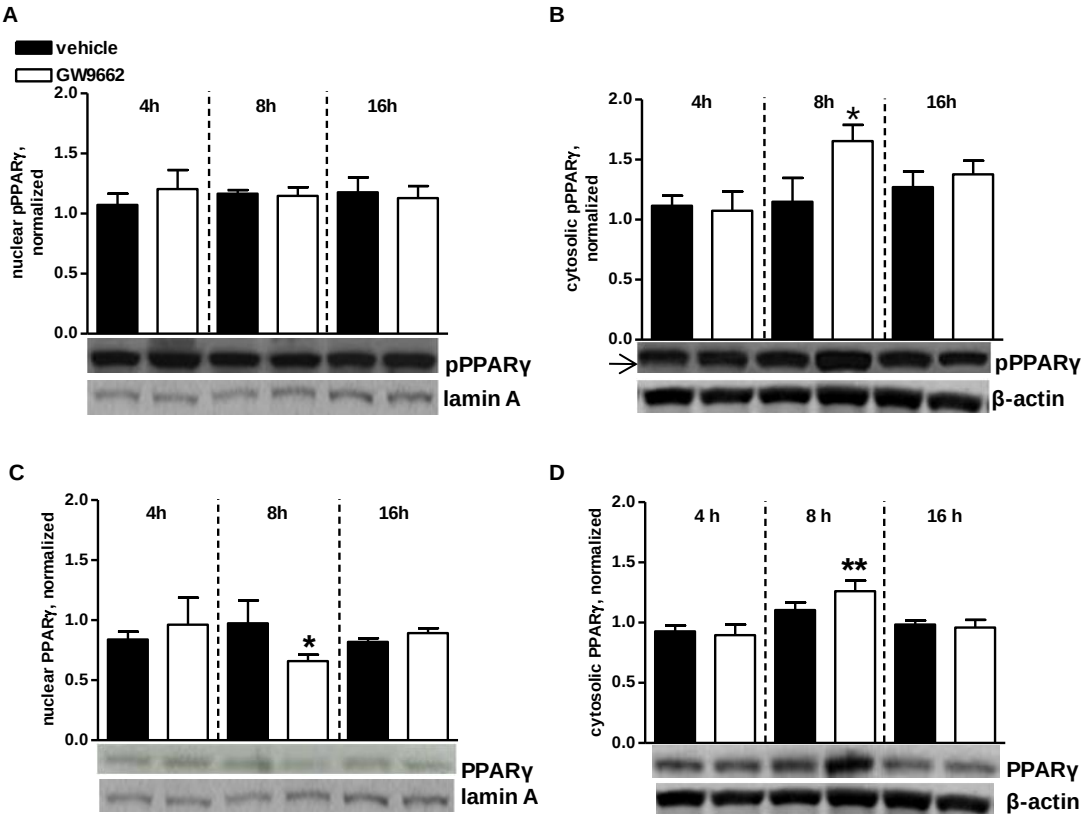


Fig. 4.4 Hippocampal nuclear ERK2 activity is modulated by PPARγ. A–D, Quantitative immunoblot of hippocampal total and pPPARγ in nuclear and cytoplasmic compartments from RSG-treated Tg2576 ICV infused with vehicle or GW9662. ICV injection of GW9662 analyzed by one-way ANOVA detected no effect on nuclear pPPARγ at any time point (**A**) ($F(6,20) = 0.49$), but did result in a significant increase in cytosolic pPPARγ by 8 h (**B**) ($F(6,24) = 3.16$). **C**, **D**, One-way ANOVA and Dunnett's *post hoc* analysis revealed that ICV injection of GW9662 led to a significant decrease in nuclear PPARγ levels 8 h after infusion (**C**), with a concomitant increase in cytosolic PPARγ (**D**) ($F(6,30) = 2.83$ and 3.38) for **C** and **D**, respectively. Data normalized to RSG-treated Tg2576 and expressed as mean ± SEM. **p*_0.05, ***p*_0.01, ****p*_0.001. Data by JRR.

At the 8 hr time point revealed no net change between the nuclear and cytosolic compartments (Student's two-tailed t-test = 0.18, data not shown). These results are consistent with a model in which PPARγ phosphorylation at Ser84 might be instrumental in nuclear export or cytoplasmic retention. In summary, inhibition of CNS PPARγ with GW9662 in RSG-treated Tg2576 mice led

to a net decrease in nuclear-PPARγ concomitant with an increase in total and pPPARγ in the cytoplasm, suggesting that reversal of cognitive improvement through inhibition of PPARγ involves subcellular redistribution of the protein.

Since the maximal effect of GW9662 on nuclear PPARγ was achieved 8 hrs after ICV injection, we evaluated whether nuclear ERK2 activity was also affected at this time point. As might be expected, GW9662 antagonism of CNS PPARγ resulted in no change in total ERK2 but decreased nuclear ERK2 phosphorylation/activation (one-way ANOVA: [$F(2,14)=6.01$, $F(2,15)=0.42$ ($p<0.05$)] for total ERK and pERK, respectively). Because ERK activation and the ERK2 isoform has been shown to be necessary for FC consolidation (Atkins et al., 1998; Selcher et al., 2001), our findings that PPARγ antagonism both reverses RSG effects on FC performance and nuclear ERK activity supports our interpretation that cognitive improvement in Tg2576 with RSG treatment results from PPARγ effects on ERK2 MAPK activity in the hippocampus.

These RSG-mediated effects are consistent with the notion that RSG crosses the blood brain barrier to activate CNS PPARγ (Willson et al., 1996; Strum et al., 2007; Festuccia et al., 2008; Diano et al., 2011; Lu et al., 2011; Ryan et al., 2011). Further, RSG increased both WT and Tg2576 hippocampal PPARγ DNA binding activity, indicating that RSG effects in Tg2576 brain are not due to compromised BBB permeability. Finally, the fact that ICV administration of the specific PPARγ full antagonist GW9662 (Leesnitzer et al., 2002) reversed RSG cognitive improvement strongly implicates CNS PPARγ as the mediator of cognitive enhancement.

4.6 RSG does not affect accumulation of CNS Aβ

Lastly, we assessed whether cognitive improvement via PPARγ agonism correlates with altered Aβ accumulation. Total $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were quantified by dissolving cortical tissue directly in guanidine-HCl to extract all forms of Aβ from untreated Tg2476 and RSG-treated Tg2576 that were ICV-injected with either vehicle or GW9662. Neither one-month RSG

treatment nor acute GW9662 PPARγ inhibition (8 hrs) significantly altered total $A\beta_{1-40}$, or $A\beta_{1-42}$ (**Table 4.2**). Therefore, neither RSG PPARγ agonism nor GW9662 PPARγ antagonism influenced Aβ accumulation in this animal model. Since we are focused on elucidating cognitive rescue mechanisms downstream of Aβ toxicity, we did not further characterize effects of RSG treatment on Aβ pathology although there are reports of Aβ mechanisms (Mandrekar-Colucci et al., 2012).

Table 4.2 Quantification of total cortical Aβ in 9MO RSG-treated Tg2576

Table 4.2. No effect of RSG +/- GW9662 on total Aβ in 9MO Tg2576. Data expressed as picomoles per gram wet weight starting material.

	Tg2576	Tg2576, RSG	Tg2576, RSG	Tg2576, RSG
(pmols/ gww)			**+ vehicle**	**+ GW9662**
Aβ 42	187.6 ± 32.0	162.5 ± 39.4	195.3 ± 49.6	201 ± 58.2
Aβ 40	360 ± 65.2	444.1 ± 76.0	463 ± 120	441 ± 126

4.7 Discussion

We and others have previously shown that PPARγ agonists improve cognitive performance in mouse models of AD, mainly in hippocampus-dependent tasks affected in human AD (Kitamura et al., 1999; Hamann et al., 2002; Pedersen et al., 2006; Hort et al., 2007; Scacchi et al., 2007; Hoefer et al., 2008; Escribano et al., 2010; Rodriguez-Rivera et al., 2011). It is also well established that hippocampal ERK MAPK is required for many of these forms of learning and memory (Sweatt, 2004). In these contexts, the current study addressed the convergence of the ERK MAPK and PPARγ signaling pathways in Tg2576 mice following cognitive improvement with RSG.

Initially, we evaluated hippocampal PPARγ in Tg2576 and WT littermates either untreated or treated with oral RSG for one month between 8MO and 9MO. RSG treatment of

Tg2576 mice significantly enhanced hippocampal PPARγ DNA binding, mRNA, and protein. PPARγ phosphorylation at Ser84 has been shown to inhibit transcriptional competency (Camp and Tafuri, 1997). We found that the ratio of pPPARγ:total PPARγ in untreated Tg2576 hippocampus nuclear fractions was significantly elevated, indicative of net PPARγ inhibition, while RSG treatment normalized this ratio to WT level.

We discovered that concomitant with RSG cognitive enhancement, the hippocampal PPARγ transcriptome and proteome converge with the ERK MAPK cascade at several levels. First, the majority of PPRE-containing target genes induced by RSG treatment also contain CREs suggesting that some PPARγ target genes are also CREB target genes which themselves are highly regulated by ERK MAPK during memory consolidation (Guzowski and McGaugh, 1997; Ahi et al., 2004; Trifilieff et al., 2006). As discussed earlier, similar transcriptional cross talk has already been observed between ERK and the glucocorticoid receptor, whose response elements are present on the promoter of Ras (Strawhecker et al., 1989) and deletion of the glucocorticoid receptor results in a net decrease in hippocampal expression of Ras, Raf-1, ERK, and Egr-1 in neuronal cells, confirming that nuclear receptors can dramatically alter the expression and activation status of members of the ERK signaling cascade (Revest et al., 2005). Second, an unbiased proteomics and bioinformatics analysis of the dentate gyrus from untreated and RSG-treated Tg2576 found that ERK MAPK was a central, integrative node of the plasticity proteins augmented by RSG. It is well established that a number of PPARγ agonists activate ERK through non-genomic pathways (Gardner et al., 2005), and the fact that specific PPARγ agonism also alters multiple proteins regulated by ERK further supports an argument for transcriptional cross talk between these two proteins. Third, RSG-mediated changes in hippocampal PPARγ and ERK were reversed when RSG-enhanced Tg2576 memory consolidation was blocked by an irreversible, selective PPARγ full antagonist (GW9662), suggesting that PPARγ is the primary mediator of these changes. Thus, there is a

coordinate relationship between PPARγ transcriptional competency and pERK activation and function that is reciprocally affected in response to chronic activation, compared to acute inhibition, of PPARγ.

Finally CBP was markedly induced during RSG-mediated cognitive enhancement. CBP functions as a coactivator with HAT ability (Bugge et al., 2009; Inoue et al., 2012) to facilitate PPARγ transcription and it is also integral to the activation of CREB, whose function is critical to learning and memory (Caccamo et al., 2010). Following NMDA receptor activation, ERK is also involved in CREB phosphorylation; thus, improper ERK activity leads to the dysregulation of CREB activation. In this regard, impaired CREB activation has been shown to negatively impact hippocampus-dependent spatial reference memory similarly to the impairments observed in AD (Lee and Silva, 2009), and others have shown that accumulation of Aβ is sufficient to decrease CREB phosphorylation (Caccamo et al., 2010). Compelling work from Caccamo et al found that increasing hippocampal CBP expression in AD transgenic mice (3xTg model) rescued learning and memory deficits without affecting Aβ or tau levels (Caccamo et al., 2010), suggesting that ERK downstream signaling can be restored even after pathological features begin to accumulate. The authors noted that increasing CBP levels led to increased brain-derived neurotrophic factor (BDNF), which itself facilitates CREB activation by 1) facilitating ERK phosphorylation (Ying et al., 2002) and 2) potentiating NMDA signaling, thereby creating a feed-forward loop to enhance ERK/CREB signaling. Furthermore, BDNF levels have also been found to increase in response to physical activity and environmental enrichment, (Neeper et al., 1996; Fahnestock et al., 2012), thereby providing a molecular basis for the observed inverse correlations between mental and physical activity and AD-related cognitive dysfunction (Szekely et al., 2007; Wilson et al., 2007). These findings bolster the idea that identification and intervention during preclinical AD may be successful in restoring cognitive function and suggest a potential target to restore CREB signaling.

From our data, we elaborate on one of many examples for convergent PPARγ and ERK pathway integration: RSG treatment impinged upon the protein SUMOylation system. Protein SUMOylation often leads to the functional inhibition of the target protein, e.g., MEK, the upstream kinase activator of ERK (Kubota et al., 2011). This post-translational inhibitory modification is reversibly regulated by the SENP family of SUMO proteases. A scenario can be considered in which increased Tg2576 hippocampal protein SUMOylation (McMillan et al., 2011) leads to inhibition of MEK, thereby preventing proper ERK activation during memory consolidation. Elevated SUMOylation could also account for the observed reduction in PPARγ transcriptional activity (Floyd and Stephens, 2012) as well as the PPARγ hippocampal co-regulator PGC1-α (Rytinki and Palvimo, 2009) and the ERK target CBP (Kuo et al., 2005). RSG-mediated induction of SENP8 gene expression could conceivably contribute to disinhibition of the PPARγ transcriptome and the ERK MAPK cascade. Likewise, RSG-induction of CBP, cyclin-dependent kinase 2, and nucleosomal assembly protein 1-like 1 would further contribute to PPARγ and ERK-dependent transcription by providing transcription co-regulators and enhancing ERK nuclear translocation (Okada et al., 2011; Plotnikov et al., 2011). This hypothetical scenario built upon the observed PPARγ transcriptome supports our model that PPARγ agonism serves to integrate the ERK and PPARγ signaling pathways to facilitate hippocampal memory consolidation.

Analysis of the Tg2576 hippocampal proteome from untreated versus RSG-treated animals also supports the notion that PPARγ agonism serves to integrate the ERK and PPARγ signaling pathways. We found that RSG led to the up-regulation of 147 proteins and down-regulation of 67 proteins in Tg2576 dentate gyrus that can be functionally categorized into energy, transport, biosynthesis, synaptic structure or plasticity; consistent with reports of many proteins affected in human AD hippocampus found via similar approaches (Sultana et al., 2007; Di Domenico et al., 2011). Again, several of the identified proteins were related to the ERK

MAPK cascade (e.g., GluR2, mGluR5, PKCγ) (Neary et al., 1999; Schroeter et al., 2007; Menard and Quirion, 2012).

GW9662 antagonism of PPARγ in RSG-treated AD mice mimics the effect of ERK MAPK inhibitors on contextual FC in WT rodents (Atkins et al., 1998) further supporting the model that PPARγ can harnesses a dysregulated ERK MAPK pathway to overcome AD-like cognitive deficits in Tg2576 mice. At the biochemical level, GW9662 reversed the effects of RSG on nuclear PPARγ and ERK activity in Tg2576 hippocampus with a time course that suggests GW9662 interferes with FC consolidation through effects on ERK via PPARγ.

GW9662 also led to elevated cytoplasmic pPPARγ, indicating that GW9662 reversed RSG effects on nuclear PPARγ and promoted its cytosolic redistribution. Since PPARγ Ser84 phosphorylation also promotes the rapid turnover of PPARγ through targeted ubiquitination, SUMOylation, and proteosomal degradation (Genini and Catapano, 2006), this may account for the relatively rapid recovery (16 hrs) from GW9662. While our methodology cannot address PPARγ nuclear/cytosolic shuttling or turnover, it can be said that GW9662 reversal of RSG cognitive improvement leads to reduced PPARγ nuclear localization and increased inhibitory phosphorylation accompanied by reduced nuclear ERK activity.

The ERK MAPK cascade has been shown to regulate PPARγ both through phosphorylation and nuclear/cytosolic trafficking via interaction with MEK-ERK complexes which themselves shuttle in and out of the nucleus (Burgermeister et al., 2007; von Knethen et al., 2010). We found that RSG increased nuclear ERK activity concomitant with a decrease in ERK-mediated pPPARγ. This at first appears illogical but one possible consequence of RSG cognitive enhancement is concurrent effects on overall ERK activity as well as ERK substrate selectivity. We suggest that following RSG treatment, pERK performs many functions, some of which are in series and in parallel with PPARγ such that not all pERK directly affects PPARγ phosphorylation because some pERK is executing additional cognitive-enhancing functions. An

alternative mechanism might be the up-regulation of phosphatases that act upon PPARγ that lead to decreased pPPARγ. Our observation that serine/threonine protein phosphatase 1 (PP1) α and γ gene transcripts are up-regulated in RSG-treated Tg2576 (Table 1) is consistent with this mechanism, although the PPARγ phosphatase has yet to be identified. Alternatively, we must consider that interaction with pERK may allow PPARγ to exert non-genomic effects in a manner similar to that observed in the estrogen receptor (Foster et al., 2008; Witty et al., 2012), and therefore the phosphorylation status or transcriptional competency of PPARγ may not be relevant to the cognitive enhancing effects of PPARγ agonism. This idea will be expanded on significantly in chapter 6.

Although many examples of TZDs increasing pERK exist in the literature, the mechanism remains poorly defined (Gardner et al., 2003; Kim et al., 2003; Rosa et al., 2008). The following model attempts to integrate our data within a framework of potential relationships with the ERK MAPK cascade and ERK molecular mechanisms gleaned from the annotated literature. RSG cognitive enhancement may reflect a feed forward loop that begins with RSG-mediated PPARγ target gene induction, e.g., CK2α (Table 4.1), which in turn stimulates ERK nuclear translocation (Plotnikov et al., 2011). We detected decreased PP2A by mass spectrometry similar to TZD (pioglitazone) effects during adipocyte differentiation (Altiok et al., 1997). Since PP2A specifically dephosphorylates and inactivates pERK (Alessi et al., 1995; Hu et al., 2009; Puustinen et al., 2009), decreased PP2A would be predicted to lead to a net increase in pERK as we found (Fig 4.2E). These results suggest potential coordinate effects of decreased PP2A and increased CK2α on nuclear ERK activity. Furthermore, cross-regulatory feed forward loops have been extensively described in that some transcription factors induced by PPARγ also bind to the PPARγ gene promoter to increase its expression. Our finding of increased PPARγ transcripts and protein in RSG-treated Tg2576 support this notion. PPARγ, in turn, may then mediate the induction of other transcription factors and target genes that

integrate the PPARγ transcriptome with the ERK MAPK cascade. One example of this comes from the C/EBP-PPARγ field (Wu et al., 1995; Wu et al., 1999; Lefterova et al., 2008).

Enhanced cognition in AD mice with RSG PPARγ agonism, coupled with our finding that neither PPARγ agonism *nor* antagonism affected WT performance, positions this nuclear receptor as a potential therapeutic target for the human disease. This idea is strengthened by the fact that PPARγ is dysregulated in AD brain and certain polymorphisms in the PPARγ gene are associated with increased risk for the disease (Kitamura et al., 1999; Scacchi et al., 2007). Furthermore, our discovery that the hippocampal PPARγ transcriptome and proteome converge with the ERK MAPK cascade at several levels, combined with the reciprocal effects of RSG and GW9662 on PPARγ and ERK activity and localization, suggest a multifaceted regulatory relationship warranting further investigation.

CHAPTER 5
THE PPARγ-pERK COMPLEX

5.1 PPARγ recruitment to active ERK during memory consolidation is required for Alzheimer's disease-related cognitive enhancement

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that manifests as cognitive impairment and has a tremendous economic and social burden, as well as a tragic prognosis for increasing incidence in a burgeoning aging population (Thies et al., 2013). Many studies have suggested that a key causative factor in AD dementia is amyloid beta (Aβ) derived from the amyloid precursor protein (APP). Prompted by the realization that insulin resistance is another recognized risk factor in AD(van Himbergen et al., 2012) and that insulin resistance is a comorbidity in both diabetes and AD(Talbot et al., 2012), many studies have investigated insulin sensitizer therapies as therapeutic targets for AD (Craft, 2012). Many of these target the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), a validated therapeutic target in type 2 diabetes, which regulates expression of many genes critical to insulin sensitivity (Wu et al., 1999). While many large-scale clinical trials for dementia due to AD failed to show efficacy of PPARγ agonism, evolving consensus considers their ineffectiveness likely due to testing in late stage disease, a fate similar to many other AD drug candidates (Becker and Greig, 2013). In contrast, clinical trials on patients with mild cognitive impairment (MCI) obtained positive outcomes using insulin sensitizers (Stockhorst et al., 2004; Watson et al., 2005; Risner et al., 2006; Sato et al., 2011). Thus, prior to overt neurodegeneration, insulin sensitizers may impinge upon signaling axes to modulate memory in early AD (Watson and Craft, 2004; Craft et al., 2012).

It is established that PPARγ agonism enhances cognition in AD animal models (Pedersen et al., 2006; Jiang et al., 2008a; Landreth et al., 2008; Escribano et al., 2009;

Rodriguez-Rivera et al., 2011) and that ERK is essential for several forms of hippocampus-dependent learning and memory that are impaired in AD (Dineley et al., 2002b; Hamann et al., 2002; Westerman et al., 2002; Sweatt, 2004; Dineley et al., 2007; Hort et al., 2007; Hoefer et al., 2008). Our work using the PPARγ agonist rosiglitazone (RSG) to enhance cognition in the Tg2576 mouse model of AD found convergence between the hippocampal PPARγ and ERK signaling pathways (Denner et al., 2012b). Since proper ERK2 activity is requisite for hippocampus-dependent learning and memory in rodents (Atkins et al., 1998; Selcher et al., 2001) we speculated that PPARγ may serve to reign in dysregulated ERK2 to enhance hippocampal cognition. Here we show that RSG cognitive enhancement leads to increased recruitment of PPARγ to activated, phosphorylated ERK (pERK) in a multiprotein complex during memory consolidation for a hippocampus-dependent cognitive task. Acute inhibition of hippocampal PPARγ, which blocks this type of memory consolidation, also prevented the increased recruitment of PPARγ to pERK indicating that formation of this protein complex is requisite for memory formation. We also show that these complexes correlate with cognitive reserve in humans with AD and AD model animals. Further, we demonstrate the ability to reconstitute PPARγ-pERK association using *in vitro* recombinant protein pull-down assays, revealing that these two proteins have intrinsic properties for direct association.

5.2 PPARγ associates with pERK *in vivo* in multiprotein complexes

While previous reports have described many binding partners for PPARγ (Miyamoto-Sato et al., 2010) and pERK (Yoon and Seger, 2006; von Kriegsheim et al., 2009), our observations regarding convergence of these signaling axes during cognitive enhancement with RSG (Denner et al., 2012b) led us to test whether PPARγ and pERK (pERK2) were associated with each other in multiprotein complexes. We found that pERK multiprotein complexes immunoprecipitated (IP) from Tg2576 hippocampal extracts (**Fig. 5.1A**) also contained PPARγ (**Fig. 5.1B**). We established that the pERK IP exhibited a linear input-output relationship up to

750 µg input protein (r^2=0.991, **Fig. 5.1C**). In reciprocal studies, PPARγ Ips (**Fig. 5.1D)** contained pERK (**Fig. 5.1E**) and the PPARγ IP exhibited linearity up to 500 µg input (r^2=0.849, **Fig 5.1F**). Given the narrower confidence intervals with the pERK Ips, we developed a quantitative method to assess the ratio of PPARγ:pERK in pERK Ips using 200 µg hippocampal protein (See Materials and Methods, **Fig. 7.2A**), thereby ensuring our Ips were within the linear range and exhibited high reproducibility (individual animal coefficients of variation <4.8%, **Fig. 7.2B**).

5.3 PPARγ-pERK complexes correlate with cognitive reserve in humans and an AD animal model

We next examined the ratio of PPARγ:pERK in post-mortem human brain samples from AD and age-matched control subjects and found a significant correlation between nuclear PPARγ:pERK in AD brain and mini-mental state exam (MMSE), a measure of cognitive reserve (**Fig. 5.2A, B**). No correlation was found between MMSE score and the PPARγ:pERK ratio in the aged-matched control group. In agreement, we observed similar relationships in mouse hippocampus where the PPARγ:pERK ratio correlated with cognitive performance in Tg2576 but not in WT mice (**Fig. 5.2C**). Since RSG treatment alleviated Tg2576 cognitive deficits and the amount of hippocampal nuclear PPARγ in complex with pERK correlated with better hippocampus-dependent cognitive performance, we tested whether RSG treatment simply led to an increase in the steady-state PPARγ:pERK ratio and found it was not affected by RSG treatment in either WT or Tg2576 mice (**Fig. 5.2D**) leading to the conclusion that the cognitive enhancing effects of PPARγ agonism were not due to increased constitutive formation of PPARγ-pERK complexes. These observations provide the first evidence for a physical interaction between PPARγ and pERK, and provide a molecular mechanism for the convergence of these two pathways in RSG-mediated cognitive enhancement in the Tg2576 mouse model for AD.

Figure 5.1

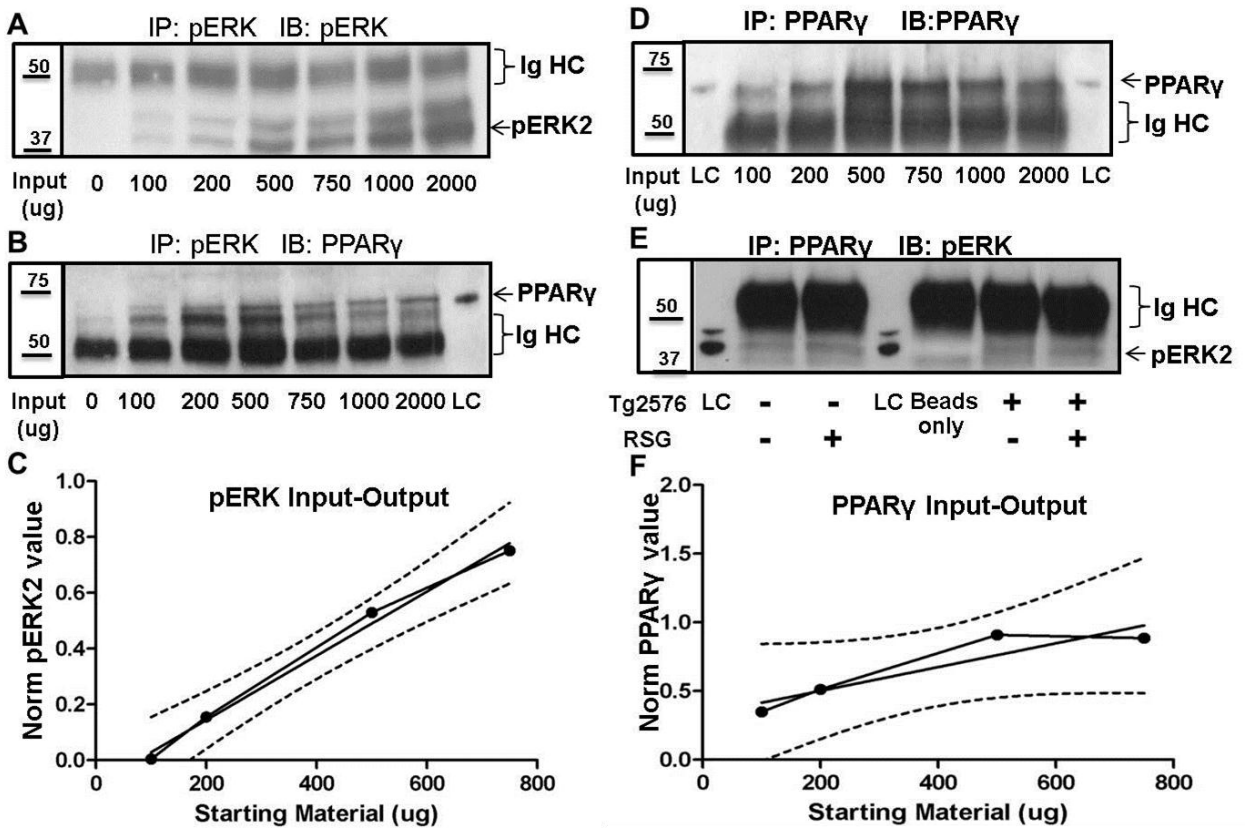


Fig. 5.1 PPARγ associates with pERK *in vivo* in Tg2576 hippocampal multiprotein complexes. **A,B***,* Western blots for pERK and PPARγ in pERK immunoprecipitates (Ips) from Tg2576 using anti-pERK-conjugated sepharose beads with increasing input of hippocampal nuclear extract. **C.** Input-output IP linear relationship for pERK IPs (r^2=0.991 up to 750 ug input]. Densitized western blot values were normalized to the loading control described in the methods and Fig. 2A. Dotted lines represent the 95% confidence intervals. IgHC= immunoglobulin heavy chain, LC = loading control. In reciprocal studies, **D**, **E**, PPARγ and pERK2 western blots of PPARγ Ips from Tg2576 using anti-PPARγ-conjugated magnetic beads with increasing input of hippocampal nuclear extract confirmed that these two proteins participate in a multiprotein complex. **F,** PPARγ IP input-output maintains a linear relationship for PPARγ (r^2=0.849 up to 500 ug input). Given the narrower confidence intervals with the pERK Ips, we utilized this approach for subsequent experiments to examine the PPARγ-pERK complex.

Figure 5.2

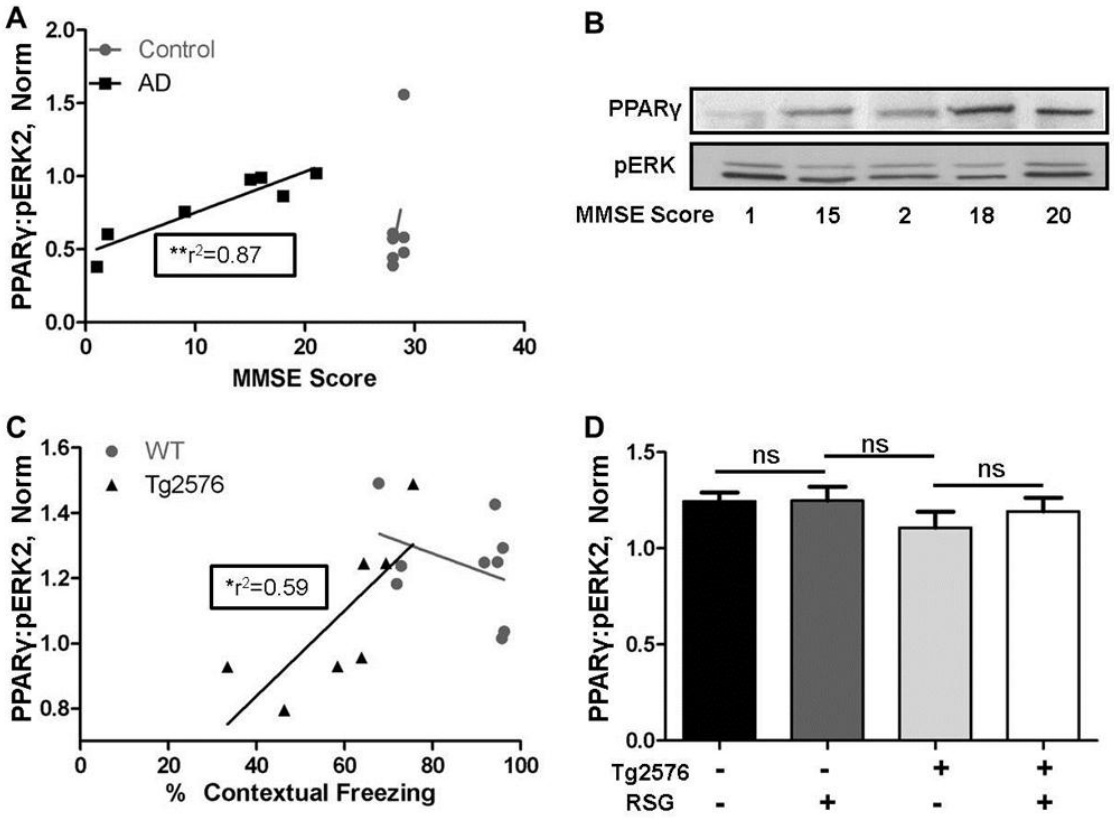


Fig. 5.2 PPARγ:pERK2 ratios in human AD brains and Tg2576 hippocampus correlate with cognitive performance. A, Correlation between AD human brain PPARγ:pERK2 ratios and MMSE, a measure of cognitive reserve in humans (n=7, $r^2 = 0.87$, P = 0.003, power > 80% (Cohen, 1992)). No correlation was found between complex ratios and cognitive reserve in control human brains (n=7). **B,** Western blots for PPARγ and pERK as a function of MMSE score. **C,** Correlation between Tg2576 hippocampal PPARγ:pERK2 ratios and contextual freezing, a measure of cognitive reserve in mice (n=7, $r^2 = 0.59$, P = 0.043). No correlation was found between complex ratios and cognitive reserve in control, WT hippocampus (n=9). ***D,*** Ratio of hippocampal PPARγ:pERK2 in WT (Tg2576-) and Tg2576 treated with (+) or without (-) RSG. No significant interaction between genotype or treatment on PPARγ:pERK2 ratios. Two-way ANOVA, n=7-12/group, $P = 0.565$, $F_{1,34} = 0.3375$. Densitometric analysis of the western blots are presented as mean ± s.e.m. ns = non-significant.

5.4 PPARγ recruitment to pERK protein complexes is increased during memory consolidation in RSG-treated Tg2576

Since 1) RSG treatment enhances hippocampus-dependent cognition in both AD animal models and some humans with AD (Watson and Craft, 2004; Pedersen et al., 2006; Jiang et al., 2008a; Landreth et al., 2008; Escribano et al., 2009; Rodriguez-Rivera et al., 2011), 2) ERK phosphorylation-dependent activation is necessary for hippocampal memory consolidation (Atkins et al., 1998; Sweatt, 2004), and 3) PPARγ associates with pERK in protein complexes, we analyzed the dynamics of these complexes during memory consolidation. RSG-treated and –untreated 9MO Tg2576 and WT littermates were subjected to two-pair fear conditioning training, wherein acquisition of the task was unaffected (Rodriguez-Rivera et al., 2011), and then sacrificed 4 hours later (**Fig. 5.3A**) at a timepoint that correlated with the peak effect of PPARγ agonism on fear conditioning consolidation (Denner et al., 2012b). Animals that were not exposed to the training chamber context served as controls. RSG-treated Tg2576 subjected to fear conditioning training exhibited significantly increased PPARγ:pERK ratios in both the nuclear (**Fig. 5.3B**) and non-nuclear fractions (**Fig. 5.3C**) compared to untreated Tg2576. Two-way ANOVA revealed a significant interaction between RSG treatment and training in regards to the Tg2576 PPARγ:pERK ratio, demonstrating that PPARγ agonism facilitated PPARγ recruitment to pERK during Tg2576 memory consolidation.

To establish the specificity of RSG induction of PPARγ recruitment to pERK during memory consolidation, we performed intracerebroventricular (ICV) injection of the PPARγ antagonist GW9662 4 hours prior to fear conditioning training, an intervention that does not affect acquisition but blocks RSG-mediated cognitive enhancement (Denner et al., 2012b) and is much more rapid than genetic intervention (Ryan et al., 2011). In a complementary manner,

PPARγ antagonism blocked training-induced increased recruitment of PPARγ to pERK in the nuclear fraction (**Fig. 5.3D**) with a similar trend in the non-nuclear fraction (**Fig. 5.3E**). In agreement with previous reports that PPARγ agonism does not affect WT cognitive performance (Denner et al., 2012b), the nuclear (**Fig. 5.3F**) and non-nuclear (**Fig. 5.3G**) hippocampal PPARγ:pERK2 ratios were unaffected by RSG treatment or GW9662 during WT memory consolidation. Two-way ANOVA and post hoc analysis revealed an interaction between RSG treatment and GW9662 ICV injection on Tg2576 PPARγ:pERK ratios indicating that RSG and GW9662 had significant effects on the complexes. Taken together, these results suggest that PPARγ agonism with RSG facilitates the association of hippocampal PPARγ with pERK to restore proper memory consolidation in the Tg2576 mouse model of AD.

We next investigated whether RSG treatment had an effect on other members of the ERK cascade. An essential mediator of ERK activation is phosphorylation by the upstream binding partner and kinase, MEK (dual specificity mitogen-activated protein kinase kinase 1/2) (Canagarajah et al., 1997) which is essential for fear conditioning (Shalin et al., 2004). Indeed, we found MEK associated with pERK, an effect enhanced by RSG (**Fig. 5.3H**). We next tested for pERK binding to p90RSK (ribosomal S6 kinase protein 1α, MAPK-activated protein kinase-1a), a downstream pERK binding partner and effector kinase (Gavin and Nebreda, 1999; Smith et al., 1999) also required for memory consolidation (Morice et al., 2013). RSK was associated with pERK, again increased in response to RSG agonism of PPARγ (**Fig. 5.3I**).

Figure 5.3

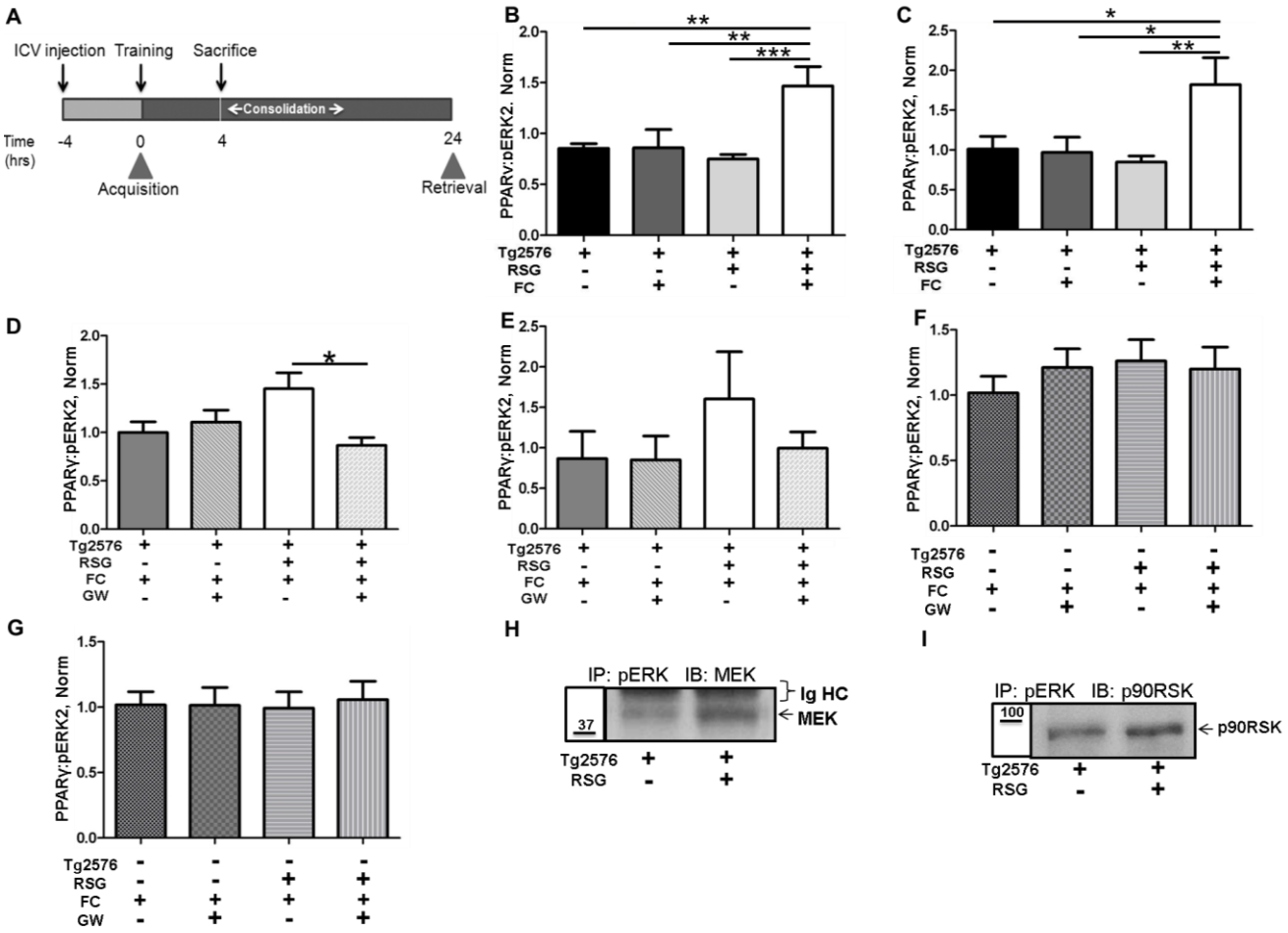


Fig. 5.3 PPARγ agonism increases recruitment of PPARγ to pERK during memory consolidation in Tg2576. **A,** Experimental paradigm: Tg2576 mice fed control (-) or RSG (+) diet were either naïve (FC-) or trained in the fear conditioning task (FC+), then sacrificed 4 hr post-training during consolidation to determine hippocampal PPARγ:pERK2 ratios. For PPARγ antagonism studies, four hr prior to training, vehicle (GW-) or GW9662 (GW+) were ICV administered and ratios determined 4 hr after training. Effects of RSG and fear conditioning on **B,** nuclear ratios (two-way ANOVA, n=7-8/group, $F_{1,\ 26}$ = 11.28, p = 0.002, 0.025, 0.002 for interaction, treatment, and training, respectively) and **C,** non-nuclear ratios (two-way ANOVA, n=7/group, $F_{1,24}$ = 8.155, p = 0.009, 0.064, 0.015 for interaction, treatment, and training, respectively). Effects of PPARγ antagonism on **D,** nuclear ratios (two-way ANOVA, $F_{1,40}$ = 5.705, P = 0.022, 0.121, 0.559 for interaction, treatment, and ICV, respectively) and **E,** non-nuclear ratios (two-way ANOVA, ns, $F_{1,\ 31}$ = 1.016). Neither RSG treatment nor GW9662 antagonism had any effect on WT PPAR:pERK2 ratios in the **F,** nuclear (two-way ANOVA, P = 0.41, ns, $F_{1,37}$ = 0.694) or **G,** non-nuclear (two-way ANOVA, P = 0.78, ns, $F_{1,24}$ = 0.074) fractions. pERK association with **H,** MEK or **I,** p90RSK. *P < 0.05; **P ≤ 0.01.

5.5 PPARγ and pERK recombinant proteins directly associate *in vitro*

To further understand the interaction between PPARγ and pERK, we next tested whether these proteins had the intrinsic ability to directly associate in the absence of other proteins. We used recombinant GST-tagged pERK (GST-pERK2) and PPARγ proteins in an *in vitro* glutathione bead pull down assay in an attempt to reconstitute the *in vivo* interaction detected by hippocampal co-IP. We found increasing amounts of input PPARγ resulted in increased GST-pERK pull-down of PPARγ (**Fig. 5.4A**) in a linear response (**Fig. 5.4B**). Control reactions demonstrated that PPARγ only associated with the beads in the presence of pERK, suggesting that the observed PPARγ signal was due to a direct association between the two proteins. When similar binding studies were performed with PPARγ and GST-tagged non-phosphorylated ERK2, no association with PPARγ was detected (**Fig. 5.4C**). Thus, ERK activation/phosphorylation is necessary for PPARγ binding, providing an intriguing level of specificity to these complexes.

5.6 Discussion

Identification of the molecular mechanisms that contribute to memory impairment in AD elucidate therapeutic strategies for the ever-expanding population of humans diagnosed with the disease (Thies et al., 2013). In the past several years, many studies have shown that agonists of PPARγ enhance memory in some patients (Watson et al., 2005; Risner et al., 2006; Sato et al., 2011) and in genetic AD mouse models in tasks that require intact ERK MAPK signaling, e.g., associative learning in the contextual fear conditioning paradigm and spatial navigation in the Morris water maze (Pedersen et al., 2006; Jiang et al., 2008a; Landreth et al., 2008; Escribano et al., 2009; Rodriguez-Rivera et al., 2011). This is not surprising, as stimulation of PPARγ by either RSG or pioglitazone (PIO) has been shown to affect production of Aβ, the hallmark pathological feature of AD, through suppression of both β-

Figure 5.4

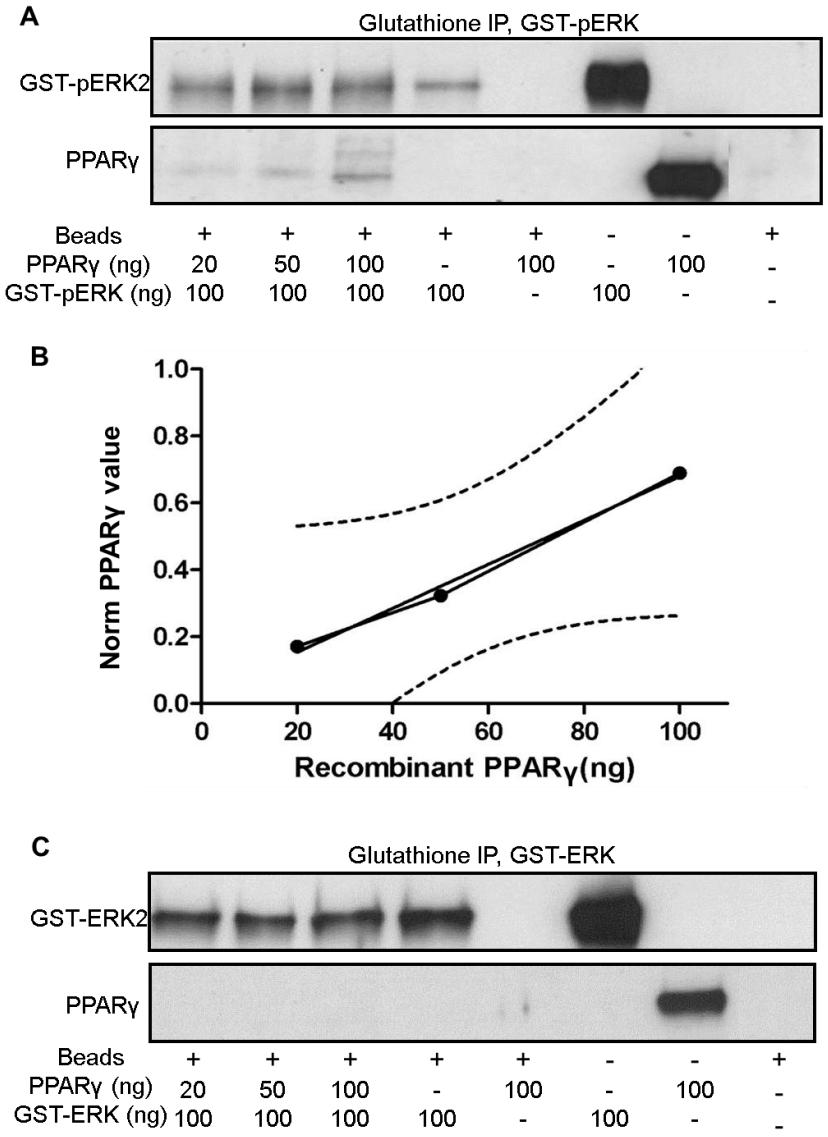


Fig. 5.4 PPARγ and pERK recombinant proteins associate *in vitro*. A, Western blot for pERK (top) and PPARγ (bottom) following incubation of recombinant human GST-pERK2 with increasing amounts of human PPARγ followed by glutathione bead affinity isolation. **B,** Input-output relationship for PPARγ pull down from GST-pERK2 IP. Dotted lines represent 95% confidence intervals. **C,** Western blot for nonphosphorylated ERK (top) and PPARγ (bottom) following incubation of recombinant GST-nonphosphorylated ERK2 with increasing amounts of human PPARγ followed by glutathione bead affinity isolation.

secretase and APP expression, as well as promoting APP ubiquitination and subsequent degradation (Landreth et al., 2008).

Our finding that RSG treatment led to cognitive enhancement in Tg2576 AD mice regardless of its efficacy for normalizing peripheral insulin resistance and hyperglycemia (Rodriguez-Rivera et al., 2011) suggested that CNS PPARγ was playing a direct role in cognitive enhancement. Still, the therapeutic mechanism by which PPARγ agonism led to improved cognition remains poorly understood. It is well established that consolidation after a learning event is an essential phase in the formation of new memories, a process that requires dynamic phosphorylation-dependent activation of hippocampal ERK (Atkins et al., 1998; Sweatt, 2004; Trifilieff et al., 2007). Prior studies in our lab demonstrated that PPARγ-mediated cognitive enhancement linked the hippocampal PPARγ and ERK MAPK signaling pathways by promoting the transcription of PPRE-containing PPARγ target genes and CRE-containing ERK-regulated target genes (Denner et al., 2012b). Given that ERK/CREB/CBP/CRE-dependent signaling is requisite for hippocampal memory consolidation (Atkins et al., 1998; McGaugh, 2000b; Vecsey et al., 2007), the current study investigated whether PPARγ agonism can directly modulate ERK to enhance this process.

Here we found that PPARγ agonism induced recruitment of PPARγ to pERK during memory consolidation, and that these complexes correlated with cognitive reserve in human AD and in a genetic AD mouse model. The fact that hippocampal PPARγ association with pERK during memory consolidation increased only in RSG-treated Tg2576 implies that PPARγ-mediated effects on ERK happen selectively during AD-related hippocampal dysfunction. In this regard, we observed that acute pharmacological antagonism of PPARγ with GW9662 only blocked hippocampal memory consolidation in RSG-treated Tg2576 (Denner et al., 2012b) via prevention of hippocampal PPARγ association with pERK during this process. Thus, we conclude that hippocampal PPARγ activity is necessary to enhance formation of complexes

during consolidation. Direct binding of recombinant PPARγ and pERK *in vitro* suggests an intrinsic affinity that may underlie the cognitive enhancing activity of RSG.

Cognitive reserve in AD is a measure of the ability of the brain to resist damage inflicted by AD pathology (Sperling et al., 2011). The observation that 9MO Tg2576 on average perform poorly in the hippocampus-dependent contextual fear conditioning task, while individual animals vary considerably, led us to hypothesize that if the ratio of PPARγ:pERK was relevant to cognitive performance, individual human or animal ratios would correlate with their respective performance. Indeed, we found in humans with AD that cognitive performance, assessed by the MMSE, positively correlated with the ratio of PPARγ:pERK. In further support of this hypothesis, we found that Tg2576 contextual freezing behavior, a reflection of cognitive performance, also positively correlated with the ratio of PPARγ:pERK. Notably, neither age-matched human controls nor WT littermates to Tg2576 exhibited such a correlation. Coupled with our previous observation that a subset of hippocampal PPARγ target genes are also CREB/CBP target genes (Denner et al., 2012b) that are known to be regulated by ERK MAPK during memory consolidation (Guzowski and McGaugh, 1997; Ahi et al., 2004), these data suggest that PPARγ participation in a pERK complex may serve a compensatory role to re-establish proper ERK signaling that is disrupted by AD pathology.

Nuclear receptor interaction with ERK is not unprecedented as exemplified by the estrogen (Hashimoto et al., 2012), glucocorticoid (Strawhecker et al., 1989; Revest et al., 2005), and progesterone (Vicent et al., 2009) receptors. As discussed in chapter 3, nuclear receptors maintain a common domain structure and given the abundance of other nuclear receptors that interact with ERK, it is likely that PPARγ does so as well. Furthermore, it is becoming evident that ERK can be regulated through protein-protein interactions via well-defined protein motifs. ERK has been shown to interact with numerous proteins, including Elk-1 and p90rsk (Sheridan et al., 2008; Eldar-Finkelman and Eisenstein, 2009), through both ERK-exclusive docking sites

known as DEF sites, defined by an F X (F/Y) P amino acid motif and generally located C-terminal to an ERK phosphorylation site (Sheridan et al., 2008) and the more general MAPK recognition D sites, defined by the amino acid sequence K/R K/R K/R $X_{(1-5)}$ L/I X L/I (Remenyi et al., 2005; Garai et al., 2012).

We identified putative DEF and D sites within the N-terminus of PPARγ at amino acids $FHYG_{119-122}$ and $RRTIRLKL_{136-143}$, respectively. It is noteworthy that proteins that contain both D and DEF sites generally exhibit more specific and higher affinity interaction with ERK than those that contain only one (Jacobs et al., 1999). Our observations that PPARγ contains consensus sequences predicted to mediate direct interaction with ERK have intrinsic properties to associate *in vitro* provide a potential mechanism for their interaction during cognitive enhancement. Further, binding of target proteins to the N-terminus of nuclear receptors stabilizes the intrinsic disorder of this domain (Khan et al., 2012) by initiating a coupled folding process which allows the receptor to adopt a functionally active conformation (Hill et al., 2012), suggesting an additional mechanism whereby pERK may facilitate complex stability by conferring order to the PPARγ N-terminal domain. Furthermore, protein binding in the intrinsically disordered N-terminal region is highly specific and typically low affinity, making this relationship ideal for transient, reversible protein-protein interactions (Hill et al., 2012) such as that observed in the PPARγ-pERK complex during memory consolidation. Thus, it should be noted that the efficiency of the observed interaction appears low; only 5-10% of the input PPARγ is pulled down with pERK. That said, multi-valent protein complexes are often stabilized *in vivo* by recruited protein partners. In contrast, the *in vitro* stability of recombinant protein-protein interactions can be relatively low due to the absence of these biologically relevant protein partners. Thus, the apparently low affinity interaction in our study may be more stable in the presence of additional stabilization proteins *in vivo*.

Our finding that PPARγ association with pERK *in vivo* was increased in RSG-treated Tg2576 only during memory consolidation suggests a dynamic ligand-dependent (RSG) mechanism for recruitment of pERK and other signaling partners (e.g. MEK and p90RSK). The conformational change conferred upon PPARγ through ligand binding (Choi et al., 2011) may make the ERK docking domains within PPARγ more accessible and could therefore increase the binding. A follow up experiment that included a physiologically relevant amount of RSG along with the recombinant proteins may shed light on the feasibility of this interpretation.

Also noteworthy is that we did not detect PPARγ pull-down with un-phosphorylated recombinant ERK. ERK interacts with substrate DEF sites via a hydrophobic pocket adjacent to the kinase active site cleft (Lee et al., 2004) that is exposed following MEK phosphorylation (Canagarajah et al., 1997). This phosphorylation-induced conformational change in ERK may account for our observation of a direct interaction in *in vitro* reconstitution studies between recombinant PPARγ and pERK2 that was not recapitulated with non-phosphorylated ERK2. Taken together, our data suggest that PPARγ and pERK directly interact *in vitro* and *in vivo* and this interaction contributes to cognitive enhancement with RSG treatment.

Since PPARγ agonism improved performance in an ERK-dependent learning and memory task, and since PPARγ has a higher affinity for phosphorylated ERK compared to non-phosphorylated ERK, MEK is likely an important mediator of PPARγ-pERK recruitment. While our studies did not directly address the involvement of MEK, as its role likely preceded the memory consolidation time point we examined, our findings do indicate that MEK is likely a dynamic component of the PPARγ-pERK complex, since we detected increased steady state MEK in pERK IP material from RSG-treated Tg2576 hippocampus yet decreased MEK when we probed pERK IP material at the 4 hour memory consolidation time point (**Fig. 5.5**).

Figure 5.5

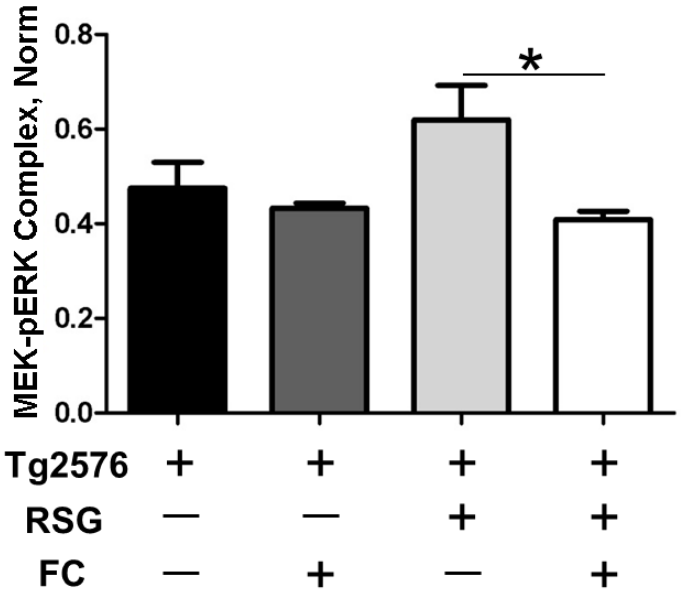


Fig. 5.5 MEK association with pERK is dynamic in RSG-treated Tg2576. Hippocampi from naïve and FC-trained Tg2576 +/- RSG were examined for MEK association in pERK Ips. Two way ANOVA ($F(1, 21) = 7.090$, $p = 0.015$) found significance in the effect of FC training on MEK-pERK association and also found a trend toward a RSG effect ($F(1, 21) = 3.167$, $p = 0.09$). Therefore, at steady state RSG treatment increases MEK association with pERK, but during memory consolidation, MEK dissociates from the complex, suggesting that RSG influences the dynamics of pERK activation.

MEK has been demonstrated to shuttle both ERK and PPARγ between nuclear and cytosolic compartments (Burgermeister and Seger, 2007); accordingly, MEK may play an important role not only in regulating the PPARγ-pERK complex but also in mediating its cellular location. Alternatively, PPARγ-pERK association may facilitate the downstream activity of pERK through improved pERK-dependent phosphorylation activation of Elk-1 and p90RSK, an integral mediator of the CRE/CREB/CBP gene transcription cascade necessary for hippocampal memory consolidation (Frodin and Gammeltoft, 1999; Ahi et al., 2004). Our data indicated that Tg2576 RSG treatment increased the amount of p90RSK in pERK IP material and that ICV administration of GW9662 reversed this (Fig. S5). Thus, we propose a model in which ligand-activated PPARγ restores dysfunctional ERK-dependent signaling to facilitate memory

consolidation through the recruitment of binding partners that facilitate the activity and localization of pERK (**Fig. 5.6**).

Figure 5.6

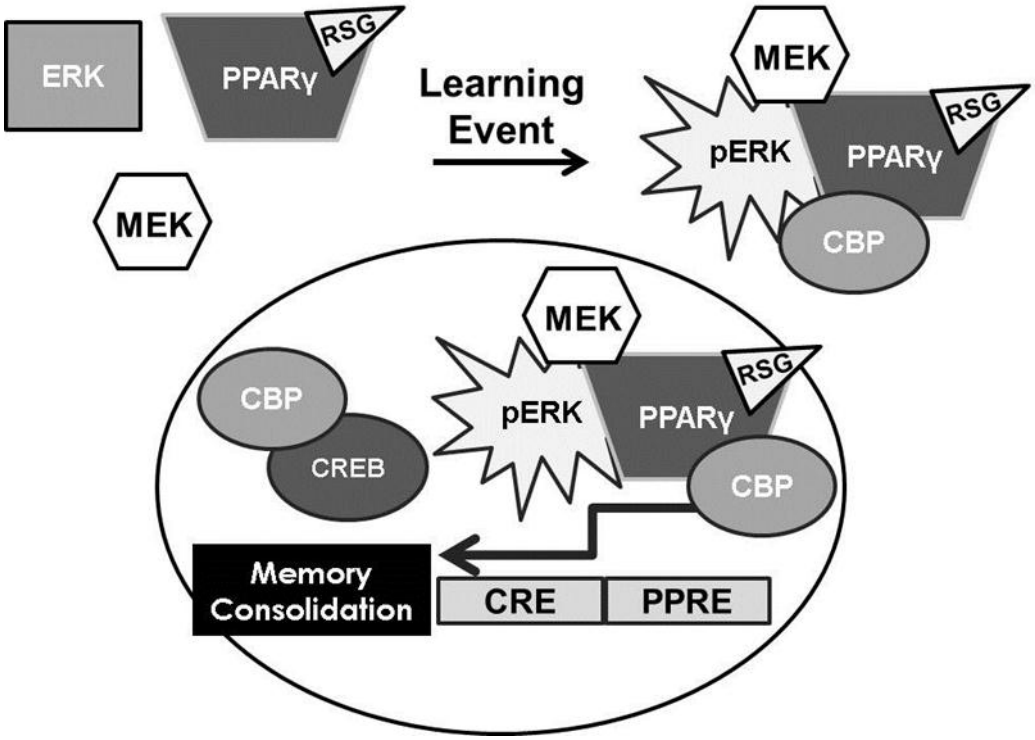


Fig 5.6 Working model for PPARγ-mediated enhancement of memory consolidation in AD. In the cognitively impaired Tg2576 AD model mice, ligand-bound PPARγ is recruited by MEK, allowing it to interact with activated ERK following a learning event. The complex recruits a number of other transcriptional regulatory proteins, ultimately increasing ERK downstream efficiency, including CRE-mediated gene transcription as well as activation of p90RSK and Elk-1. This process enhances memory consolidation and rescues the deficient ERK signaling resulting from AD-like pathology.

Memory formation begins with an acquisition phase followed by a consolidation phase in the ensuing hours to form a memory trace that can be retrieved at a later time. ERK phosphorylation is required for memory through transcriptional regulation of target genes essential for coding a new memory trace. In the Tg2576 model of AD, ERK is dysregulated and unable to properly function in new memory formation. Thus, we propose a model in which ligand-activated PPARγ restores dysfunctional ERK-dependent signaling to facilitate memory consolidation through the recruitment of binding partners to a pERK multiprotein complex (Fig. 6). One potential binding partner in this process is the histone acetyltransferase CBP which serves as a transcriptional cofactor for both CREB and PPARγ (Vecsey et al., 2007; Bugge et al., 2009), and may be a convergent central node between the PPARγ and ERK pathways (Denner et al., 2012b). This possibility will be discussed in detail in the next chapter. The identification of these novel PPARγ-pERK complexes provides unique opportunities for newly targeted therapeutics to improve memory in AD and warrants further investigation.

CHAPTER 6
FURTHER DISCUSSION AND FUTURE DIRECTIONS

6.1 Progressive insulin dysregulation in Tg2576 reveals therapeutic windows

As already discussed in depth, insulin resistance increases the risk for developing Alzheimer's disease (AD) (Schrijvers et al., 2010) and is a hallmark of diabetes. Recent clinical trials on patients with mild cognitive impairment (MCI) indicated that insulin sensitizers as well as intranasal insulin provided significant cognitive benefit (Stockhorst et al., 2004; Watson et al., 2005; Risner et al., 2006; Sato et al., 2011; Craft et al., 2012). Thus, like insulin, insulin sensitizers appear to impinge upon components of the insulin signaling axis and expression of genes that can potentially modulate memory in MCI and early AD (Watson and Craft, 2004). The focus of the present work has been on RSG-mediated agonism of PPARγ and the subsequent restoration of cognition in 9MO Tg2576 via rescue of a dysregulated ERK signaling cascade, suggesting that intervention via PPARγ agonism during this stage of disease progression provides a specific therapeutic window. It is noteworthy, then, that different interventions on the insulin signaling cascade can also affect ERK-dependent hippocampal memory during other windows of disease progression. When we evaluated 13MO Tg2576, we found that PPARγ agonism no longer facilitated cognitive function. However, we also found that a key regulator of energy metabolism within the insulin signaling network, AMP-activated kinase (AMPK), was downregulated at this time and that AMPK agonism with the insulin sensitizer metformin (MFM) significantly improved cognition. Interestingly, MFM intervention in 9MO Tg2576 did not improve their cognitive performance, indicating that Tg2576 exhibit cognitive deficits with concomitant dysregulation of hippocampal insulin signaling that is ameliorated with mechanistically distinct insulin sensitizers in an age- and disease stage-specific manner. These findings define therapeutic windows within the AD continuum and have clinical relevance for

predicting AD patient populations responsive to distinct insulin sensitizer therapies. The fact that these various interventions seemingly have highly specific effective windows highlights the need for more accurate diagnosis and staging of AD pathology.

Our initial evaluation of insulin resistance in Tg2576 assessed peripheral insulin and glucose regulation by directly measuring serum insulin and glucose as well as performing the glucose tolerance test (Rodriguez-Rivera et al., 2011). These measures showed that 5MO Tg2576 are normoglycemic and normoinsulinemic with the emergence of peripheral insulin resistance and hyperinsulinemia by 9MO. We previously reported that 9MO Tg2576 respond to cognitive enhancement with the insulin sensitizer RSG while 13MO do not (Rodriguez-Rivera et al., 2011; Denner et al., 2012a). These observations suggested that a different aspect of the insulin signaling pathway might be dysregulated at late-stage cognitive impairment, thereby providing another therapeutic opportunity at later stage disease. An alternative integrator of insulin resistance and ERK signaling is AMPK (Ali and Fonseca, 2012); therefore, we evaluated the status of hippocampal AMPK in 9MO and 13MO Tg2576 and WT littermates. We found that AMPK in 9MO Tg2576 was equivalent to WT but up-regulated in 13MO Tg2576 hippocampus concomitant with down-regulation of the activating phosphorylation on Thr172 (**Figure 6.1A, B**).

We then tested whether one-month treatment with the insulin sensitizing AMPK agonist MFM affected 9MO or 13MO Tg2576 and WT littermate performance in 2-pair FC. During the training phase for FC, no detectable differences were noted between WT and Tg2576 treated or untreated at any age as determined with two-way repeated measures ANOVA followed by Tukey post hoc pairwise comparison (data not shown). As expected (Dineley et al., 2002a), Tg2576 performed equivalently to WT for freezing to the cue and MFM had no effect in this task (data not shown). As predicted from hippocampal AMPK dysregulation at 13MO, multiple comparisons tests detected that MFM only affected 13MO Tg2576 cognitive performance (**Figure 6.1C**). Untreated (CTRL) 9MO and 13MO Tg2576 performed significantly worse than

WT, and one month MFM treatment had no effect on WT or 9MO Tg2576. It should be noted that 13MO Tg2576 CTRL group freezing was significantly worse than 9MO Tg2576 CTRL group ($p<0.001$, Student's two-tailed t-test), indicating age-dependent cognitive decline. Although one month MFM treatment improved 13MO Tg2576 contextual freezing compared to untreated Tg2576, complete rescue did not occur. Therefore, AMPK dysregulation predicts a therapeutic response to MFM treatment although only a partial cognitive rescue was achieved.

Hippocampal ERK2 is a critical mediator of FC consolidation (Atkins et al., 1998; Selcher et al., 2001; Ahi et al., 2004), and we previously showed that ERK2 is hyper-activated in 13MO Tg2576 (Dineley et al., 2001b). Since we observed hypo-functional AMPK at 13MO and AMPK is a negative regulator of ERK activity in neurons (Tillu et al., 2012), we hypothesized that MFM cognitive improvement impinged upon ERK in 13MO Tg2576 hippocampus. Quantitative immunoblot evaluation of ERK2 in the hippocampus of 13MO WT and Tg2576 untreated or MFM-treated demonstrated again that ERK2 protein is not significantly affected in 13MO Tg2576 hippocampus (Dineley et al., 2001b). However, ERK2 activation as detected by Thr202/Tyr204 phosphorylation was elevated at 13MO in Tg2576 hippocampus and that MFM treatment reduced this; however, in a manner similar to its effect on contextual fear conditioning, MFM did not completely reverse ERK2 hyperphosphoylation (**Figure 6.1D**). MFM had no effect on WT ERK2. Since two-way ANOVA detected both a genotype and interaction effect, MFM selectively affected Tg2576 hippocampal pERK. Furthermore, MFM-mediated normalization of ERK2 activity coincided with cognitive enhancement in 13MO Tg2576.

The criteria for staging and diagnosis of AD were recently revised to reflect new knowledge regarding biomarker profiles for the disease. With refinement of clinical staging comes the realization that many of the mechanistically-conceived animal models are more representative of preclinical AD and possibly MCI (Hernandez et al., 2010; Ferretti et al., 2011) which is consistent with the finding that recent AD clinical trials using the insulin sensitizers RSG and

Figure 6.1

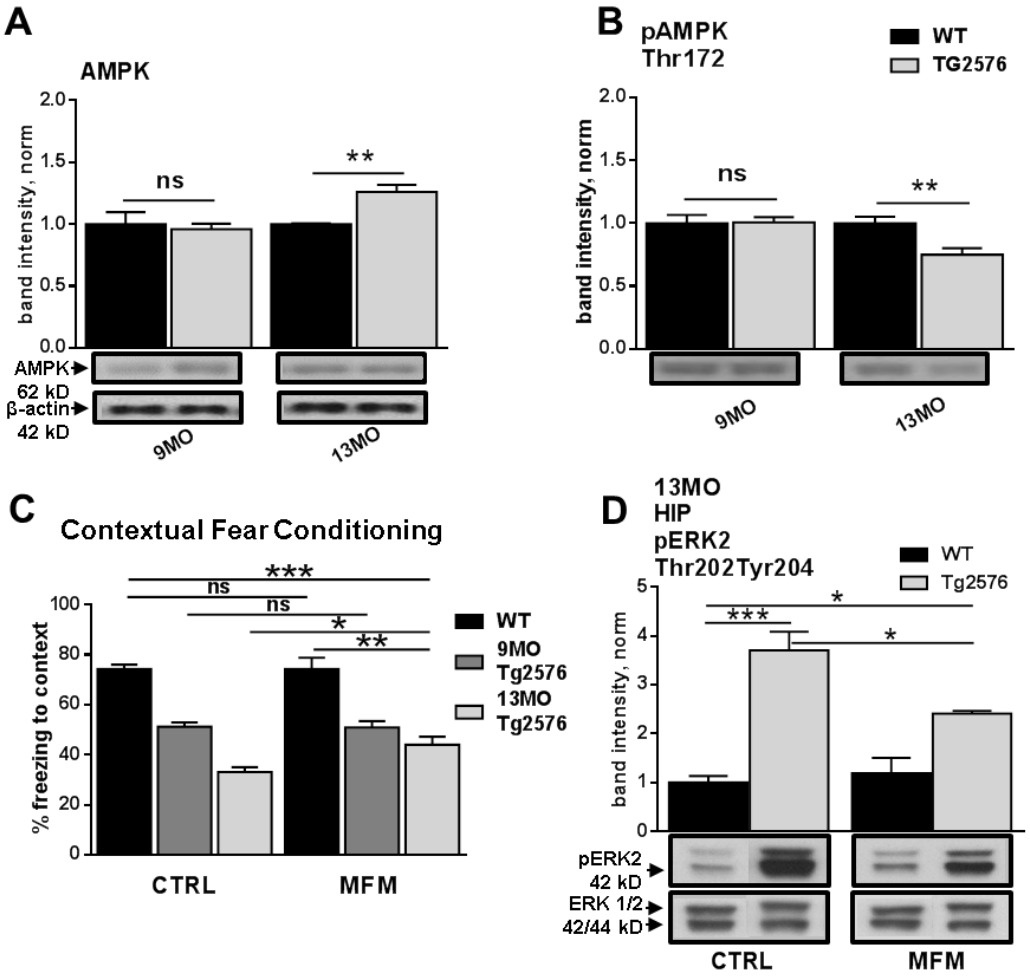


Figure 6.1 Targeting the energy sensor, AMPK, in 13MO Tg2576 enhances HIP-dependent cognitive function by decreasing ERK hyper-activation. **A,** Total AMPK is up regulated in 13MO, but not 9MO, Tg2576 HIP (Students two-tailed t-test). **B,** Phosphorylation activation of AMPK is decreased in 13MO, but not 9MO, Tg2576 HIP (Students two-tailed t-test). pAMPK:AMPK=1.05 and 0.59 for 9MO and 13MO Tg2476, respectively. **C,** One month MFM treatment partially rescued 13MO Tg2576 contextual freezing deficits. 13MO MFM-treated Tg2576 was significantly different from both untreated 13MO Tg2576 and WT. Two-way ANOVA and Tukey post hoc analysis; $F(3,16)=3.830$ ($p=0.06$), =3.825 (0.06), =60.35 ($p<0.0001$) for interaction, treatment, and genotype, respectively. Untreated 9MO and 13MO Tg2576 performed significantly worse than WT ($p<0.0001$). One month MFM treatment had no effect on WT or 9MO Tg2576; $F=1.305$ ($p=0.259$), =1.303 (0.259), =56.34 ($p<0.0001$) for interaction, treatment, and genotype, respectively. **D,** MFM treatment ameliorated ERK hyper-activation in 13MO Tg2576 HIP with no effect on WT. Two-way ANOVA and Tukey's multiple comparisons test $F(1,8)=8.32$ ($p=0.02$), =58.0 ($p<0.0001$), =4.53 ($p=0.7$) for interaction, genotype, and treatment, respectively. Tg2576 data reported as normalized to WT; pERK2:ERK2 ratios were 1.0, 1.2, 4.1, 2.6 for WT, WT+MFM, Tg2576, Tg2576+MFM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

MFM that target PPARγ and AMPK, respectively, mainly provide cognitive benefit during early stage disease and possibly in an additional subset of patients with peripheral insulin resistance (Watson et al., 2005; Risner et al., 2006; Gold et al., 2010; Sato et al., 2011; Craft, 2012). Thus, stage-dependent studies of animal models can help predict positive therapeutic responses in future clinical trials by informing upon the disease stage profile that matches a particular therapeutic intervention.

6.2 Determining the site that mediates the PPARγ-pERK interaction

One of the most obvious next steps in furthering this work is to determine the site(s) that mediates the PPARγ-pERK interaction. Chapter 5 briefly touched on our identification of two potential interaction sites on PPARγ1, amino acids $FHYG_{119\text{-}122}$ and $RRTIRLKL_{136\text{-}143}$, which respectively represent putative DEF and D sites, motifs known to be recognized by ERK (Jacobs et al., 1999). Thus, the first experiment I would suggest is to determine if either (or both) of these sites mediate the PPARγ- pERK interaction by utilizing peptides that mimic these sites in order to competitively inhibit protein complex formation *in vitro*. This experiment will require the recapitulation of the *in vitro* IP experiment described in chapter 5.5 wherein recombinant PPARγ is incubated with a recombinant GST- pERK2 and purified via glutathione bead assay. This group will serve as our positive control as we have already determined that PPARγ binds to pERK under these conditions (Fig 5.4). Group 2 will stage the same interaction with the addition of a peptide mimicking the putative DEF site, while group 3 will contain a peptide mimicking the putative D site. Finally, the fourth experimental group will incubate PPARγ and pERK together in the presence of both the D and DEF site peptides. If the PPARγ-pERK interaction is exclusively mediated by either the DEF or D site, we should observe abolished PPARγ pull down in groups 2 or 3, respectively, while complex formation would be sustained in the other. Because group 4 would contain both inhibitory peptides, we would not expect to observe any PPARγ-pERK interaction in this group if either peptide (group 2 or group

3) successfully disrupted the complex independently. Conversely, if both the DEF and D sites are required for complex formation, we might expect to observe decreased binding in both groups 2 and 3 (relative to group 1) and abolished PPARγ-pERK interaction in group 4. It is also conceivable that the PPARγ-pERK interaction is not mediated by either of these sites, in which case we would expect to see comparable PPARγ pull down across all four groups.

I hypothesize that inhibition of the DEF site (group 2) mediates the PPARγ-pERK interaction, as DEF sites are specific to ERK interactions while D sites can be recognized by other members of the MAPK family (Sheridan et al., 2008). Furthermore, D site motifs have commonly been found in MAPK binding partners and substrates, whereas transcription factors (i.e. Elk-1, Fos) more commonly exhibit DEF sites (Eldar-Finkelman and Eisenstein, 2009). Although there is no reason to anticipate a problem, it may also be necessary to include control IPs of pERK alone and in the presence of each peptide in order to ensure that the peptides do not affect the glutathione pull down. Likewise, it may be pertinent to examine the effects of the peptides on the PPARγ recombinant protein to ensure that peptide binding does not disrupt the PPARγ antibody interaction. This experiment provides a highly controlled environment in which to investigate the location of the physical interaction between PPARγ and pERK.

6.3 Observing the PPARγ-pERK interaction in a cell line

Once the PPARγ-pERK interaction site has been determined, it may be informative to investigate complex formation in a cell line, which would provide a less controlled and therefore less synthetic environment to study this interaction. Our studies suggest that ERK activation during memory consolidation is required for PPARγ-pERK complex formation. In order to investigate whether ERK activation drives this interaction, we could co-transfect CHO cells with both WT human EGF receptor and PPARγ. These cells will then transiently express PPARγ (Savage et al., 2002) and addition of EGF to this system will stimulate the ERK MAPK cascade and culminate in the phosphorylation/activation of ERK (Ahsan et al., 2009) – cellular events

comparable to those which take place during fear conditioned learning. We can also include a control group which expresses empty vector in place of the EGF receptor that will not facilitate ERK activation when EGF is added. Homogenization and subsequent pERK immunoprecipitation from these cells will allow for the assessment of baseline PPARγ-pERK binding, while immunoprecipitation from the EGFR-transfected cells following EGF stimulation will determine whether ERK activation affects PPARγ binding. Based upon the steady-state complex work described in the previous chapter, I do not anticipate that ERK activation will be sufficient to increase PPARγ-pERK binding. Conversely, I hypothesize that we will be able to drive complex formation by treating our EGFR-transfected cells with both EGF and RSG, as this will result in both ERK stimulation and PPARγ conformation change, exposing the ERK-docking site(s) and in essence, recreating what we believe are the physiological events that take place during memory consolidation in RSG-treated Tg2576.

Assuming that these predictions are correct, we can then repeat these conditions (e.g. CHO cells co-transfected with PPARγ and EGFR, stimulated with EGF and treated with RSG) while also treating the cells with the inhibitory peptide determined in the recombinant protein experiment described in 5.1. If we have correctly identified the binding site for PPARγ and pERK, addition of this peptide should result in a significant reduction (or even complete abolishment) in PPARγ-pERK complexes that can be immunoprecipitated from this sample compared to those from the uninhibited, RSG-treated, and EGF stimulated cells. To the same end, an alternative approach would be to transfect the CHO cells with a mutant PPARγ with alterations to the ERK binding sequence. Rather than competitively inhibiting complex formation with a peptide, the mutant PPARγ would not have an ERK binding site at all, and therefore we would expect that no PPARγ-pERK complex would form, even in the presence of both RSG and EGF. This data will be necessary to permanently disrupt the PPARγ-pERK complex *in vivo*, an idea that will be discussed in the next section.

An additional advantage of examining this protein complex in a cell line is that this environment would include additional stabilization proteins and binding partners not present in the recombinant protein experiment. This is particularly important given our earlier conjecture that the stability of the PPARγ-pERK complex may be greatly affected by scaffolding and co-activator proteins, and a cell line would offer a quick and efficient means by which to study candidate binding partners. Use of the same PPARγ and EGFR co-tranfected CHO cells described above, we could drive formation of the complex (using RSG) and then perform mass spectrometry on our immunoprecipitated samples in the hopes of identifying additional binding partners. This experiment would be similar to that described in chapter 4 where we identified dentate gyrus proteins altered by RSG treatment (Denner et al., 2012b). Here though, rather than a broad examination of all the proteins in the dentate gyrus, we would be identifying the specific proteins that are in complex with pERK. There are disadvantages to this approach, of course, in that CHO cells are not a neuronal cell line and thus the available co-activator proteins may differ from those in the hippocampus. It may be a worthwhile approach to perform these experiments in cultured hippocampal neurons to more closely mimic the natural environment in which this interaction occurs. That said, the proposed mass spectrometry analysis could also be performed on 9MO Tg2576 hippocampus following FC training to provide the most accurate environment; however, this approach is far more expensive, time consuming, and work intensive than the cell line approach. At least for the initial pilot experiments, a cell line would provide an easily repeatable and relatively cheap method to screen for potential PPARγ-pERK binding partners.

Going in a slightly different direction, it might also be informative to treat our CHO cells with Aβ to see if the dynamics of complex formation are affected. Although likely unrelated to the binding site, this experiment might serve to shed light on how Aβ oligomers and/or subsequent plaques disrupt or dysregulate complex formation and could thereby provide some

insight as to why RSG treatment ceases to alleviate Tg2576 cognitive deficits after roughly 9-10 months of age as disease pathology continues to progress and accumulate. This, of course, is an entirely separate endeavor.

6.4 Disruption of the PPARγ-pERK binding site *in vivo*

Since we know that 9MO Tg2576 exhibit significant cognitive deficits that can be restored with RSG treatment (Denner et al., 2012b) and that disruption of the PPARγ-pERK complex prevents this rescue (chapter 5), interference with the region of PPARγ that binds to pERK should prevent RSG-mediated cognitive rescue in these mice. The proposed recombinant protein and cell line experiments should yield a competitive inhibitory peptide that would serve this purpose (*N.B.* In the interest of brevity, for the purposes of this hypothetical experiment let us assume that the PPARγ-pERK interaction is mediated at the putative DEF site proposed in section 6.1). However, to use these peptides in a living animal introduces a number of complications. To begin, such peptides have a very short half-life and thus the appropriate timing of their delivery would be imperative to the successful disruption of complex formation. Presently, all we know is that PPARγ and pERK are bound 4 hours after a learning event but we do not know when this interaction begins or how long it lasts. Furthermore, even if we knew the exact time after a learning event that complex formation began, the brief window during which our peptide would be effective may only delay the formation of the complex by several minutes and would likely not have a demonstrable effect on behavior. Second, the peptide's short half-life complicates its delivery method. Because ICV delivery requires anesthesia and recovery time, such a route is not practical for a compound with a limited window of effect. Other less invasive routes of administration (e.g. intraperitoneal, IV, or IM) would introduce our inhibitory peptide into the periphery and interfere with any pERK binding partner with the same DEF site; these non-specific effects could have disastrous consequences (Eldar-Finkelman and Eisenstein, 2009) and profoundly affect animal health and/or behavior. Furthermore, while the

small size of our peptide would allow it to cross the blood-brain barrier, we would not know what percentage actually reached the brain without substantial investigation, and certainly we would not know how much reached the hippocampus. As such, utilizing the inhibitory DEF peptide is both a logistical and technical nightmare and therefore an undesirable approach.

With this in mind, I propose that rather than interfering with the natural DEF site, we instead utilize embryonic transfection to produce Tg2576 expressing a mutant PPARγ with an altered DEF site motif that cannot bind to pERK. We would be able to screen for a competent mutant with normal PPARγ transcriptional function but an inability to bind pERK using the cell line approach described in 6.2, thereby significantly reducing the risk of this experiment not working. Such an approach would guarantee that PPARγ and pERK could not interact but would not prevent PPARγ ligand-binding or the resulting conformational change. In this case, if RSG treatment did not restore cognition we could be certain that direct binding of PPARγ and pERK mediates the cognitive improvement and that RSG simply serves to facilitate the conformation-dependence of this interaction. While certainly involved, this would conclusively show that RSG-mediated cognitive rescue is mediated by a direct PPARγ-pERK interaction. This would not only confirm our findings described in chapter 5, but would also define the mechanism of cognitive rescue.

6.5 Non-genomic actions of PPARγ

Because the formation of the PPARγ-pERK complex seems dependent upon ligand-activation of PPARγ via RSG, one might assume that this effect is transcription dependent. However, there is ample evidence for non-genomic actions of PPARγ and its agonists which suggests that this interaction may be entirely independent of PPARγ target gene transcription. The first account of the non-genomic actions of PPARγ ligands came from the observation that certain PPARγ agonists inhibit cellular growth in cell types that do not contain the PPARγ

receptor (Palakurthi et al., 2001). Research in PPARγ-/- and PPARγ+/+ embryonic stem (ES) cells demonstrated that TZD treatment could equally arrest the cell cycle in the G1 phase in both cell types, indicating a transcription-independent mechanism for this action (Palakurthi et al., 2001). Interestingly, it is well established that various PPARγ agonists can activate members of the MAPK family, including ERK, within minutes of TZD treatment and therefore in a manner far too rapid to require protein synthesis (Lennon et al., 2002; Gardner et al., 2003; Teruel et al., 2003). This work has been conducted in a multitude of cell types, and the different PPARγ agonists (e.g. RSG, PIO, troglitazone, ciglitazone) seem to have differential and tissue-specific effects (Gardner et al., 2005), further supporting the concept of non-genomic actions of PPARγ since these effects are independent of PPARγ transcriptional competency.

Non-genomic signaling is not uncommon for nuclear receptors; while traditional hormone actions require hours or even days to occur following ligand activation, some steroid effects can be observed within minutes (Losel and Wehling, 2003), suggesting the involvement of alternative signal transduction pathways. Such actions have been demonstrated in a number of other receptors in the nuclear receptor family. For example, the glucocorticoid receptor-mediated stress response occurs very rapidly and activation of GR causes increases expression levels and activity of the ERK MAPK pathway, ultimately leading to increased expression of the downstream ERK-mediated immediate early gene Egr-1 (Revest et al., 2005). Similarly, activation of the vitamin D receptor, another member of the nuclear receptor superfamily, can enhance the PKC pathway, ultimately culminating in the rapid activation of the ERK MAPK cascade through Raf (Marcinkowska et al., 1997). Finally, the activation of the progesterone receptor through ligand binding has been shown to cause the rapid maturation of *Xenopus* oocytes arrested in the G2 phase of the cell cycle, even in enucleated cells that cannot undergo transcription (Masui and Markert, 1971). Furthermore, progesterone activates both PI3K and ERK (Losel and Wehling, 2003), suggesting that these rapid actions are mediated by protein

kinase signaling cascades. Indeed, the progesterone receptor interacts with the SH3 domain of the tyrosine protein-kinase Src and induces the stimulation of Raf and ultimately ERK (Losel and Wehling, 2003). Thus, it is a recurring theme that nuclear receptors can initiate second messenger cascades that impinge upon kinase pathways to exert rapid systemic effects. In light of these examples, it is reasonable to suggest that formation of the PPARγ-pERK complex during memory consolidation, while dependent on PPARγ conformational change conferred by its agonism, does not require PPARγ-mediated transcription and instead exerts non-genomic effects to facilitate the downstream actions of ERK.

6.6 MEK as a mediator of the PPARγ-pERK complex

The non-genomic effects of PPARγ agonists may provide some insight into the unanswered questions that remain regarding other members of the PPARγ-pERK protein complex. My work suggests that MEK is somehow involved in formation of the PPARγ-pERK complex (Fig. 5.5). In resting cells, both ERK and MEK are localized to the cytosol but translocate to the nucleus upon cellular stimulation (Yoon & Seger, 2006). ERK can remain in the nucleus for up to 3 hours, but the MEK primary sequence contains a nuclear export signal (NES) resulting in its rapid export back to the cytosol (Jaaro et al., 1997). In contrast, most nuclear receptors contain a nuclear localization signal (NLS) that forces them to the nucleus upon ligand binding; PPARγ is an exception, as no NLS has been discovered (Burgermeister and Seger, 2007). Still, most evidence suggests that in resting cells, PPARγ is primarily located in the nucleus (Berger et al., 2000) and recent evidence suggests that PPARγ is exported from the nucleus by MEK upon mitogenic stimulation (Burgermeister and Seger, 2007). This nuclear export inhibits the genomic function of PPARγ by preventing its transcription of nuclear target genes (Burgermeister and Seger, 2007) but has the added effect of facilitating PPARγ interaction with cytosolic and membrane proteins (Burgermeister et al., 2007). Other nuclear receptors, including the estrogen and androgen receptors, are known to associate with adapter

proteins in the cytoplasm that ultimately impinge upon the Src/Ras/ERK pathway (Song et al., 2005), supporting the hypothesis that the PPARγ-pERK complex exerts its memory enhancing effects through non-genomic pathways.

The idea of redistribution of ligand-bound PPARγ to the cytosol, combined with evidence for non-genomic signaling by nuclear receptors does well to ameliorate the somewhat paradoxical observation that ERK-mediated phosphorylation of PPARγ is generally considered inhibitory (Hu et al., 1996; Camp and Tafuri, 1997; Shao et al., 1998; Camp et al., 1999) while PPARγ agonists facilitate ERK activation (Gardner et al., 2005) and therefore the likelihood of ERK-mediated PPARγ phosphorylation. As already noted, it is important to consider that the downstream effects of PPARγ-pERK complex formation may be entirely independent of PPARγ transcriptional competency, therefore making both the effects and status of PPARγ phosphorylation in the hippocampus irrelevant. It may be that MEK shuttles RSG-bound PPARγ into the cytosol, where it can 1) activate ERK via non-genomic pathways and 2) recruit and directly bind the newly activated ERK at ERK interaction site(s) exposed due to the ligand-conferred conformation change in PPARγ. This complex may then translocate to the nucleus where ERK is able to exert its downstream effects on proteins important to learning and memory such as p90RSK or Elk-1. That our studies indicate an increased MEK-pERK steady-state association during RSG treatment that is disrupted during memory consolidation (Fig. 5.5) suggests that PPARγ increases the potential for ERK activation and also prolongs ERK activity in the nucleus during memory consolidation. To clarify, MEK is believed to facilitate the export of ERK from the nucleus (Burgermeister et al., 2007). As such, a secondary consequence of PPARγ-pERK complex formation may be the prevention of MEK binding to ERK due to interference with the MEK-ERK binding site by PPARγ. This idea is supported by my finding that MEK association with pERK is decreased at the 4 hour post-consolidation time point (Fig. 5.5) and the fact that MEK is unlikely to interact with PPARγ and pERK simultaneously

(Burgermeister et al., 2007). Interestingly, ERK recognizes and interacts with a MEK D site motif (Eldar-Finkelman and Eisenstein, 2009; Garai et al., 2012). Therefore, if the PPARγ-pERK interaction is mediated by a PPARγ D site (a possibility described in section 6.1), this interaction could preclude ERK-MEK binding and subsequent shuttling. Similarly, if the PPARγ-pERK interaction is mediated by a DEF site, the pERK conformation may prevent surface exposure of the ERK D site interaction domain and thereby thwart MEK binding in this manner. Either scenario would prolong ERK-mediated transcription in the nucleus and would thereby provide a mechanism for enhanced memory consolidation resulting from hippocampal PPARγ agonism without a dependence on PPARγ transcription.

It is noteworthy that PPARγ antagonism with GW9662 prevents both cognitive rescue and formation of the hippocampal PPARγ-pERK complex in Tg2576. Generally, antagonists of nuclear receptor genomic activity do not affect the non-genomic activity of the receptor (Losel and Wehling, 2003). However, antagonism with GW9662 inhibits PPARγ ligand binding and thus conformation change. As the cognitive enhancing effects of RSG seem to be mediated by direct interaction of PPARγ and pERK, disruption of this protein complex would likely also prevent non-genomic actions. This is particularly true if, in fact, PPARγ-pERK interaction prevents ERK from being removed from the nucleus, since GW9662 would prevent PPARγ from binding to pERK, likely restoring shuttling of ERK by MEK.

Investigation of this mechanism would require examination of the MEK-PPARγ interaction that likely precedes PPARγ-pERK binding since ERK and PPARγ are localized to different cellular compartments in quiescent cells (Yoon & Seger, 2006; Burgermeister & Seger, 2007). Such a study could be accomplished in a very similar manner to the present IP work substituting MEK for pERK and observing PPARγ association. That said, tissue harvest would likely need to be accomplished in a much narrower window following the initiation of memory consolidation (e.g. within 15 minutes to an hour of FC training) as ERK phosphorylation via MEK

occurs rapidly during this phase (Sweatt, 2004; Trifilieff et al., 2007). The proposed cell line IP and mass spectrometry experiments outlined in 6.2 may be useful in highlighting the role of MEK in this protein complex.

6.7 CBP may also play a critical role in PPARγ-pERK complex formation

Given that our work found CBP was increased in response to RSG treatment in Tg2576 (Denner et al., 2012b) and that CBP is a known mediator of both PPARγ (Bugge et al., 2009; Inoue et al., 2012) and ERK (Caccamo et al., 2010), further characterization of CBP's role in the PPARγ-pERK complex is required. Other researchers have shown that CBP forms a complex with p90RSK in response to increased insulin exposure (Frodin and Gammeltoft, 1999; Wang et al., 2003); thus, this CBP-RSK complex is likely elevated in both Tg2576 and diabetic/hyperinsulinemic AD subjects. The authors note that formation of the CBP-RSK complex inhibits CRE-dependent gene transcription (Frodin and Gammeltoft, 1999), and this may be one reason that hyperinsulinemic individuals exhibit a higher incidence of AD-like cognitive dysfunction than other individuals. Notably, the association between CBP and RSK is substantially decreased via activation of ERK upon mitogenic stimulation (Frodin and Gammeltoft, 1999), suggesting that restoring proper ERK activation should decrease CBP-RSK complexes and increase free CBP and p90RSK. To reiterate a point from earlier, increased CBP has been shown to rescue learning and memory deficits in the 3xTg AD mouse model without affecting Aβ or tau levels/deposition (Caccamo et al., 2010). Our own findings that RSG treatment 1) restores cognition, 2) facilitates formation of the PPARγ-pERK complex during memory consolidation, 3) increases CBP levels, and 4) does not affect total Aβ40 or Aβ42 levels (Denner et al., 2012b) suggest that the PPARγ-pERK complex may directly enhance CBP activity. Since increased CBP is known to increase levels of BDNF and this increase facilitates ERK phosphorylation (Ying et al., 2002), this may be a feed-forward mechanism to enhance ERK/CREB nuclear activity. As such, formation of the PPARγ-pERK complex may serve to

directly facilitate proper ERK activation, which subsequently disrupts CBP-RSK complexes and increases CBP activity, ultimately facilitating CRE-dependent gene transcription and memory consolidation.

With this in mind, it may be pertinent to examine the presence of hippocampal CBP-RSK complexes in Tg2576 and how they are affected by RSG treatment. I would hypothesize that RSG treatment decreases CBP-RSK association during memory consolidation, via facilitation of ERK activity. This could be investigated using our familiar IP technique to reciprocally IP both CBP and p90RSK and immunoblot for the other. However, this presents a number of challenges, most notably determining the proper time point to examine this interaction. If disruption of CBP-RSK complexes allows free CBP to facilitate ERK activity, one would expect that disruption of CBP-RSK occurs prior to PPARγ-pERK complex formation and therefore several time points should be examined within the first 4 hours of consolidation. Conversely, in respect to CBP's coactivator and HAT functions, RSG-bound PPARγ may be responsible for the initial recruitment and increased activity of CBP, which facilitates ERK phosphorylation through increased BDNF activity, thereby increasing the likelihood of PPARγ-pERK complex formation. Given the murky nature of the chain of events in PPARγ-pERK complex formation, it may be more relevant to simply examine the association of CBP with pERK during memory consolidation at the 4 hour time point, wherein CBP association with the PPARγ-pERK complex would presumably be elevated in RSG treated animals. This idea is supported by my observation that hippocampal p90RSK is increased in Tg2576 following RSG treatment, suggesting that RSG facilitates downstream ERK activity.

6.8 Why are WT mice unaffected by PPARγ agonism/antagonism?

One of the most notable outcomes of the experiments described herein was that unlike their age-matched Tg2576 littermates, WT mice did not exhibit improved cognition following

RSG treatment, nor did they suffer any impairment in response to GW9662. While our studies did not directly address the reasons for this, we can postulate a number of theories. We concluded earlier that PPARγ agonism rescued cognition in Tg2576 by harnessing a dysregulated ERK signaling pathway – one that is entirely functional in WT animals. Thus, PPARγ agonism may not affect WT simply because this mechanism represents a redundant pathway to ensure homeostatic ERK function. To put it another way, if ERK is already functioning properly, PPARγ interaction with pERK may not have any discernible effect on cognition due to a ceiling effect (e.g. PPARγ cannot improve on an optimally functioning ERK system). Indeed, our studies did find that pERK and PPARγ still interact in WT (Fig. 5.2); there was simply no correlation with cognition as all WT subjects were cognitively intact.

On the other hand, RSG may not affect WT because PPARγ is already saturated with endogenous ligand in this system, whereas Tg2576, who are deficient in fatty acid metabolism and may be unable to utilize endogenous ligand may be more likely to bind RSG. However, this scenario does not seem likely for several reasons. First, WT mice treated with RSG exhibit increased CNS PPARγ DNA binding compared to controls (Fig. 4.1A), suggesting that RSG does bind PPARγ even in a 'normally' functioning system. Also, similar to RSG-treated Tg2576, WT animals treated with RSG exhibit increased adiposity and edema compared to controls, and these are primary side effects of TZD-mediated PPARγ agonism. Thus, it seems that WT mice do experience effects of RSG and therefore must be binding it with some affinity. Finally, the fact that GW9662 does not decrease WT cognitive function suggests that PPARγ regulation of ERK is not a primary mechanism in 'normal' cognition but is only utilized when ERK signaling becomes dysregulated. Once again, this suggests a ceiling effect of optimal ERK functioning in WT that is unaffected by PPARγ; it is noteworthy that Tg2576, who exhibit dysregulated ERK signaling and that we propose rely on PPARγ to restore ERK function exhibit a marked reduction in cognition following GW9662 treatment. It may be informative to examine a cohort of

Tg2576 on control diet who receive GW9662 to determine whether this treatment reduces variability amongst this group (to recall a point from earlier, while Tg2576 as a group perform poorly on the contextual fear task and also exhibit low PPARγ:pERK ratios, on an individual basis there are animals that exhibit substantially higher freezing and have elevated PPARγ:pERK ratios. Therefore, if we examine the Tg2576 control diet group treated with GW9662, I would hypothesize a reduction in variability compared to Tg2576 that received vehicle, with all members of the group expressing low freezing and low PPARγ:pERK).

6.9 Does PPARγ impinge on other points in the ERK cascade?

The majority of the work highlighted here suggests that PPARγ directly enhances ERK activity through a protein-protein interaction. We must acknowledge, though, that PPARγ agonism may also impinge upon the ERK cascade at earlier stages to facilitate this enhancement. As discussed above, MEK directly binds to and shuttles PPARγ from the nucleus and it is entirely feasible that this mechanism is enhanced by RSG in a manner similar to our hypothesized ERK interaction (i.e. PPARγ ligand-bound conformation may make binding to MEK more likely). If this is the case, increased PPARγ shuttled to the cytosol would subsequently increase the likelihood of interaction with pERK during memory consolidation and therefore the intial PPARγ-MEK interaction may be causal in RSG-mediated memory enhancement.

Also possible is that RSG enhances upstream synaptic transmission which results in increased intracellular calcium, amplified PKA/PKC activity, and therefore ultimately initiates the upstream ERK cascade members (e.g. Ras, Raf) indirectly. Studies performed in aged Wistar rats demonstrate that RSG treatment restores population spike amplitude and EPSP slope to levels comparable to WT (Wang et al., 2012), indicating that PPARγ agonism normalizes the cellular response to stimulation. Unfortunately, the mechanism by which PPARγ normalized

EPSPs in this study was not defined, and therefore this area requires further study before any conclusions can be drawn. PPARγ may have the ability to enhance presynaptic glutamate release, facilitate calcium transmission, or even enhance PKA/PKC/CaM/CaMKII signaling (**Figure 6.2**); this is of course speculation. Still, assuming PPARγ contributes to proper synaptic transmission, we would expect to see a domino effect down the signaling cascade beginning with restored influx of intracellular calcium in response to normal EPSPs. This, of course, would enhance CaM/CaMKII signaling, PKA/PKC activity, and facilitate the GDP to GTP exchange between Ras and Raf1, all of which would facilitate ERK nuclear function. In reality, all of these factors (e.g. PPARγ-pERK interaction, MEK-PPARγ shuttling, and enhanced calcium transmission) likely contribute to RSG-mediated cognitive enhancement.

6.10 Final remarks

Of course, there are limitations to the work presented here. Animal models of AD are imperfect, as they are genetic manipulations that do not fully recapitulate the full spectrum of the human disease; however they do confer some aspects of the disease pathophysiology that are thought to play a causal role. As such, there are a number of transgenic mouse models of AD (Gotz et al., 2004), including those that lead to aberrant accumulation of β-amyloid (Tg2576, PDAPP, presenilin conditional KO/APP, PS1/APP, CRND8, PGDF-APP_{SW}) and mutant tau (P301S, rTg4510, 3xTg-AD). Many interventions have been deemed successful in nearly all of these models, only to fail or have unanticipated side effects when employed in human trials. Thus, finding a true candidate for successful human intervention will require not only the discovery of a compound that has disease-modifying properties, but also an understanding of the underlying mechanism for these actions as well as an understanding of the appropriate disease-stage for which it is efficacious. Therefore, animal models, although imperfect, are necessary and informative if used appropriately for preclinical development.

Figure 6.2

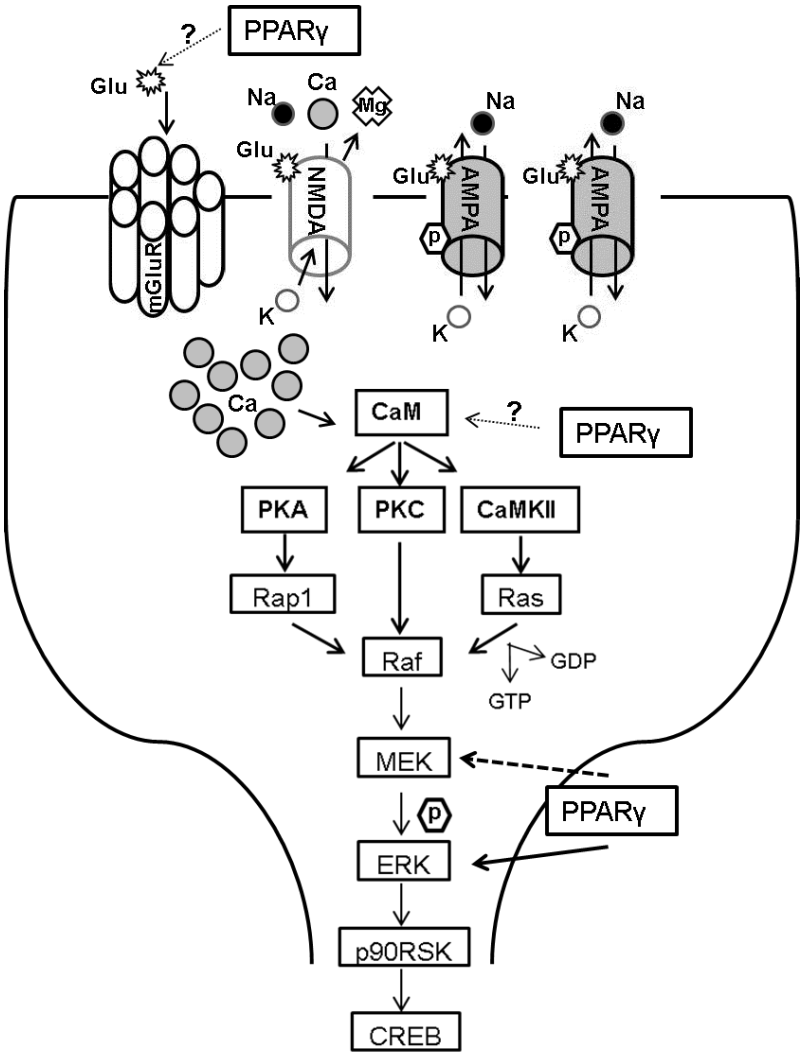


Figure 6.2 PPARγ may impinge upon ERK signaling at multiple points. We have demonstrated that PPARγ directly interacts with pERK and likely facilitates its downstream activity. Given the established shuttling relationship between PPARγ and MEK, it is likely that this interaction contributes to PPARγ mediated cognitive rescue. It may also be possible that PPARγ normalizes population spike amplitude and EPSPs (Wang et al., 2012) by impinging upon presynaptic glutamate release (either directly or indirectly through an intermediary) or enhancing the cellular response to intracellular calcium by facilitating CaM or subsequent PKA/PKC/CaMKII signaling. It is also conceivable that all of these mechanisms contribute to PPARγ-mediated cognitive enhancement and such possibilities warrant further investigation.

With this in mind, it is important to emphasize that at this time, the Tg2576 model utilized for these studies provides one of the best-characterized AD mouse models from both a behavioral and biological perspective. By 9MO, Tg2576 do not exhibit overt neurodegeneration but show signs of

synaptic dysfunction and structural changes in the CA1 region of the hippocampus (Perez-Cruz et al., 2011) and also exhibit impairment in hippocampus-dependent memory tasks. Thus, 9MO Tg2576 recapitulate a relevant and diagnosable transition from preclinical to MCI stage of human AD and therefore the study of this model is well suited to the NIA goal of identifying early stage biomarkers and potential therapeutics at a stage that precedes significant neuronal structural damage. As such, PPARγ agonism to selectively impinge upon the ERK MAPK cascade represents a disease modifying intervention for humans. Furthermore, given the adverse side effects attributed to RSG full agonism of PPARγ, it will be important to test alternative TZDs such as pioglitazone as well as next-generation PPARγ non-agonist and partial agonist ligands (Choi et al., 2010; Choi et al., 2011; Vidovic et al., 2011).

Our identification of the mechanism of PPARγ-mediated cognitive rescue – that is restoration of a dysfunctional ERK pathway through direct protein-protein interaction – provides a novel means by which impaired learning and memory can be therapeutically targeted. Thus, compounds that facilitate formation of the PPARγ-pERK complex may have applications not just to AD cognitive dysfunction, but also to other learning and memory disorders. Furthermore, because nuclear receptors maintain common structure, this mechanism could possibly be exploited through the agonism of other nuclear receptors that have the ability to modify ERK activity, such as GR (Revest et al., 2005). This idea is supported by the fact that RSG has been shown to prevent GR down-regulation in the hippocampus (Escribano et al., 2009) suggesting that the agonism of one nuclear receptor can modulate the activity of another. This approach may yield a therapeutic that indirectly facilitates PPARγ-pERK complex formation and therefore avoids the negative side effects associated with TZD treatment.

Finally, future endeavors that identify other members of this protein complex will be crucial to the development of highly specific therapeutic targets. It is conceivable that directly facilitating the downstream effects of PPARγ-pERK complex formation will yield an even greater

therapeutic effect or expand the window during which such treatment is effective. While the PPARγ-pERK complex does not represent a cure for AD, its discovery is certainly a positive step toward combating the devastating behavioral and mental changes conferred by this disease.

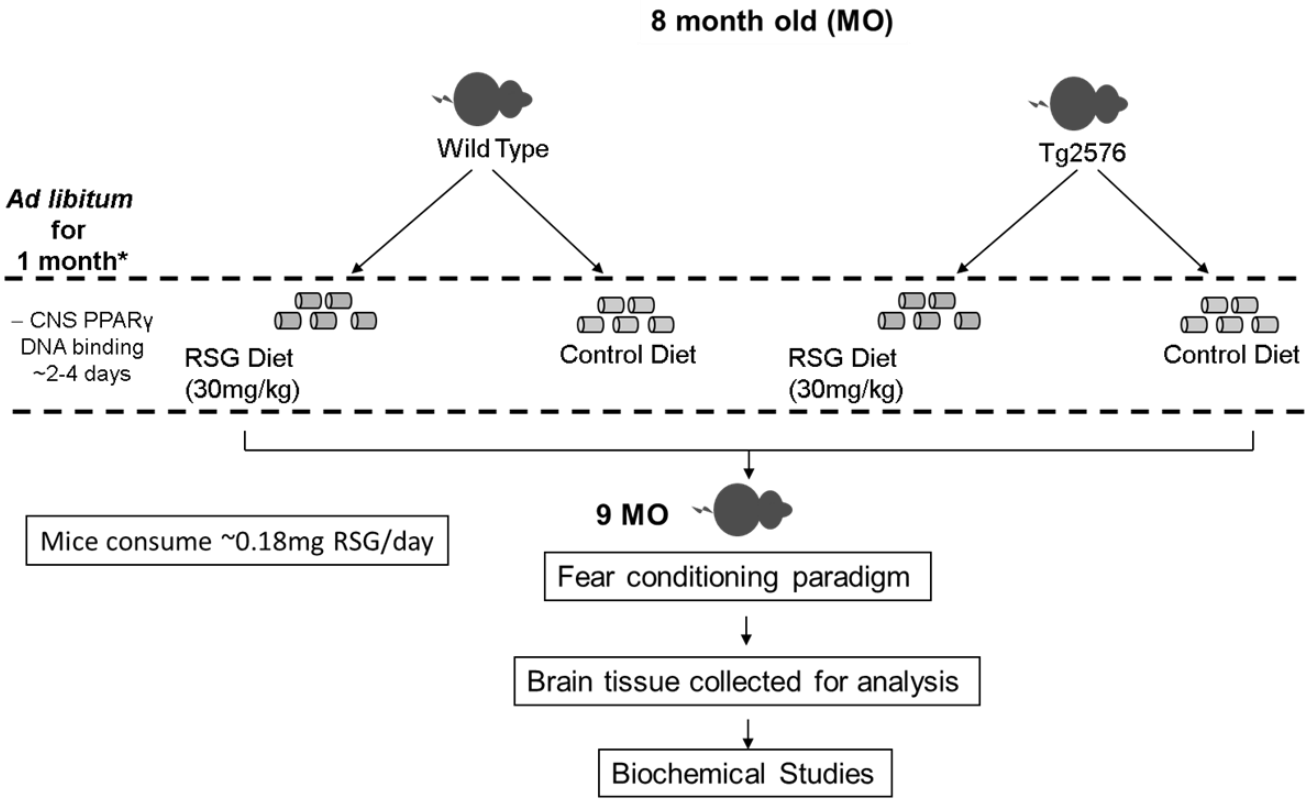
CHAPTER 7

MATERIALS AND METHODS

Animals. Tg2576 mice were bred in the University of Texas Medical Branch at Galveston (UTMB) animal care facility by mating hemizygous Tg2576(Hsiao et al., 1996) males with B6SJl/F1J females (Jackson Laboratory Stock#100012). Mice were housed, n ≤ 5 per cage, with food and water *ad libitum*. UTMB operates in compliance with the USDA Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and Institutional Animal Care and Use Committee approved protocols. Genotyping services were outsourced (Transnetyx) and determined from tail clip biopsies obtained at weaning and sacrifice to determine and validate genotypes, respectively. All animal manipulations were conducted during the lights-on phase (0600–1800 h).

Rosiglitazone treatment. Male and female 8 months old (8MO) Tg2576 and WT littermates were fed control or 30mg/kg RSG diet (Bio-Serv) for 30 days, as previously described(Rodriguez-Rivera et al., 2011). Mouse food intake and body weights were monitored during the 30-day period and no significant differences were observed by genotype or treatment group (data not shown). Additionally, age-related animal mortality rates were similar between groups. Animals were randomly assigned to receive control or RSG feed and sample sizes were balanced by sex and genotype. Experimenters were blinded to treatment groups during key data acquisition and analysis steps. A schematic representation of the overall treatment and testing protocol is provided in **Figure 7.1** below.

Figure 7.1 Schematic representation of RSG treatment for Tg2576 and WT littermates



Antibodies. Antibodies to pERK (9101, 1:1,000), ERK (9102, 1:1,000), MEK1/2 (9122, 1:1,000), RSK (9333, 1:1,000), anti-rabbit HRP secondary (7074, 1:20,000), anti-pERK conjugated sepharose bead slurry (3510) were from Cell Signaling Technologies. Additional reagents included anti-PPARγ (Millipore 07-466, 1:500) and Phospho-Ser84 PPARγ (1:500; MAB3632) and anti-PPARγ conjugated magnetic bead slurry (Affinity Life Sciences, custom preparation). B-Actin (1:5000; A5441) was obtained from Sigma. Lamin A/C (1:100; SC-20681) was obtained from Santa Cruz Biotechnology. HRP-conjugated anti-mouse IgG (1:50,000; NA931V) and anti-rabbit IgG (1:100,000; NA934V) were obtained from GE Healthcare.

Fear conditioning. Behavioral experiments were performed during the lights-on phase (0600-1800 hr) in the UTMB Rodent *In Vivo* Assessment Core (directed by KT Dineley) within the UTMB Center for Addiction Research (directed by Dr. Kathryn Cunningham). Two-pair fear conditioning (FC) training and FC hippocampus-dependent contextual testing was performed on awake and alert subjects 4 h after ICV injection. Based upon power analyses of previous data, ten (WT) to 20 (Tg2576) mice per group (male and female) were trained in the FC chamber following our standard FC protocol, as described previously (Dineley et al., 2002). Twenty-four hours later, mice were returned to the training chamber for testing in the hippocampus-dependent contextual FC paradigm. Hippocampus-dependent contextual learning was assessed by quantifying freezing behavior when the animals were placed back into the training chamber. Cued FC was not included in this study since Tg2576 are not deficient in the hippocampus-independent cued FC task and RSG treatment has no effect on WT or Tg2576 performance in this task (Dineley et al., 2002a; Rodriguez-Rivera et al., 2011; Denner et al., 2012b). Freezing behavior was analyzed using automated software (FreezeFrame/View) from digitally recorded videos (Actimetrics).

Contextual fear conditioning is amenable to the testing of manipulations hypothesized to disrupt memory consolidation (e.g., GW9662) as fear conditioning training is achieved in a single training session as opposed to those cognitive tasks that require repeated training sessions, e.g., Morris water maze(Westerman et al., 2002). We previously established that Tg2576 have intact perception as they exhibit similar shock threshold to WT animals (described below), and also freeze comparably to WT in response to shock during training (Rodriguez-Rivera et al., 2011; Denner et al., 2012b).

Shock threshold. Approximately 9 animals per group were subjected to shock threshold test to assess shock sensitivity, as described previously (Dineley et al., 2002). Briefly, a

sequence of single foot shocks was delivered to animals placed on the same electrified grid used for fear conditioning. Initially, a 0.1 Mv shock was delivered for 1 s, and the animals' behavior was evaluated for flinching, jumping, and vocalization. At 30 s intervals the shock intensity was increase by 0.1 Mv up to 0.7 Mv and then returned to 0 Mv in 0.1 Mv increments at 30 s intervals. Threshold to vocalization, flinching, and then jumping was quantified for each animal by averaging the shock intensity at which each animal manifested a behavioral response to the foot shock.

Sacrifice. Under deep anesthesia (1 ml Avertin [Fluka Analytical 90710] working solution [125 ul, 1.0 g Avertin/Ml tert-amyl-alcohol + 9.88Ml 0.9% NaCl]), animals were sacrificed by transcardial perfusion with ice cold PBS containing protease and phosphatase inhibitors (Sigma-Aldrich P8340 protease inhibitor cocktail, 30 Mm NaF, 10 Mm Na_3VO_4, 1 Mm PMSF [added fresh to perfusion buffer every 30 min]). This is in contrast to previous work wherein animals were sacrificed via decapitation without perfusion(Rodriguez-Rivera et al., 2011). Whole brains were extracted and hippocampi dissected in ice-cold saline (110 Mm sucrose, 60 Mm NaCl, 3 Mm KCl, 1.25 Mm sodium phosphate monobasic monohydrate, 28 Mm sodium bicarbonate, 5 Mm D-glucose, 1 Mm L-ascorbic acid, 1 Mm $MgCl_2$, 1 Mm $CaCl_2$). All samples were frozen on dry ice and stored at -80°C until use.

Intracerebroventricular (ICV) injection. The PPARy antagonist GW9662 (Sigma-Aldrich) and vehicle (1% dimethyl sulfoxide) were directly infused into the lateral ventricles using a modified free-hand method(Clark et al., 1968; Taglialatela et al., 2009; Denner et al., 2012b). Using aseptic technique, mice were anesthetized (isoflurane, 1–4 %) and the skull was exposed with a small incision along the midline to locate bregma(Paxinos et al., 1985). A 26G needle was inserted 3 mm deep at 1 mm anterior and 1 mm lateral to bregma. GW9662 (32.5 pmol) or

vehicle were delivered by an electronic programmable microinfuser (Harvard Apparatus) at 3 ul/min for 1 min. The needle was stabilized for 1 min after infusion to ensure complete delivery. Doses and delivery rates were determined based on previous work utilizing GW9662 to antagonize PPARγ function in the CNS(Bjorklund et al., 2012; Denner et al., 2012b). ICV injections were performed 4 hr prior to fear conditioning training and 8 hours prior to animal sacrifice and tissue harvest. This time point was chosen based on our previous work demonstrating the GW9662 peak effect on PPARγ was 8 hr post injection(Rodriguez-Rivera et al., 2011) which also corresponds to the timeframe for hippocampal ERK-mediated memory consolidation(McGaugh, 2000b; Trifilieff et al., 2007).

Protein extraction. Nuclear extracts were isolated from hippocampi at 4°C using the ActiveMotif Nuclear Extract Kit (#40010) then stored at -80°C. The resultant extracts were comprised of nuclei (nuclear) and a separate fraction comprised of the remaining cellular components (non-nuclear). Total protein concentrations in extracts were determined using a BCA protein assay kit (Thermo Scientific #23225).

Quantitative immunoprecipitation. Hippocampal extracts were thawed on ice and 200ug (nuclear) or 500ug (non-nuclear) of protein was suspended in 500µl extract buffer (25 Mm HEPES, 0.1 % Triton X-100, 10 % glycerol), supplemented with 0.02 M Sigma protease inhibitor cocktail (P8340), 0.02 M NaF, and 0.02 M sodium orthovanadate. Ten µl of anti-pERK conjugated sepharose bead slurry (Cell Signaling #3510) or 10µl anti-PPARγ conjugated magnetic bead slurry (Affinity Life Sciences) was added and this mixture was allowed to incubate on a rotating shaker at 4°C for ~18 hr. All remaining steps were performed at 4°C unless otherwise noted. Following incubation, sepharose bead samples were pelleted by centrifugation (14,000 x g, 1 min) or magnetic samples were isolated using a magnetic stand; in

each case the supernatant was then removed. The pelleted beads were washed by resuspension in extract buffer for 20 min then centrifuged (14,000 x g, 1 min) or placed in the magnetic stand to isolate washed beads. Bead wash was repeated 4 times. Protein was eluted in 30 µl 2x Laemmli sample buffer (20 % SDS, 20 % glycerol, 1 M Tris, 5 % β-mercaptoethanol, 8 M urea, ddH_2O, bromophenol blue) and incubated for 5 min at 95-100°C. One final bead pelleting step was performed to avoid loading beads onto SDS-PAGE gels.

Quantitative immunoblot for experiments described in chapter 4. Using our previously described method (Dineley et al., 2001b), 10–40µg (DC Protein Assay, Bio-Rad) of nuclear or cytosolic hippocampal extract from individual animals was resolved by SDS-PAGE, transferred to PVDF membrane (Immobilon, Millipore), then probed with the appropriate primary and secondary antibodies. Protein bands were detected by chemiluminescence (Advance ECL, GE Healthcare) and film exposures in the linear range for the antigenantibody combination were developed with a Kodak imager (Kodak). Band densities were measured with ImageJ (NIH) and normalized to control level. Normalized control values were determined for each immunoblot by averaging control values, dividing each control and test sample density by the average of the control set, and then determining the average and SEM for control and test samples for n = 6–10 animals/group. All blots were sequentially probed for PPARγ phosphorylated on Ser84, PPARγ, ERK phosphorylated on Thr202/Tyr204, ERK, then lamin or actin for normalization.

Quantitative immunoblot following immunoprecipitation. Extracted proteins were resolved by SDS-PAGE (BioRad, 7.5 % Mini-PROTEAN® TGX™) and electroblotted onto nitrocellulose. In order to quantitatively compare between immunoblot film bands, a crude whole-brain lysate (20 µg/well) prepared from ~40 C57Bl/6J control mice was included in triplicate on each gel as an internal standard (further described below).

Following electrophoresis and transfer, each membrane was blocked (2 % Advanced ECL blocking solution, GE Healthcare), and incubated with primary and secondary antibodies. Samples were visualized via chemiluminescence using the Amersham ECL Western blotting reagent system, according to the manufacturer's instructions (GE Healthcare). Exposure to Amersham Hyperfilm ECL (GE Healthcare) was performed to obtain band intensities within the linear range of the antibody combinations used.

Immunoblot (IB) membranes were scanned at 300 dpi and numeric band density and background values were acquired using ImageJ software (NIH). The numeric values for the loading control protein from each of the three identical C57Bl/6J internal standards was averaged (loading control or LC), and all other samples (e.g. PPARγ or pERK2 from the immunoprecipitation) were normalized LC average. The PPARγ value in our control sample was chosen as a normalization value in order to remain in the linear range of the samples we were investigating. Because Ips were loaded with 200 µg or 500 µg of protein, a standard loading control such as actin would have generated a signal too intense to accurately quantify the PPARγ and pERK that is present. Thus, this method allows for more precise quantitative comparison between different gels and across different experiments. After normalizing PPARγ and pERK2 protein density values for each sample, the amount of PPARγ that co-IP'ed with pERK was determined by taking the ratio of normalized PPARγ and normalized pERK2. This step corrected for any variation in IP efficiency. Thus, the final value represents the relative amount of PPARγ that is associated with pERK2 in a given sample normalized to a low abundance protein whose expression level is not subject to the effects of the pharmacological manipulations utilized.

Typically, our quantification and normalization procedure calculated as follows (**Fig. 7.2A**):

$$\frac{\{(PPAR\gamma_{RawValue})/[Avg(LC1+LC2+LC3)]\}}{\{(pERK42_{RawValue})/[Avg(LC1+LC2+LC3)]\}} = \text{Normalized PPARγ:pERK}$$

Quadruplicate runs on six individual animals using this approach yielded a coefficient of variation between 1% and 4.8% for the four PPARγ:pERK ratios calculated; thus, demonstrating reproducibility and accuracy of immunoprecipitation (**Fig. 7.2B**).

Recombinant protein and *in vitro* GST pull-down assay. *In vitro* recombinant protein association studies were performed using Pierce glutathione agarose beads (Thermo Scientific, 16100), recombinant human PPARγ (Randox Life Sciences, RCP9207), GST-tagged (N terminal) recombinant human active ERK2 (R&D Systems, 1230-KS), and GST-tagged (N terminal) recombinant human ERK2 (Sino Biological Inc., 10030-H09B). All steps were performed at 4°C unless otherwise specified. Glutathione beads were suspended in 250ul 1x TBS (0.02 M Tris, 0.14 M NaCl) and incubated with 100 ng of recombinant PPARγ and 100 ng of either recombinant GST-pERK or GST-ERK (GST-Terk) protein on a rocker overnight.

Controls were prepared to include all possible combinations of glutathione beads and recombinant proteins. Beads were pelleted by centrifugation (700 x g, 2 min) and the supernatant removed. Samples were washed (4 x 1 min) in 1x TBS, followed by centrifugation (700 x g, 2 min) to pellet beads. Bound proteins were eluted with 2x sample buffer (30 % glycerol, 2 % SDS, 62.5 Mm Tris Ph 6.8, bromophenol blue) and heated for 5 min at 95-100°C.

Figure 7.2

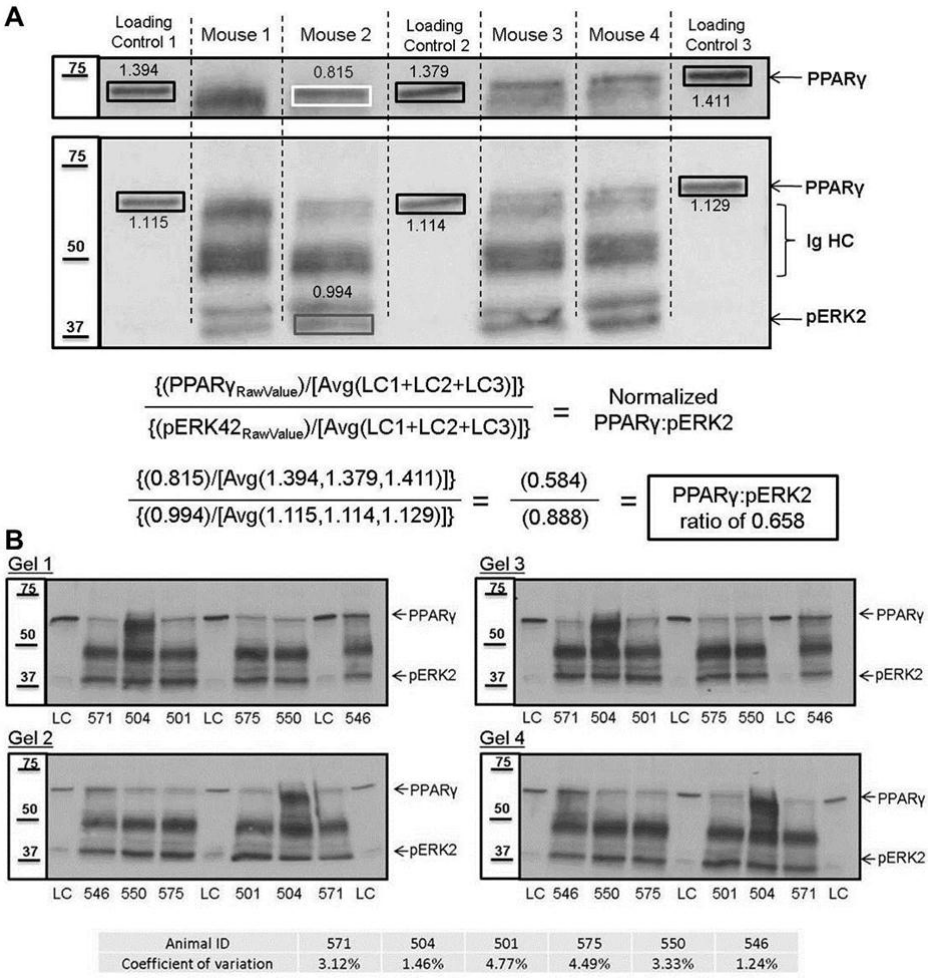


Figure 2 Quantification method to determine PPARγ:pERK2 ratios. **A,** Shown is an example Western blot for PPARγ and pERK in pERK Ips from 4 individual mice. For quantification across multiple immunoblots of IP material, a homogenate prepared from pooled brains from C57Bl/6J mice was used as a loading control (LC) and was resolved in triplicate (lanes 1, 4, 7) on each SDS-PAGE gel. In this figure, pERK Ips from 4 individual mice (lanes 2, 3, 5, 6) are depicted. For the data described herein, immunoblots for PPARγ or pERK2 from the Ips were normalized relative to the LC. PPARγ in the LC lanes was chosen as the normalization protein because it tracked in the linear range with IP'ed PPARγ and pERK2 for their respective exposures. After acquiring normalized values for IP'ed PPARγ and pERK2 proteins for each individual animals' hippocampal extract, the amount of PPARγ that co-IP's with pERK was calculated by taking the ratio of normalized PPARγ to normalized pERK2. In the example above, Mouse 2 has a hippocampal PPARγ:pERK2 ratio of 0.658 **B,** PPARγ:pERK2 ratios are highly reproducible. Western blots of PPARγ and pERK in 4 independent pERK Ips from 6 individual animals (lanes 2, 3, 4, 6, 7, 8) and the triplicate loading control (LC; lanes 1, 5, 9) resolved by 4 separate gels. The PPARγ:pERK2 ratios were calculated as in Fig. 2*A* and the coefficient of variation for each individual animal was determined. All replicate Ips yielded a coefficient of variation $\underline{\leq}$4.8 %.

Human brain tissue. Frozen human cortex was acquired from the Oregon Brain Bank at Oregon Health and Science University (OHSU) in Portland, OR as previously described(Bjorklund et al., 2012). Briefly, all donor subjects were enrolled and evaluated in studies at the NIH-sponsored Layton Aging and AD Center (ADC) at OHSU. Subjects were evaluated for neurological and neuropsychological competency annually and subsequently assigned a clinical dementia rating (CDR) by an experienced clinician. AD subjects were diagnosed by a clinical team consensus conference, met National Institute for Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorder Association diagnostic criteria for clinical AD, had a CDR > 1.0, and AD status was confirmed at autopsy following informed consent. All tissue was examined by a neuropathologist to confirm neurodegenerative pathology including neurofibrillary tangles and neuritic plaques. Amyloid score was assessed using standardized Consortium to Establish a Registry for Alzheimer's Disease criteria (0 = no plaques, 1 = sparse plaques, 2 = moderate plaques, 3 = dense plaques), and a Braak stage (0-6 with 6 being the most severe) indicative of the level and location of hyper-phosphorylated tau tangles. In addition to the pathological information detailed above, demographic data including age, sex, and mini mental state examination (MMSE) score were received along with the frozen tissue. Individual subject data is provided in **Table 7.1**

DNA binding assays. Eight micrograms of nuclear extract was assayed for PPARγ binding to the PPRE with TransAM ELISA kit (Active Motif) according to the manufacturer's instructions. Data are reported as mean +/- SEM normalized to WT signal.

RNA extraction and PPARγ1 and PPARγ2 reverse transcriptase-PCR. Hippocampi from WT mice were dissected out and stored in RNAlater RNA protection solution (Ambion,

catalog #AM7024) for further analysis. TotalRNAwas isolated from the tissue using RNAqueous-Micro Kit (Ambion) following the manufacturer's instructions. RNA sample quality and quantity were analyzed using Agilent 2100 Bioanalyzer and Nanodrop ND1000, respectively. One microgram of total RNA was synthesized into Cdna using Transcriptor High Fidelity Cdna Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions and subjected to PCR with primers (Sigma-Genosys) specific for PPARγ1 and PPARγ2 transcripts. PCR (25 cycles) was performed (pERKinElmer PE2400) under the following conditions: 94°C, 30 s; 58°C, 20 s; 72°C, 20 s. PCR products were analyzed in 2% agarose gels in Tris-acetate-EDTA buffer with base pair marker.

Quantitative PCR. Individual hippocampi were collected from 4 animals (male and female) of each group (WT untreated, untreated Tg2576, RSG-treated Tg2576) and suspended in 20-fold excess (w/v) TRIzol (Invitrogen). The tissue was homogenized in a 1 ml Dounce homogenizer on ice and RNA extracted according to the manufacturer's instructions. Quality control assessment of total RNA was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies) as well as *A*260/*A*280 and *A*260/ *A*230 nm ratio analyses using NanoDrop technology (Thermo Scientific). Cdna was synthesized from 5 µg of hippocampal mRNA using Superscript III (Invitrogen) according to the manufacturer's instructions. Individual animal mRNA was quantified for a custom array of predominantly PPRE-containing PPARγ genes on 1 µl of Cdna using a Roche LightCycler 480 and LightCycler 480 SYBR Green I Master reagent (Roche Applied Science) in the University of Texas Medical Branch Mo- lecular Genomics Core Facility. All oligos (Table 1) were purchased from Integrated DNA Technologies Δ*C*T values were calculated by subtracting the average *C*T of three housekeeping genes (*GAPDH*, *Rpl19*, and *Bpol*) from each gene of interest and the ΔΔ*C*T method (Applied Biosystems) was used to

calculate fold-change values between treatment groups. –Δ*C*T values are shown (Figs. 4.1*B*, 4.2*D*) to indicate increased number of mRNA transcripts.

Quantitative mass spectrometry. Stable isotope labeling was used to quantify differential protein expression as previously described (Sadygov et al., 2010; Starkey et al., 2010). Briefly, the dentate gyrus from 10 mice each of Tg2576 fed control or RSG diet were homogenized in TRIzol and the protein pellet resuspended in guanidine. Following reduction and alkylation, proteins were digested with trypsin and peptides desalted with SepPack C18 cartridges. Dried peptides were then treated with immobilized trypsin (Applied Biosystems) in normal water ($H_2{}^{16}O$) or heavy water ($H_2{}^{18}O$) for trypsin-mediated exchange of oxygen atoms from water onto the C-terminus of peptides. Desalted peptides were then pooled to prepare a mixture of ^{16}O-labeled peptides from control-fed mice and ^{18}O-labeled peptides from RSG-fed mice. To reduce the sample complexity and increase the depth of analysis into the proteome, the peptide mixture was resolved into 60 fractions using strong cation exchange chromatography.

Two-dimensional liquid chromatography-tandem mass spectrometry. Each SCX fraction was injected onto a C18 peptide trap (Agilent), desalted, and eluted peptides separated on a reversed phase nano-HPLC column with a linear gradient over 120 min at 200 nl/min. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were performed with a LTQ linear ion trap mass spectrometer (ThermoFinnigan) equipped with a nanospray source. The mass spectrometer was coupled online to a ProteomX nano-HPLC system (ThermoFinnigan). The mass spectrometer was operated in the data-dependent tripleplay mode. In this mode, the three most intense ions in each MS survey scan were automatically selected for moderate resolution zoom scans which were followed by MS/MS. Each of the

peptide mixtures was repetitively analyzed by nano-HPLC-MS/MS three times. The acquired MS/MS spectra were searched with SEQUEST algorithm performed on the Bioworks 3.2 platform (ThermoFinnigan) using conservative filtering criteria of Sp≥300, ΔCn≥0.12, and Xcorr of 1.9, 2.0 and 3.0 for data from a singly, doubly or triply charged precursor ions, respectively. The zoom scan data were used to calculate the relative abundance ratios of ^{18}O-labeled peptide/ ^{16}O-unlabeled peptide pairs using MassXplorer (Sadygov et al., 2010). Peptides with charge >3, false discovery rate >3%, ^{18}O/^{16}O ratios <0.1 or >10, and reversed sequences were removed from further analysis. Calculated peptide ratios were $\log_2$ transformed and mean centered before statistical analysis. Significance was determined by assessed using the Wilcoxon rank-sum test with Benjamini-Hochberg false discovery rate correction for multiple testing comparisons as indicated (Benjamini and Hochberg, 1995).

Data were then analyzed through the use of the extensively curated Ingenuity Pathways Analysis (Ingenuity Systems) with a significance cutoff of $p \leq 0.05$ and ≥20%change in protein expression. Functional Analysis using Gene Ontology classifiers identified the biological functions that were most significant to the dataset. Right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function assigned to that dataset is due to chance alone. Network Analysis generates a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. All lines are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. Nodes are displayed with various shapes that represent the functional class of the gene product.

Total β-amyloid quantification. Cortex from 18 Tg2576 and 18 Tg2576 RSG-treated (male and female) was homogenized in 8x (volume by wet weight) 5 M guanidine HCl, 50 Mm

Tris HCl, Ph 8.0. Signal Select colorimetric sandwich ELISA (BioSource) for either human Aβ1–40 or Aβ1–42 was used in comparison to a standard curve.

Statistics. Data are reported as mean ± SEM. Statistical analyses were conducted using GraphPad Prism6. Where indicated, one- or two-way ANOVA was performed for group analyses followed by Tukey's, Bonferroni's, or Dunnet's *post hoc* comparison. Where appropriate, Student's *t* test was used for pairwise comparison. Correlations were determined by Pearson correlation test for linearity, and coefficient of variation was assessed by calculating the average percent deviation from the respective group mean. Significance was set to $p < 0.05$.

Table 7.1. Demographic and cognitive data for control and AD subject cortical samples

Case#	Diagnosis	Age at Onset	Age	Sex	PMI	Braak	Plaque	MMSE
1008	Control	---	77.4	F	12	0	4	>25
1525	Control	---	88.7	F	3	1	4	29
1029	Control	---	73	F	4	0	4	>25
767	Control	---	86	F	8	2	4	>25
1775	Control	---	85	M	38.5	3	3	28
1013	Control	---	>89	M	6	1	0	29
1052	Control	---	87.7	M	8	2	1	29
1766	AD	57.3	63	F	3.5	6	1	18
1770	AD	70.2	82	F	6.5	6	1	15
1811	AD	87.3	>89	M	18	6	2	21
1774	AD	n/a	>89	M	3.25	6	1	2
1742	AD	48.6	64	M	9.25	6	1	1
1777	AD	n/a	67	F	20.5	6	3	9
1827	AD	n/a	>89	F	5	6	2	16

n/a = not available

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VITA

Jordan Blaine Jahrling was born in Washington D.C. on November 17, 1986 to Peter Becker Jahrling and Daria Lynne Baldovin-Jahrling. He completed his high school education at Middletown High School in Middletown, Maryland in 2004. That same year he began his tenure at Emory University from which he ultimately earned a B.S. in Neuroscience and Behavioral Biology in May of 2008. During his undergraduate, Mr. Jahrling gained laboratory experience at the United States Army Medical Research Institute for Infectious Diseases (USAMRIID). After completing his B.S., Mr. Jahrling entered the Neuroscience Graduate Program at the University of Texas Medical Branch in pursuit of his Ph.D. Mr. Jahrling married Dr. Christina Palazzotta Jahrling in October of 2012. In 2011, he received the Peyton and Lydia Schapper Endowed Scholarship for the demonstration of outstanding professional and personal leadership in the fields of gerontology and health promotion. In 2013, Mr. Jahrling received the Dr. and Mrs. Seymour Fisher Award for Academic Excellence in Neuroscience for his first-author manuscript "Cognitive enhancement following PPARγ agonism is mediated by a hippocampal pERK-PPARγ complex during memory consolidation in Tg2576 Alzheimer's mice". He was also awarded the Leroy Olson Ph.D. Endowed Scholarship for merit and high academic standards and the Jen Chieh and Katherine Huang Scholarship for research excellence in neuroscience.

Education

B.S., May 2008, Emory University, Atlanta, Georgia

Publications

Jordan B Jahrling, Caterina M Hernandez, Larry A Denner, Kelly T Dineley "PPARγ associates with pERK during memory consolidation in rosiglitazone-treated Tg2576 Alzheimer's mice" Submitted for review to *Journal of Neuroscience* September 2013

Larry A Denner, Jennifer Rodriguez-Rivera, Jordan B Jahrling, J Russ Carmical, Sigmund J Haidacher, Rovshan Sadygov, Jonathan M Starkey, Heidi Spratt, Bruce A Luxon, Thomas G Wood, Kelly T Dineley "Cognitive Enhancement with Rosiglitazone Links the Hippocampal PPARγ and ERK MAPK Signaling Pathways" J Neurosci. 2012 Nov 21;32(47):16725-35

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Abstracts

ENDO 2013, San Francisco, June 15-18 2013. Kelly T Dineley, Jordan Jahrling, Wei Song, Caternina Hernandez, Sigmund Haidacher, Patrick R Griffin and Larry Denner "Next generation PPARγ modulators enhance memory in the Tg2576 model of Alzheimer's disease". Sunday, June 16, 2013: 11:15 AM-12:45 PM. Session: OR23-Metabolic & Stress Receptors in Energy Homeostasis

Alzheimer's Association International Conference, Vancouver, BC, Canada July 14-18 2012 Jordan B Jahrling, Larry Denner, Kelly T Dineley "PPARγ agonism promotes the formation of a nuclear pERK-PPARγ complex that mediates cognitive rescue in Tg2576 Alzheimer's mice"

2012 Keystone Symposia on Molecular and Cellular Biology, Whistler, BC, Canada. Kelly T Dineley, Jordan B Jahrling, Larry Denner "PPARγ agonism promotes the formation of nuclear PPARγ-pERK complexes that mediates cognitive rescue in Tg2576 Alzheimer's mice"

ENDO 2012: The Endocrine Society's 94th Annual Meeting & Expo. Larry Denner, Jordan B Jahrling, Kelly T Dineley "PPARγ Agonism Promotes the Formation of Nuclear PPARγ-pERK Complexes That Mediate Cognitive Rescue in Tg2576 Alzheimer's Mice"

Society for Neuroscience 2012 Oct 13-17, New Orleans, LA. Jordan B Jahrling, Larry A Denner, Kelly T Dineley "PPARγ Agonism Promotes the Formation of a Nuclear Hippocampal pERK-PPARγ Complex during the Memory Consolidation Phase that Mediates Cognitive Rescue in Tg2576 Alzheimer's Mice"

2011 Alzheimer's Association Annual Conference, Baylor College of Medicine, Houston, TX. Jordan B Jahrling, Jennifer Rodriguez-Rivera, Larry Denner, Kelly T