Adiponectin alters hippocampal synaptic function: a mechanism for amelioration of synaptic deficits in a mouse model of Alzheimer's disease

by

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Abstract

The hormone adiponectin, the most abundant adipokine, is an important regulator of metabolic function, and recent evidence suggests that adiponectin plays an essential role in the cross-talk between adipose tissue and brain function. Additionally, adiponectin receptors in the brain may serve as novel targets for central nervous systems disorders, including Alzheimer's disease (AD). In the current study, we provide evidence that adiponectin receptor signaling directly influences hippocampal synaptic function. First, we established that adiponectin receptors are located at the synapse, with AdipoR1 particularly enriched in the synaptic fraction. Next, we found that aged adiponectin knockout mice display deficits in behavioral tasks including novel object recognition. The behavioral alterations were mirrored by a reduction in hippocampal synaptic plasticity and basal synaptic transmission, and synaptic deficits were rescued by slice incubation with the adiponectin receptor agonist, AdipoRon. Our results, along with emerging results from other groups, suggested that adiponectin may support neuronal processes and that a reduction in adiponectin or adiponectin receptor signaling may lead to an AD-like pathology. Therefore, we hypothesized that activation of adiponectin receptors may rescue synaptic deficits in a mouse model of AD. We found evidence for dysregulation of adiponectin and AdipoR1 expression in the 3xTg AD model and that slice incubation in adiponectin or the adiponectin receptor agonist, AdipoRon, could rescue synaptic deficits. The rescue of some synaptic parameters by AdipoRon appeared to be via activation of 5' AMP-activated protein kinase (AMPK), and rescue was associated with alterations in glycogen synthase kinase beta (GSK3β) and glutamatergic receptors. Taken together, the results from the current study indicate that adiponectin receptor signaling influences hippocampal synaptic

function. This work contributes to a growing body of evidence which suggests a therapeutic potential for adiponectin receptor agonists in the prevention or treatment of AD.

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Chapter 1: Introduction to Dissertation

The hormone adiponectin is an adipokine with an emerging role in central nervous system disorders. Over the past ten years, our understanding of adiponectin as a neuroactive adipokine has greatly improved. However, much remains unknown regarding the effects of adiponectin receptor signaling in various regions of the brain. The first suggestions that adiponectin may play a neuroprotective or pathological role in Alzheimer's disease (AD) arose from various groups in the early 2010s (Thundyil et al. 2012; van Himbergen et al. 2012; Wan and Little 2014). As adiponectin is one of the major physiological regulators of insulin sensitivity, and insulin resistance in the brain is present in AD, it was hypothesized that adiponectin receptor signaling in the brain may improve insulin sensitivity and thus reduce AD pathology (Thundyil et al. 2012). Importantly, adiponectin receptors are expressed throughout the brain, with particularly high expression in the hippocampus (Yamauchi et al. 2003; Neumeier et al. 2007; Thundyil et al. 2012), an essential area for learning and memory and a key region in AD neuropathology. However, seemingly paradoxically, higher adiponectin serum concentrations were associated with an increased risk of AD, particularly in women (van Himbergen et al. 2012). In contrast, the pre-clinical studies at the time indicated a neuroprotective role of adiponectin against neurotoxic insults (Chan et al. 2012; Qiu et al. 2011; Zhang et al. 2011).

At the initiation of the current project, very little was known regarding the direct physiological effects of adiponectin in the brain or whether an adiponectin receptor agonist could influence neuronal processes. Thus, the initial aim of the dissertation project was to elucidate the role of adiponectin in hippocampal synaptic function and cognition. I hypothesized that a lack of adiponectin, through the use of adiponectin-knockout mice, would lead to memory deficits along

with alterations in synaptic plasticity, although I also considered the possibility that the lack of adiponectin may lead to the opposite effects (i.e. and enhancement of memory and synaptic plasticity). I found memory deficits along with synaptic alterations in aged adiponectin knockout mice (Chapter 3) (Bloemer et al. 2019), indicating that adiponectin signaling may contribute to hippocampal neuronal function. Concurrently, other groups reported findings that were in line with a positive role for adiponectin receptor signaling in synaptic functionality and memory. For example, adiponectin receptor knockdown led to an AD-like phenotype in terms of hippocampal-based memory deficits along with hippocampal neurodegeneration (Kim et al. 2017). Based on my results along with the emerging studies from other groups, I next hypothesized that enhancement of adiponectin receptor signaling may provide a therapeutic avenue for the prevention or treatment of AD.

At the initiation of the second part of my project, evidence was accumulating that adiponectin receptor signaling may ameliorate various pathologies of AD including Aβ/tau-mediated toxicity (Ali et al. 2015) and brain insulin resistance (Ng et al. 2016). Additionally, a peripherally injected adiponectin receptor agonist improved memory deficits in an AD mouse model (Shah et al. 2017). However, it was unknown whether enhanced adiponectin receptor signaling can directly influence synaptic deficits which are characteristic of AD and contribute to AD pathology (Mango et al. 2019). As my initial data indicated that adiponectin receptors are found at the synapse, and that acute activation of these receptors may enhance long-term potentiation (LTP), I hypothesized that an adiponectin receptor agonist would rescue synaptic deficits in an AD mouse model. To test this hypothesis, I utilized acute hippocampal slices from 3xTg mice, which express three mutations associated with familial AD (Chapter 4). Once rescue of LTP by an adiponectin receptor agonist was established, I hypothesized that the synaptic effects may be

mediated by 5' AMP-activated protein kinase (AMPK), because (1) AMPK is a major downstream signaling molecule for adiponectin receptors (Yamauchi et al. 2007) (2) other studies have indicated that AMPK is responsible for neuroprotective effects of adiponectin (Ali et al. 2015; Ng et al. 2016; Shah et al. 2017), and (3) AMPK is a key regulator of synaptic function (Yu et al. 2016; Marinangeli et al. 2018). From my results, I concluded that some, but not all, of the synaptic alterations induced by adiponectin receptor signaling appear to be mediated by AMPK. Additional studies are required to determine whether adiponectin receptor agonists can ameliorate AD pathology and improve synaptic deficits *in vivo* and to address other limitations of the current study (Chapter 5). This work contributes to a growing body of evidence which suggests a therapeutic potential for adiponectin receptor agonists in the prevention or treatment of AD.

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Chapter 2: Review of adiponectin and its therapeutic potential in Alzheimer's disease

1 Introduction

Adiponectin, the most abundant adipokine, was first described approximately twenty-five years ago. Since then, our understanding of the physiological role of adiponectin in diverse processes including insulin sensitivity, inflammation, and vascular processes, has greatly improved. Adiponectin's pleiotropic effects have prompted great interest in the potential for adiponectin receptor agonists in the therapeutic treatment of complex metabolic disorders including diabetes, obesity, and cardiovascular disease. More recently, research on adiponectin's role in the brain has produced a similar interest in the potential for targeting adiponectin signaling pathways for central nervous system disorders, especially those with overlapping components of metabolic dysfunction. In this review, I will discuss the current understanding of adiponectin and its receptors, adiponectin's role in the brain, and the therapeutic potential for adiponectin receptor agonists in Alzheimer's disease (AD).

2 Overview of adiponectin and the adiponectin receptors

2.1 Structure of adiponectin

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30) (Scherer et al. 1995), AdipoQ (E. Hu, Liang, and Spiegelman 1996), adipose most abundant gene transcript 1 (apM1) (K. Maeda et al. 1996), and gelatin-binding protein of 28 kDa (GBP28) (Nakano et al. 1996), is a 244-amino acid glycoprotein (247 amino acids in mice) composed of four regions, including a short N-terminal signal sequence, a variable sequence that differs between species, a collagenous domain, and the C-terminal globular domain (Figure 2.1) (Berg,

Combs, and Scherer 2002). The N-terminal signal sequence is responsible for the targeting of adiponectin to the endoplasmic reticulum (Radjainia et al. 2012), whereas the globular domain is essential for the binding of adiponectin to the adiponectin receptors (Otvos et al. 2011). The amino acid sequence from 149-166 (in human adiponectin) located in the globular domain may be the primary active site which binds to receptors (Otvos et al. 2011), although different oligomeric forms of adiponectin may lead to unique signaling pathways (Tsao et al. 2003). The globular domain is structurally similar to the complement component 1q, and adiponectin also has significant homology with tumor necrosis factor alpha (TNFα) and the brain-specific factor cerebellin (E. Hu, Liang, and Spiegelman 1996). Based on its structure, adiponectin is considered to be part of the complement 1q family (Kadowaki and Yamauchi 2005).

Full-length adiponectin (fAd) circulates in various multimeric forms including trimers, hexamers, and multimers containing 12 or more monomers (Waki et al. 2003). These forms are also referred to as low molecular weight (LMW), middle molecular weight (MMW), and high molecular weight (HMW) adiponectin, respectively. fAd is thought to circulate exclusively in these multimeric forms, as adiponectin monomers have not been observed under native conditions (Yu Wang et al. 2008). Additionally, globular adiponectin (gAd), which is detected in low levels in plasma and circulates as a trimer, is formed from cleavage by leukocyte elastase, which is secreted by activated neutrophils (Waki et al. 2005). The various forms of adiponectin appear to have diverse yet overlapping physiological activities and target tissues (T Yamauchi and Kadowaki 2008), somewhat complicating functional studies of the role of adiponectin in biological processes.

Multimerization of adiponectin occurs through formation of covalent and non-covalent interactions. Trimers can be considered the basic unit of circulating adiponectin, and these are

formed by hydrophobic interactions between specific amino acid residues in the globular domain and further stabilized by collagenous domain interactions (Waki et al. 2003). The fAd trimer has been described as a "ball-and-stick-like" structure consisting of a spherical globular head with a stalk representing the collagenous domains (Yu Wang et al. 2008). Trimers associate into hexameric and HMW adiponectin through disulfide bonding of the Cys36 residue (Cys39 in murine adiponectin) as mutations at this cysteine residue preclude the formation of adiponectin hexamers and HMW forms (Pajvani et al. 2003; Waki et al. 2003). Adiponectin hexamers are structurally described as parallel trimers with a main stalk and globular heads creating a "Y" shape (Tsao et al. 2003). HMW adiponectin has proven difficult to structurally characterize due to its heterogenous composition (i.e. 12-, 15-, and 18+ -mers) (Yu Wang et al. 2008). Importantly, other post-translational modifications including hydroxylation and glycosylation appear to be important for proper functionality of multimers, as bacterial expressed adiponectin shows altered structural and functional properties (Richards et al. 2006), highlighting the importance of the use of mammalian-expression systems for studying adiponectin.

2.2 Production and clearance of adiponectin

Adiponectin is produced primarily by adipocytes, although low levels of production have also been detected in some other tissues including the placenta (Caminos et al. 2005), airway epithelial cells (Miller et al. 2009), and the hypothalamus (Kaminski et al. 2014). Adiponectin plasma levels are quite high at 2-20 μg/mL on average in adults (Labcorp reference values), with females displaying circulating levels up to 2-fold higher than males (Pajvani et al. 2003; Yang et al. 2001). Plasma levels of adiponectin are markedly reduced in states of obesity and insulin resistance (Arita et al. 1999; Weyer et al. 2001). Secretion of adiponectin is tightly regulated within adipocytes, and oligomerization occurs prior to release. In the endoplasmic reticulum

(ER), the chaperone ER protein of 44 kDa (ERp44) is responsible for retaining trimeric adiponectin, which is thought to allow for further oligomerization (Z. V Wang et al. 2007). Conversely, the oxidoreductase Ero1-Lα is responsible for mediating the release of adiponectin through disrupting the interaction between adiponectin and ERp44 (Z. V Wang et al. 2007). The balance between ERp44 and Ero1-Lα appears to regulate the plasma ratio of the oligomeric forms of adiponectin (Qiang, Wang, and Farmer 2007). The ER protein disulfide-bond A oxidoreductase-like protein (DsbA-L) also promotes multimerization and secretion of adiponectin (M. Liu et al. 2008), possibly though interactions with Ero1-Lα (M. Liu et al. 2015).

A number of mediators alter the secretion and oligomerization of adiponectin. The inflammatory cytokine TNFα, which is upregulated in obesity, is a powerful negative regulator of secretion and oligomerization of adiponectin, likely due to reduced expression of the chaperones Ero1-Lα, ERp44, and DsbA-L (He et al. 2016). Testosterone reduces plasma levels of HMW adiponectin without altering mRNA levels of adiponectin (Nishizawa et al. 2002; A. Xu et al. 2005), suggesting alterations in secretion of adiponectin multimers. This may explain the sexual dimorphism present in adiponectin serum concentrations. The direct influence of insulin on adiponectin secretion is unclear, as some studies have found and an increase in adiponectin secretion (Blümer et al. 2008; Scherer et al. 1995) while others have found a reduction (Fasshauer et al. 2002), but the differential effects may be time-based. An acute increase in insulin may lead to an increase in adiponectin production whereas chronic hyperinsulinemia may lead to a reduction.

Expression of adiponectin is controlled by various transcription factors including peroxisome proliferator-activated receptor gamma (PPARγ) and forkhead box protein O1 (FOXO1). PPAR response elements are found on the adiponectin promoter, and PPARγ is a major regulator of the

gene expression of adiponectin. The PPARγ agonist class of drugs, used in treatment of diabetes, leads to an increase in adiponectin production (N. Maeda et al. 2001; Iwaki et al. 2003). The PPARγ agonists may specifically increase the HMW form of adiponectin through increased expression of ERp44 and Ero1-Lα (Bodles et al. 2006; Qiang, Wang, and Farmer 2007). FOXO1 increases the gene expression of adiponectin by interacting with CCAAT/enhancer-binding protein alpha (C/EBPα) to form a transcription complex at the adiponectin promoter. Sirtuin 1, which is reduced in states of obesity, positively regulates the formation of the FOXO1-C/EBPα complex and upregulates adiponectin gene transcription (Qiao and Shao 2006). Negative regulators of adiponectin gene expression include TNFα (Hector et al. 2007) and interleukin-6 (IL-6) (Fasshauer et al. 2003).

Mechanisms for adiponectin clearance are not fully elucidated, but clearance through the liver appears to be the major pathway for the breakdown of adiponectin (Halberg et al. 2009). Degradation in circulation seems unlikely due to the stability of plasma adiponectin (Pischon, Hotamisligil, and Rimm 2003), although low levels of excretion of native adiponectin through the kidneys cannot be ruled out. Interestingly, different oligomeric forms of adiponectin appear to be cleared at different rates in mice based on half-life values of exogenously administered adiponectin ($T_{1/2}$ of ~32 minutes for adiponectin trimers, and $T_{1/2}$ of ~85 minutes for HMW adiponectin, with no difference between $T_{1/2}$ in male vs. female mice) (Halberg et al. 2009). In a human study which did not account for differences in oligomeric forms of adiponectin, the $T_{1/2}$ of adiponectin in adult females was indirectly calculated to be approximately 2.5 hours (Hoffstedt et al. 2004). Plasma adiponectin levels show diurnal and ultraradian variations with fluctuations within approximately ±20% of the mean concentration, and nadir occurring early in the morning (Yildiz et al. 2004; Gavrila et al. 2003). Current evidence suggests that reduction in

plasma levels of adiponectin related to metabolic dysfunction may be primarily due to alterations in production and secretion rather than enhanced clearance (Halberg et al. 2009).

2.3 Adiponectin receptors

The receptors for adiponectin include AdipoR1, AdipoR2, T-cadherin (also called Cadherin-13), and AdipoR3 (also called PAQR3) (Figure 2.2) (Garitaonandia et al. 2009). AdipoR1-3 are in the Progestin and AdipoQ receptor (PAQR) family of proteins that contains at least 11 receptors (Kupchak et al. 2009), whereas T-cadherin is unique a member of the T-cadherin superfamily which lacks the transmembrane and cytoplasmic domains and is instead anchored through glycosylphosphatidylinositol (GPI) (Sternberg et al. 2017).

AdipoR1 and AdipoR2 are plasma membrane receptors that have a unique membrane topology characterized by seven transmembrane domains with an intracellular N-terminal and an extracellular C-terminal (Toshimasa Yamauchi et al. 2003). Thus, their structure is similar to the G-protein coupled receptors, but their orientation in the membrane is flipped, and they do not interact with G-proteins. AdipoR1 and AdipoR2 have been the most well-studied adiponectin receptors, and these receptors are essential for the established metabolic effects of adiponectin including regulation of insulin sensitivity, anti-inflammatory effects, and anti-atherosclerotic effects (Fang and Judd 2018).

The crystal structures of AdipoR1 and AdipoR2 were determined recently (Figure 2.3). The transmembrane domains of these receptors are arranged in a clockwise manner to create a cavity which contains three histidine residues (Tanabe et al. 2015). A zinc ion binds these highly conserved residues and likely functions to stabilize the structure of the receptors, although the zinc ion may also be important for signal transduction in AdipoR2 (Tanabe et al. 2015). The

intracellular residues are quite different between AdipoR1 and AdipoR2, which may relate to distinct signaling pathways for these receptors. In contrast, the main extracellular adiponectin binding site appears very similar between AdipoR1 and AdipoR2 (Tanabe et al. 2015); however, slight differences in residues here may cause AdipoR1 and AdipoR2 to have different affinities for adiponectin. Scatchard plot analysis has suggested that AdipoR2 has two intermediate affinity binding sites (K_d 0.8 µg/ml and K_d 6.7 µg/ml), whereas AdipoR1 has a high affinity binding site (K_d 0.06 µg/ml) and a low affinity binding site (K_d 329 µg/ml) (Toshimasa Yamauchi et al. 2003). It was suggested that gAd binds with a high affinity to AdipoR1 and an intermediate affinity to AdipoR2, and that fAd has an intermediate affinity for AdipoR2 and a low affinity for AdipoR1. However, oligomeric differences in affinity for the receptors are not elucidated. It is important to note that the affinity data was obtained using bacterially expressed adiponectin, which has since been found to display altered functional and structural properties (Richards et al. 2006). Importantly, one study found that mammalian expressed full-length adiponectin, but not bacterially expression full-length adiponectin, binds to T-cadherin (Hug et al. 2004). Thus, a re-examination of the affinity of adiponectin for AdipoR1 and AdipoR2, along with examination of differences in binding affinities for different oligomeric forms is needed.

AdipoR3 is located in the Golgi apparatus rather than the plasma membrane, where it regulates cholesterol homeostasis (D. Xu et al. 2015) and is thought to function as a tumor suppressor (X. Wang et al. 2012). AdipoR3 also appears to modulate energy homeostasis as demonstrated by a study utilizing AdipoR3 knockout mice (L. Wang et al. 2013), but an *in vivo* role for adiponectin-mediated signaling via this receptor is not established.

T-cadherin is not structurally similar to the AdipoRs, but instead appears to function as an adiponectin binding protein which allows for accumulation of adiponectin at the site of action in vascular endothelium and skeletal muscle (Toshimasa Yamauchi et al. 2014). In cardiomyocytes, T-cadherin is required for the association of adiponectin with cardiac tissue and thus is essential for adiponectin-mediated cardioprotection (Denzel et al. 2010). T-cadherin binds hexameric and HMW adiponectin but does not appear to bind trimeric and globular adiponectin (Hug et al. 2004). Scatchard plot analysis revealed an intermediate binding affinity for T-cadherin to purified serum adiponectin (K_d 0.5 μg/ml), without differentiating for various forms (Fukuda et al. 2017).

2.4 Adiponectin receptor signaling

AdipoR1 and AdipoR2 appear to have diverse but overlapping downstream signaling pathways (Figure 2.4). The adaptor protein, APPL1 [Adaptor protein containing a Pleckstrin homology (PH) domain, Phosphotyrosine binding (PTB) domain and Leucine zipper motif] interacts with the intracellular domain of AdipoR1 and AdipoR2 to mediate signal transduction (Mao et al. 2006). APPL2 is an isoform of APPL1 which shares 54% homology in amino acid sequence and acts as a negative regulator of adiponectin signal transduction. APPL1 and APPL2 are similar in structure and contain an N-terminal Bin/Amphiphysin/Rvs (BAR) domain, a central PH domain, and a C-terminal PTB domain (Miaczynska et al. 2004). However, APPL1 and APPL2 appear to bind to the AdipoRs via different domains. It is suggested that APPL1 binds to AdipoR1 via its PTB domain based on yeast two-hybrid assays and further supported by co-immunoprecipitation experiments (Mao et al. 2006). Despite determination of the crystal structure of APPL1, the exact mechanism for this interaction remains unclear (Li et al. 2007). In contrast, APPL2 appears to bind to AdipoR1 via the BAR domain, as deletion of this domain leads to loss of this

interaction (Wang et al. 2009). Despite interactions via different domains, APPL2 binding to AdipoR1 competitively inhibits the binding of APPL1 (Wang et al. 2009). Additionally, APPL2 and APPL1 form heterodimers via interaction of the BAR domains, which prevents APPL1 binding to AdipoRs (Wang et al. 2009). Thus, Wang et al. proposed the 'Yin and Yang' concept for the influence of APPL1 and APPL2 on adiponectin receptor signaling. The major downstream modulators of AdipoR1 and AdipoR2 activation are 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α (PPARα) (Toshimasa Yamauchi et al. 2007).

AMPK is recognized as an important mediator of energy homeostasis (Hardie, Ross, and Hawley 2012). AdipoR-mediated AMPK activation can occur through APPL1-dependent and APPL1-independent mechanisms. In myocytes, adiponectin-induced activation of APPL1 leads to activation of protein phosphatase 2A (PP2A) and inactivation of protein kinase Cζ (PKCζ), which thus results in dephosphorylation of liver kinase B (LKB1) at Ser307 (Deepa et al. 2011). This allows translocation of LKB1 which activates AMPK via phosphorylation at Thr172 (Zhou et al. 2009). Adiponectin can also lead to activation of AMPK though an APPL1 independent mechanism via calcium signaling from intracellular or extracellular locations (Iwabu et al. 2010). In myocytes, adiponectin induces activation of phospholipase C leading to production of inositol 3-phosphate (IP3), causing calcium release and activating Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), which then activates AMPK (Deepa et al. 2011) via phosphorylation (Woods et al. 2005).

AdipoR2 signaling increases the expression of PPARα ligands thus increasing acetyl CoA oxidase (ACO) and uncoupling proteins (UCPs), which play a role in fatty acid oxidation (Ruan and Dong 2016). The exact mechanisms underlying activation of PPARα in response to AdipoR

signaling is unclear. In general, PPARα is activated by fatty acid derivatives such as those formed during lipolysis (Pawlak, Lefebvre, and Staels 2015). Some evidence suggests that activation of PPARα is dependent on activation of AMPK (Yamauchi et al. 2007), while other evidence suggests that activation of PPARα occurs in an AMPK independent manner (Yoon et al. 2006).

Activation of ceramidase through AdipoR1 and AdipoR2 is also an important physiological action of these receptors (Holland et al. 2011). The enzyme ceramidase is responsible for converting potentially harmful ceramides into the sphingosines including sphingosine 1-phosphate (S1P) (Holland and Summers 2008). Recently, it was determined that AdipoR2 contains intrinsic ceramidase activity via a tunnel which allows ceramide entry followed by hydrolysis (Vasiliauskaité-Brooks et al. 2017). Although AdipoR1 lacks this structural component, it also appears to possess ceramidase activity through unknown mechanisms (Vasiliauskaité-Brooks et al. 2017). The ceramidase promoting activity of adiponectin receptors is proposed to be independent of AMPK activation and represents an additional mechanism by which adiponectin receptor signaling can regulate energy homeostasis (Holland et al. 2011; Kadowaki and Yamauchi 2011).

3 Adiponectin in the brain

3.1 The source and oligomeric distribution of adiponectin in the brain

The adiponectin level in the cerebral spinal fluid (CSF) is as little as 0.1-1% of the serum concentration, (Neumeier et al. 2007; Kos et al. 2007; Kusminski et al. 2007). Trimeric and hexameric adiponectin are the primary oligomers found in the CSF (Figure 2.5) (Ebinuma et al. 2007; Kusminski et al. 2007). HMW and globular adiponectin may also be present but at very

low levels (Ebinuma et al. 2007). Females display slightly higher levels of CSF adiponectin compared to males (Neumeier et al. 2007; Qi et al. 2004). However, there does not appear to be a difference in the distribution of adiponectin oligomers in CSF for males and females. This may be because in serum, the difference in distribution of oligomers is thought to be due to a higher proportion of HMW adiponectin in women (Aso et al. 2006), and HMW adiponectin is not present in significant quantities in the brain.

Although the source of adiponectin in the brain has been controversial, current evidence suggests that the adiponectin found in the brain is derived from production in the periphery by adipocytes. Initially, it was believed that adiponectin does not cross the blood brain barrier (BBB), after a report that radiolabeled bacterially expressed globular adiponectin injected by intravenous route was not detected in the brain (Spranger et al. 2006). However, a different group showed that administration of wild-type mammalian-expressed adiponectin by intravenous route led to a 2-fold increase in adiponectin serum levels along with a 3-fold increase in adiponectin CSF levels (Qi et al. 2004), and another group has demonstrated that labeled adiponectin is detected in the brain (Neumeier et al. 2007). Additionally, in adiponectin knockout (APN-KO) mice, trimeric adiponectin injected via intravenous route was detected in the CSF following administration, indicating entry through the BBB (Yau et al. 2014). Interestingly, brain adiponectin levels may fluctuate independently from peripheral levels, as a physical activity induced a 25% increase in adiponectin levels in hippocampal tissue in the absence of significant alterations in serum adiponectin levels (Yau et al. 2014). Further studies have revelated that adiponectin in the CSF is positively correlated with the CSF albumin/serum albumin ratio, (Neumeier et al. 2007; Schön et al. 2019) which indicates BBB permeability.

Although some studies have failed to detect adiponectin mRNA in total brain tissue (Neumeier et al. 2007; Spranger et al. 2006), the possibility for localized production of adiponectin within specific areas of the brain cannot be ruled out. Adiponectin mRNA has been detected in murine pituitary cells (Rodriguez-Pacheco et al. 2007; Wilkinson et al. 2007) and porcine hypothalamus (Kaminski et al. 2014). The possibility for intrathecal synthesis of adiponectin has also been suggested (Thundyil et al. 2012).

Thus, the current consensus in the field is that peripherally produced adiponectin crosses the BBB and is the main source of CNS adiponectin. It is possible that the previously mentioned study failed to detect radiolabeled adiponectin due to its production in a bacterial expression system or due to insufficient injection amount.

3.2 Adiponectin receptors in the brain

Adiponectin receptors are expressed throughout the brain where they play a role in various processes such as neurogenesis and regulation of energy homeostasis (Qi et al. 2004; Yau et al. 2014). AdipoR1 and AdipoR2 are expressed widely in the brain (Toshimasa Yamauchi et al. 2003; Neumeier et al. 2007) including in the brain microvasculature (Spranger et al. 2006), cortex (Rastegar et al. 2019), hypothalamus (Hoyda et al. 2007), brainstem (Fry et al. 2006), pituitary glands (Rodriguez-Pacheco et al. 2007; Wilkinson et al. 2007), and hippocampus (Rastegar et al. 2019). In general, AdipoR1 appears to have a higher expression level in the brain compared to AdipoR2 (Thundyil et al. 2012), and these receptors are expressed in both neurons and glial cells. AdipoR1 is more highly expressed in neurons compared to astrocytes and microglia, whereas AdipoR2 is more highly expressed astrocytes and microglia compared to neurons (Thundyil et al. 2012; Guillod-Maximin et al. 2009; Jian et al. 2019). T-cadherin is also

widely expressed in the brain, in both neurons and astrocytes (Huang et al. 2003; Takeuchi et al. 2002). T-cadherin shows especially high expression levels in the amygdala, hippocampus, and cortex, where it may play a role in the maintenance of neuronal circuitry (Takeuchi et al. 2002). Thus, adiponectin receptors are widely expressed throughout the brain.

3.3 Considerations for adiponectin activity in the brain

Based on the concentration of endogenous adiponectin found in the CSF and the reported dissociation constants for adiponectin receptors, it is unclear whether baseline levels of adiponectin oligomers found in the brain are sufficient to activate the receptors. One possibility is that CSF levels do not accurately reflect adiponectin levels in the extracellular fluid (ECF) (De Lange 2013). The concentration of adiponectin in ECF would more accurately reflect the concentration at the site of action for adiponectin receptors located on neuronal and glial cells in the brain (De Lange 2013). Thus, sampling of ECF could reveal the local concentrations of adiponectin in various brain regions. Another possibility is that the reported dissociation constants for adiponectin receptors may be inaccurate due to the use of bacterially-produced forms of adiponectin in the original study (Toshimasa Yamauchi et al. 2003), as bacterially produced adiponectin shows alterations in structural and functional properties (Hug et al. 2004; Richards et al. 2006). Additionally, the oligomeric distribution of adiponectin in the brain differs substantially from that in plasma, and dissociation constants for various oligomeric forms of adiponectin are unknown.

Although local levels of adiponectin in specific brain regions are not known, adiponectin uptake into specific areas of the brain which express adiponectin receptors has been suggested (Neumeier et al. 2007; Schön et al. 2019). In one rodent study, after 2 weeks of running, hippocampal adiponectin was about 25% higher with no significant change in circulating

adiponectin levels, suggesting a local increase in adiponectin levels in hippocampal tissue (Yau et al. 2014). A recent study in humans looked at running-induced alterations of CSF levels of 174 cytokines and found that adiponectin displayed the largest degree of exercise-induced change. Approximately 20-40% reduction in CSF adiponectin was observed after a 90-minute run in healthy adults. Authors suggested that the acute reduction in adiponectin CSF levels indicates adiponectin uptake by the brain, since the observed change in adiponectin CSF levels greatly exceeded that expected based on BBB permeability (Schön et al. 2019). Uptake by the brain occurs for some hormones such as insulin in response to physical activity (Blázquez et al. 2014). Taken together, the results of Yau and Schön suggest a potential uptake of adiponectin into brain tissue in response to exercise.

Potential mechanisms for uptake from the CSF are unclear. In the circulation, adiponectin appears to be concentrated at some target tissues through interaction with T-cadherin. In T-cadherin knockout mice, circulating adiponectin levels are increased whereas adiponectin association with cardiac tissue is no longer detected (Denzel et al. 2010), suggesting an important role of T-cadherin in concentrating adiponectin at cardiac tissue. T-cadherin is highly expressed in various regions of the brain, including the hippocampus, an important area for learning and memory (Rivero et al. 2015). Of the adiponectin oligomers found in the brain, current evidence suggests that hexameric but not trimeric adiponectin binds to T-cadherin (Hug et al. 2004). Thus, the possibility of a yet-to-be discovered binding protein for adiponectin in the brain has also been suggested (Neumeier et al. 2007). Much remains unknown regarding adiponectin in the brain and whether there are mechanisms in place to increase adiponectin concentrations at target tissues in response to physiological events.

4 The therapeutic potential of adiponectin in Alzheimer's disease (AD)

4.1 Introduction to adiponectin and AD

Adiponectin is well-known for its pleiotropic actions including increased insulin sensitivity, anti-inflammation, and improved vascular health (Fang and Judd 2018). Thus, adiponectin signaling pathways have been studied for potential beneficial effects in various diseases including diabetes, obesity, and atherosclerosis. Recent evidence suggests that adiponectin signaling pathways may also be beneficial in central nervous system disorders including stroke, depression, anxiety, and Alzheimer's disease (AD) (Bloemer et al. 2018). Adiponectin appears to have neuroprotective effects against amyloid beta (Aβ) mediated toxicity (Chan et al. 2012), and targeting adiponectin signaling pathways for the treatment for AD has been proposed (Ng and Chan 2017). However, the role of adiponectin in AD is controversial, because some studies have uncovered a positive association between adiponectin serum levels and the risk for Alzheimer's disease (Une et al. 2011; Shafique, Ishtiaque, and Arain 2014). Despite this, recent scientific literature supports a neuroprotective role of adiponectin receptor signaling in AD (Ng and Chan 2017). AD is a complex disease with multiple pathological mechanisms (Hara, McKeehan, and Fillit 2019), many of which may be targeted by adiponectin receptor signaling. Neuroinflammation, dysregulated autophagy, brain insulin resistance, vascular alterations, and synaptic dysfunction, along with Aβ /tau mediated toxicity are all drivers of AD pathology (Hara, McKeehan, and Fillit 2019) which may be rescued by adiponectin receptor signaling pathways. Thus, there has recently been a high level of interest in studying adiponectin or adiponectin receptor agonists in AD.

4.2 Evidence for altered adiponectin levels in AD

There are contrasting reports on the association between serum adiponectin levels and cognitive impairment or AD. The first major report of an association between serum adiponectin and AD came from the Framingham Heart Study cohort. The Framingham Heart study, which was launched in 1948, is one of the largest population health studies. This study found that in women, elevated circulating adiponectin levels are associated with an increased risk of developing AD (van Himbergen et al. 2012). This association was not observed in men, but the lack of association might have been due to inadequate sample size. Authors of this study noted that the results were surprising, since adiponectin improves insulin sensitivity and brain insulin signaling dysregulation occurs in AD. Thus, they suggested that adiponectin levels may be increased as a protective response to brain pathology. In a small study of Japanese patients, adiponectin serum levels were increased in both mild cognitive impairment (MCI) and AD patients (Une et al. 2011). Additional studies have found an increase (Khemka et al. 2014; Waragai et al. 2016), decrease (Teixeira et al. 2013), or no change (Bigalke et al. 2011; Dukic et al. 2016; Warren, Hynan, and Weiner 2012) in adiponectin serum levels in MCI or AD. Interestingly, adiponectin gene polymorphisms associated with lower plasma adiponectin levels have been linked to a higher risk of late-onset AD (Z. Yu et al. 2015).

The reason for differences in the study results for associations between adiponectin levels and MCI/AD is unknown, but many factors influence circulating adiponectin levels. For example, adiponectin serum levels have a strong negative correlation with body mass index (Lubkowska et al. 2015), and weight loss often precedes a diagnosis of AD (Cova et al. 2016). However, in the Framingham Heart study, the correlation between increased adiponectin serum levels and AD was still present after controlling for body mass index and weight change. Adiponectin serum

levels are also increased by intake of omega-3 polyunsaturated fatty acids (Mostowik et al. 2013) and certain medications including acetylcholinesterase inhibitors used to treat AD (Pákáski et al. 2014), the anti-diabetic thiazolidinediones (J. G. Yu et al. 2002), and lipid lowering drugs such as niacin (Plaisance et al. 2009; Westphal et al. 2007), fenofibrate (Rosenson 2009), and statins (Nakamura et al. 2007). Physical activity also a major factor in serum adiponectin levels, with fasting adiponectin levels increasing by more than two-fold following initiation of an exercise program in overweight males in one study (Kriketos et al. 2004). The majority of correlative studies of adiponectin serum levels and AD have not documented these factors as possible confounders, so there is a possibility that this may account for discrepancies among studies, especially in those with a small sample size. Of note, the largest sample size by far was found in the Framingham study, whereas the other studies have mostly been in small populations.

It is currently unknown whether differences in specific oligomeric isoforms of adiponectin may be responsible for alterations in serum levels in MCI/AD populations, or whether the ratio is similar to that found in healthy individuals. Currently, one study has looked into the correlation between HMW adiponectin in serum and incidence of dementia but found no correlation (Kitagawa et al. 2016). Total adiponectin levels and other oligomers were not measured, so it is possible the lack of correlation was due to a lack of association between total serum adiponectin and dementia in the study population.

A recent study provided a more in-depth assessment of adiponectin serum, CSF, and brain tissue levels in MCI and AD (Waragai et al. 2016). Waragai et al. reported a significant increase in serum adiponectin in MCI and AD compared to age-matched controls along with a non-significant upward trend with progression of MCI to AD (Figure 2.6). In contrast, a reduction in CSF adiponectin was observed in AD, while a non-significant downward trend was observed in

MCI, compared to age-matched controls (Figure 2.6). This suggests that alterations in serum adiponectin levels do not correlate with changes in CSF adiponectin levels in MCI/AD. Additionally, CSF adiponectin levels positively correlated with scores on the mini-mental status exam and inversely correlated with the severity of hippocampal atrophy in AD, suggesting that higher CSF adiponectin translates to a lower degree of pathology. Upon further dissection of adiponectin levels in the brain, the adiponectin trimer was significantly decreased in AD brains compared to normal controls, suggesting a possible downregulation of adiponectin signaling in AD. Interestingly, adiponectin was co-localized with neurofibrillary tangles (NFTs) in AD brains. The authors suggested that adiponectin may be sequestered by tau into NFTs in AD, leading to a reduction in free adiponectin trimers, a reduction in CSF adiponectin, and a reduction in adiponectin signaling (Waragai et al. 2016). It is possible that the concurrent increase in serum adiponectin may be a compensatory mechanism to increase adiponectin receptor signaling. It remains unclear whether the alterations in adiponectin levels reflect an active role in AD pathology, or rather a passive consequence of AD.

Very few studies have looked into alterations in adiponectin receptors in Alzheimer's disease. Using approximately 6-month old transgenic male AD mice [APP(swe)/Presen(e9d)], one group observed a reduction of hippocampal AdipoR1 expression relative to control mice but no change in AdipoR2 expression (Várhelyi et al. 2017). Evaluation of the effects of normal aging on brain adiponectin parameters in male rats (approximately 3-months, 9-months, and 24-months) revealed a reduction of adiponectin levels in the hippocampus with increasing age, along with an increase in AdipoR1 expression in the hippocampus (Yuan Sun et al. 2018). Thus, there appears to be age-related alterations in brain adiponectin signaling and changes in adiponectin receptor expression in models of AD.

Taken together, dysregulation of adiponectin seems to occur in MCI/AD. Whether this directly contributes to pathological processes involved in AD remains unclear. Some groups have suggested that adiponectin may contribute to AD pathology (Wan and Little 2014), however, the vast majority of studies suggest that increasing adiponectin signaling can serve as a therapeutic approach in AD (Ng and Chan 2017; Forny-Germano, De Felice, and Do Nascimento Vieira 2019).

4.3 Influence of adiponectin signaling on various AD pathologies

4.3.1 Adiponectin and insulin signaling

Insulin resistance in the brain is a prominent feature of AD (L. S. S. Ferreira et al. 2018), and adiponectin is a potent insulin sensitizing adipokine which reduces hepatic gluconeogenesis and increases insulin signaling in peripheral tissues (Fang and Judd 2018). Post-mortem brains from AD patients show biochemical signs of insulin resistance including increased phosphorylation of the insulin receptor substrate 1 (IRS-1) at Ser616 and Ser636, reduction in phosphorylation of Akt, and reduced insulin receptor (IR) expression (Talbot et al. 2012; Moloney et al. 2010; Yarchoan et al. 2014). There are a number of mechanisms by which insulin resistance can lead to AD-like pathologies, however, central to many of these hypotheses is that dysregulation of insulin signaling in the brain leads to increased activation of glycogen synthase kinase 3-beta (GSK3β), thus enhancing production of Aβ and pathogenic phosphorylation of tau (Hooper, Killick, and Lovestone 2008). Animal models of central insulin resistance such as intracerebral streptozotocin strongly mimic the AD phenotype including memory deficits, synaptic plasticity deficits, increased Aβ, and reduced cholinergic signaling in the brain (De La Monte and Wands 2008). Thus, traditional anti-diabetic drugs have been repurposed for treatment of AD in pre-

clinical studies and clinic trials (Boccardi, Murasecco, and Mecocci 2019), including the peroxisome proliferator-activated receptor gamma (PPARγ) agonists which increase the expression of adiponectin (Govindarajulu et al. 2018).

Adiponectin knockout mice show brain insulin resistance, demonstrated by increased pIRS1 Ser616 along with impaired ability of insulin to increase phosphorylation of Akt following intra-hippocampal injection of insulin (Ng et al. 2016). In the periphery, one mechanism by which adiponectin can directly improve insulin sensitivity is by inhibition of serine phosphorylation of IRS1 and promotion of Akt phosphorylation (C. Wang et al. 2007). Thus, a similar mechanism may be responsible for alterations in brain insulin signaling in adiponectin knockout mice. The adaptor protein APPL1, which is highly expressed in the brain (Mao et al. 2006), may also play a key role in cross-talk between adiponectin and insulin receptor signaling pathways (Ruan and Dong 2016). Following injection of pathogenic Aβ, pAkt Ser473 and pGSK3β Ser9 were reduced in the hippocampus, an effect which was rescued by treatment with the adiponectin receptor agonist osmotin (Ali et al. 2015). In neuronal cell culture, mammalian trimeric adiponectin enhanced insulin sensitivity, an effect which was blocked by the AMPK inhibitor compound C or transfection with siAdipoR1, but not blocked by transfection with siAdipoR2 (Ali et al. 2015). Therefore, the absence of adiponectin leads to brain insulin resistance, and adiponectin signaling may improve brain insulin signaling through AdipoR1-AMPK mediated pathway. Because brain insulin resistance appears to contribute to the pathophysiology of AD, enhancing insulin sensitivity is one mechanism by which adiponectin receptor signaling could reduce AD pathology.

4.3.2 Adiponectin and vascular health

Although vascular dementia and AD are recognized as distinct types of dementia, there is considerable overlap between the two, and intra-brain vascular dysfunction is recognized as a contributor to the development of late-onset AD (Iturria-Medina et al. 2016). In post-mortem AD brains, cerebrovascular dysfunction is observed including reduction in cerebral blood flow and alterations in microvasculature integrity (Attems and Jellinger 2014). Carriers of the apolipoprotein-E (APOE) ε4 allele, which is a well-known genetic risk factor for AD, are also at a higher risk of vascular diseases (Elosua et al. 2004). Therefore, there appears to be a significant vascular component to AD pathology.

Adiponectin reduces the development of vascular atherosclerosis, and there is a direct association between adiponectin and vascular function which is independent of insulin sensitivity (Fernández-Real et al. 2004). In the APOE knockout mouse model of atherosclerosis, which produces a similar but less severe phenotype compared to APOE4 models of AD (Safieh, Korczyn, and Michaelson 2019; Avdesh et al. 2011), adenovirus-mediated increase in adiponectin expression reduced atherosclerotic lesions and prevented the transformation of macrophages to foam cells (Okamoto et al. 2002). Both AdipoR1 and AdipoR2 mediated signaling are involved in the prevention of atherosclerotic lesions. Mechanisms by which adiponectin suppresses foam cell formation include AdipoR1-mediated suppression TNFα expression and AdipoR2-mediated suppression of scavenger receptor A type 1 (Tian et al. 2012).

Although the effects of adiponectin receptor signaling on vascular dysfunction in AD models is mostly unexplored, in ischemic stroke models adiponectin reduces cerebral infarct size and improves neurological function (Nishimura et al. 2008; Miao et al. 2013). Interestingly, it was

recently reported that APOE4 carriers have an increased serum concentration of adiponectin relative to other APOE alleles, although the association was relatively weak (Royall et al. 2017). This is in line with evidence that higher serum adiponectin levels are associated with AD as a protective response (van Himbergen et al. 2012). In the future, potential neuroprotective effects of adiponectin or an adiponectin receptor agonist in models of AD characterized by vascular dysfunction such as APOE4 transgenic mice should be explored.

4.3.3 Adiponectin and neuroinflammation

Chronic inflammation in the brain is a major feature of AD (Kinney et al. 2018). Although it was previously thought that inflammation may be a consequence of AD rather than a pathological driver, recent studies have highlighted the role of inflammation as a driving force for neurodegeneration in AD (Cribbs et al. 2012; Sudduth et al. 2013). Acute inflammation in the brain has a neuroprotective function, but in states of chronic inflammation, excessive activation of microglia leads to neurotoxicity (Kinney et al. 2018). In early stages of AD, activated microglia remove Aβ plaques through phagocytosis (Bolmont et al. 2008). However, with disease progression, microglia are unable to process Aβ effectively but continuously release inflammatory cytokines (Hickman, Allison, and El Khoury 2008). Some of the inflammatory cytokines which are linked to AD progression include TNFα as well as interleukin-1β (IL-1β) (Liao et al. 2004).

Adiponectin signaling leads to a reduction in inflammation, an effect that may be mediated by both AdipoR1 and AdipoR2 in peripheral macrophages (Mandal et al. 2011). Adiponectin knockout mice show exacerbated neuronal inflammation along with increased expression of TNFα, which was ameliorated through treatment with globular adiponectin (Piccio et al. 2013).

Similarly, adiponectin knockout mice show enhanced neuroinflammation related to increased microglia activation following lipopolysaccharide (LPS) challenge (Nicolas et al. 2017). In a cell culture model, globular adiponectin reduced LPS-induced upregulation of TNFα and IL-1β by an AdipoR1 dependent mechanism. Globular adiponectin also suppressed activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Nicolas et al. 2017). Thus, adiponectin receptor signaling appears to reduce neuroinflammation through a reduction in microglial activation, which could be beneficial in AD to reduce the pathogenic overactivation of microglia.

Recently, it was reported that adiponectin protects against Aβ oligomer-induced release of TNFα and IL-1β in microglial cells, an effect which was dependent on AMPK activation, as the AMPK inhibitor Compound C blocked the effects of adiponectin (Jian et al. 2019). The protective effects of adiponectin were also inhibited by siAdipoR1 but not siAdipoR2, suggesting that the reduction in cytokine release is dependent on an AdipoR1-AMPK pathway. Furthermore, adiponectin treatment of microglial cells protected hippocampal neurons from reduced viability caused by Aβ oligomer-induced release of inflammatory cytokines (Jian et al. 2019). Thus, adiponectin receptor signaling in microglial cells leads to a reduction in release of neurotoxic cytokines from microglia in response to Aβ oligomers. In an animal model, adiponectin deficiency exacerbated the upregulation of inflammatory cytokines TNFα and IL-1β in the brain of 5xFAD mice (Jian et al. 2019). Taken together, microglial adiponectin receptor signaling, particularly through AdipoR1, may reduce neuroinflammation in AD.

4.3.4 Adiponectin and autophagy

In AD, dysregulation of macroautophagy is thought to contribute to the accumulation of Aβ and hyperphosphorylated tau (Uddin et al. 2018). Macroautophagy is a process that contributes to homeostasis through encapsulation of damaged cellular components into an autophagosome, which can then transport the components to the lysosome for degradation and recycling (Glick, Barth, and Macleod 2010). In AD, dysregulation of autophagy may occur via decreased induction of autophagy and impaired formation of the autophagosome and autophagolysosome (Uddin et al. 2019). Additionally, in AD, the retrograde transport of autophagolysosomes may be inhibited leading to build-up of these autophagy intermediates (Nixon et al. 2005). Interestingly, autophagy in the presynaptic compartment may regulate neurotransmitter release (Nikoletopoulou and Tavernarakis 2018), which could have implications in glutamate-induced excitotoxicity. Drugs which stimulate autophagy, such as rapamycin, have shown promising therapeutic potential in pre-clinical studies (Uddin et al. 2018).

The role of adiponectin in brain autophagy has not yet been explored, however, adiponectin appears to increase autophagy in various peripheral tissues through AdipoR1/2 dependent mechanisms. Adiponectin was found to directly stimulate autophagy in skeletal muscle cells indicated by conversion of LC3-1 to LC3-2, which serves as a marker for autophagosome formation, and this effect was blocked by the AMPK inhibitor compound C (Y. Liu et al. 2015). In one study, adiponectin failed to improve insulin sensitivity in autophagy-deficient myoblasts (Ahlstrom et al. 2017), indicating autophagy is one mechanism for adiponectin-induced reduction in insulin resistance. Adiponectin-induced autophagy may protect against exogenous insults in hepatic cells, as adiponectin protected hepatocytes from ethanol-induced apoptosis (Nepal and Park 2013) and acetaminophen-induced mitochondrial damage (Lin et al. 2014)

through activation of autophagy. In rat chondrocytes, adiponectin suppressed hydrogen peroxide induced apoptosis by a mechanism partially dependent on activation of AMPK. Adiponectin also decreased phosphorylation of mammalian target of rapamycin (mTOR) (J. Hu et al. 2017), a well-known regulator of autophagy.

Thus, adiponectin appears to promote autophagy in various tissues, possibly through an AMPK-mTOR mediated pathway. Because dysregulation of autophagy is observed in AD, and the mTOR inhibitor rapamycin reduces AD pathology, adiponectin may also reduce AD pathology through autophagy-dependent mechanisms.

4.3.5 Adiponectin and sphingolipids

Sphingolipids are a class of cell-membrane lipids which are especially enriched in brain tissues and serve as important signaling molecules (Futerman and Hannun 2004). In AD, the balance between sphingolipids is altered such that there is an increase in pro-apoptotic ceramide and a decrease in the neuroprotective sphingosine 1-phosphate (S1P) (Czubowicz et al. 2019). The balance between ceramide and S1P influences various AD pathologies including the processing of amyloid precursor protein (APP), cell death signaling, inflammation, insulin resistance, neurotransmission, and autophagy (Czubowicz et al. 2019). In post-mortem AD brains, reduced levels of S1P in the hippocampus correlate with increasing Braak stage, indicating an inverse relationship between S1P and progression of AD (Couttas et al. 2014).

Activation of AdipoR1 and AdipoR2 increases ceramidase activity which reduces ceramide by converting it to sphingosine, which can then be converted to S1P. Interestingly, this increase in ceramidase activity appears to be independent of AMPK activation (Holland et al. 2011). In the peripheral tissues, adiponectin-induced ceramidase activity may be important for improving

glucose tolerance, increasing whole body insulin sensitivity, and opposing hepatic steatosis (Z. V. Wang and Scherer 2016). Whether ceramidase activity and activation of the traditional downstream signaling molecules of AdipoRs such as AMPK represent parallel or overlapping pathways in the pleiotropic actions of adiponectin is not entirely clear (Reibe-Pal and Febbraio 2017).

The ability of adiponectin receptor signaling to reduce ceramide content and increase the formation of S1P in the brain has not yet been explored. This presents another promising avenue by which adiponectin receptor signaling may reduce AD pathology.

4.3.6 Adiponectin and neurotransmission

AD is associated with synaptic changes including alterations in glutamatergic neurotransmission, which contributes to deficits in cognition and synaptic processes (Kandimalla and Reddy 2017). Synaptic alterations may be in part due to Aβ toxicity, as Aβ increases presynaptic release probability (Abramov et al. 2009), promotes downregulation of the synaptic surface expression of glutamatergic receptors (Miñano-Molina et al. 2011), and directly impairs long-term potentiation (LTP) (Chen et al. 2000) in hippocampal synapses. LTP, which has been termed the cellular correlate of learning and memory, is a type of synaptic plasticity characterized by an activity-dependent increase in synaptic strength, and impairment of LTP is a characteristic feature of animal models of AD (Mango et al. 2019).

Very recent evidence indicates that adiponectin rescues deficits in hippocampal LTP in the 5xFAD model of AD (M. Wang, Jo, and Song 2019), indicating a direct role of adiponectin receptor signaling at glutamatergic synapses. Furthermore, adiponectin incubation promoted surface expression of the glutamatergic receptors, N-methyl-D-aspartate (NMDA) and α-amino-

3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in hippocampal slices (M. Wang, Jo, and Song 2019). This indicates that adiponectin receptor signaling plays a role in glutamatergic receptor expression or trafficking, likely through activation of adiponectin receptors at hippocampal synapses. This is important, since glutamatergic receptor downregulation is observed in post-mortem AD brains (Yuehua Wang et al. 2000). In the dentate gyrus of the rat hippocampus, intracerebroventricular administration of adiponectin led to an increase in NMDAR-dependent LTP and a reduction in NMDAR-dependent long-term depression (LTD), suggesting that adiponectin receptor signaling may influence both processes (Pousti et al. 2018). Thus, adiponectin receptor signaling appears to play a role in synaptic processes, although the contribution of specific adiponectin receptors is not established. Although adiponectin receptors are found in hippocampal neurons, the synaptic localization of these receptors is unclear.

4.3.7 Adiponectin and Aβ pathology

Aβ plaques and NFTs are pathological hallmarks of AD. In the amyloid cascade hypothesis, the aggregation of pathogenic Aβ is thought to initiate the cascade of pathological events leading to clinical AD (Allsop and Mayes 2014). Although the current focus in pre-clinical research is beginning to shift away from the amyloid hypothesis in favor of a more comprehensive model, Aβ remains an important facet of the disease pathology.

The ability of adiponectin or adiponectin receptor agonists to protect neurons against Aβ-induced cytotoxicity has been studied in cell culture models. Adiponectin protected against Aβ-induced cytotoxicity in oxidative stress conditions in a neuroblastoma model by a mechanism dependent on AdipoR1 and AdipoR2 (Chan et al. 2012). It was suggested that the protection was mediated by suppression of the activation of NF-kB, thereby reducing neuroinflammation (Chan et al.

2012). The adiponectin receptor agonist osmotin also protected against an $A\beta_{1–42}$-induced reduction in cell viability in hippocampal cell culture (Ali et al. 2015). Thus, adiponectin receptor signaling may reduce neurotoxicity associated with pathogenic Aβ.

In addition to the neuroprotective effects, adiponectin receptor signaling (adiponectin or osmotin) also reduces expression of $A\beta_{1–42}$ (Ng et al. 2016; Shah et al. 2017). The reason for reduced expression following osmotin treatment appeared to be due to increased expression of the alpha secretase ADAM10, which is important in the non-amyloidogenic pathway of APP, an effect which was mediated by AMPK (Shah et al. 2017). In The APP/PS1 mouse model of AD, osmotin reduced expression of $A\beta_{1–42}$ along with increased expression of ADAM10 and soluble APPα (Shah et al. 2017). Taken together, adiponectin receptor signaling may reduce neurotoxicity associated with pathogenic Aβ and also reduce its expression in favor of the non-amyloidogenic pathway.

4.3.8 Adiponectin and tau pathology

Tau is a microtubule-associated protein which becomes abnormally phosphorylated and aggregates into NFTs in AD (Pooler et al. 2013). The progression of AD in terms of cognitive decline correlates will with progression of NFTs (Nelson et al. 2012). Hyperphosphorylation of tau is one of the first steps in abnormal tau processing along with oligomerization, spread, and aggregation (Šimić et al. 2016). Thus, therapies which target the abnormal processing of tau are proposed as potential treatments for AD (Šimić et al. 2016).

Adiponectin receptor signaling may reduce pathogenic tau phosphorylation through inactivation of GSK3β. The adiponectin receptor agonist, osmotin, reduced hippocampal pTau Ser413 and increased pGSK3β Ser9, which indicates reduced activation of GSK3β, following

intracerebroventricular injection of $A\beta_{1-42}$ (Ali et al. 2015). However, AMPK, which is an essential mediator of adiponectin receptor signaling, can phosphorylate tau and is implicated as a potential pathogenic mediator in AD (Thornton et al. 2011). Thus, further research is required to determine the how adiponectin receptor signaling influences tau phosphorylation.

Unexpectedly, adiponectin is co-localized with NFTs in post-mortem AD brains (Waragai et al. 2016). The implications of the co-localization are not clear, although the authors suggested that sequestration of adiponectin by tau may reduce neurotoxicity. Clearly, more studies are required to understand the potentially complex interactions between tau and adiponectin.

4.3.9 Utilization of adiponectin receptor agonists in models of AD

There are numerous potential mechanisms by which adiponectin receptor signaling may decrease AD pathology (Figure 2.7). However, only few studies have investigated the ability of an adiponectin receptor agonist to reduce overall AD pathology in rodent models. In one study, the adiponectin receptor agonist osmotin ameliorated neurotoxicity following intracerebroventricular injection of $A\beta_{1-42}$ (Ali et al. 2015). A single dose of osmotin administered by intraperitoneal route 3 or 40 days after $A\beta_{1-42}$ administration led to normalization of spontaneous alternation behavior during Y-maze along with reductions in Aβ and pTau. In a separate study by the same group, chronic osmotin treatment reduced neuropathological deficits in the APP/PS1 mouse model of AD (Shah et al. 2017). Treatment with osmotin improved performance in the Morris Water Maze task, a hippocampal based memory task, and improved synaptic plasticity (Shah et al. 2017). However, data for osmotin permeability through the BBB and resulting brain concentrations were not measured in either study, and it is unclear whether these effects are the direct result of adiponectin receptor activation in the brain. Future studies should confirm

whether the neuroprotective actions of osmotin are due to AdipoR1 and/or AdipoR2 signaling in the brain. Whether other adiponectin receptor agonists or adiponectin can reduce neuropathology in animal models of AD is not yet elucidated.

4.4 Considerations for development of adiponectin receptor agonists in AD

The development of small molecule compounds which activate adiponectin receptors is of great interest for adiponectin research. Utilization of the protein adiponectin as a therapeutic agent has a number of limitations including: (1) requirement of production in a mammalian expression system for normal functionality (Richards et al. 2006), (2) a relatively short half-life in serum (30-150 minutes) (Halberg et al. 2009; Hoffstedt et al. 2004), (3) potential differences between physiological effects of the various oligomeric forms of adiponectin (Lara-Castro et al. 2006), and (4) need for parenteral administration. Thus, development of small molecule agonists for adiponectin receptors is desirable.

Currently, there are two adiponectin receptor agonists which have been primarily studied in animal models: osmotin and AdipoRon. Osmotin is a 26-kDa antifungal protein produced by various plants which is typically isolated from the tobacco plant for research purposes (Anil Kumar et al. 2015). Osmotin shows structural and functional similarity to adiponectin (Miele, Costantini, and Colonna 2011) and acts as an agonist at AdipoR1 and AdipoR2, but it is not orally active (Ahmad et al. 2019). AdipoRon is a potent small-molecule agonist of AdipoR1 and AdipoR2 (Okada-Iwabu et al. 2013), which is orally active (Yamashita et al. 2018) and commercially available. Both AdipoRon and osmotin cross the BBB (Nicolas et al. 2018; Amin et al. 2017), and one group has demonstrated improved BBB penetration for osmotin through the use of magnetic nanoparticles (Amin et al. 2017).

Screening of a natural product library of 10,000 compounds revealed four agonists of AdipoR1, four agonists of AdipoR2, and one agonist of both AdipoR1 and AdipoR2 (Yiyi Sun et al. 2013), although it does not appear that these compounds have been tested as adiponectin receptor agonists in subsequent studies thus far. Recently, efforts have also been made to develop novel peptide adiponectin receptor agonists which selectively bind to AdipoR1 (Kim et al. 2018). When considering clinical utility in central nervous system disorders such as AD, the ideal adiponectin receptor agonist would likely be a small molecule which can be taken orally and cross the BBB to reach target brain areas, such as AdipoRon.

Potential adverse effects of adiponectin receptor agonists should be considered when developing these agents for clinical use. Adiponectin receptor signaling may reduce bone mineral density, as demonstrated in mice which overexpress adiponectin (Ealey et al. 2008). Likewise, the thiazolidinediones, a class of medications used to treat diabetes which increase adiponectin expression levels, carry an increased risk of fractures (Zhu, Jiang, and Ding 2014). Additionally, adiponectin receptor signaling appears to influence the female reproductive system. Although most studies indicate that adiponectin promotes fertility (Dobrzyn et al. 2018), the females in one transgenic mouse model which overproduces adiponectin show impaired fertility (Combs et al. 2004). Thus, the potential adverse effects of adiponectin receptor agonists should be carefully evaluated prior to the clinical use of these agents.

5 Conclusion

In summary, adiponectin is an important adipokine which influences numerous physiological processes and is increasingly recognized for its ability to influence the brain. Its role in neurodegeneration has been somewhat controversial due to a positive correlation between serum

adiponectin levels and AD. However, preclinical studies of adiponectin in various facets of AD pathology have indicated neuroprotective effects of enhanced adiponectin receptor activation. A number of key questions remain regarding adiponectin in the brain and the potential for adiponectin receptor agonists to reduce pathology, including the following: (1) How are adiponectin levels in the brain and CSF regulated? (2) What are the endogenous concentrations of adiponectin in extracellular fluid in various regions of the brain? (3) Are there physiologically relevant interactions between T-cadherin and adiponectin in the brain? (4) Why are adiponectin serum and CSF levels altered in AD? (5) What is the physiological relevance of adiponectin-tau co-localization in AD? And (6) How does adiponectin influence synaptic dysfunction and ultimately cognition in AD? Thus, there is much to learn regarding adiponectin in the brain and its therapeutic potential in AD, and further research on this topic will allow for a more comprehensive understanding of the numerous biological effects of adiponectin.

6 Figures

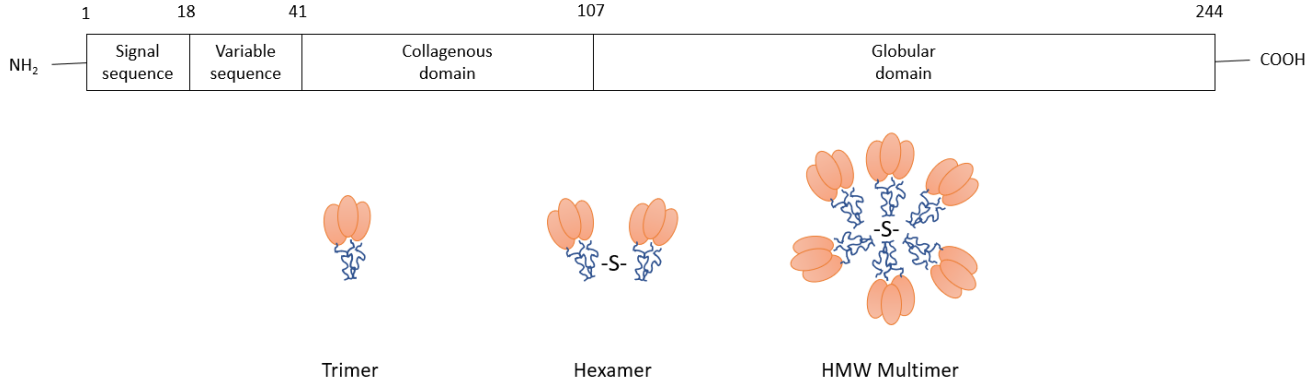

Figure 2.1 Structure of Adiponectin.

Human adiponectin contains 244 amino acids and consists of a N-terminal signal sequence which targets adiponectin to the endoplasmic reticulum, a variable sequence which contains a cysteine residue (Cys36) required for the formation of hexamers and high molecular weight multimers, a collagenous domain which contains a number of conserved proline and lysine residues required for physiological function, and a globular domain which is required for adiponectin binding to the AdipoRs. Adiponectin circulates as trimers, hexamers, and high molecular weight (HMW) multimers.

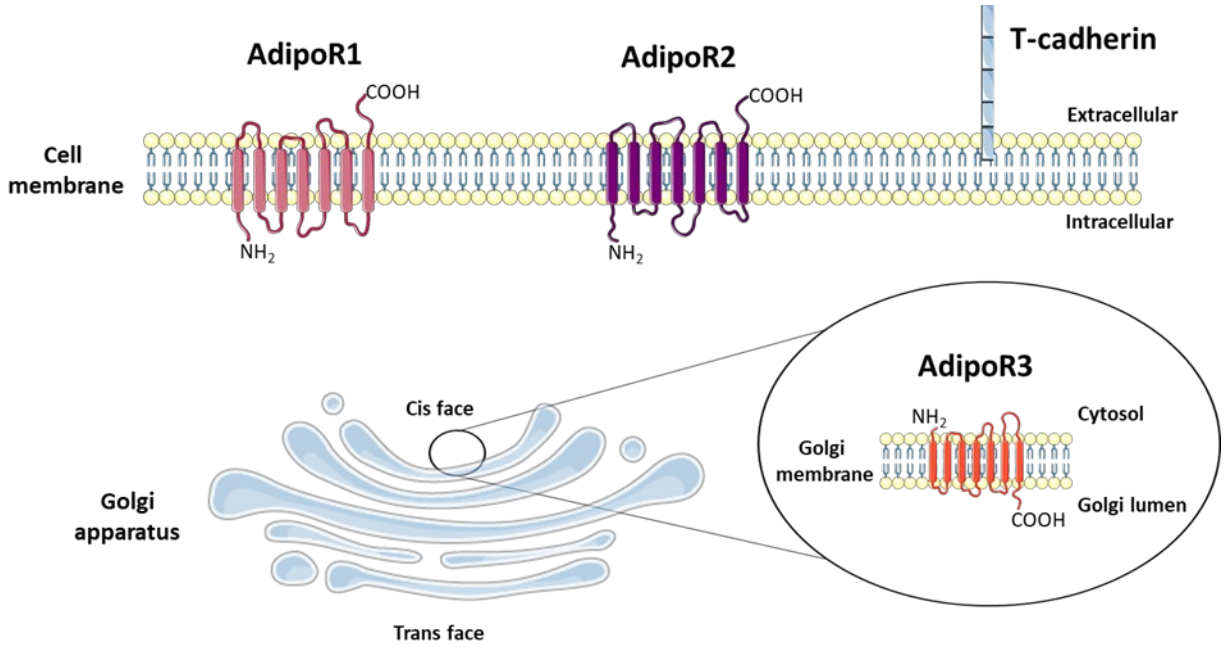

Figure 2.2 Adiponectin Receptors.

The receptors for adiponectin include AdipoR1, AdipoR2, T-cadherin (also called Cadherin-13), and AdipoR3 (also called PAQR3). AdipoR1-3 are 7-transmembrane receptors in the Progestin and AdipoQ receptor (PAQR) family of proteins, whereas T-cadherin is unique a member of the T-cadherin superfamily which lacks the transmembrane and cytoplasmic domains and is instead anchored through glycosylphosphatidylinositol. AdipoR1, AdipoR2, and T-cadherin are cell membrane receptors, whereas AdipoR3 is found in the cis face of the Golgi apparatus. AdipoR1 and AdipoR2 have a unique membrane topology with an intracellular N-terminal and an extracellular C-terminal. AdipoR3 has a cytosolic N-terminal and a C-terminal facing the Golgi apparatus lumen.

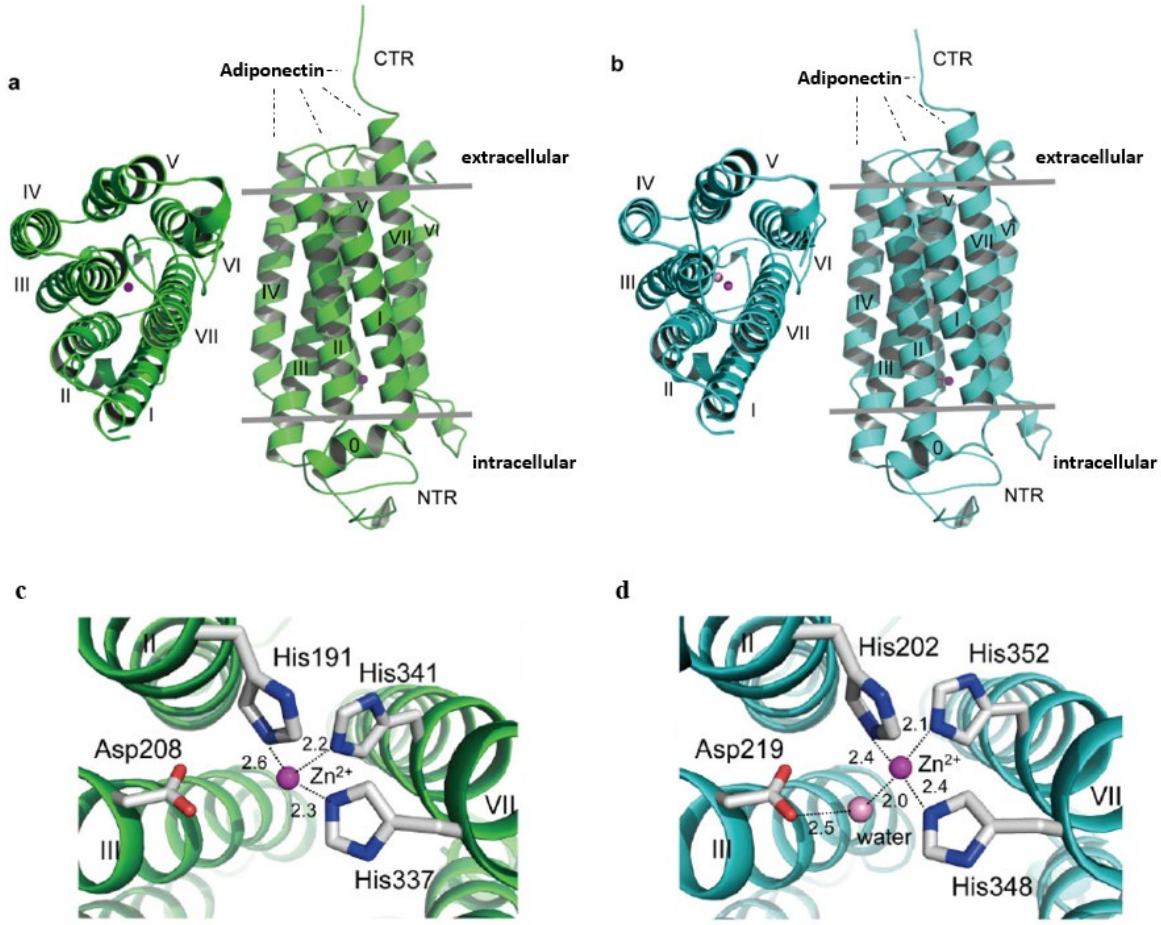

Figure 2.3 Crystal structures of AdipoR1 and AdipoR2.

a, The 2.9-Å resolution structure of AdipoR1. **b,** The 2.4-Å resolution structure of AdipoR2. The structures (a,b) are viewed from the extracellular side (left) and parallel to the membrane (right). The N-terminal intracellular region (NTR), helix 0, transmembrane helices I–VII, and the C-terminal extracellular region (CTR) of AdipoR1 and AdipoR2 are indicated. AdipoR1 and AdipoR2 appear to share the same adiponectin binding site, and experimental data indicate that AdipoR1 may recognize adiponectin via various residues in its extracellular face including residues found in the three extracellular loops as well as the C-terminal turns of helix VII. **c,** Coordination of the zinc ion by three His residues of AdipoR1 **d**, Coordination of the zinc ion by

three His residues of AdipoR2. A water molecule is also coordinated to the zinc ion in AdipoR2 and is fixed by an Asp residue. The structures (c,d) are viewed from the cytoplasmic side. In (a-d), the position of the zinc ion is represented as a magenta sphere. The second, pink sphere present in the AdipoR2 structure (b,d) represents the water molecule. Reprinted with minor adaptations by permission from Springer Nature: Nature, Crystal structures of the human adiponectin receptors, Hiroaki Tanabe et al., Copyright 2015.

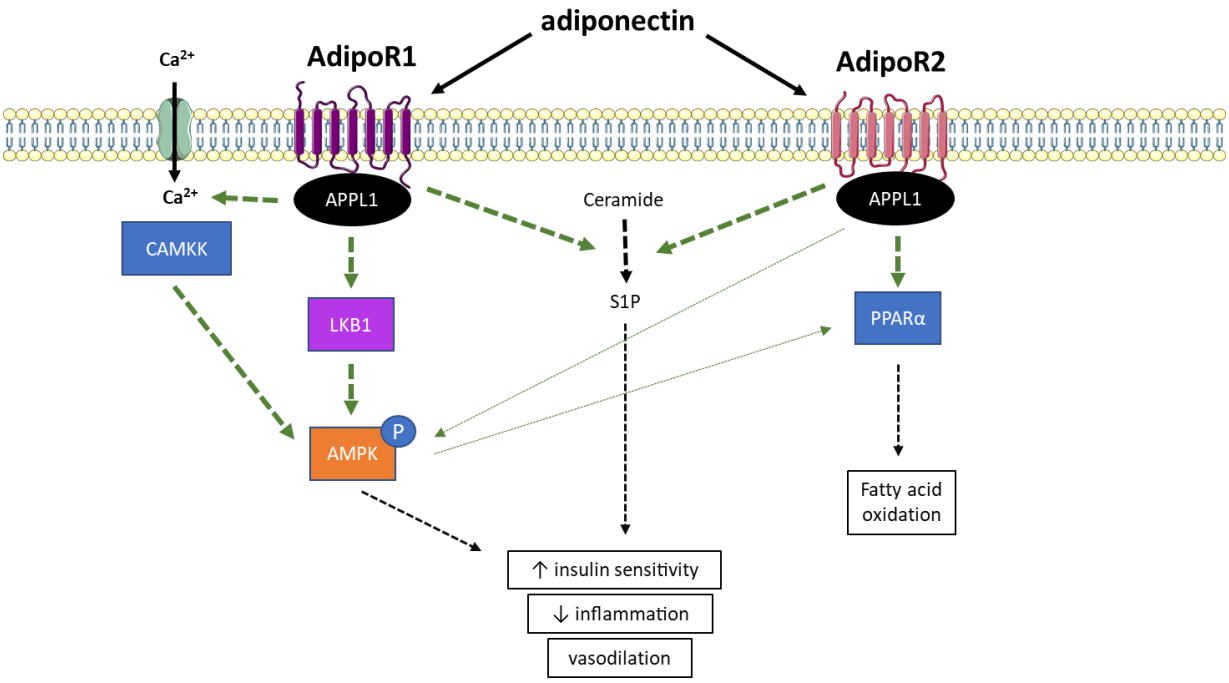

Figure 2.4 Adiponectin receptor signaling.

The adaptor protein, APPL1 (Adaptor protein containing a Pleckstrin homology domain, Phosphotyrosine binding domain and Leucine zipper motif) interacts with the intracellular domain of AdipoR1 and AdipoR2 to mediate signal transduction. The major downstream modulators of AdipoR1 and AdipoR2 activation are 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-α (PPARα). Activation of AdipoR1 leads to translocation of LKB1 which activates AMPK via phosphorylation at Thr172. Activation of AdipoR1 is also associated with calcium influx and activation of calcium/calmodulin-dependent protein kinase kinase (CAMKK). AdipoR2 signaling activates PPARα leading to fatty acid oxidation. Evidence suggests that activation of PPARα may be dependent on activation of AMPK in some tissues. AdipoR1 and AdipoR2 activation lead to an increase in ceramidase activity which favors the formation of sphinosine-1-phosphate (S1P). Formation of S1P and activation of AMPK are responsible for many of the

physiological effects of adiponectin including increased insulin sensitivity, reduced inflammation, and vasodilation.

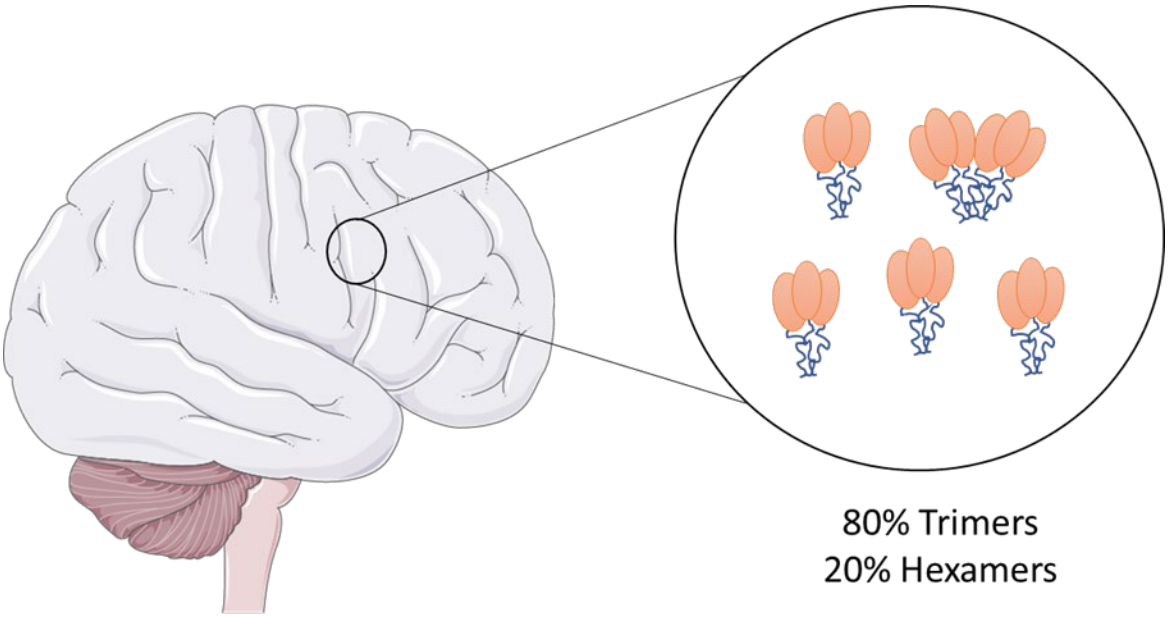

Figure 2.5 Adiponectin oligomer distribution in cerebral spinal fluid.

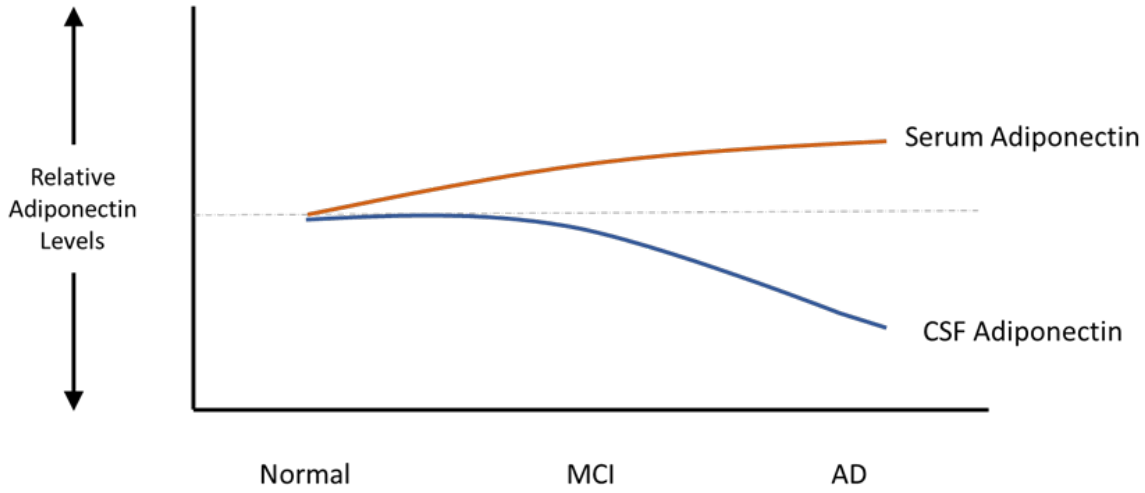

Figure 2.6 Adiponectin level trends in mild cognitive impairment and Alzheimer's disease.

Graph shows relative adiponectin levels in cerebral spinal fluid (CSF) and serum in MCI and AD versus age-matched controls, based on findings by Waragai et al. 2016. Graph represents overall trends and is not to scale.

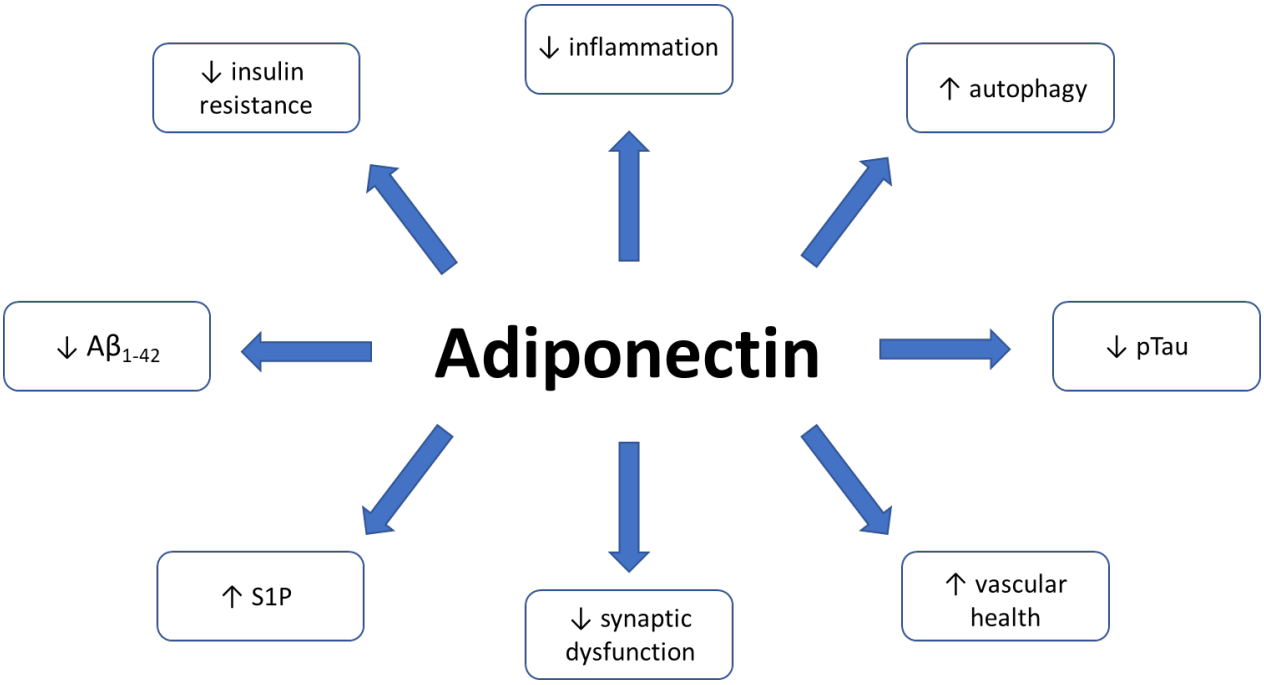

Figure 2.7 Potential mechanisms by which adiponectin may reduce Alzheimer's disease pathology.

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Chapter 3: Adiponectin knockout mice display cognitive and synaptic deficits

1 Abstract

Adiponectin is an adipokine that has recently been under investigation for potential neuroprotective effects in various brain disorders including Alzheimer's disease, stroke, and depression. Adiponectin receptors (AdipoR1 and AdipoR2) are found throughout various brain regions, including the hippocampus. However, the role of these receptors in synaptic and cognitive function is not clear. Therefore, the goal of the current study was to evaluate synaptic and cognitive function in the absence of adiponectin. The current study utilized 12-month old adiponectin knockout (APN-KO) mice and age-matched controls to study cognitive and hippocampal synaptic alterations. We determined that AdipoR1 and AdipoR2 are present in the synaptosome, with AdipoR2 displaying increased presynaptic versus postsynaptic localization, whereas AdipoR1 was enriched in both the presynaptic and postsynaptic fractions. APN-KO mice displayed cognitive deficits in the novel object recognition and Y-maze tests. This was mirrored by deficits in long-term potentiation (LTP) of the hippocampal Schaefer collateral pathway in APN-KO mice. APN-KO mice also displayed a reduction in basal synaptic transmission and an increase in presynaptic release probability. Deficits in LTP were rescued through hippocampal slice incubation with the adiponectin receptor agonist, AdipoRon, indicating that acute alterations in adiponectin receptor signaling influence synaptic function. Along with the deficits in LTP, altered expression of key presynaptic and postsynaptic proteins involved in glutamatergic neurotransmission were observed in APN-KO mice. Taken together, these results indicate that adiponectin is an important regulator of cognition and synaptic

function in the hippocampus. Future studies should examine the role of specific adiponectin receptors in synaptic processes.

2 Introduction

Adiponectin is an important adipokine known for its role in modulation of metabolic processes including enhancement of insulin sensitivity (Kadowaki et al. 2006). In recent years, it has become increasingly clear that adiponectin is an essential player in the cross-talk between adipose tissue and brain function. Adiponectin is produced by adipocytes and circulates in various forms including trimers, hexamers, high molecular weight complexes, and a globular form (Waki et al. 2003). Trimers, hexamers, and globular adiponectin are detectable in cerebral spinal fluid (Neumeier et al. 2007), and there is evidence that trimers are the most biologically relevant form in the brain (Kusminski et al. 2007). Adiponectin receptors, which include AdipoR1, AdipoR2, and T-cadherin, are expressed in various regions of the brain, including the hippocampus, an important area for learning and memory processes (Thundyil et al. 2012). Notably, altered adiponectin plasma levels are associated with neurological disorders, including Alzheimer's disease (AD) and major depressive disorder (Cao et al. 2018). Additionally, studies have shown a positive correlation between serum adiponectin levels and cognitive performance in post-menopausal women (Franciscis et al. 2017) and middle-aged adults at risk of type 2 diabetes (Cezaretto et al. 2018). While adiponectin signaling in the brain appears to contribute to processes including hippocampal neurogenesis and energy expenditure (Bloemer et al. 2018), the role of adiponectin in synaptic and cognitive function is still unknown.

To better understand the role of adiponectin in hippocampal synaptic function, we first identified the synaptic localization of the adiponectin receptors AdipoR1 and AdipoR2. Next, we

investigated the effects of adiponectin on cognitive and synaptic functioning by comparing aged adiponectin knockout mice (APN-KO) to age-matched controls using behavioral and electrophysiological techniques. We determined that the adiponectin receptor agonist AdipoRon rescues deficits in synaptic plasticity in the APN-KO mice. Finally, we identified alterations in key synaptic proteins that may underlie the cognitive and synaptic deficits observed in APN-KO mice.

3 Materials and Methods

Animals

Adiponectin knockout (B6;129-*Adipoq*^{tm1Chan}/J) and control (B6129SF2/J) male mice were obtained from The Jackson Laboratory (stock #008195 and #101045, respectively) and aged to twelve months. Mice were group-housed with free access to food and water in a temperature- and humidity-controlled colony room with a 12:12 light/dark cycle (lights on at 6AM). Behavioral experiments were performed starting at approximately 3 hours into the light cycle. All procedures were carried out in accordance with NIH guidelines and approved by Auburn University Animal Care and Use Committee.

Chemicals

AdipoRon, 2-(4-benzoylphenoxy)-*N*-[1-(phenylmethyl)-4 piperidinyl]acetamide (Cayman Chemical), was dissolved in dimethyl sulfoxide (DMSO) and stored as a stock solution (50mM). For electrophysiology experiments, AdipoRon was diluted in artificial cerebral spinal fluid (ACSF) to a final concentration of 15μM or 50μM (Zhang et al. 2017). All other chemicals were obtained from Millipore Sigma, unless otherwise specified.

Synaptic Fractionation

Hippocampal synaptosomes were prepared as previously described (Vaithianathan et al. 2005; Parameshwaran et al. 2012; Johnson, Chotiner, and Watson 1997), followed by synaptic fractionation (Pacchioni et al. 2009). In brief, hippocampi were homogenized in ice-cold modified KREBS (mKREBS) buffer containing (in mM): 118.5 NaCl, 4.7 KCl, 1.18 MgSO4, 2.5 $CaCl_2$, 1.18 KH_2PO_4, 24.9 $NaHCO_3$, and 10 dextrose and adjusted to pH 7.4. The homogenate was filtered through three layers of nylon filter (100 μm) and then through a low-protein binding filter (5 μm). The filtered particulate was centrifuged at 1,000x g at 4°C for 15 minutes and the pellet contained the synaptosomal fraction. Synaptosomes were resuspended in isolation buffer (20 mM HEPES, 100 mM NaCl, 0.5% Triton X-100, pH 7.2), incubated for 15 minutes, and centrifuged at 12,000x g for 20 minutes. The supernatant contained the non-postsynaptic density (non-PSD) fraction. The pellet (PSD fraction) was incubated in buffer (20 mM HEPES, 0.15 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1% SDS, pH 7.5) for 1 hour, then centrifuged for 15 minutes at 10,000x g. The supernatant contained the PSD fraction, and the pellet was discarded. All buffers were supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (ThermoFischer Scientific). Protein concentration was estimated by BCA assay (Pierce BCA Protein Assay Kit ThermoFischer Scientific) and samples were stored at −20°C until use.

Immunoblotting

Following euthanasia, hippocampal tissue was extracted and homogenized in lysis buffer (Neuronal Protein Extraction Reagent, ThermoFischer Scientific) containing a protease and phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail, ThermoFischer Scientific). Total protein was estimated by bicinchoninic acid (BCA) assay (Pierce BCA Protein

Assay Kit, ThermoFischer Scientific) and stored at −20°C until use. Samples were mixed thoroughly with 4x Laemmli buffer, heated, and were loaded into a handcast 10% acrylamide gel. Electrophoresis was performed using the Mini-PROTEAN 3 system (Bio-Rad). Proteins were electro-transferred to PVDF membranes (Amersham Hybond P, GE Healthcare) at 70V over 2 hours via wet tank transfer utilizing the Mini-PROTEAN 3 system (Bio-Rad). Next, the PVDF membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline, 0.1% v/v Tween 20 (TBST) for 2 hours. Membranes were washed with TBST and incubated with primary antibody overnight at 4°C. Additional washing steps were performed, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. Primary antibodies and secondary antibodies were diluted in 5% BSA in TBST and utilized at the concentrations indicated in Table 1. Immunoreactivity was visualized using enhanced chemiluminescence (Amersham ECL Select or Amersham ECL Prime, GE Healthcare) in a FluorChem Q imager system (Proteinsimple). Density of immunoreactivity for each band was measured using AlphaView software (Proteinsimple) and values were normalized to the beta actin levels of corresponding lanes.

Novel Object Recognition

To measure recognition memory, novel object recognition (NOR) was performed as previously described (Wu et al. 2019) with some modifications. Novel object recognition was performed in a 40 x 40 cm box made of plexiglass. Prior to the experiment, mice were habituated to an empty box for 10 minutes on 2 consecutive days. During the familiarization phase, two identical objects (either Object A, plastic cube measuring 4 cm^3 or Object B, cylindrical cone 5 cm in diameter) were placed in the northeast and northwest quadrants positioned 11cm from the walls. During the test phase 24 hours later, one of the familiar objects was replaced by a novel object. Objects and

the arena were cleaned with 70% ethanol between animals to minimize odor cues. During the familiarization and testing phases, mice were placed into the center of the box facing away from the objects and allowed to freely explore for 4 minutes. Recordings were scored by blinded reviewers. Exploration of the objects was defined as time spent with the snout orientated towards the object at a distance of ≤2 cm of the object. Results were expressed as discrimination index (DI), (T novel − T familiar)/(T familiar + T novel).

Y-maze Test

Spatial recognition memory utilizing a two-trial Y-maze task was performed as described previously (Parameshwaran et al. 2012). Briefly, the Y-maze apparatus consisted of three arms separated by 120°. Visual cues were placed around the Y-maze. The two trials were separated by a 3-hour intertrial interval to assess spatial recognition memory. During the first trial (acquisition), mice were allowed to explore two arms of the maze for 10 minutes; the starting arm in which they were initially placed, and a second arm referred to as the familiar arm; the third (novel) arm was closed. During the second trial (retention test), mice were placed back in the starting arm and allowed to explore for 6 min with free access to all three arms (the novel arm was opened). The arena was cleaned with 70% ethanol between animals to minimize odor cues. Recordings were scored by blinded reviewers, and the total number of entries and time spent in each arm were measured.

Hippocampal slice preparation

Animals were euthanized with carbon dioxide, and 350 μm-thick transverse slices were prepared using a Leica VT1200S Vibratome (Leica Microsystems, Wetzlar, Germany). Slices were incubated at room temperature in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM

KCl, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM dextrose, pH 7.4) saturated with 95% O_2/5% CO_2 until transfer to the recording chamber. In animals which were behaviorally tested, one week elapsed between behavioral testing and electrophysiology experiments.

Extracellular field potential recording

After at least one hour of incubation, slices were transferred into a recording chamber for electrophysiological measurements as previously described (D. Bhattacharya et al. 2015; Parameshwaran et al. 2013; Hunsberger et al. 2016; S. Bhattacharya et al. 2017) with continuous ACSF perfusion at 34°C. A bipolar stimulating electrode (MicroProbes, Gaithersburg, MD) was placed in the Schaffer collateral pathway. An extracellular recording pipette drawn with the PC-10 Dual-Stage Glass Micropipette Puller (Narishige, Amityville, NY) and filled with ACSF (2–6 MΩ) was placed in the stratum radiatum of CA1 to record field excitatory postsynaptic potentials (fEPSPs). For paired pulse facilitation (PPF), pairs of stimuli were separated by varying intervals. Ratios of fEPSP slopes from the second stimulus (fESPS2) to fEPSP slopes from the first stimulus (fESPS1) were calculated and plotted as a function of interstimulus intervals. Basal synaptic transmission, represented by input-output responses, was determined as the slope of fEPSPs and plotted as a function of fiber volley amplitude. For LTP experiments, stimulus intensity was set at 50% of the amplitude at which initial population spike appeared. LTP was induced after at least 10 min of stable baseline recording using a Theta Burst Stimulation (TBS) protocol (10 bursts of stimuli, each of four pulses at 100 Hz, interburst interval of 200 ms, and 20s intervals between individual sweeps), and recording was continued for 60 minutes post-TBS (Kerr and Abraham 1995; Eckhardt et al. 2000; Parameshwaran et al. 2013). LTP was measured as an average of fEPSP slopes from 50-60 minutes after the end of induction. For the induction

data analysis, sweep analysis was computed by normalizing the amplitude of the first fEPSP of sweeps 2-5 with the amplitude of the first fEPSP of the first sweep. The data were recorded online using the WinLTP software (University of Bristol, UK). Standard off-line analyses of the data were conducted using Prism software (GraphPad Prism version 8, San Diego California, USA).

Statistical analysis

Statistical analysis was performed using Prism 8 software. Data was assessed by one-way analysis of variance or two-tailed, unpaired and paired t-tests, where appropriate. Tukey post-hoc comparisons were used to compare groups when analysis of variance indicated significant effects except where expected effects were assessed with planned pairwise comparisons. Results were considered significantly different when $p<0.05$. All data are presented as means ± SEM.

4 Results

4.1 AdipoR1 and AdipoR2 are expressed in hippocampal synapses

Though adiponectin receptors are found throughout the brain including the hippocampus, cortex, hypothalamus, and brainstem (Thundyil et al. 2012), whether adiponectin receptors are expressed at the synapse, the functional unit of communication between neurons, is unknown. To determine whether AdipoR1 and AdipoR2 receptors are expressed at the hippocampal synapse in wild-type mice, a synaptosomal isolation procedure followed by immunoblotting was used to compare synaptic versus total hippocampal expression. Synaptic fractionation efficiency was confirmed by immunoblotting for PSD95, which is enriched in the postsynaptic faction, and SNAP25, which is enriched in the presynaptic fraction. In wild-type mice, AdipoR1 expression in various

fractions differed such that the AdipoR1 receptor was significantly enriched in the synaptosome compared to the total hippocampal lysate [$F(3,8) = 9.751$, $p = 0.0048$; Fig. 1A]. When the synaptosomal fraction was further isolated into the postsynaptic density (PSD) and non-PSD fractions, with the non-PSD fraction containing predominately presynaptic material, there was no significant difference in AdipoR1 expression in postsynaptic versus presynaptic fractions. However, each was about three times higher compared to the total lysate (Figure 3.1A), indicating that AdipoR1 receptors may play a role in both presynaptic and postsynaptic processes. Expression level of AdipoR2 in the synaptosome was similar to expression in total hippocampal lysate. However, AdipoR2 expression was significantly higher in the presynaptic fraction compared to the postsynaptic fraction, implying a potential presynaptic role of this receptor [$F(3,8) = 38.23$, $p = <0.0001$; Figure 3.1B].

4.2 APN-KO mice display deficits in NOR and Y-maze tests

Since adiponectin receptors are expressed in hippocampal synapses, we hypothesized that these receptors may play a role in synaptic processes and ultimately cognition and that loss of adiponectin may lead to learning and memory deficits. To determine whether cognitive deficits are present in aged adiponectin knockout mice, we performed hippocampal-dependent NOR (Wu et al. 2019; Antunes and Biala 2012) and Y-maze (Parameshwaran et al. 2012) tests. Using a 24-hour separation between the familiarization and test phase for the NOR task to test long term recognition memory (Guzmán-Ramos et al. 2012), control mice were found to spend significantly more time interacting with the novel object compared to the familiar object [$F(1,14) = 7.622$, $p = 0.0153$; Figure 3.2A], whereas the APN-KO mice showed no preference for the novel object [$F(1,14) = 0.3167$, $p = 0.5825$; Figure 3.2A]. This was confirmed by comparing the discrimination index, which revealed a significant impairment in the APN-KO mice compared to

controls in overall performance in NOR [$F(1,14) = 5.88$, $p = 0.0294$; Figure 3.2B]. There was no significant difference in exploration time between groups during familiarization [$F(1,14) = 0.4044$, $p = 0.5825$; Figure 3.2C], indicating that the lack of preference for the novel object in the APN-KO mice was not due to deficits in exploratory behavior.

To determine whether APN-KO mice also display deficits in short-term spatial recognition memory, we utilized a two-trial Y-maze task with an intertrial interval of 3 hours. The was no difference in number of total arm entries between groups during testing, indicating a similar level of exploratory behavior [$F(1,14) = 0.008$, $p = 0.9299$; Figure 3.3A]. While the control mice showed a significant preference for entries into the novel arm [$F(1,14) = 9.356$, $p = 0.0085$; Figure 3.3B], the APN-KO did not discriminate between the familiar and novel arms [$F(1,14) = 0.0092$, $p = 0.9251$; Figure 3.3B]. Likewise, the APN-KO mice did not demonstrate discrimination between arms in terms of percentage time spent in the novel arm, whereas the control mice strongly preferred the novel arm [$F(1,14) = 11.11$, $p = 0.0049$; Figure 3.3C]. Taken together, this data implies cognitive impairment in the APN-KO mice.

4.3 Deficits in basal synaptic transmission in APN-KO mice are rescued by slice incubation with AdipoRon

To determine whether cognitive impairment in the APN-KO mice is linked to alterations in basal synaptic transmission, hippocampal slices were used to measure the fEPSP responses at increasing stimulus intensities. We observed an alteration in fEPSPs over a range of stimulus intensities between groups [$F(3,18) = 13.73$, $p < 0.0001$; Figure 3.4A]. fEPSP slope was reduced in APN-KO mice compared to controls, implying deficits in baseline glutamatergic synaptic transmission. These deficits were rescued by slice incubation with AdipoRon. To determine

whether the deficits in basal synaptic transmission in APN-KO mice are due to alterations in presynaptic axon recruitment, stimulus intensity versus fiber volley (FV) amplitude was compared and found to be similar between groups [$F(3,18) = 0.8927$, $p = 0.4639$; Figure 3.4B], suggesting that the changes in basal synaptic transmission are not due to changes in axon recruitment. Further supporting this, when controlling for FV amplitude, the fEPSP remained significantly lower in the APN-KO mice, an effect which was rescued by AdipoRon exposure [$F(3,16) = 7.756$, $p = 0.002$; Figure 3.4C]. Taken together, this suggests that the reduction in basal synaptic transmission is not due to a decreased in number of presynaptic axons recruited or a reduction in excitability of the presynaptic axons.

4.4 APN-KO mice display alterations in presynaptic release probability

To determine whether alterations in presynaptic release probability could account for the changes in basal synaptic transmission, we evaluated paired pulse facilitation (PPF), a type of short-term plasticity that depends on residual calcium build-up in the presynaptic terminal. Surprisingly, we found a decrease in PPF in the APN-KO mice [$F(3,12) = 11.36$, $p = 0.0008$; Figure 3.4D], which indicates an increase in presynaptic release potential. AdipoRon incubation reduced the presynaptic release potential. To investigate whether changes in presynaptic proteins involved in glutamate release may account for the alteration in PPF in APN-KO mice, the hippocampal expression of vesicular glutamate transporter 1 (VGLUT1), which is responsible for uptake of glutamate into synaptic vesicles, and synaptosome associated protein 25 (SNAP25), which plays an essential role in release of neurotransmitters, was evaluated. While SNAP25 expression did not differ between the groups [$T(6) = 0.2513$, $p = 0.81$; Figure 3.4E], VGLUT1 expression was significantly increased in APN-KO mice [$T(6) = 3.176$, $p = 0.0192$; Figure 3.4E], suggesting that

an increase in vesicular glutamate storage may underlie the increase in glutamate release observed in APN-KO mice.

4.5 Deficits in LTP in APN-KO mice are rescued through slice incubation with AdipoRon

We next examined whether cognitive impairments in APN-KO are associated with alterations in synaptic plasticity by measuring LTP, which has been termed the cellular correlate of learning and memory (Lüscher and Malenka 2012). Using hippocampal slices, we determined that APN-KO mice display deficits in LTP induced by theta burst stimulation in the Schaeffer collateral pathway. These deficits were rescued by the adiponectin receptor agonist, AdipoRon [$F(3,15) = 4.899$, $p = 0.0144$; Figure 3.5B), suggesting adiponectin receptor signaling may directly influence glutamatergic synaptic processes in the hippocampus.

A reduction in LTP could indicate alterations in the strength of the signaling during LTP induction (Lüscher and Malenka 2012). To assess for alterations during LTP induction we evaluated fEPSP amplitude during theta burst stimulation (Parameshwaran et al. 2013). When the first fEPSP from each sweep was normalized to the first fEPSP of the first sweep, we found a reduction in the fEPSP amplitude in the APN-KO mice compared to controls, indicating that alterations in LTP may be in part due to reduced synaptic activation during LTP induction. Incubation with AdipoRon incubation restored the fEPSP amplitude during induction [$F(3,12) = 9.989$, $p = 0.0014$; Figure 3.5C].

4.6 Reduced expression of glutamatergic receptor subunits in APN-KO mice

Since altered expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) or N-methyl-D-aspartate receptor (NMDAR) subunits can lead to changes in the

induction and maintenance of LTP (Lüscher and Malenka 2012), we next evaluated hippocampal expression of key subunits via Western Blot. The AMPAR subunit GluA1 was reduced in APN-KO mice [$T(4) = 5.526$, $p = 0.0052$; Figure 3.5D], which may contribute to deficits in LTP along with deficits in basal synaptic transmission. Additionally, there was a reduction in expression of GluN1 [$T(6) = 3.465$, $p = 0.0134$; Figure 3.5D] and GluN2A [$T(6) = 3.046$, $p = 0.0226$; Figure 3.5D], the primary NMDAR subunits found in the postsynaptic zone (Groc et al. 2006; Steigerwald et al. 2000). No change in expression of GluN2B was observed in APN-KO mice [$T(6) = 0.3035$, $p = 0.7717$; Figure 3.5D]. Taken together, our data indicates that reduced expression of glutamatergic receptor subunits may account for the alteration in synaptic plasticity in APN-KO mice.

4.7 Dose-dependent effects of AdipoRon on LTP and basal synaptic transmission

To determine whether the effects of AdipoRon are dose-dependent, we also tested a higher dose of AdipoRon. When we compared the effects of 15 μM and 50 μM AdipoRon on hippocampal slices from APN-KO mice and controls and measured LTP, we found a significant difference among groups [$F(5, 22) = 4.248$, $p = 0.0075$; Figure 3.6A]. Interestingly, although 15 μM AdipoRon led to an increase in LTP in APN-KO hippocampal slices, the 50 μM concentration was unable to significantly increase LTP, although a slight upward trend was observed. In the control group, there were no significant differences with either concentration of AdipoRon, although a trend towards decreased LTP was observed with increasing concentrations. Similar effects were observed for analysis of the FV amplitude versus fEPSP slope. There was a significant difference between groups [$F(5, 23) = 5.686$, $p=0.0015$; Figure 3.6B], and although 15 μM AdipoRon led to an increase in basal synaptic transmission in APN-KO hippocampal slices, the 50 μM concentration was unable to significantly increase basal synaptic transmission.

Additionally, in the control groups, there was no statistically significant difference with any AdipoRon concentration, although a downward trend was noted with increasing concentrations. For PPF, no statistically significant dose-dependent effects were observed at any interstimulus interval (data not shown). Thus, dose-dependent effects are observed for AdipoRon in the rescue of LTP and basal synaptic transmission.

4.8 Altered phosphorylation of downstream signaling molecules in APN-KO mice

To identify potential mechanisms by which a reduction in adiponectin receptor signaling might result in synaptic alterations, specifically alterations in LTP, we evaluated expression of key downstream signaling molecules for AdipoR1 (Figure 3.7). Phosphorylation of AMP-activated protein kinase (AMPK), a major downstream signaling molecule for the AdipoR1 receptor (Bjursell et al. 2007) and important regulator of LTP (Marinangeli et al. 2018), was significantly reduced in APN-KO mice [$T(6) = 2.855$, $p = 0.029$; Figure 3.8A]. AMPK leads to inactivation of glycogen synthase kinase 3beta (GSK3β) through phosphorylation of Ser9, which enhances LTP (Zhu et al. 2007). In APN-KO mice, the pGSK3β/GSK3β ratio was reduced [$T(6) = 5.45$, $p = 0.0016$; Figure 3.8B], indicating increased activation of GSK3β. The activity of cAMP response element-binding protein (CREB) is reduced by GSK3β (Beurel, Grieco, and Jope 2015) and CREB is important in promoting the expression of glutamatergic receptor subunits (Middei et al. 2013; Lakhina et al. 2015). In APN-KO mice, the pCREB/CREB ratio was reduced [$T(6) = 4.39$, $p = 0.0046$; Figure 3.8C], indicating lower activity of CREB. Thus, the deficits in LTP in APN-KO mice may be related to a reduction in AdipoR1-mediated signaling.

5 Discussion

The key findings of the current study are that adiponectin receptors are expressed in hippocampal synapses, and adiponectin receptor signaling influences synaptic processes (Figure 3.7). Expression patterns of the adiponectin receptors in the hippocampal synaptosome indicate a potential role for AdipoR1 in both presynaptic and postsynaptic compartments, and a role for AdipoR2 in the presynaptic compartment. This led us to hypothesize that the adiponectin receptors may play a direct role in synaptic function and ultimately learning and memory. In APN-KO mice, signaling through both AdipoR1 and AdipoR2 are presumably reduced. In the current study, reduced adiponectin receptor signaling led to deficits in NOR and Y-maze tasks. In accordance with this, 18-month old APN-KO display impaired performance in the Morris Water Maze task (Ng et al. 2016), a hippocampal-dependent memory task. In an Alzheimer's disease (AD) model, treatment with the adiponectin receptor agonist, osmotin, improves performance in the Y-maze spontaneous alternations test (Ali et al. 2015). Thus, our findings are in line with prior studies which indicate a positive role for adiponectin receptor signaling in learning and memory. Interestingly, AdipoR1 and AdipoR2 may play distinct roles in learning and memory processes. Knockdown of AdipoR1 leads to impaired spatial memory and development of an AD-like pathology (Kim et al. 2017). In contrast, AdipoR2, but not AdipoR1, knockout mice show impaired contextual fear extinction (Zhang et al. 2017), implying a role for AdipoR2 in specific learning paradigms. The differential expression of adiponectin receptors in various compartments observed in the current study may relate to the different roles of these receptors in learning and memory processes.

The deficits in basal synaptic transmission observed in the current study indicate deficits in glutamatergic signaling in the hippocampus in APN-KO mice. The relationship between the slope of the fEPSP and the FV amplitude is the ideal indicator of basal synaptic transmission,

because it represents the postsynaptic response after controlling for the activation of presynaptic fibers (Sweatt 2010). Various factors may contribute to a reduction in basal synaptic transmission in glutamatergic synapses including reduction in the release of glutamate, altered glutamate uptake, or decreased expression of glutamatergic receptors, specifically AMPARs (Sweatt 2010). In the current study, the reduction in basal synaptic transmission may be related to a reduction in expression of the AMPAR subunit, GluA1. One possible explanation for the reduction in GluA1 expression could be due to the increase in presynaptic release probability observed in APN-KO mice. Alternatively, an absence of AdipoR1 signaling could lead to reduced activation of CREB and thus reduced expression of GluA1 (Lakhina et al. 2015). A recent study showed that adiponectin receptor signaling may directly influence the trafficking of GluA1-containing AMPARs and increase surface expression in an AD model (Wang, Jo, and Song 2019). It is also possible that the prolonged absence of adiponectin receptor signaling leads to reduction in surface expression of GluA1-containing AMPARs, which may promote receptor degradation.

In the current study, the increase in presynaptic release potential in APN-KO mice was mirrored by an increase in VGLUT1 expression. VGLUTs are responsible for uptake of glutamate into synaptic vesicles in presynaptic terminals, and VGLUT1 is the primary isoform found in the hippocampus (Fremeau et al. 2001). Expression level of VGLUT is directly correlated to the glutamate fill state of presynaptic vesicles and release probability in hippocampal neurons (Wilson et al. 2005; Herman et al. 2014). No change was observed in the level of SNAP25, which facilitates synaptic transmission by mediating synaptic vesicle exocytosis (Antonucci et al. 2016). Thus, the increase in presynaptic release potential observed in the current study may be due to an increase in vesicular glutamate packaging.

In addition to altering basal synaptic transmission and presynaptic release probability, adiponectin receptor signaling also influences synaptic plasticity. The reduction of LTP in APN-KO mice indicates an impairment in synaptic strengthening, which also occurs in neurodegenerative disorders such as AD (Bliss, Collingridge, and Morris 2014). Importantly, incubation with an adiponectin receptor agonist restores LTP, indicating that the deficits in APN-KO mice are reversed with acute restoration in adiponectin receptor signaling. However, an important concentration-dependent effect was observed for AdipoRon in that an increased concentration did not rescue deficits in LTP or basal synaptic transmission. This implies a possible bell-shaped curve for the effects of AdipoRon on LTP and basal synaptic transmission. Adiponectin receptor signaling has been shown to enhance LTP in other models, as adiponectin incubation restored deficits in LTP in an AD model (Wang, Jo, and Song 2019), which was associated with altered surface expression of glutamatergic receptor subunits. Thus, acute adiponectin receptor signaling appears to improve LTP deficits. However, AdipoRon did not increase LTP in control mice in our study, indicating that there may be a ceiling effect related to the ability of adiponectin receptor signaling to enhance synaptic plasticity.

The reduction in LTP in APN-KO mice in the current study may be related to reduction in expression of glutamatergic receptor subunits. The induction of NMDAR-dependent LTP involves concurrent activation of the glutamatergic receptors AMPA and NMDA (LYNCH 2004; Park et al. 2014). Specifically, during LTP induction, activation of AMPARs leads to removal of Mg^{2+} blockage from NMDARs to allow Ca^{2+} influx and subsequent downstream signaling (Lüscher and Malenka 2012). This downstream signaling pathway induces alterations AMPAR expression and trafficking which are responsible for maintenance of LTP (Lüscher and

Malenka 2012). Thus, in the current study, reduced expression of AMPAR and NMDAR subunits may be responsible for the impairments in LTP induction and maintenance.

The alterations in LTP and glutamatergic receptor subunits in APN-KO mice may be the due to reduced activation of downstream signaling molecules of adiponectin receptors. In the current study, we focused our efforts on downstream signaling of AdipoR1, because our synaptic fractionation indicated that AdipoR1 may be enriched in the synapse. AMPK, a downstream signaling molecule of AdipoR1 and an important energy sensing molecule, is highly expressed in the brain (Ramamurthy and Ronnett 2012). Pharmacological inhibition of AMPK impairs LTP (Yu et al. 2016; Marinangeli et al. 2018), however, there is also evidence that overactivation of AMPK impairs LTP, implying that a fine balance is required (Potter et al. 2010). Thus, it is possible that the higher dose of AdipoRon failed to rescue LTP deficits in the APN-KO group due to overactivation of AMPK, which could also explain the trend towards reduction of LTP in control hippocampal slices with increasing concentrations.

AMPK inhibits the activation of GSK3β (Yu et al. 2016), another important regulator of synaptic plasticity (Bradley et al. 2012). In the current study, we observed a reduction in AMPK phosphorylation, indicating reduced activity, and a reduction of GSK3β phosphorylation, which indicates increased activity, in APN-KO mice. This AMPK-GSK3β signaling pathway has been proposed to lead to AD-like cognitive deficits in states of adiponectin deficiency (Ng et al. 2016). We also observed a reduction in pCREB in APN-KO mice, which may be a result of increased GSK3β activity (Beurel, Grieco, and Jope 2015). Importantly, CREB activation leads to upregulation of glutamatergic receptor subunits, so the reduction in pCREB could account for the reduced expression of glutamatergic receptor subunits in APN-KO mice. Future studies should determine whether treatment with AdipoRon restores phosphorylation levels of these

signaling proteins in APN-KO mice. Although the findings from the current study suggest a synaptic role of adiponectin, it is important to note that an influence of adiponectin receptor signaling on glial cells and/or non-synaptic neuronal locations cannot be ruled out. Based on the current study, we hypothesize that adiponectin receptor activation influences hippocampal processes via influencing glutamatergic synapses, however, a direct effect of adiponectin receptor activation on glutamatergic synapses must be confirmed in future mechanistic studies.

In conclusion, our results indicate that adiponectin receptors are present in hippocampal synapses where they influence synaptic processes and ultimately cognitive function. Absence of adiponectin leads to cognitive deficits, reduced basal synaptic transmission, increased presynaptic release probability, impaired synaptic plasticity, and altered glutamatergic receptor expression. The role of specific adiponectin receptors in synaptic processes warrants further investigation. Additionally, our study supports investigation into the use of adiponectin receptor agonists in conditions associated with synaptic dysfunction and reduced adiponectin receptor signaling.

6 Table

Table 3.1 Summary of antibodies and working conditions used in the experiments.

Antibodies	Species	Source	Catalogue #	Dilution
Primary antibodies				
AdipoR1	Rabbit	Abcam	ab126611	1:200
AdipoR2	Mouse	Santa Cruz	sc-514045	1:200
SNAP25	Mouse	Santa Cruz	sc-136267	1:2000
PSD95	Rabbit	Cell Signaling Technology	3409	1:1000
VGLUT1	Rabbit	Cell Signaling Technology	12331	1:500
GluA1	Rabbit	Cell Signaling Technology	13185	1:750
GluN1	Rabbit	Cell Signaling Technology	5704	1:500
GluN2A	Rabbit	Cell Signaling Technology	4205	1:500
GluN2B	Rabbit	Cell Signaling Technology	4207	1:500
AMPK	Rabbit	Cell Signaling Technology	5832	1:1000
pAMPK (Thr 172)	Rabbit	Cell Signaling Technology	2531	1:500
GSKβ	Rabbit	Cell Signaling Technology	12456	1:1000
pGSK3β (Ser 9)	Rabbit	Cell Signaling Technology	5558	1:1000
CREB	Rabbit	Cell Signaling Technology	4820	1:1000
pCREB (Ser 133)	Rabbit	Cell Signaling Technology	9198	1:500
Beta actin	Rabbit	Cell Signaling Technology	8457	1:2000
Secondary antibodies				
Anti-mouse IgG	N/A	Santa Cruz	sc-516102	1:2000
Anti-rabbit IgG	Goat	Cell Signaling Technology	7074	1:5000

7 Figures

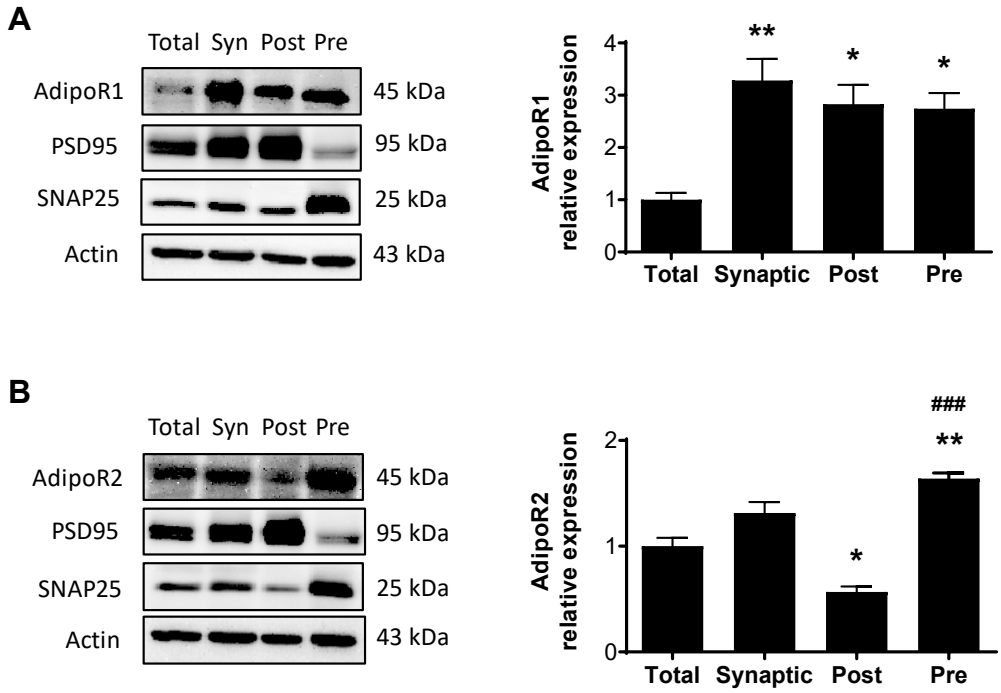

Figure 3.1 Synaptosomal expression of AdipoR1 and AdipoR2.

(A) Representative immunoblot showing AdipoR1 immunoreactivity normalized to beta actin in hippocampal fractions (B) Representative immunoblot showing AdipoR2 immunoreactivity normalized to beta actin in hippocampal fractions. Hippocampal lysate was divided into total (Total) and synaptosomal (Syn) fractions. The synaptosome was further fractionated into the PSD (Post) and non-PSD (Pre) fractions. Synaptic fractionation efficiency is represented by immunoreactivity of PSD95 and SNAP25 for Post and Pre, respectively. 25µg of protein was loaded per lane. Bars represent mean ± SEM from 3 independent experiments, hippocampi from 2 mice were pooled for each experiment (n = 6 mice); *indicates significant difference versus Total; #indicates significant difference versus Post; *$p<0.05$, **$p<0.01$, ###$p<0.001$. Tukey's post hoc test was used for multiple comparisons.

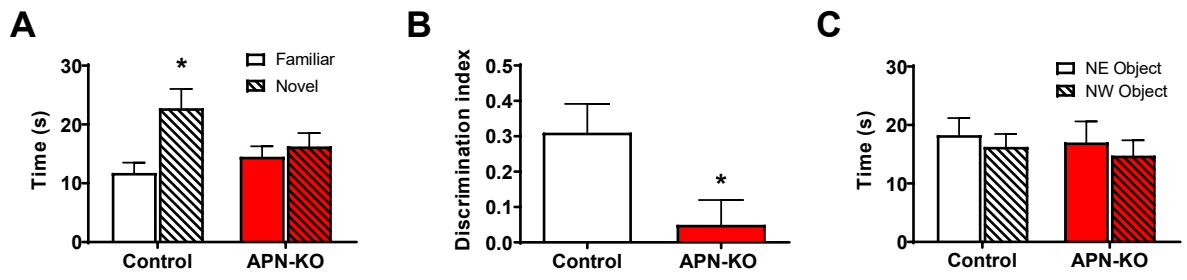

Figure 3.2 APN-KO mice display deficits in novel object recognition test.

(A) Time spent interacting with the familiar versus novel object during testing (B) Discrimination index calculated as $(T_N - T_F) / (T_N + T_F)$ where T_N is time spent with the novel object and T_F is time spent with the familiar object (C) Time spent interacting with the object in the northeast (NE) and northwest (NW) quadrants during familiarization. Bars represent mean ± SEM; *$p<0.05$; n = 8 mice per group.

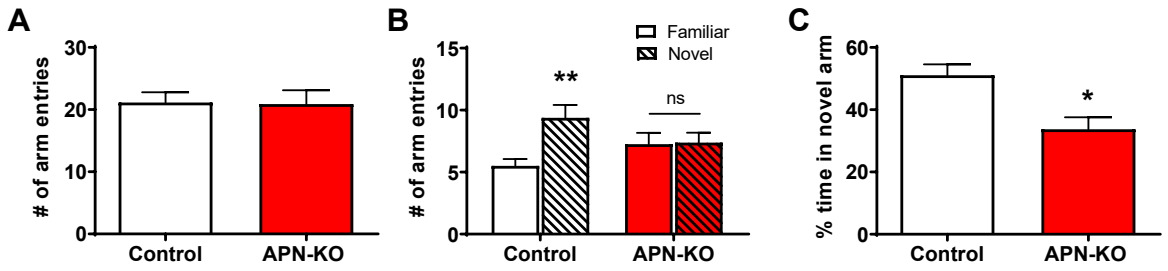

Figure 3.3 APN-KO mice display deficits in two-trial Y-maze task.

(A) Total number of arm entries during testing (B) number of arm entries into the familiar and novel arms during testing (C) percent time spent in novel arm during testing. Bars represent mean ± SEM; *p<0.05, **p<0.01; n = 8 mice per group.

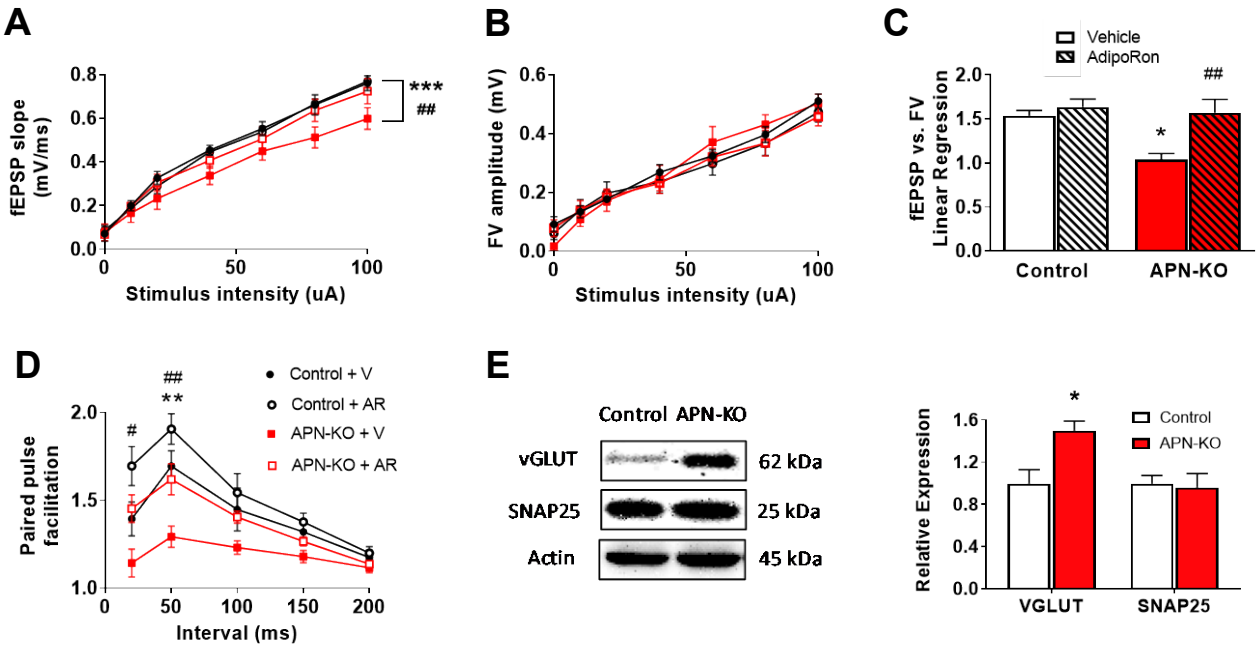

Figure 3.4 APN-KO mice display alterations in basal synaptic transmission and paired pulse facilitation which are rescued through incubation with AdipoRon.

For A-D, hippocampal slices were prepared from Control and APN-KO mice and incubated for 2 hours in either ACSF containing 0.03% DMSO [Vehicle (V)] or ACSF containing 15 µM of AdipoRon and 0.03% DMSO [AdipoRon (AR)] prior to recording. (A) Input-output curve of fEPSP slope measured at increasing stimulus intensities (B) Input-output curve of FV amplitude measured at increasing stimulus intensities (C) Slope of the linear regression line of best fit from plotting fEPSP slope versus FV amplitude (D) Paired-pulse facilitation expressed as the change of ratio of the second stimulus fEPSP to the first stimulus fEPSP slope plotted as a function of interstimulus interval (E) Representative immunoblot showing vGLUT1 and SNAP25 relative expression normalized to beta-actin in hippocampal lysate. Symbols/bars represent mean ± SEM; *indicates significant difference between APN-KO and Control, #indicates significant difference between APN-KO and APN-KO + AR; */#p<0.05, **/##p<0.01, ***p<0.001; for A-D, n = 5-6

slices from 4 mice per group; for E, n = 4 mice per group; for A-C, Tukey's post hoc test was used for multiple comparisons; for D, planned pairwise comparisons were performed for individual data point analysis for Control versus APN-KO, APN-KO versus APN-KO + AR, and Control versus APN-KO + AR.

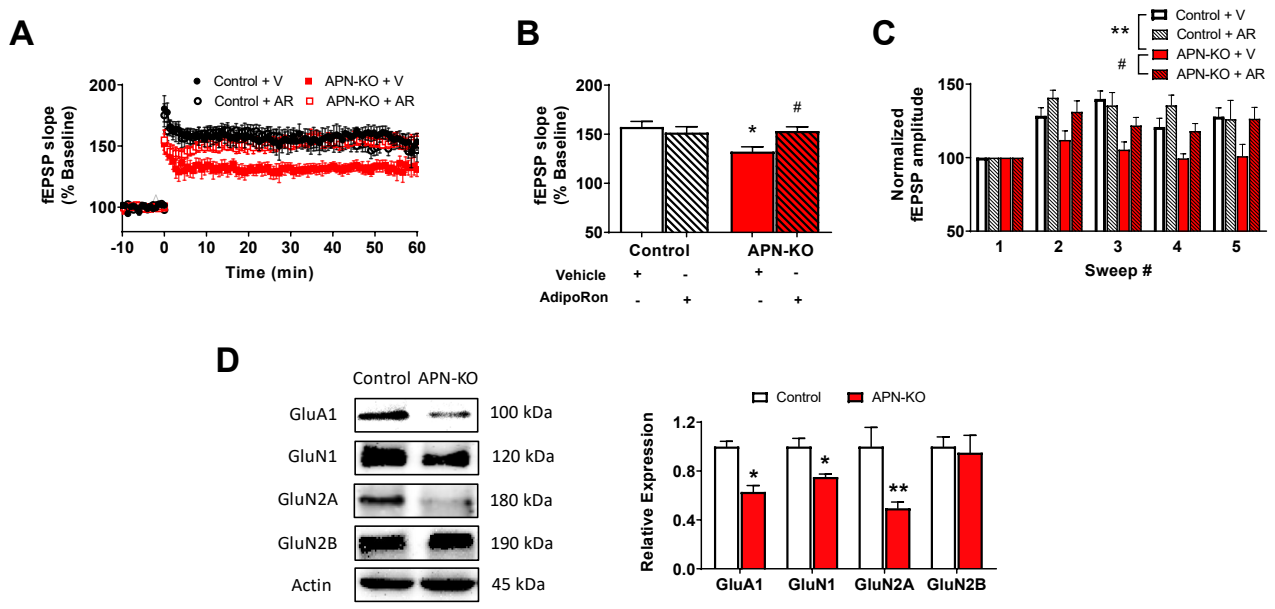

Figure 3.5 Deficits in LTP in APN-KO mice are associated with reduced expression of glutamatergic receptor subunits.

For A-C, hippocampal slices were prepared from Control and APN-KO mice and incubated for 2 hours in either ACSF containing 0.03% DMSO [Vehicle (V)] or ACSF containing 15 µM of AdipoRon and 0.03% DMSO [AdipoRon (AR)] prior to recording. (A) LTP graph represents fEPSP slope before and after induction by TBS (B) LTP bar graph shows fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels (C) Sweep analysis computed by normalizing the amplitude of the first fEPSP of sweeps 2-5 with the amplitude of the first fEPSP of sweep 1 during LTP induction (D) Representative immunoblot showing GluA1, GluN1, GluN2A, and GluN2B relative expression normalized to beta-actin in hippocampal lysate. 40µg of protein was loaded per lane. Bars represent mean ± SEM; *indicates significant difference between APN-KO and Control, #indicates significant difference between APN-KO and APN-KO + AR; */#p<0.05, **/##p<0.01; for A-C, n = 5-6 slices from 4

mice per group; for D, n = 3-4 mice per group; Tukey's post hoc test was used for multiple comparisons.

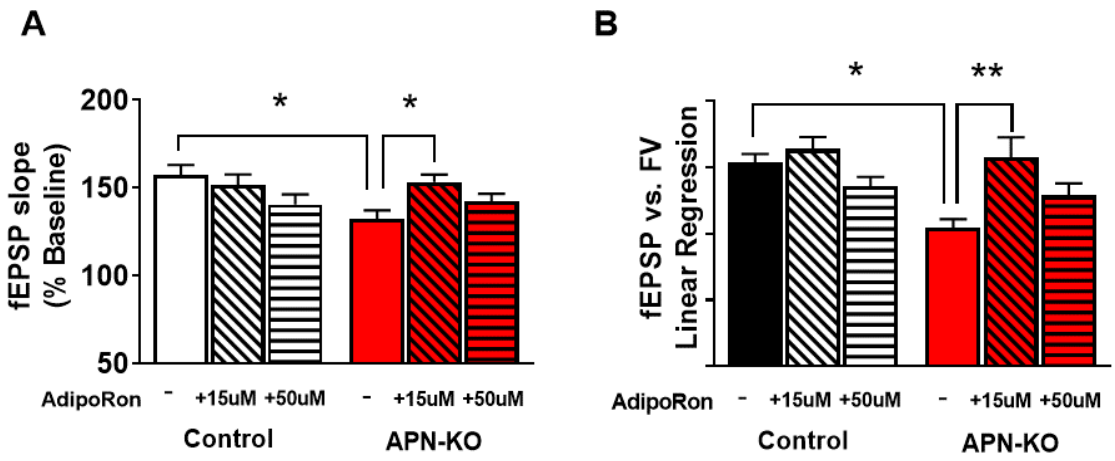

Figure 3.6 Dose-dependent effects of AdipoRon on LTP and basal synaptic transmission. Hippocampal slices were prepared from Control and APN-KO mice and incubated for 2 hours in either ACSF containing 0.03% DMSO [Vehicle (V)] or ACSF containing AdipoRon and 0.03% DMSO [AdipoRon (AR)] prior to recording. (A) LTP bar graph shows fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels (B) Slope of the linear regression line of best fit from plotting fEPSP slope versus FV amplitude. Symbols/bars represent mean ± SEM; *$p<0.05$, **$p<0.01$; n = 4-6 slices from 3-4 mice per group; Tukey's post hoc test was used for multiple comparisons.

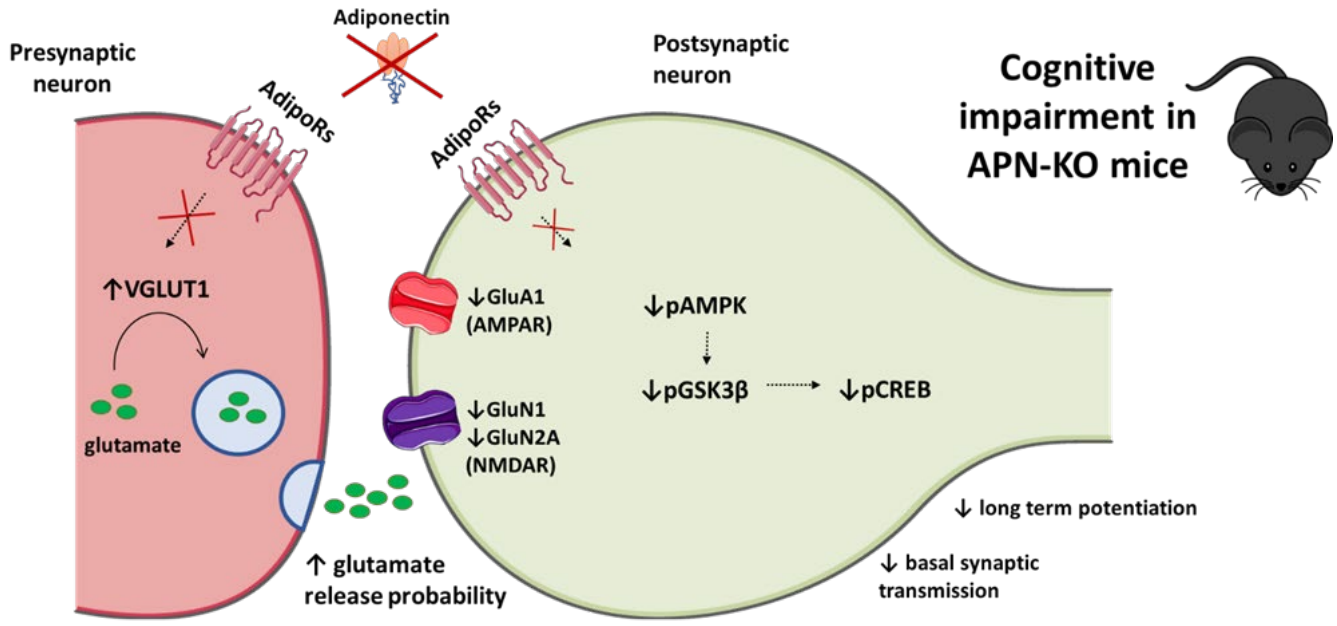

Figure 3.7 Hippocampal alterations in APN-KO mice.

APN-KO mice display cognitive deficits and alterations in hippocampal synaptic function. An increase in protein level of VGLUT1 was observed in APN-KO mice, which may account for the increase in glutamate release probability. A reduction in protein level of GluA1, GluN1, and GluN2A was observed in APN-KO mice, which may account for the reduction in long-term potentiation and basal synaptic transmission. Concurrently, a reduction in phosphorylation of AMPK, GSK3β, and CREB was observed in APN-KO mice.

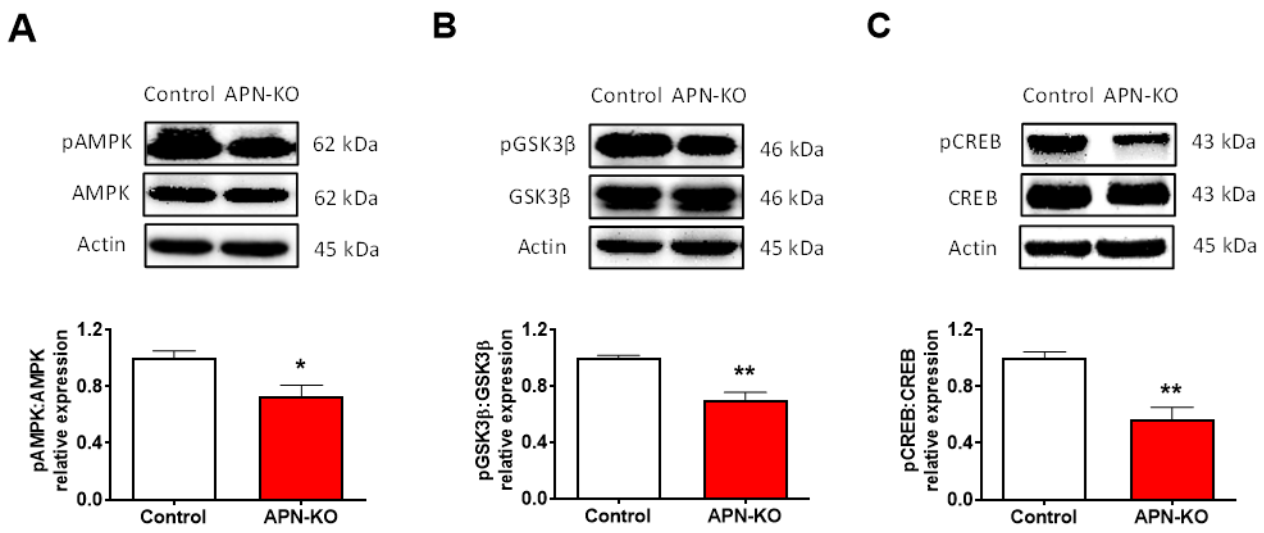

Figure 3.8 Altered phosphorylation of downstream signaling molecules in APN-KO mice. Representative immunoblots showing (A) pAMPK/AMPK relative expression ratio (B) pGSK3β/GSK3β relative expression ratio and (C) pCREB/CREB relative expression ratio, in hippocampal lysate. 40µg of protein was loaded per lane. Bars represent mean ± SEM; *$p<0.05$, **$p<0.01$; n = 4 mice per group.

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Chapter 4: Synaptic deficits in the 3xTg mouse model of Alzheimer's disease are rescued by an adiponectin receptor agonist via activation of AMPK

1 Abstract

The hormone adiponectin alters brain function through enhancing neurogenesis, regulation of energy homeostasis, and regulation of synaptic processes. Recent reports indicate that use of adiponectin or adiponectin receptor agonists may protect against Alzheimer's disease (AD) and reduce AD pathology. Adiponectin may contribute to various facets of protection against AD by enhancing insulin sensitivity, regulating autophagy, reducing neuroinflammation, and promoting clearance of amyloid beta. However, the mechanisms by which adiponectin receptor signaling improves synaptic dysfunction are unclear. In the current study, we utilized acute hippocampal slices from 3xTg-AD mice and age-matched controls to evaluate the ability of the adiponectin receptor agonist, AdipoRon, to rescue synaptic deficits in an AD model. We found that hippocampal slice incubation in AdipoRon or adiponectin restores long-term potentiation (LTP) and basal synaptic transmission in the Schaffer collateral pathway of 3xTg-AD mice, and that this improvement appeared to be dependent on activation of AMPK. AdipoRon was unable to enhance LTP or basal synaptic transmission in the presence of the AMPK inhibitor, Compound C, while the AMPK activator, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), restored these parameters in 3xTg-AD hippocampal slices. AdipoRon also altered presynaptic parameters, by a mechanism which did not appear to be solely dependent on AMPK. Our results indicate that acute restoration of adiponectin receptor signaling recues deficits in synaptic plasticity and basal synaptic transmission through activation of AMPK. Furthermore, we provide evidence that dysregulation of adiponectin receptor signaling may occur in AD based on altered

expression of adiponectin and the adiponectin receptor, AdipoR1, in the hippocampus of 3xTg-AD mice. Restoration of adiponectin receptor signaling may protect against AD pathology, which warrants further investigation into the possibility of utilization of adiponectin receptor agonists in prevention or treatment of AD.

2 Introduction

Adiponectin is a hormone which influences brain processes including hippocampal neurogenesis and brain energy homeostasis (Yau et al. 2014; Qi et al. 2004). There appears to be an association between altered adiponectin levels and Alzheimer's disease (AD), and recent reports indicate that adiponectin levels in the cerebral spinal fluid (CSF) are reduced in AD (Waragai et al. 2016, 2017). Additionally, adiponectin polymorphisms associated with lower adiponectin levels are linked to an increased risk of AD (Z. Yu et al. 2015). Animal studies indicate a neuroprotective role of adiponectin receptor signaling against AD, as reduction in adiponectin receptor expression in the brain leads to an AD-like pathology (Kim et al. 2017), and the adiponectin receptor agonist, osmotin, reduces neuropathology in animal models of AD (Ali et al. 2015; Shah et al. 2017). Thus, there is a recent interest in the use of adiponectin or adiponectin receptor agonists in the prevention or treatment of AD.

AD is characterized by numerous pathological alterations including neuroinflammation, dysregulated autophagy, brain insulin resistance, vascular alterations, synaptic dysfunction, and amyloid-beta (Aβ)/tau mediated toxicity (Hara, McKeehan, and Fillit 2019). There is evidence that adiponectin receptor signaling in the brain may reduce many of these pathologies including neuroinflammation (Jian et al. 2019), brain insulin resistance (Ng et al. 2016), vascular dysfunction (J. Yu et al. 2019), and Aβ/tau-mediated toxicity (Ali et al. 2015). Synaptic

dysfunction is a key component of AD pathology, and an impairment in long-term potentiation (LTP) is a characteristic feature of animal models of AD (Mango et al. 2019). Adiponectin receptor signaling influence synaptic processes, as a recent study found that intracerebroventricular administration of adiponectin enhanced LTP in the perforant pathway (Pousti et al. 2018). Thus, the goal of the current study was to determine whether acute adiponectin receptor activation can rescue synaptic impairments in a mouse model of AD. Utilizing hippocampal slices from 8-9-month-old 3xTg-AD and control mice, we evaluated the ability of AdipoRon, an agonist at the adiponectin receptors AdipoR1 and AdipoR2, to rescue synaptic deficits. We also determined whether inhibition of 5' AMP-activated protein kinase (AMPK), a major downstream signaling molecular of AdipoR1, could prevent the effects of AdipoRon on synaptic processes.

3 Materials and Methods

Animals

3xTg-AD and non-Tg control mice were obtained from The Jackson Laboratory and bred at an in-house facility. Mice were group-housed with free access to food and water in a temperature- and humidity-controlled colony room with a 12:12 light/dark cycle. Female 3xTg and non-Tg control mice were used for all experiments, due to the lack of phenotypic traits in male transgenic mice (The Jackson Laboratory). For electrophysiology experiments, brain slices from 8-9-month-old mice were used. All procedures were carried out in accordance with NIH guidelines and approved by Auburn University Animal Care and Use Committee.

Chemicals

AdipoRon, 2-(4-benzoylphenoxy)-*N*-[1-(phenylmethyl)-4 piperidinyl]acetamide (Cayman Chemical), was dissolved in dimethyl sulfoxide (DMSO) and stored as a stock solution, then diluted in artificial cerebral spinal fluid (ACSF) on the day of the experiment to a final concentration of 15μM (Zhang et al. 2017). Mammalian-produced, trimeric adiponectin (ProspecBio) was diluted in ACSF on the day of the experiment to a final concentration of 10 nM (J. Sun et al. 2016). AICAR, 5-amino-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide (Cayman Chemical) was diluted in ACSF to a final concentration of 1 mM (Marinangeli et al. 2018). Compound C, also known as dysmorphin, (Cayman Chemical) was dissolved in DMSO and stored as a stock solution, then diluted in ACSF on the day of the experiment to a final concentration of 10 μM (Marinangeli et al. 2018). All other chemicals including those used to make ACSF were obtained from Millipore Sigma, unless otherwise specified.

Hippocampal slice preparation

Mice were euthanized with carbon dioxide, and 350 μm-thick coronal slices were prepared using a Leica VT1200S Vibratome (Leica Microsystems, Wetzlar, Germany). Slices were incubated at room temperature in ACSF (124 mM NaCl, 2.5 mM KCl, 1.5 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM dextrose, pH 7.4) saturated with 95% O_2/5% CO_2 until transfer to the recording chamber. For all electrophysiology experiments, DMSO was including in the ACSF at a final concentration of 0.025% v/v. Hippocampal slices for immunoblotting were incubated in drug containing ACSF solution for 2 hours, then stored at at −80°C until use.

Extracellular field potential recording

After at least two hours of incubation, slices were transferred into a recording chamber for electrophysiological measurements as previously described (D. Bhattacharya et al. 2015;

Parameshwaran et al. 2013; Hunsberger et al. 2016; S. Bhattacharya et al. 2017) with continuous ACSF perfusion at 34°C. A bipolar stimulating electrode (MicroProbes, Gaithersburg, MD) was placed in the Schaffer collateral pathway. An extracellular recording pipette drawn with the PC-10 Dual-Stage Glass Micropipette Puller (Narishige, Amityville, NY) and filled with ACSF (2–6 MΩ) was placed in the stratum radiatum of CA1 to record field excitatory postsynaptic potentials (fEPSPs). For paired pulse facilitation (PPF), pairs of stimuli separated by varying intervals. Ratios of fEPSP slopes from the second stimulus ($fESP_2$) to fEPSP slopes from the first stimulus ($fESP_1$) were calculated and plotted as a function of interstimulus intervals. For readily-releasable pool (RRP) experiments, train stimulation (40 pulses, 10ms interstimulus-interval) was applied and fEPSP slopes from pulses 2-40 were normalized to the first pulse (Parameshwaran et al. 2012). Basal synaptic transmission, represented by input-output responses, was determined as the slope of fEPSPs at various stimulus intensities and plotted as a function of fiber volley amplitude (Parameshwaran et al. 2012). For LTP experiments, stimulus intensity was set at 50% of the amplitude at which initial population spike appeared. LTP was induced after at least 10 min of stable baseline recording using a theta burst stimulation (TBS) protocol (10 bursts of stimuli, each of four pulses at 100 Hz, interburst interval of 200 ms, and 20s intervals between individual sweeps), and recording was continued for 60 minutes post-TBS (Kerr and Abraham 1995; Eckhardt et al. 2000; Parameshwaran et al. 2013). LTP was measured as an average of fEPSP slopes from 50-60 minutes after the end of induction and represented as the percentage increase from baseline. For the LTP induction data analysis, sweep analysis was computed by normalizing the amplitude of the first fEPSP of sweeps 2-5 with the amplitude of the first fEPSP of the first sweep. Train analysis was computed by normalizing the amplitude of the fEPSP 2-10 for each train with the amplitude of the first fEPSP of each train. For

depotentiation (DP) experiments, DP was induced using a low frequency stimulation (LFS) protocol (1200 pulses, 2 Hz for 10 min), 60 minutes after LTP induction (Sanderson et al. 2012; Park et al. 2019). DP was measured as an average of fEPSP slopes from 50-60 minutes after the end of DP induction and represented as the percentage decrease from baseline. Baseline was measured as the average fEPSP slopes during the 10 minutes preceding DP induction. The data were recorded online using the WinLTP software (University of Bristol, UK). Standard off-line analyses of the data were conducted using Prism software (GraphPad Prism version 8, San Diego California, USA).

Immunoblotting

Hippocampal tissue was homogenized in lysis buffer (Radioimmunoprecipitation assay buffer, ThermoFischer Scientific) containing protease and phosphatase inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail, ThermoFischer Scientific). Total protein was estimated by bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit, ThermoFischer Scientific) and stored at −20°C until use. Samples were mixed thoroughly with 4x Laemmli buffer, heated, and loaded into a handcast 10% acrylamide gel. Electrophoresis was performed using the Mini-PROTEAN 3 system (Bio-Rad). The proteins were electro-transferred to PVDF membranes (Amersham Hybond P, GE Healthcare) at 70V over 2 hours via wet tank transfer utilizing the Mini-PROTEAN 3 system (Bio-Rad). Next, the PVDF membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline, 0.1% v/v Tween 20 (TBST) for 2 hours.

Membranes were washed with TBST and incubated with primary antibody overnight at 4°C. Additional washing steps were performed, followed by incubation in horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. Primary antibodies and secondary

antibodies were diluted in 5% BSA and utilized at the concentrations indicated in Table 1. Immunoreactivity was visualized using enhanced chemiluminescence (Amersham ECL Select or Amersham ECL Prime, GE Healthcare) in a FluorChem Q imager system (ProteinSimple). Density of immunoreactivity for each band was measured using AlphaView software (ProteinSimple), and beta actin was used as a loading control.

Enzyme-linked immunosorbent assay (ELISA)

Whole blood samples were collected from 4-month, 8-month, and 12-month-old mice. All samples were collected at the same time of day to reduce the impact of diurnal variations on adiponectin levels. Samples were collected in tubes and left to clot at room temperature for 90 minutes. Next, samples were centrifuged at 2000 x g for 15 minutes at room temperature. The supernatant (serum) was collected immediately following centrifugation and stored at -80°C until use. Assay was performed following the standard instructions provided by the company (Cayman Chemical, Adiponectin (mouse) ELISA Kit). Briefly, this kit utilized a double-sandwich antibody technique. The capture antibody was a monoclonal antibody for mouse adiponectin, and a biotin-labeled polyclonal anti-mouse antibody was used for detection. Adiponectin concentration was determined by spectrophotometry at 450nm following the addition of streptavidin-horseradish peroxidase and the appropriate substrate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. Data was assessed by one-way or two-way analysis of variance (ANOVA), with or without repeated measures where appropriate. For two-way ANOVA group x treatment, group refers to 3xTg-AD or Control, while treatment refers to the various drugs used for incubation. Tukey post-hoc comparisons

were used to compare groups when analysis of variance indicated significant effects. Results were considered significantly different when $p<0.05$. All data are presented as means ± SEM.

4 Results

4.1 3xTg-AD mice show deficits in basal synaptic transmission which are rescued by AdipoRon

To determine whether AdipoRon rescues deficits in glutamatergic basal synaptic transmission in the Schaffer collateral pathway of the hippocampus in 3xTg-AD mice, field excitatory post-synaptic potentials (fEPSPs) were measured at increasing stimulus intensities. Two-way ANOVA (group x treatment) revealed an interaction effect at higher stimulus intensities [$F(1,18) = 4.679$, $p=0.0442$ at 160μA; $F(1,18) = 4.654$, $p=0.0447$ at 180μA; $F(1,18) = 6.289$, $p=0.022$ at 200 μA; Figure 4.1A]. Specifically, AdipoRon incubation increased the fEPSP slope in the 3xTg-AD group ($p=0.021$ at 160 μA, $p=0.0264$ at 180 μA, $p=0.0235$ at 200 μA), but AdipoRon incubation had no effect at any stimulus intensity in the Control group ($p >0.9$). Of note, fEPSP slope was reduced in 3xTg-AD compared to Control ($p = 0.0282$ at 180 μA, $p=0.0047$ at 200 μA) indicating deficits in baseline glutamatergic synaptic transmission, while no significant difference in Control versus 3xTg-AD + AdipoRon was observed at any stimulus intensity ($ps>0.8$).

To determine whether the deficits in basal synaptic transmission in 3xTg-AD mice are due to deficits in presynaptic axon recruitment, stimulus intensities versus fiber volley (FV) amplitudes were compared and found to be similar between groups and treatments at all stimulus intensities (Figure 4.1B), suggesting that the changes in basal synaptic transmission are not due to changes in axon recruitment.

Evaluation of the FV amplitude versus fEPSP slope provides a measure of basal synaptic transmission while controlling for axon recruitment, and thus is the ideal measure of overall basal synaptic transmission (Sweatt 2010). Two-way ANOVA (group x treatment) revealed an interaction effect [$F(1,16) = 8.988$, $p=0.0085$; Figure 4.1C]. Specifically, AdipoRon incubation increased the linear regression slope in the 3xTg-AD hippocampal slices ($p=0.0029$) indicating an increase in basal synaptic transmission, but AdipoRon incubation had no effect in the Control group ($p=0.999$). Of note, the linear regression slope was reduced in the 3xTg-AD group compared to Control ($p=0.012$), while no significant difference was observed in Control versus 3xTg-AD + AdipoRon ($p=0.9264$). The increase in synaptic transmission by AdipoRon incubation in the 3xTg-AD group, but not the Control group, indicates a selective improvement in glutamatergic neurotransmission in AD pathology.

4.2 AdipoRon incubation alters presynaptic parameters in 3xTg-AD and control mice

To determine whether alterations in basal synaptic transmission could be related to alterations in presynaptic glutamate availability, we evaluated paired pulse facilitation (PPF) and depletion of the readily releasable pool (RRP). PPF is a type of short-term plasticity that depends on residual calcium build-up in the presynaptic terminal, whereas the RRP represents glutamatergic synaptic vesicles which are available for immediate release. Two-way ANOVA (group x treatment) did not reveal an interaction effect, indicating a similar effect of AdipoRon in both Control and 3xTg-AD groups. Main effects of treatment [$F(1, 20) = 12.47$; $p=0.0021$] and group [$F(1, 20) = 6.009$; $p=0.0235$] were observed at the lowest interstimulus interval (Figure 4.2A). There was a significant reduction in PPF at 20 ms ($p=0.0346$) and 50 ms ($p=0.011$) in 3xTg-AD compared to Control. AdipoRon treatment significantly increased PPF at 20 ms ($p=0.0065$) and 50 ms ($p=0.0017$) in 3xTg-AD mice to a level which was not significantly different than Control

(p=0.8697 and p=0.8636, respectively). As there is an inverse relationship between PPF and presynaptic release probability, this indicates that 3xTg-AD mice have an increased presynaptic release probability, and that AdipoRon leads to a reduction in presynaptic release probability.

For the RRP analysis, two-way ANOVA (group x treatment) did not reveal an interaction effect [$F(1,18) = 2.398$, $p=0.1389$; Figure 4.2B], indicating a similar effect of AdipoRon in both Control and 3xTg-AD groups. A main effect of treatment was observed [$F(1,18) = 8.358$, $p=0.0097$], with AdipoRon leading to more rapid depletion of the RRP in both 3xTg-AD and control. In the 3xTg-AD group, the depletion of the RRP was significantly slower compared to Control ($p<0.01$), indicating a larger size of the RRP of glutamate in presynaptic hippocampal neurons in 3xTg-AD.

Taken together, this indicates that the 3xTg-AD group has a higher level of presynaptic glutamate availability, which may indicate an increased propensity for glutamatergic excitotoxicity, and AdipoRon reduces glutamate availability in both 3xTg-AD and Control. Thus, in contrast to the actions of AdipoRon on basal synaptic transmission, the actions of AdipoRon on presynaptic parameters do not appear to be selective for AD pathology.

4.3 AdipoRon rescues LTP deficits in 3xTg-AD mice but does not alter DP

To determine whether AdipoRon incubation rescues deficits in synaptic plasticity in 3xTg-AD mice, we measured LTP and DP in hippocampal slices. Hippocampal LTP is highly correlated with performance on hippocampal-based cognitive tests, and thus LTP has been termed the cellular correlate of learning and memory (Lüscher and Malenka 2012). For LTP analysis two-way ANOVA (group x treatment) revealed an interaction effect [$F(1,20) = 12.15$, $p=0.0023$; Figure 4.3B], as AdipoRon incubation increased LTP in the 3xTg-AD group ($p<0.001$) but not in

Control (p=0.9627). LTP was reduced in the 3xTg-AD group compared to Control (p<0.001), but there was no significant difference between 3xTg-AD + AdipoRon and Control (p=0.8816). This indicates that AdipoRon rescued LTP in the 3xTg-AD group to a level comparable to Control LTP.

To determine whether alterations are present during the induction of LTP, we evaluated fEPSP amplitude during TBS (Parameshwaran et al. 2013). When the first fEPSP from each sweep was normalized to the first fEPSP of the first sweep, we did not find any significant interaction effect or main effects of treatment or group (Figure 4.3C). Likewise, when the fEPSPs within the train was normalized to the first fEPSP of the train, we did not find any significant interaction effect or main effects (Figure 4.3D). This indicates that the alterations in LTP might not be due to changes during the induction period.

DP is a type of synaptic plasticity in which a low frequency stimulation leads to synaptic weakening at a synapse that has previously undergone LTP (Bashir and Collingridge 1994). To determine whether AdipoRon incubation can protect against enhanced DP in 3xTg-AD mice, we utilized a low frequency stimulation protocol to induce DP following sixty minutes of LTP recording. We found no significant interaction effect (group x treatment) [$F(1,15) = 0.5873$, $p=0.4553$] and a main effect of group [$F(1,15) = 15.02$, $p=0.0015$] but not treatment [$F(1,15) = 0.02383$, $p=0.8794$] (Figure 4.3F). Specifically, DP was significantly enhanced (a larger reduction from baseline) in the 3xTg-AD group and the 3xTg-AD + AdipoRon group compared to Control ($p=0.0191$ and $p=0.045$, respectively). Thus, AdipoRon incubation fails to normalize DP in the 3xTg-AD hippocampal slices.

4.4 Trimeric adiponectin is similar to AdipoRon in amelioration of synaptic alterations in 3xTg-AD mice

To validate that the actions of AdipoRon are similar to that of adiponectin, we incubated slices from 3xTg-AD mice in trimeric adiponectin (3xTg-AD + APN) and compared the effects to 3xTg-AD and 3xTg-AD + AdipoRon. Trimeric adiponectin was utilized because it is the major form of adiponectin found in CSF (Ebinuma et al. 2007). The concentration of adiponectin utilized (10nM) is similar to that found in CSF and was chosen based on a previous study which evaluated the effects of adiponectin on hypothalamic neurons following brain slice incubation (J. Sun et al. 2016).

First, we validated that adiponectin incubation enhances basal synaptic transmission in 3xTg-AD hippocampal slices by assessing the slope of the linear regression for FV amplitude versus fEPSP slope. We found a significant difference among groups [$F(2,11) = 8.278$, $p=0.0064$; Figure 4.4A]. Adiponectin incubation led to a significant increase in basal synaptic transmission ($p=0.0103$) and was not significantly different than AdipoRon incubation ($p=0.9974$). Next, we determined whether adiponectin leads to enhancement of hippocampal LTP. We found a significant difference between groups [$F(2,15) = 20.44$, $p<0.001$; Figure 4.4B], with adiponectin increasing LTP ($p<0.001$) in 3xTg-AD mice. There was no significant difference between adiponectin and AdipoRon incubation ($p=0.6848$). This implies that the dose AdipoRon utilized is similar to endogenous adiponectin in terms of enhancement of basal synaptic transmission and LTP in 3xTg-AD hippocampal slices.

Next, we determined the effects of adiponectin on presynaptic parameters in hippocampal slices from 3xTg-AD mice. For PPF, there was a significant treatment effect [$F(2,13) = 15.49$,

p<0.001; Figure 4.4C], and adiponectin incubation led to a significant increase at lower interstimulus intervals (p=0.0073 and p=0.0013 at 20 and 50 ms, respectively), whereas there was no difference between AdipoRon and adiponectin incubation at any interstimulus interval (ps>0.25). Likewise, for RRP, there was a significant treatment effect [F(2,14) = 4.956, p=0.0236; Figure 4.4D], with adiponectin incubation leading to a more rapid depletion of glutamate (p<0.001), whereas there was no significant difference between adiponectin and AdipoRon incubation (p=0.8431). This implies that AdipoRon is similar to adiponectin in reduction of presynaptic release probability and increased depletion of the RRP in 3xTg-AD hippocampal slices. Thus, the concentration of AdipoRon used for incubation of hippocampal slices appears to have a similar effect as a physiological concentration of adiponectin.

4.5 AdipoRon incubation alters phosphorylation of AMPK, GSK3β, and GluA1 in 3xTg-AD hippocampal slices

To investigate potential signaling mechanisms associated with synaptic alterations following AdipoRon incubation, we performed Western blot analysis following hippocampal slice incubation. We first evaluated AMPK, a major downstream signaling molecular for adiponectin receptors, particularly AdipoR1. Two-way ANOVA (group x treatment) did not reveal an interaction effect [F(1,12) = 2.456, p=0.143; Figure 4.5A), although there were main effects of group [F(1,12) = 29.82; p<0.001] and treatment [F(1,12) = 12.45, p=0.0042]. Specifically, there was a significant reduction in pAMPK in 3xTg-AD compared to Control (p<0.0016). AdipoRon incubation significantly increased pAMPK in 3xTg-AD (p=0.0165) to a level that was not significantly different than Control (p=0.5420).

Next, we evaluated GSK3β, a key player in the pathophysiology of AD (Hooper, Killick, and Lovestone 2008), which is phosphorylated and thus inactivated following activation of adiponectin receptors (Ali et al. 2015). Two-way ANOVA (group x treatment) revealed an interaction effect [$F(1,20) = 4.716$, $p=0.0421$; Figure 4.5B). Upon further analysis, there was an increase in pGSK3β following AdipoRon incubation in 3xTg-AD ($p=0.0477$) but not in Control ($p=0.9944$). Additionally, there was a reduction in baseline pGSK3β in 3xTg-AD compared to Control ($p=0.0018$) although there was no significant difference in 3xTg-AD + AdipoRon and Control ($p=0.4583$). Thus, we hypothesized that AdipoRon incubation may be affecting synaptic parameters in the Schaffer Collateral pathway of the hippocampus via activation of AMPK and inhibition of GSK3β.

Next, we considered whether AdipoRon incubation may be altering phosphorylation of glutamatergic receptor subunits involved in the maintenance of LTP. GluA1 is a subunit of the glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and an increase in phosphorylation of Ser831 increases conductance of the AMPARs and thus may enhance basal synaptic transmission as well as LTP (Lee et al. 2003). Two-way ANOVA (group x treatment) revealed a significant interaction effect [$F(1,12) = 9.494$, $p=0.0095$; Figure 4.5C). AdipoRon incubation led to a significant increase in GluA1 Ser831 phosphorylation in the 3xTg-AD group ($p<0.001$) but not in Control ($p=0.5382$). There was a reduction in baseline pGluA1 in 3xTg-AD compared to Control ($p=0.0486$). We also looked for alterations in phosphorylation of GluA1 Ser845 following AdipoRon incubation, as an increase in phosphorylation leads to increased open channel probability of GluA1, but we found no changes (data not shown). Thus, AdipoRon incubation may be enhancing basal synaptic transmission and LTP in part via increased phosphorylation of GluA1 Ser831.

Because AdipoRon treatment increased phosphorylation of AMPK, and AMPK is an important mediator of synaptic function (Marinangeli et al. 2018), we hypothesized that an AMPK inhibitor may prevent AdipoRon-induced alternations in synaptic function in 3xTg-AD mice. We utilized the AMPK inhibitor Compound C (CC) to test this hypothesis, and we also utilized the AICAR, an AMPK activator, to serve as a positive control. Thus, we compared electrophysiology parameters following slice incubation in the following groups: 3xTg-AD, 3xTg-AD + AdipoRon, 3xTg-AD + AdipoRon + CC, and 3xTg-AD + AICAR. To determine whether the activation or inhibition of AMPK might influence synaptic parameters in the Control group, we then compared electrophysiological parameters following slice incubation in the following groups: Control, Control + AdipoRon, Control + CC, and Control + AICAR. Since our initial data indicated that AdipoRon induces presynaptic changes in the Control group, we also added on a Control + AdipoRon + CC group for presynaptic parameters.

4.6 AdipoRon fails to increase basal synaptic transmission in the 3xTg-AD group in the presence of an AMPK inhibitor

First, we evaluated the ability of AdipoRon to enhance basal synaptic transmission in the presence of CC in 3xTg-AD mice. There was a significant treatment effect among groups [$F(3,20) = 7.494$, $p=0.0015$; Figure 4.6A], and AdipoRon failed to enhance basal synaptic transmission in the presence of CC ($ps>0.5$ at all stimulus intensities). The AMPK activator, AICAR, significantly enhanced basal synaptic transmission at higher stimulus intensities ($p=0.0413$, $p=0.0283$, and $p=0.0175$ at 160, 180, and 200 μA respectively). As expected, there was no significant treatment effect among groups for FV analysis [$F(3,20) =0.4866$, $p=0.6954$; Figure 4.6B]. In the FV amplitude versus fEPSP slope analysis for overall changes in basal synaptic transmission, there was a significant treatment effect among groups [$F(3,18) = 11.11$;

p<0.001; Figure 4.6C]. Again, AdipoRon failed to enhance basal synaptic transmission in the presence of CC (p=0.704), while AICAR increased basal synaptic transmission (p<0.001). This provides evidence that AdipoRon may be enhancing basal synaptic transmission via activation of AMPK.

To determine whether activation or inhibition of AMPK may independently alter basal synaptic transmission in Control hippocampal slices, we compared slice incubation of AdipoRon, AICAR, and CC. There were no significant treatment effects in fEPSP slope, FV amplitude, or fEPSP slope versus FV amplitude linear regression analyses among groups [F(3,18) = 0.6887, p=0.5706; Figure 4.6D], [F(3,18) = 0.4849, p=0.6970; Figure 4.6E], and [F(3,18) = 0.05152, p=0.9841; Figure 4.6C], respectively. Thus, our results indicate that AMPK activation selectively enhances basal synaptic transmission in 3xTg-AD hippocampal slices rather than acting as a general enhancer of synaptic activity, which aligns with the AdipoRon incubation data.

4.7 AdipoRon retains the ability to alter RRP in the presence of an AMPK inhibitor

To determine whether AdipoRon is able to alter presynaptic parameters in the presence of an AMPK inhibitor in 3xTg-AD hippocampal slices, we evaluated PPF and depletion of the RRP. For PPF, there was a significant treatment effect among groups [F(3,20) = 3.417, p=0.0372; Figure 4.7A]. Although AdipoRon increased PPF at 20 and 50 ms (p=0.0159 and p=0.0062), AdipoRon failed to significantly increase PPF in the presence of CC (p=0.2668 and p=0.2690), although a trend towards increased PPF was observed. Interestingly, AICAR failed to significantly increase PPF at any interstimulus interval (ps>0.5). As AMPK activation alone was insufficient to increase PPF, this may indicate that AMPK is not the major mechanism by which

AdipoRon increases PPF. However, since AdipoRon was unable to significantly increase PPF in the presence of an AMPK inhibitor, perhaps AMPK is contributing to the activity of AdipoRon.

For the RRP analysis in 3xTg-AD, we found a significant treatment effect among groups [$F(3,19) = 5.052$, $p=0.0097$; Figure 4.7B]. AdipoRon maintained the ability to enhance the depletion of the RRP in the presence of CC ($p=0.0013$), while there was no significant change following AICAR incubation ($p=0.9543$). Although AdipoRon retained the ability to enhance depletion in the presence of CC, there was also a significant difference in AdipoRon and AdipoRon + CC ($p=0.0019$) indicating a reduction the influence of AdipoRon in the presence of CC. Since AMPK activation alone did not alter RRP this may indicate that AMPK is not the major mechanism by which AdipoRon alters RRP. Although AdipoRon was able to enhance the depletion of the RRP in the presence of CC, it was less effective compared to AdipoRon alone.

Next, we determined whether activation or inhibition of AMPK altered PPF or RRP in Control hippocampal slices. For PPF, there was no significant treatment effect [$F(4,25) = 1.294$, $p=0.2990$; Figure 4.7B] but, there was a significant treatment effect for depletion of the RRP [$F(4, 25) = 6.071$, $p=0.0015$; Figure 4.7C]. AdipoRon maintained the ability to significantly enhance depletion of the RRP in the presence of CC ($p=0.0494$), and AICAR did not significantly alter the RRP ($p>0.99$). This suggests that AdipoRon is altering RRP by a mechanism which is not dependent on AMPK activation in Control hippocampal slices.

4.8 AdipoRon fails enhance LTP in 3xTg-AD mice in the presence of an inhibitor of AMPK

Next, we determined whether AdipoRon alters synaptic plasticity in 3xTg-AD hippocampal slices in the presence of an AMPK inhibitor. There was a significant treatment effect [$F(3, 22) = 19.61$, $p<0.0001$; Figure 4.8B], and AdipoRon was unable to enhance LTP in the presence of an

AMPK inhibitor (p=0.9869). Additionally, the AMPK activator enhanced LTP (p<0.001) to a level that was not significantly different than AdipoRon alone (p=0.8288). To determine whether inhibition or activation of AMPK might alter LTP in Control hippocampal slices, we compared the effects of drug incubation, and found no significant differences among groups [F(3,18) = 0.9239, p=0.4493; Figure 4.8D]. Thus, in 3xTg-AD hippocampal slices, AMPK activation or AdipoRon significantly enhances LTP, and AdipoRon is unable to enhance LTP in the presence of an AMPK inhibitor. This implies that AdipoRon may be enhancing LTP via a mechanism dependent on AMPK.

4.9 Inhibition of AMPK blocks AdipoRon-induced increased phosphorylation of GSK3β and GluA1

To determine whether the use of an AMPK inhibitor could prevent the AdipoRon-induced enhancement of GSK3β and GluA1 phosphorylation in 3xTg-AD hippocampal slices, we performed Western blot following slice incubation. First, we confirmed prevention of AdipoRon-induced AMPK phosphorylation in the presence of CC, as well as phosphorylation of AMPK via AICAR in 3xTg-AD hippocampal slices. There was a significant treatment effect [F(3,20) = 15.95, p<0.001; Figure 4.9A]. CC prevented AdipoRon-induced increase in AMPK phosphorylation (p=0.01), while AICAR enhanced phosphorylation of AMPK (p<0.001).

Next, we evaluated the ability of CC to prevent the AdipoRon-induced increase in phosphorylation of GSK3β. We found a significant treatment effect among groups [F(3, 20) = 7.178, p=0.0019; Figure 4.9B]. The increase in phosphorylation of GSK3β was prevented by CC incubation (p=0.0074), and AICAR increased phosphorylation of GSK3β (p=0.0479) in 3xTg-

AD hippocampal slices. This implies that AdipoRon-induced enhancement of GSK3β phosphorylation and thus inactivation of GSK3β is dependent on activation of AMPK.

We next determined whether enhancement of GluA1 phosphorylation following AdipoRon incubation is also dependent on AMPK activation. We found a significant treatment effect among groups [$F(3,20) = 26.27$, $p<0.001$; Figure 4.9C]. Phosphorylation of GluA1 by AdipoRon incubation was prevented by co-incubation in CC ($p<0.001$). AICAR alone was able to enhance phosphorylation of GluA1 ($p=0.003$), although AdipoRon cause a more profound increase compared to AICAR ($p=0.0224$). Thus, the ability of AdipoRon to enhance Ser9 phosphorylation of GSK3β and Ser831 phosphorylation of GluA1 appears to be dependent on activation of AMPK.

4.10 Altered adiponectin and adiponectin receptor expression in 3xTg-AD mice

To evaluate whether adiponectin levels or adiponectin receptor expression is altered in the hippocampus of 3xTg-AD mice, we performed Western blot on hippocampal tissue from 4, 8, and 12-month-old Control and 3xTg-AD mice. Two-way ANOVA (group x age) for hippocampal adiponectin expression did not reveal an interaction effect [$F(2, 12) = 1.666$, $p=0.2298$; Figure 4.10B], however there was a main effect of group [$F(1, 12) = 52.27$, $p<0.001$]. Total adiponectin expression was reduced in 3xTg-AD hippocampi at 4, 8, and 12-months ($p=0.0346$, $p=0.0053$, and $p<0.001$, respectively). For AdpoR1 hippocampal expression, there was no interaction effect [$F(2, 12) = 0.2074$, $p=0.8156$; Figure 4.10C], but there was a main effect of group [$F(1, 12) = 24.82$, $p<0.001$]. The reduction in expression of AdipoR1 in 3xTg-AD hippocampi was only significant at 12-months ($p=0.0158$) although a trend toward reduced expression was observed at all ages. For AdipoR2 hippocampal expression, there was no

interaction effect [$F(2,12) = 0.2788$, $p=0.7615$; Figure 4.10D] and no significant main effects. Thus, there appears to be a reduction in expression of adiponectin and AdipoR1, but not AdipoR2, in the hippocampus of 3xTg-AD mice.

To determine whether serum levels of adiponectin are altered in 3xTg-AD mice, we performed ELISA for total adiponectin concentrations. Two-way ANOVA (group x age) was performed and did not reveal a significant interaction effect [$F(2, 21) = 1.909$, $p=0.1730$; Figure 4.10E], but there was a main effect of group [$F(1,21) = 5.323$, $p=0.0313$]. Upon further analysis, adiponectin serum levels were significantly lower in 12-month 3xTg-AD mice compared to Control mice ($p=0.0242$). This implies that serum adiponectin levels are altered in aged 3xTg-AD mice.

5 Discussion

The results of the current study provide evidence that adiponectin receptor signaling may reduce AD pathology by directly influencing synaptic function. Furthermore, we have identified AMPK as a critical player for adiponectin receptor signaling in rescuing specific synaptic deficits (Figure 4.11). We first evaluated the influence of AdipoRon on basal synaptic transmission. Deficits in basal synaptic transmission are established to occur in various animal models of AD, and deficits correlate with progression of disease (Oddo et al. 2003; Larson et al. 1999; Saganich et al. 2006). Basal synaptic transmission is influenced by many factors including release of glutamate, glutamate uptake, and the expression or functionality of glutamatergic receptors, specifically AMPARs (Sweatt 2010). In the current study, AdipoRon enhanced the phosphorylation of the GluA1 subunit of AMPAR at Ser831 in the 3xTg-AD group, which is associated with increased conductance of the receptor (Lee et al. 2003). This could be one reason for enhanced basal synaptic transmission following AdipoRon incubation. A recent study

showed an increase in surface expression of GluA1 following adiponectin slice incubation (Wang, Jo, and Song 2019), which implies that adiponectin receptor signaling alters AMPAR trafficking in addition to influencing AMPAR activity. Therefore, it seems likely that adiponectin receptor signaling enhances basal synaptic transmission in part via influencing AMPARs. Interestingly, AdipoRon enhanced depletion of presynaptic glutamate and reduced presynaptic release probability of glutamate, which could decrease basal synaptic transmission in the absence of other factors. Thus, the rescue of basal synaptic transmission by AdipoRon appears unrelated to presynaptic alterations.

In the current study, the AdipoRon-induced increase in basal synaptic transmission and GluA1 phosphorylation was inhibited by co-incubation with the AMPK inhibitor, CC. This indicates that activation of AMPK is required for both of these effects. Interestingly, incubation in AdipoRon did not significantly enhance phosphorylation of AMPK, phosphorylation of GluA1, or basal synaptic transmission in hippocampal slices from the Control group. We hypothesize that the normalization of basal synaptic transmission following AdipoRon incubation of 3xTg-AD hippocampal slices is due to restoration of normal postsynaptic adiponectin receptor signaling, and that a further enhancement of postsynaptic adiponectin receptor signaling does not yield further augmentation.

In the current study, we observed an increase in presynaptic release probability (reduction in PPF) along with a slower depletion of presynaptic glutamate in the 3xTg-AD mice. This implies increased presynaptic glutamate availability, suggesting an increased potential for glutamatergic excitotoxicity (Marsh and Alifragis 2018). In animal models of AD, there are differing findings regarding the influence of AD pathology on presynaptic glutamate release probability, which may be time-dependent (Mango et al. 2019). Some have proposed a two-stage model in which

increased glutamatergic availability and glutamatergic hyperexcitability occurs initially, followed by glutamatergic hypoactivity (Olney, Wozniak, and Farber 1997; Butterfield and Pocernich 2003). However, paradoxically, increased glutamate availability may co-exist with impaired synaptic transmission (Larson et al. 1999; Mango et al. 2019), which is observed in the current study. The increase in presynaptic glutamate release and availability in the 3xTg-AD group was reduced by AdipoRon incubation, and AdipoRon also reduced presynaptic glutamate availability in the Control group. Our data suggests that activation of AMPK is not the primary mechanism by which AdipoRon influences presynaptic parameters, but inhibition of AMPK may alter the presynaptic effects of AdipoRon.

The mechanism by which adiponectin receptor signaling alters presynaptic parameters is unclear from the current study. However, we previously found increased expression of the vesicular glutamate transporter (VGLUT) in adiponectin knockout mice (unpublished data). VGLUT is responsible for packaging of glutamate into presynaptic vesicles for eventual release, and an increase in VGLUT is correlated with enhanced presynaptic glutamate availability (Wilson et al. 2005; Herman et al. 2014). Thus, adiponectin receptor signaling may lead to alterations glutamate availability by influencing presynaptic glutamatergic vesicles. One possible mechanism by which adiponectin receptor signaling could influence presynaptic glutamatergic vesicles is by regulation of autophagy. Enhancement of autophagy in presynaptic neuronal terminals via inhibition of mammalian target of rapamycin (mTOR) leads to a reduction in the number of neurotransmitter vesicles and a reduction in neurotransmitter release (Hernandez et al. 2012). Furthermore, autophagy processes are impaired in AD and in aging (Nixon et al. 2005; Rubinsztein, Mariño, and Kroemer 2011). Therefore, whether adiponectin receptor signaling

reduces presynaptic glutamate availability through enhancement of autophagy warrants further investigation.

Deficits in LTP are an integral component of the synaptic dysfunction observed in AD pathology (Nisticò et al. 2012). In the current study, we observed that AdipoRon enhances LTP in the hippocampal Schaffer collateral pathway in the 3xTg-AD mice without affecting Control LTP. Other groups have also reported an influence of adiponectin receptor signaling on LTP. Adiponectin-mediated enhancement of LTP *in vivo* has been shown in the hippocampal perforant pathway of the hippocampus in adult rats (Pousti et al. 2018). The effects of adiponectin receptor signaling on LTP may be dose-dependent, as we previously found that AdipoRon reduced LTP in the hippocampal Schaffer collateral pathway in control mice at higher concentrations (unpublished data). Although the results were not available at the time of our study, a very recent report has shown that adiponectin incubation of hippocampal slices in the 5xFAD model of AD enhances LTP in the Schaffer collateral pathway (Wang, Jo, and Song 2019). Thus, adiponectin receptor signaling appears to enhance LTP in both the 3xTg-AD and the 5xFAD models of AD.

We found that inhibition of AMPK prevents AdipoRon mediated enhancement of LTP, and that the AMPK activator, AICAR, also enhances LTP in the 3xTg-AD hippocampal slices. This was supported by a reduction of hippocampal pAMPK in the 3xTg-AD which was normalized following incubation in AdipoRon. Thus, it appears that activation of AMPK is required for adiponectin receptor signaling enhancement of LTP. AMPK is an essential mediator of energy homeostasis which is highly expressed in neurons (Culmsee et al. 2001). Importantly, tight regulation of AMPK activity is essential for synaptic activation and LTP, and inhibition of AMPK or overactivation of AMPK may lead to deficits in LTP (Potter et al. 2010; Marinangeli et al. 2018). The role of AMPK in AD is controversial and while some studies have found that

activation of AMPK can reduce AD pathology (Du et al. 2015; Won et al. 2010; P. Sun et al. 2019), other studies indicate that activation of AMPK may be detrimental related the ability of AMPK to phosphorylate tau (Domise et al. 2016; Thornton et al. 2011). In our study, AdipoRon increased pAMPK in 3xTg-AD hippocampal slices to the level of Control, without significantly increasing pAMPK in the Control group.

In addition to increased activation of AMPK, AdipoRon incubation increased phosphorylation and thus inactivation of GSK3β, an effect which was absent in the presence of the AMPK inhibitor. Importantly, AMPK induced inactivation of GSK3β via Ser9 phosphorylation promotes LTP *in vivo* (D.-F. Yu et al. 2016). GSK3β is a major mediator of synaptic plasticity, as inhibition of GSK3β promotes LTP while activation of GSK3β promotes long-term depression (LTD) (Peineau et al. 2008; Beurel, Grieco, and Jope 2015). Additionally, GSK3β inactivation is associated with enhanced Ser831 phosphorylation of GluA1 (Urbanska et al. 2019), which may promote LTP via enhanced conductance of AMPARs. Thus, the AdipoRon induced inactivation of GSK3β along with increased conductance of AMPARs are potential mechanisms by which AdipoRon enhanced LTP in the current study. In contrast, AdipoRon was unable to rescue the enhancement in DP present in the 3xTg-AD hippocampal slices in the current study. This may be because GSK3β has not been shown to regulate this form of synaptic plasticity, which is dependent on activation of metabotropic glutamate receptors (Bradley et al. 2012).

Our results regarding an AdipoRon mediated increase in phosphorylation of GSK3β is in line with another study which showed an increase in hippocampal pGSK3β in an animal model of AD following injection of the adiponectin receptor agonist osmotin (Ali et al. 2015). In AD, there is a large body of evidence to suggests that overactivation of GSK3β promotes memory

impairment, synaptic impairments, and tau hyperphosphorylation (Hooper, Killick, and Lovestone 2008), and thus inhibition of GSK3β is proposed as a potential mechanism for reduction in AD pathology.

Interestingly, we found a reduction in hippocampal adiponectin expression in 3xTg-AD mice compared to control mice in the current study across various ages. This suggests a potential dysregulation of adiponectin in AD pathology. In addition to the reduction in total adiponectin expression in the hippocampus, a reduction in AdipoR1 expression was observed at 12-months, but no change in AdipoR2 expression was found at any age. This is in line with a previous study which found a reduction in AdipoR1 but not AdipoR2 expression in the hippocampi of 6-month old male APP/PS1 mice (Várhelyi et al. 2017). Somewhat unexpectedly, a reduction in serum adiponectin was observed in the 3xTg-AD mice at 12-months of age in the current study. Some clinical studies have found an increase in adiponectin serum levels in mild cognitive impairment or AD (Une et al. 2011; Khemka et al. 2014; Waragai et al. 2016), although others have found a decrease or no significant changes in serum levels (Teixeira et al. 2013; Bigalke et al. 2011; Dukic et al. 2016). It has been suggested that an increase in serum adiponectin could be a protective response to brain pathology in AD, however, an increase was not observed in the current study at any age. Taken together, our data suggests that there may a progressive dysregulation of adiponectin in the 3xTg-AD model.

In summary, the key findings from the current study are that the adiponectin receptor agonist AdipoRon rescues basal synaptic transmission and LTP in the Schaffer collateral pathway of the hippocampus in 3xTg-AD mice through a mechanism which appears to be dependent on the activation of AMPK. AdipoRon also alters presynaptic glutamate availability, but this does not appear to be solely dependent on the activation of AMPK. AdipoRon enhances Ser9

phosphorylation of GSK3β and Ser831 phosphorylation of GluA1, and these effects are blocked by inhibition of AMPK. Thus, we hypothesize that AdipoRon-mediated improvement of basal synaptic transmission and LTP are related to increased conductance of GluA1-containing AMPARs and inactivation of GSK3β following activation of AMPK. We also found evidence of adiponectin dysregulation in the 3xTg-AD mice. The current study provide evidence that adiponectin receptor signaling may directly reduce synaptic dysfunction in AD. Additional studies are required to determine whether AdipoRon can ameliorate AD pathology and improve synaptic deficits *in vivo*.

6 Table

Table 4.1 Summary of antibodies and working conditions used in the experiments.

Antibodies	Species	Source	Catalogue #	Dilution
Primary antibodies				
AMPK	Rabbit	Cell Signaling Technology	5832	1:1000
pAMPK (Thr 172)	Rabbit	Cell Signaling Technology	2531	1:500
GSKβ	Rabbit	Cell Signaling Technology	12456	1:1000
pGSK3β (Ser 9)	Rabbit	Cell Signaling Technology	5558	1:1000
GluA1	Rabbit	Cell Signaling Technology	13185	1:750
pGluA1 (Ser831)	Rabbit	Cell Signaling Technology	75574	1:500
Beta actin	Rabbit	Cell Signaling Technology	8457	1:2000
AdipoR1	Rabbit	Abcam	ab126611	1:200
AdipoR2	Mouse	Santa Cruz	sc-514045	1:200
Adiponectin	Rabbit	Abcam	ab62551	1:200
Secondary antibodies				
Anti-mouse IgG	N/A	Santa Cruz	sc-516102	1:2000
Anti-rabbit IgG	Goat	Cell Signaling Technology	7074	1:5000

7 Figures

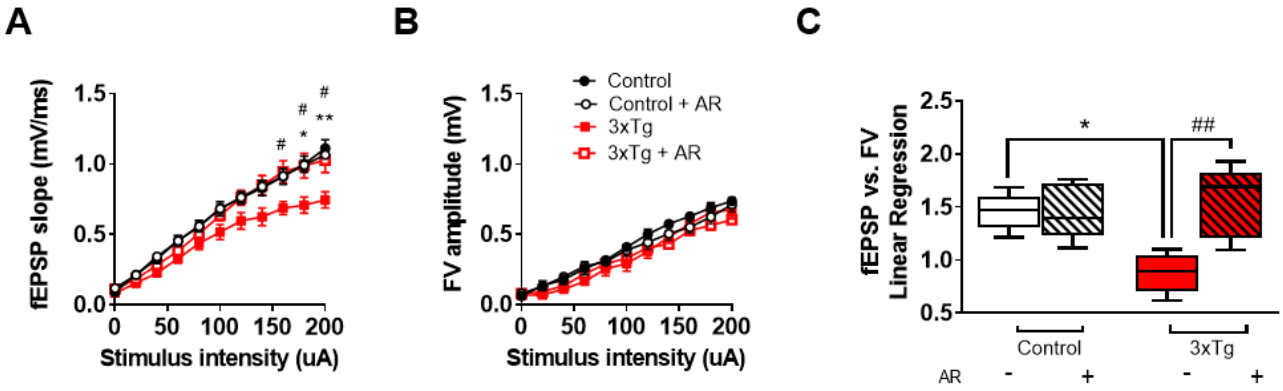

Figure 4.1 3xTg-AD mice display deficits in basal synaptic transmission which are rescued by AdipoRon incubation.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) Input-output curve of fEPSP slope measured at increasing stimulus intensities (B) Input-output curve of FV amplitude measured at increasing stimulus intensities (C) Slope of the linear regression line of best fit from plotting fEPSP slope versus FV amplitude. A-C were analyzed by two-way ANOVA (group x treatment) followed by Tukey's post hoc test was used for multiple comparisons. (*) represents significance difference in Control vs. 3xTg, ($^{\#}$) represents significant difference in 3xTg vs. 3xTg + AR; symbols/bars represent mean ± SEM; $^{*/\#}p<0.05$, $^{\#\#}p<0.01$; n = 5-6 slices from 4-5 mice per group.

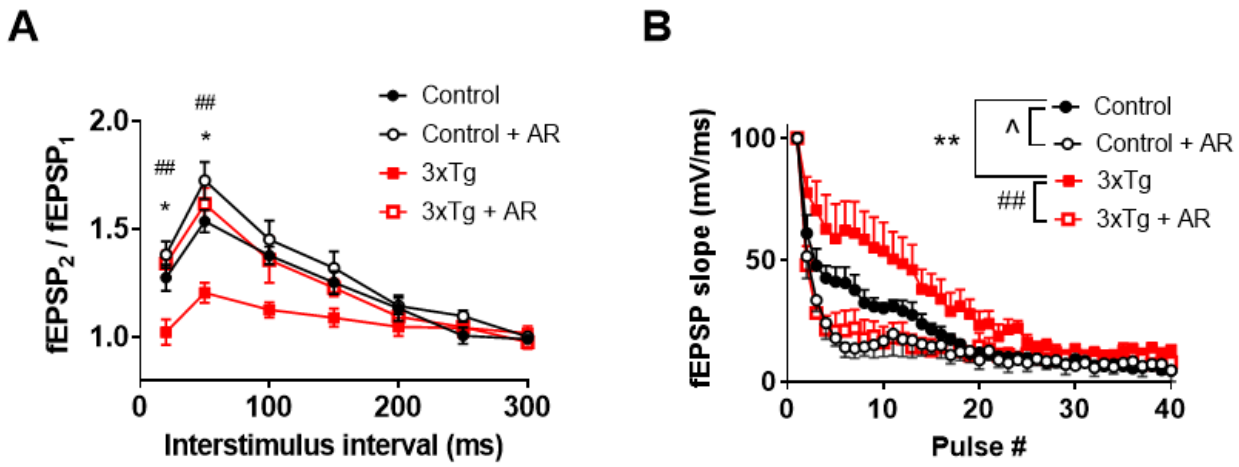

Figure 4.2 3xTg-AD mice display presynaptic alterations which are normalized by AdipoRon incubation.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) Paired-pulse facilitation expressed as the ratio of the second stimulus fEPSP slope to the first stimulus fEPSP slope plotted as a function of interstimulus interval (B) Readily-releasable pool expressed as the fEPSP slopes from stimuli 2-40 normalized to the first stimulus. A-B were analyzed by two-way ANOVA (group x treatment) followed by Tukey's post hoc test was used for multiple comparisons. (*) represents significance difference in Control vs. 3xTg, ($^{\#}$) represents significant difference in 3xTg vs. 3xTg + AR, (^) represents significance difference in Control vs. Control + AR; symbols/bars represent mean ± SEM; $^{*/\#/\wedge}p<0.05$, $^{**/\#\#}p<0.01$; n = 5-6 slices from 4-5 mice per group.

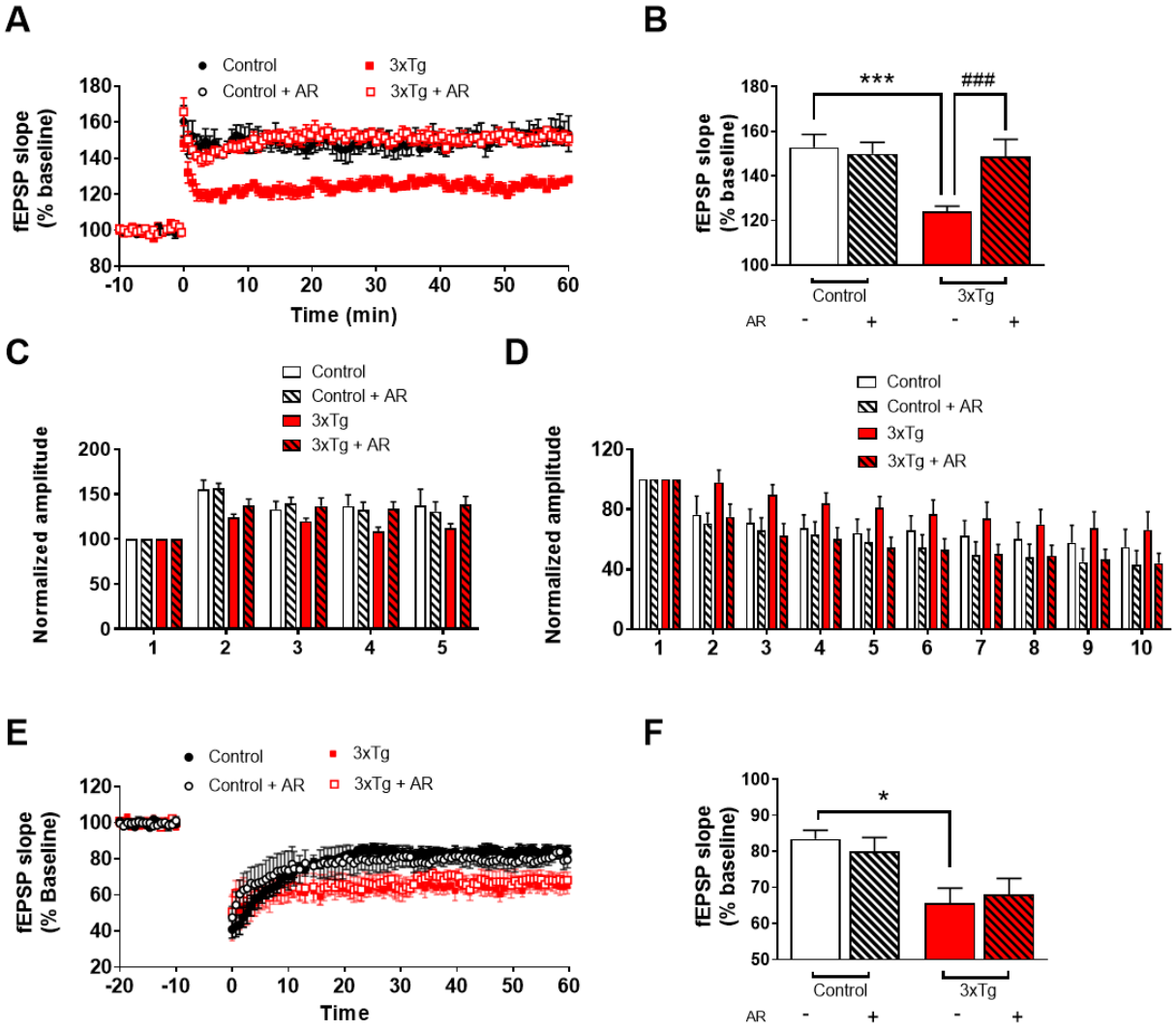

Figure 4.3 AdipoRon rescues LTP deficits in 3xTg-AD mice but does not alter DP. Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) LTP graph represents fEPSP slope before and after induction by TBS (B) LTP bar graph shows the average of fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels (C) Sweep analysis, represented by normalizing the amplitude of the first fEPSP for trains #2-5 with the first fEPSP of the first train (D) Train analysis, represented by normalizing the amplitude of fEPSPs #2-10 with the amplitude of the first fEPSP (E) DP graph represents fEPSP slope before and after LFS

(F) DP bar graph shows the average of fEPSPs recorded during the time period 50-60 min following LFS normalized to baselines levels. B, C, D, and F were analyzed by two-way ANOVA (group x treatment) followed by Tukey's post hoc test was used for multiple comparisons. Symbols/bars represent mean ± SEM; *$p<0.05$, ***$^{/\#\#\#}p<0.001$; n = 5-6 slices from 4-5 mice per group.

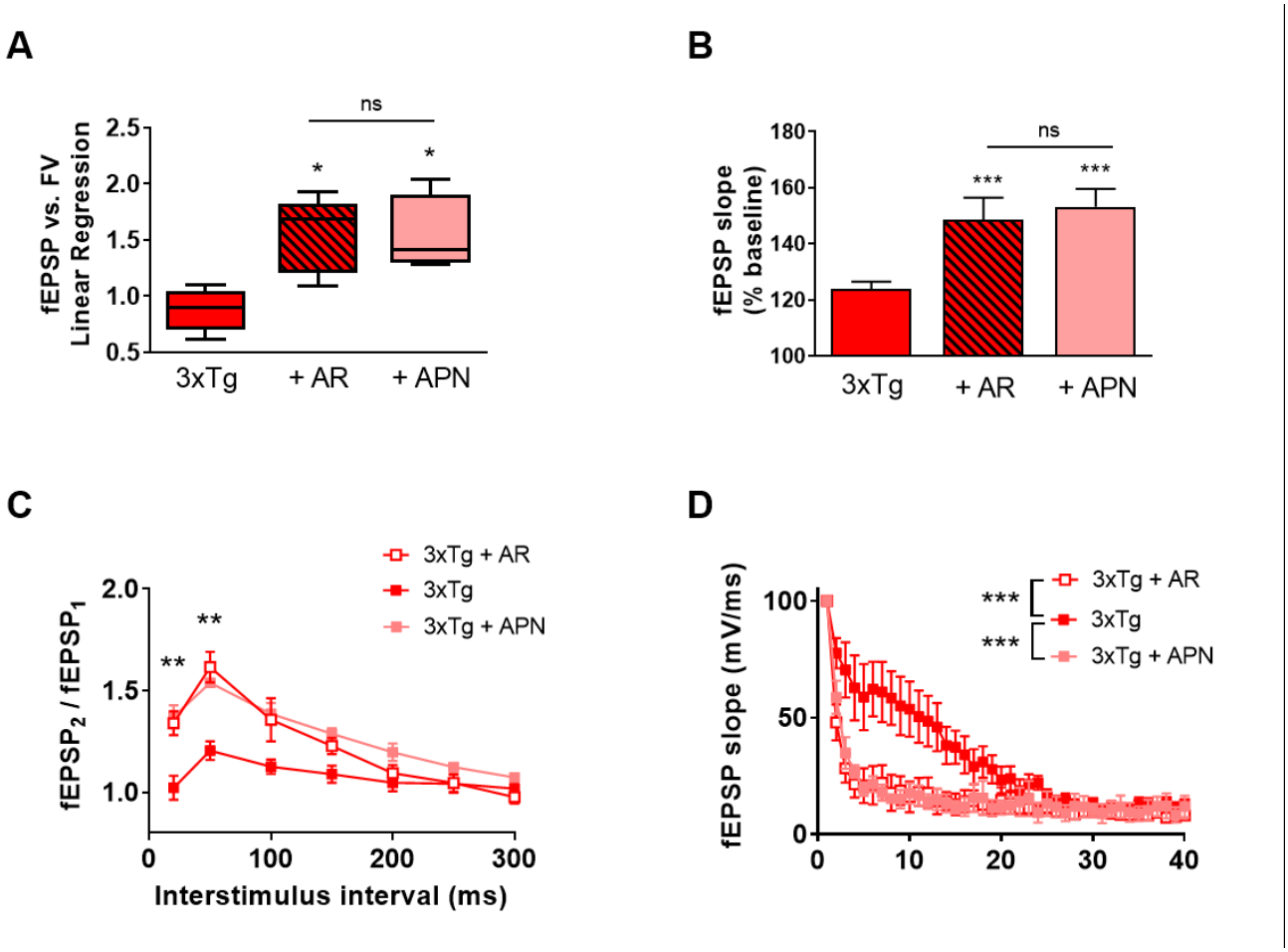

Figure 4.4 Trimeric adiponectin is similar to AdipoRon in amelioration of synaptic alterations in 3xTg-AD mice.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) Slope of the linear regression line of best fit from plotting fEPSP slope versus FV amplitude (B) LTP bar graph shows the average of fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels (C) Paired-pulse facilitation expressed as the ratio of the second stimulus fEPSP slope to the first stimulus fEPSP slope plotted as a function of interstimulus interval (D) Readily-releasable pool expressed as the fEPSP slopes from stimuli 2-40 normalized to the first stimulus. A and B were analyzed by one-way ANOVA, C and D were analyzed by two-way ANOVA

(treatment x interstimulus interval or pulse number, respectively). For all, Tukey's post hoc test was used for multiple comparisons. Symbols/bars represent mean ± SEM; *represents comparison to 3xTg; *$p<0.05$, **$p<0.01$, ***$p<0.001$; n = 5-7 slices from 4-5 mice per group, except 3xTg + APN group where n = 4 slices from 4 mice.

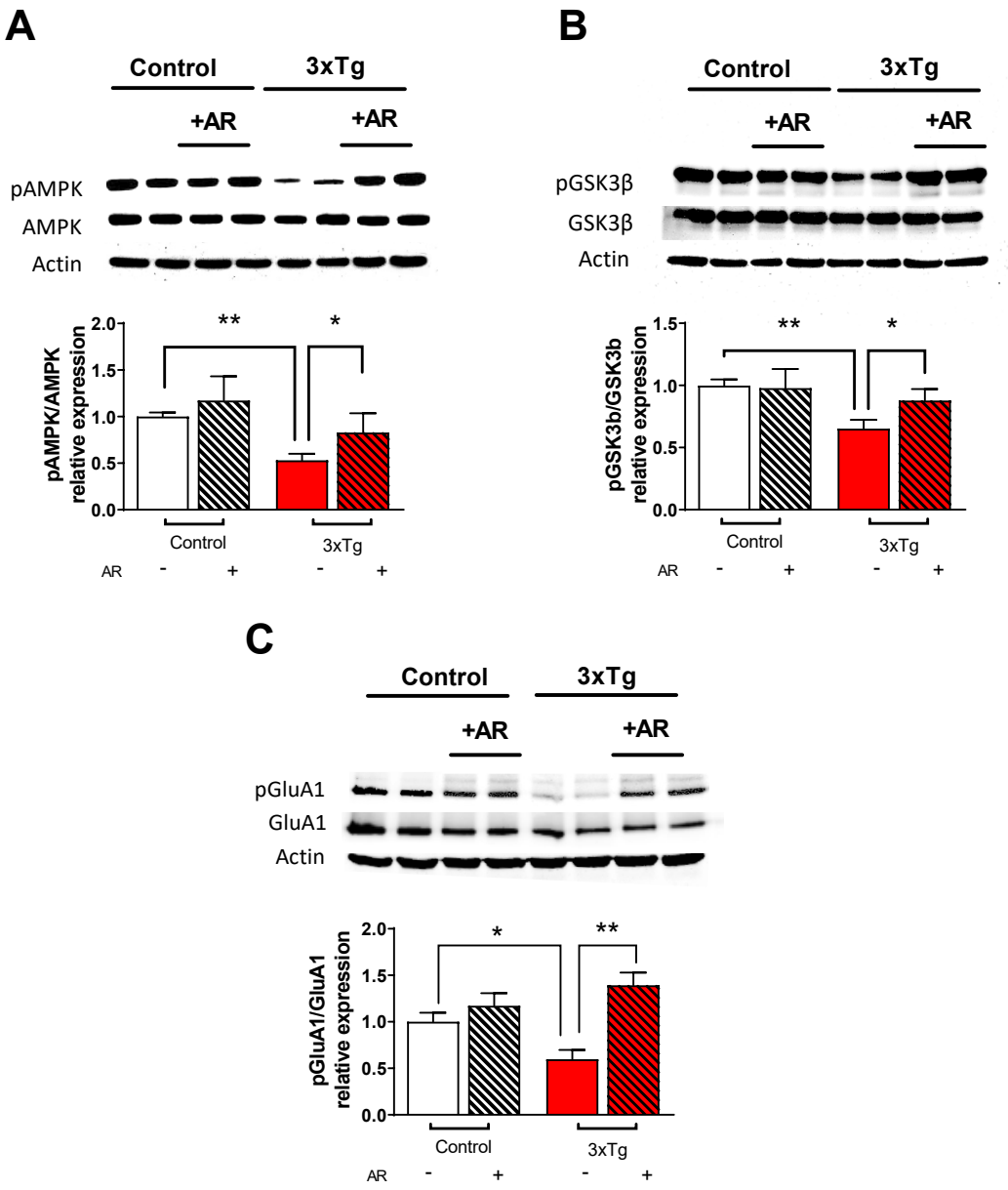

Figure 4.5 AdipoRon incubation increases pAMPK, pGSK3β, and pGluR1 Ser831 expression in hippocampal slices from 3xTg-AD mice.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution. Then, hippocampal tissue was isolated and kept at -80°C until use. Representative immunoblots showing (A) pAMPK/AMPK relative expression ratio (B) pGSK3β/GSK3β relative expression ratio and (C) pGluA1 Ser831/GluA1 relative expression

ratio, in hippocampal lysate. 40μg of protein was loaded per lane. A-C were analyzed by two-way ANOVA (group x treatment) followed by Tukey's post hoc test for multiple comparisons. *$p<0.05$, **$p<0.01$; n = 4-6 mice per group.

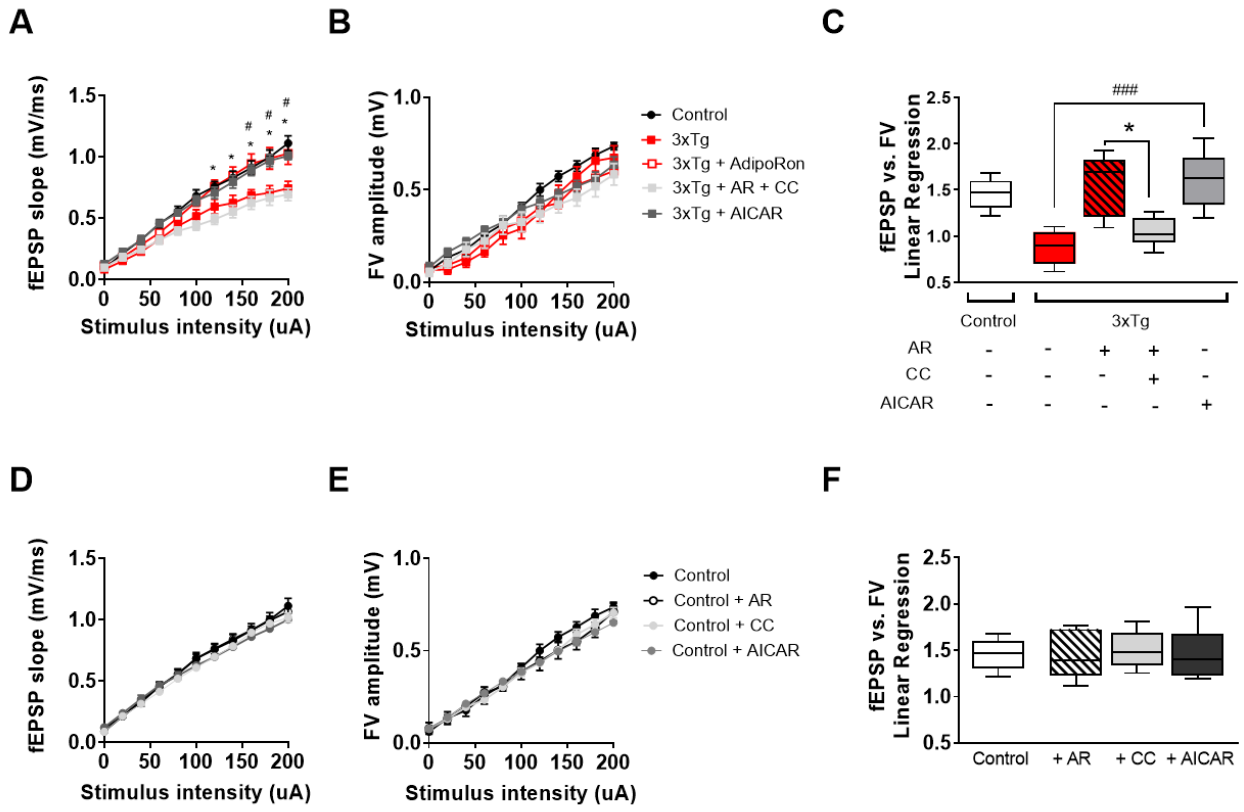

Figure 4.6 AdipoRon fails to increase basal synaptic transmission in 3xTg-AD mice in the presence of an AMPK inhibitor.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) and (D) Input-output curve of fEPSP slope measured at increasing stimulus intensities (B) and (E) Input-output curve of FV amplitude measured at increasing stimulus intensities (C) and (F) Slope of the linear regression line of best fit from plotting fEPSP slope versus FV amplitude. A, B, D, and E were analyzed by two-way ANOVA (treatment x stimulus intensity). C and F were analyzed by one-way ANOVA. For all, Tukey's post hoc test was used for multiple comparisons. For A-C, Control group is shown for visual comparison but was not included in the analysis. Symbols/bars represent mean ± SEM; (*) represents significance difference in 3xTg + AR vs. 3xTg + AR + CC, ($^{\#}$) represents significant

difference in 3xTg vs. 3xTg + AICAR; $^{*/\#}p<0.05$, $^{\#\#\#}p<0.001$; n = 5-7 slices from 4-5 mice per group.

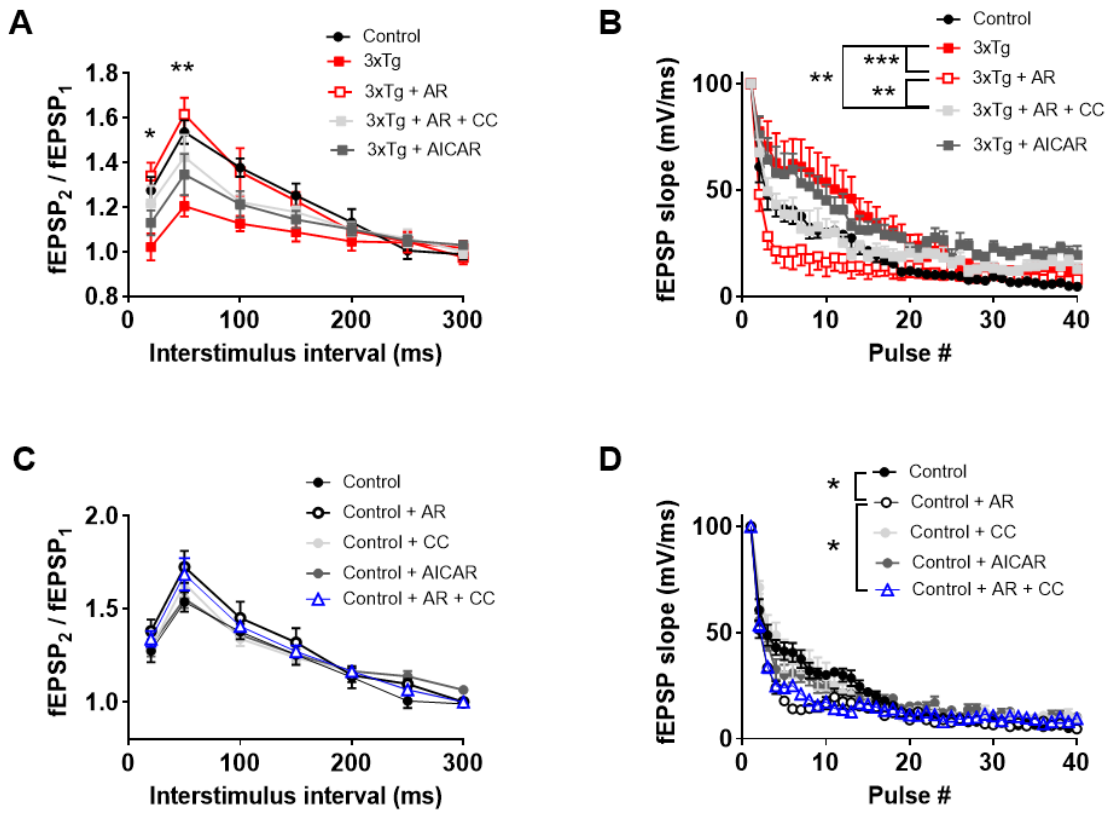

Figure 4.7 AdipoRon retains ability to alter presynaptic parameters in the presence of an AMPK inhibitor in 3xTg-AD mice.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) and (C) Paired-pulse facilitation expressed as the ratio of the second stimulus fEPSP slope to the first stimulus fEPSP slope plotted as a function of interstimulus interval (B) and (D) Readily-releasable pool expressed as the fEPSP slopes from stimuli 2-40 normalized to the first stimulus. A,C and B,D were analyzed by two-way ANOVA (treatment x interstimulus interval or pulse number, respectively). For all, Tukey's post hoc test was used for multiple comparisons. For A-B, Control group is shown for visual comparison but was not included in the analysis. Symbols/bars represent mean ± SEM; For A, (*) represents

significance difference in 3xTg vs. 3xTg + AR; *$p<0.05$, **$p<0.01$; ***$p<0.001$; n = 5-6 slices from 4-5 mice per group.

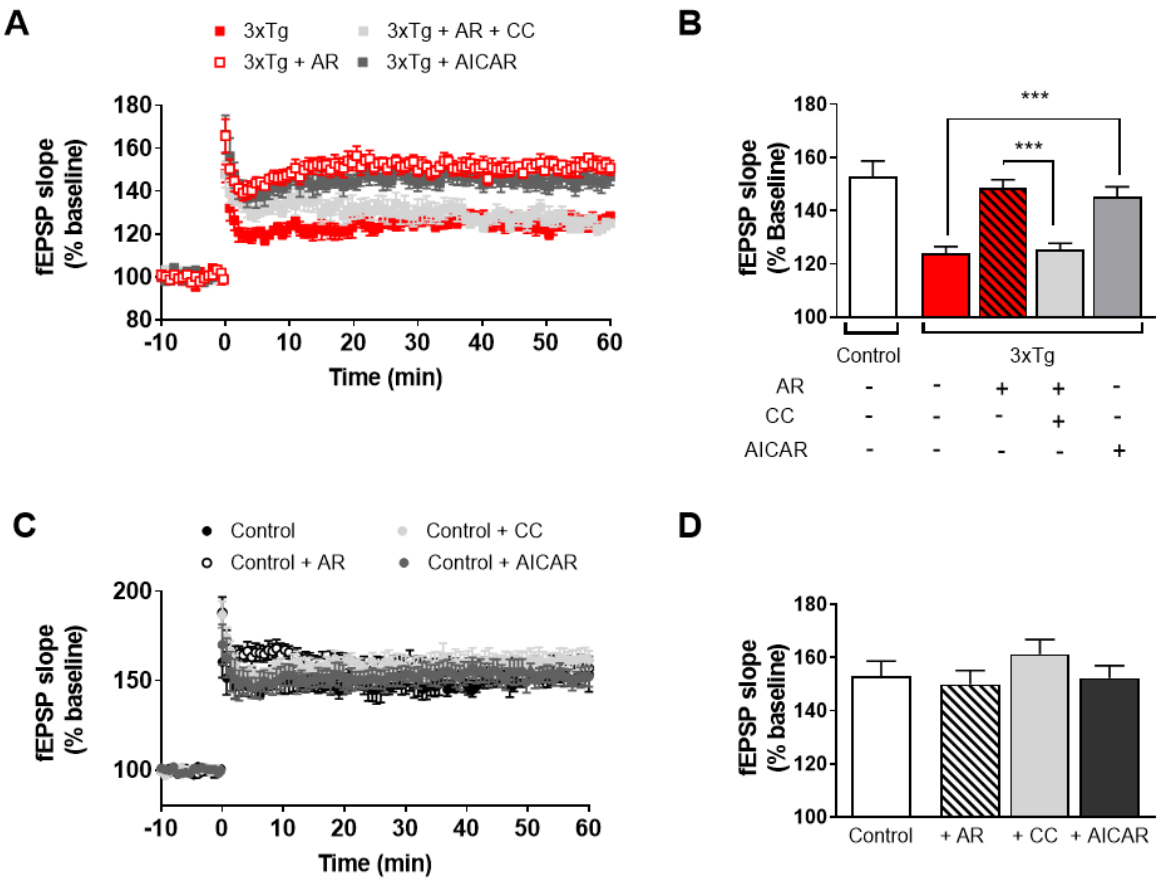

Figure 4.8 AdipoRon fails enhance LTP in 3xTg-AD mice in the presence of an AMPK inhibitor.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution prior to recording. (A) and (C) LTP graph represents fEPSP slope before and after induction by TBS (B) and (D) LTP bar graph shows the average of fEPSPs recorded during the time period 50-60 min following TBS induction normalized to baselines levels. B and D were analyzed by one-way ANOVA followed by Tukey's post hoc test. For B, Control group is shown for visual comparison but was not included in the analysis. Symbols/bars represent mean ± SEM; $^{***}p<0.001$; n = 5-7 slices from 4-5 mice per group.

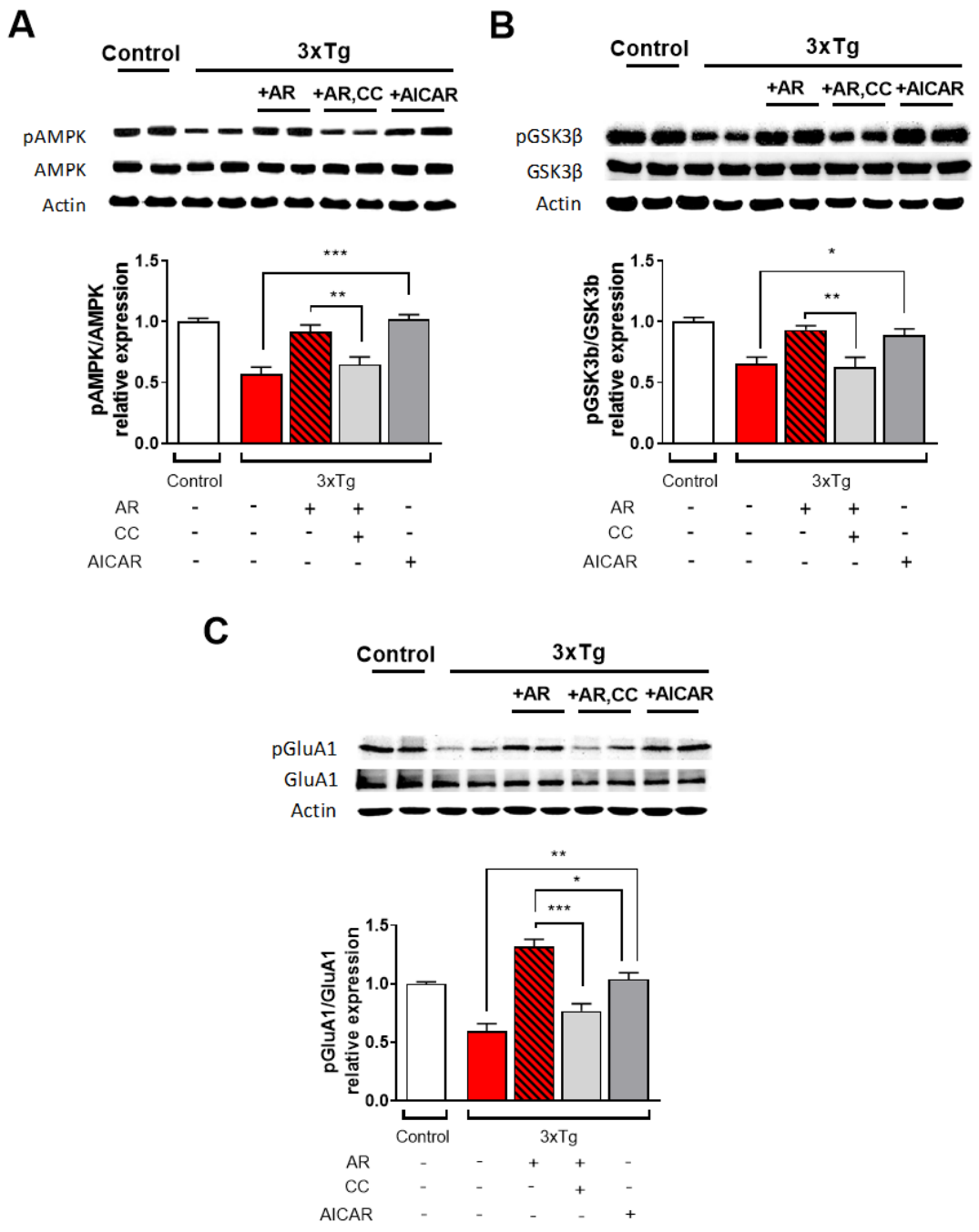

Figure 4.9 AdipoRon fails increase pAMPK, pGSK3b, and pGluA1 in 3xTg-AD hippocampal slices in the presence of an AMPK inhibitor.

Hippocampal slices were prepared from Control and 3xTg mice and incubated for 2 hours in ACSF-drug solution. Then, hippocampal tissue was isolated and kept at -80°C until use.

Representative immunoblots showing (A) pAMPK/AMPK relative expression ratio (B) pGSK3β/GSK3β relative expression ratio and (C) pGluA1 Ser831/GluA1 relative expression ratio, in hippocampal lysate. 40µg of protein was loaded per lane. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Symbols/bars represent mean ± SEM; *$p<0.05$, **$p<0.01$, ***$p<0.001$; n = 5-6 mice per group.

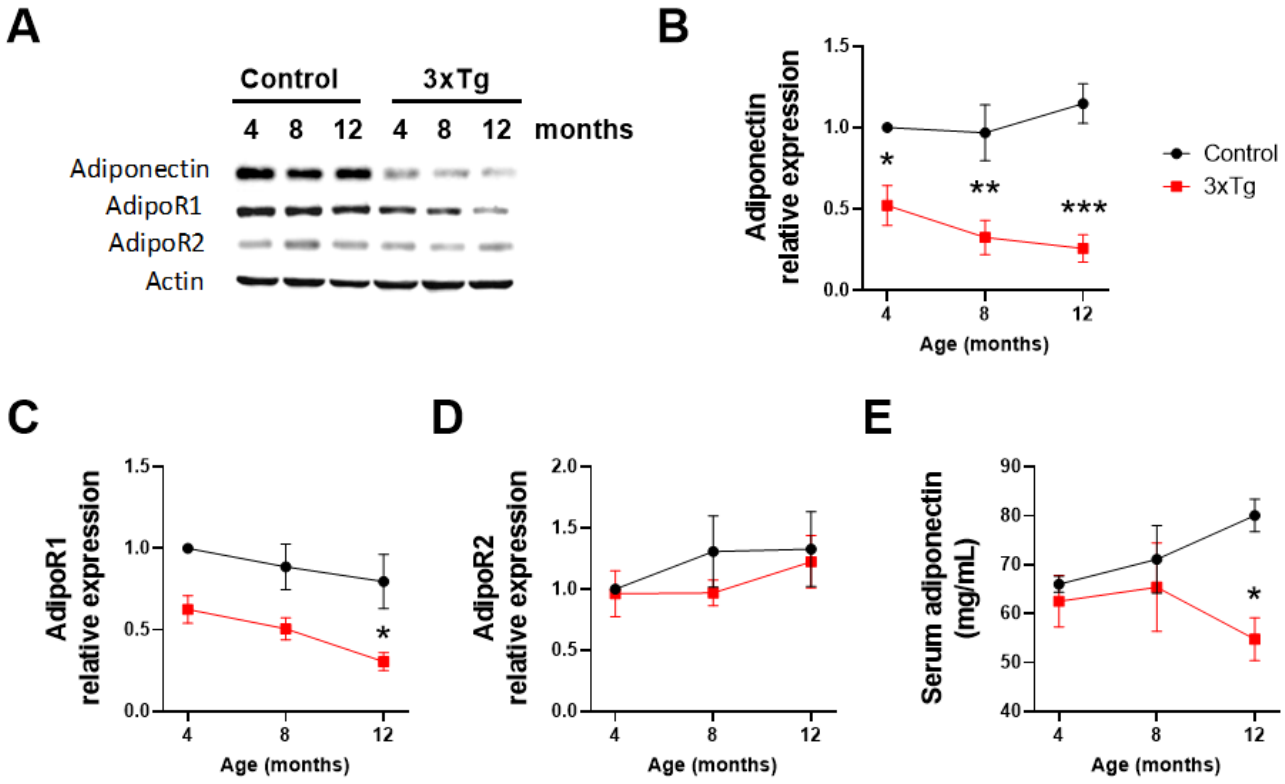

Figure 4.10 Altered adiponectin levels and adiponectin receptor expression in 3xTg-AD mice.

Hippocampal tissue (A-D) or serum (E) was isolated from 4, 8, and 12-month-old Control and 3xTg-AD mice and used for Western blot or ELISA, respectively. (A) Representative immunoblots for graphs B-D (B) adiponectin/beta-actin relative expression (C) AdipoR1/beta-actin relative expression (D) AdipoR2/beta-actin relative expression (E) serum adiponectin concentrations. For Western blot, 40µg of protein was loaded per lane. Data were analyzed by two-way ANOVA (group x age) followed by Tukey's post hoc test to compare Control versus 3xTg-AD at each age. Symbols/bars represent mean ± SEM; *$p<0.05$; for B-D, n = 3 mice per group; for E, n = 4-5 mice per group.

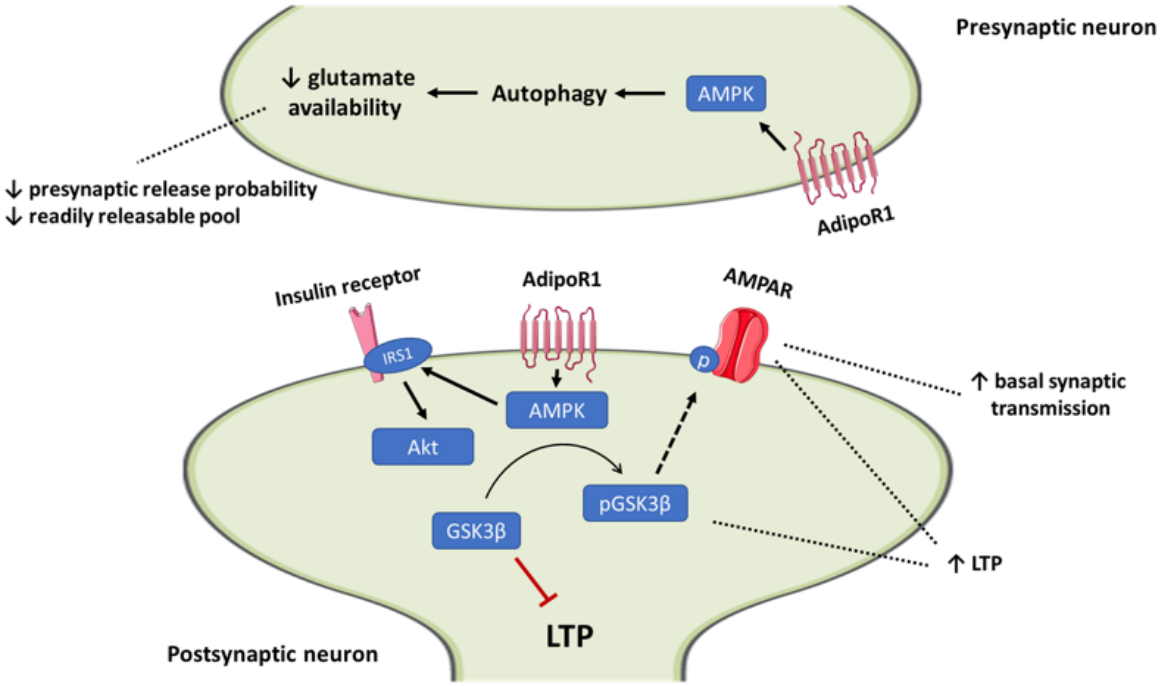

Figure 4.11 Potential mechanisms for synaptic alterations via AdipoR1-AMPK signaling. In the current study, AdipoRon led to phosphorylation of GSK3β and the GluA1 subunit of AMPAR via an AMPK-dependent mechanism. Additionally, enhancement of basal synaptic transmission and long-term potentiation were dependent of activation of AMPK. AMPK activation leads to enhancement of autophagy which reduces neurotransmitter availability in the presynaptic compartment. This is one potential mechanism for the AdipoRon-induced reduction in presynaptic release probability observed in the current study.

8 References

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Chapter 5: Conclusion and Future Directions

The current work focused on elucidating the influence of adiponectin receptor signaling on hippocampal synaptic function and determining whether an adiponectin receptor agonist could directly rescue synaptic deficits in a mouse model of Alzheimer's disease (AD). To summarize key results, (1) adiponectin receptors are located at hippocampal synapses, with AdipoR1 particularly enriched in the synaptic fraction compared to total lysate; (2) the absence of adiponectin leads to deficits in basal synaptic transmission, deficits in long-term potentiation (LTP), and an increase in presynaptic release probability; (3) synaptic alterations in the adiponectin knockout mice are normalized by acute restoration of adiponectin receptor signaling; (4) there is evidence for dysregulation of adiponectin receptor signaling in the hippocampi of 3xTg-AD mice; (5) the adiponectin receptor agonist AdipoRon, or trimeric adiponectin, enhances basal synaptic transmission and LTP in hippocampal slices from 3xTg-AD mice; (6) the rescue of some, but not all, synaptic parameters in 3xTg-AD hippocampal slices appears to be via activation of 5' AMP-activated protein kinase (AMPK); and (7) this rescue is associated with inactivation of glycogen synthase kinase beta (GSK3β) and enhanced conductance of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs).

There are a number of limitations in the current study. Ideally, sex should be considered as a biological variable in vertebrate studies as recommended by the National Institutes of Health. Only male adiponectin knockout mice were used in the study due to limited access to female mice and the potential for estrous cycles to influence behavioral data (Ter Horst et al. 2012). However, in the future, female adiponectin knockout mice should also be studied, especially since sexual dimorphism is observed for serum and CSF adiponectin levels, with females

displaying higher levels of adiponectin (Pajvani et al. 2003). In contrast, in the studies utilizing 3xTg-AD mice, only female mice were used due to the lack of phenotypic traits observed in the male 3xTg-AD mice (The Jackson Laboratory). Male mice which express an AD phenotype should also be studied to verify whether similar effects of adiponectin receptor signaling on hippocampal synaptic function are observed. A significant association between serum adiponectin levels and development of AD was only observed in females in one study (van Himbergen et al. 2012), highlighting the importance of sex differences when considering the relationship between adiponectin and AD.

Another limitation is the use of *ex vivo* hippocampal slices rather than *in vivo* hippocampal recordings. In the current study, hippocampal slices were utilized because these slices preserve synaptic circuitry and maintenance of neuronal function while providing direct access to specific pathways for studying the effects of various drugs. The use of hippocampal slices may be ideal for elucidating the molecular mechanisms of drug treatment (Chang et al. 2019), however, the results of the current study, especially rescue of LTP by AdipoRon, should be validated *in vivo* to confirm the physiological relevance of these findings.

Whether AdipoRon can rescue the behavioral deficits in the 3xTg-AD mice was not determined in the current study. Previous studies have shown that the adiponectin receptor agonist, osmotin, can rescue cognitive deficits and reduce neuropathological hallmarks in mouse models of AD (Ali et al. 2015; Shah et al. 2017). However, rescue of cognitive deficits by AdipoRon in an animal model of AD has not been validated to my knowledge, although AdipoRon does cross the blood brain barrier (Nicolas et al. 2018). As LTP in the hippocampus correlates well with performance on hippocampal based memory tests in rodent models (Lüscher and Malenka 2012), it is expected that AdipoRon could rescue behavioral deficits in mouse models of AD if

sufficient brain concentrations are achieved. The peripheral dose required to reach the brain concentrations which were utilized for hippocampal slice incubation in the current study is unknown. Future studies should evaluate pharmacokinetic parameters of AdipoRon and establish oral or parenteral dosing regimens needed to achieve adequate brain concentrations for physiological effects. If AdipoRon fails to achieve sufficient brain concentrations via oral dosing, development of other adiponectin receptor agonists with enhanced BBB permeability should be pursued.

Failure to achieve statistical significance for some analyses in the current study may be related inadequate sample size. For example, in Figure 4.3, *AdipoRon rescues LTP deficits in 3xTg-AD mice but does not alter DP*, there was not a statistically significant difference in field excitatory post-synaptic potential (fEPSP) amplitude during induction of LTP among groups. However, there appears to be trend towards reduction in the 3xTg-AD slices compared to Control and 3xTg-AD + AdipoRon for the sweep analysis (Figure 4.3C), and an increase in the 3xTg-AD slices compared to other groups for the train analysis (Figure 4.3D). Thus, whether there are alterations in LTP induction in 3xTg-AD hippocampal slices is somewhat inconclusive based on the current study. Use of power analysis to determine the sample size required to detect a difference among groups would be the ideal next step to elucidate whether there are differences in LTP induction.

Much remains unknown regarding adiponectin in the brain and the potential use of adiponectin receptor agonists in AD. Human evidence for dysregulation of adiponectin receptor signaling in AD, such as whether there are differences in receptor expression in post-mortem AD brains, and whether adiponectin receptor polymorphisms may contribute to the risk of AD, should be explored. Additional studies are required to determine whether AdipoRon can ameliorate AD

pathology and improve synaptic deficits *in vivo* and to determine whether similar outcomes are observed in both males and females. Furthermore, the role of specific adiponectin receptors responsible for synaptic alterations warrants further investigation. If one specific adiponectin receptor appears to responsible for mediating improvement in AD models, development of a receptor specific agonist may be ideal for future therapeutic use. Additional facets of adiponectin receptor signaling which may reduce AD pathology including enhancement of autophagy and receptor-mediated ceramidase activity should be explored.

Taken together, the results from the current study indicate that adiponectin receptor signaling influences hippocampal synaptic function, and these results provide evidence to support a neuroprotective role of adiponectin receptor signaling in AD. Thus, amelioration of synaptic dysfunction is another potential mechanism of adiponectin receptor signaling in reduction of AD pathology in addition to reduction of neuroinflammation (Jian et al. 2019), improved brain insulin sensitivity (Ng et al. 2016), reduced vascular dysfunction (Yu et al. 2019), and protection against Aβ/tau-mediated toxicity (Ali et al. 2015). This work contributes to a growing body of evidence which suggests a therapeutic potential for adiponectin receptor agonists in the prevention or treatment of AD.

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