Signaling pathways associated with Alzheimer's disease and possible therapeutic targets

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Abstract

Despite being first identified over a century ago, Alzheimer's disease (AD) is a complex neurological disorder that still has not been properly characterized. Most cases are sporadic in nature, with an unidentifiable cause, but early-onset familial Alzheimer's disease (FAD) is induced by genetic mutations in certain key genes. FAD mutations in the full length amyloid precursor protein (flAPP) increases production of the amyloid beta (Aβ) peptide responsible for plaque formation commonly associated with the disease, leading to neuronal death. A mutation in the PS1 gene (mPS1) results in increased APP cleavage into Aβ1-42, also leading to early AD formation. Although discoveries of FAD mutations have enabled concentrated studies into AD pathogenesis, its cause is still unknown.

In this thesis, experimental projects were designed to study how signaling pathways associated with markers of AD, including APP and PS1 gene mutations, could result in neuronal dysfunction associated with disease pathology, and how these pathways could be manipulated for use as potential therapeutic targets. Cortical neurons isolated from FAD mPS1 mice (expressing the Met146Val mPS1 protein) were analyzed to establish neuronal viability in response to Aβ1-42 insult compared to healthy neurons. mPS1 neurons were no more susceptible to cell death compared to wild-type neurons, because of an increased activation of the transcription factor nuclear factor kappa B (NF-κB) protein brought about by elevated endoplasmic reticulum (ER) calcium release due to the PS1 mutation. However, NF-κB inhibition in the mPS1 neurons caused increased pro-apoptotic protein CHOP expression leading to significantly higher cell death versus controls when neurons were exposed to Aβ1-42. Following this study, the role of the neurotrophic protein neuregulin on

cytoplasmic calcium levels of hippocampal neurons was examined, with the intent of assessing the contributioin of that signaling pathway to AD neuropathology in AD transgenic mice. Neuregulin has been shown to modify glutamatergic channels at neuronal synapses, but how this could affect cytoplasmic calcium levels in neurons was uncertain. Long term treatment (24 hours), but not short-term (1 hr), with neuregulin increased glutamatergic-induced intracellular calcium levels in hippocampal neurons, through a PI3K-mediated mechanism. This study demonstrated that inhibition of the NRG/ErbB axis could be a possible therapeutic target to reduce excitotoxic levels of calcium leading to neuronal death in AD, or enhance synaptic plasticity and memory in AD-initiated areas of deficit. Finally, interactions between the neurotrophic insulin pathway and amyloid peptides were studied using an amyloid precursor protein (APP) overexpressing mouse model, the TgCRND8 strain. Despite insulin depletion induced by streptozotocin injection, young diabetic TgCRND8 mice displayed no impairment in insulin signaling compared to controls, likely due to activation of the insulin signaling pathway by sAPPα. This indicates a possible biological role for sAPPα that prevents diabetic-induced insulin signaling impairment. Thus, the data from these three projects elucidated different components of AD pathogenesis and possibly targets of future AD treatment.

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Dedication

I'd like to dedicate this thesis to my wife Christine, for her love, support and inspiration.

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Chapter 1 - Introduction

1. Alzheimer's Disease and amyloid peptides

1.1 Background

Alzheimer's disease (AD), the leading cause of dementia, was first described in 1906 following diagnosis of patient Auguste D. by Dr. Alois Alzheimer. Psychological assessment of the patient revealed clear dementia, and she died 4 years after being brought to Dr. Alzheimer, at the age of 51. Post-mortem brain analysis using histological staining revealed neurofibrillary tangles and insoluble plaques, both of which were attributed to disease formation. In the early sixties, analysis of the dense plaque formations resulted in the first description of its major constituent as amyloid beta (Aβ) protein, (Kidd, 1963; Terry et al., 1964), which wasn't sequenced until 1985 (Masters et al., 1985). Aβ protein forms fibrous and insoluble aggregates which promote formation of the dense plaques commonly seen in AD post-mortem. Despite their identification as the probable leading cause of sporadic AD, their formation remained a mystery. A distinction between sporadic and genetically-derived early-onset familial AD (FAD) was made in the 1930s and 1940s but it wasn't until much later that FAD genetic studies revealed that the disease was caused by mutations in specific proteins, such as the full-length amyloid precursor protein (flAPP) (Goate et al., 1991) and presenilin-1 (PS1) proteins (Gustafson et al., 1998) . These protein mutations resulted in increased formation of AD hallmark Aβ, and heightened interest in the normal physiological role of flAPP and PS1 proteins, with the goal of determining how their dysfunction may lead to sporadic AD. Generation of transgenic animal models based upon FAD mutations has permitted pathological

and cellular analysis that has contributed much to our limited knowledge of the disease.

AD is a neurological condition resulting in cognitive decline and eventually death. Only a small percentage of cases (~5%) are genetically driven, with most cases being sporadic (95%). Psychological tests like the mini-mental state examination (MMSE) can give an indication of possible AD, but the only sure diagnosis is via post-mortem brain analysis. Recent advances in magnetic resonance imaging (MRI) and positron emission tomography (PET) technology may provide a means of earlier diagnosis of the disease, but could still be too late for proper treatment. Early symptoms include the inability to form new memories, while more advanced AD sufferers will demonstrate confusion, aggression, and long-term memory loss (Waldemar et al., 2007). Eventually, neuronal loss compromises fundamental physiological processes, finally resulting in death. It is always fatal with no known cure, and no effective medications. Yet even though successful therapies are frustratingly elusive, and despite a lack of evidence linking pathogenesis of sporadic and familial AD. much of the information on AD signaling has been gathered from the study of FAD mutations, and of the basal functioning of the proteins those mutations affect

1.2 Proteins associated with AD

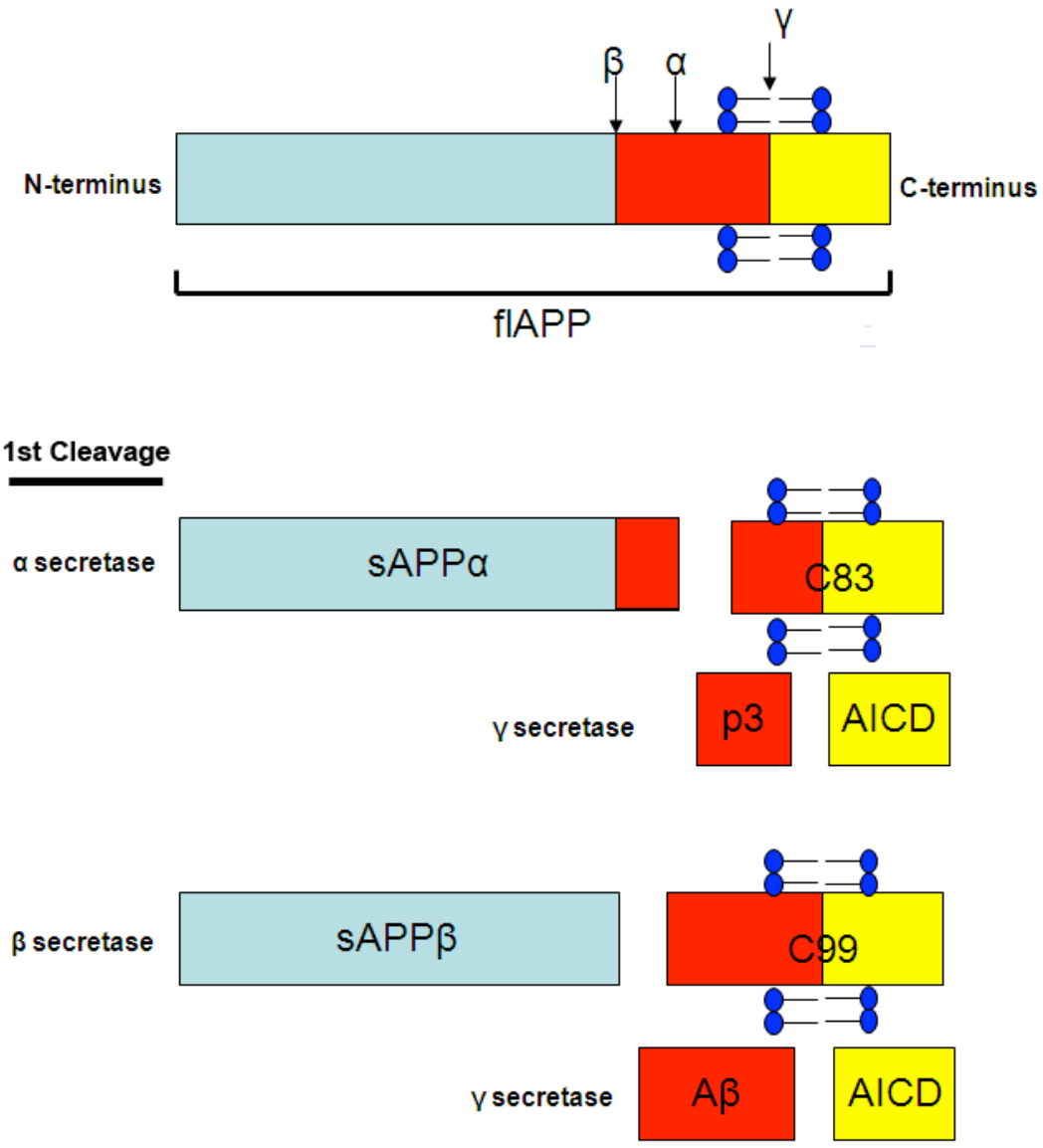
1.2.1 flAPP and cleavage byproducts

flAPP is a transmembrane protein containing distinct enzymatic cleavage sites for generation of multiple functional peptides. Isoforms of different lengths have been named for the number of amino acids they contain, including APP751, APP770, and

APP695, the latter being the most abundant form in the brain (Kitaguchi et al., 1988; Tanzi et al., 1988; Tanaka et al., 1989; Golde et al., 1990; Koo et al., 1990). However, all three isoforms are found at different ratios in the brain; cortical mRNA levels of 770:751:695 are 1:10:20 (Tanaka et al., 1989), while those in cultured astrocytes are 2:4:1 (Gray and Patel, 1993). Established extracellular binding domains for heparin, collagen, and laminin may indicate binding partners for either flAPP or resultant cleavage products (Kibbey et al., 1993; Beher et al., 1996; Mok et al., 1997). Following translation, the full-length protein is translocated through the Golgi apparatus and integrated into the plasma membrane (Turner et al., 2003). The protein can be processed at multiple cellular locations, to generate a variety of functional peptides that can work either intra- or extracellularly (Turner et al., 2003). flAPP or cleaved byproducts at the plasma membrane can be endocytosed and brought back to the Golgi apparatus for additional cleavage, or shuttled to lysosome for degradation (Turner et al., 2003).

flAPP processing is mediated by three different enzymes named for their corresponding cleavage sites (γ-, α-, and β-secretase) and the sequence in which these proteases function will produce specific byproducts to be released (Figure 1). Initially, flAPP is cleaved by either α- or β- secretase, but not both, with □ -secretase activity precluding Aβ formation (Allinson et al., 2003). □ -secretase-directed flAPP processing by A Disintegrin And Metalloproteinases (ADAM 9, 10, or 17) generates the neurotrophic protein secreted amyloid precursor protein α (sAPPα) (Allinson et al., 2003), and subsequent gamma cleavage of the C-terminal stub produces p3 and APP intracellullular C-terminal domain (AICD) fragments (Thinakaran and Koo, 2008). Primary β-secretase (BACE1) is predominantly found in the Golgi apparatus,

Figure 1: flAPP cleavage products



Products generated by α- or β- secretase, followed by γ secretase. flAPP is cleaved initially by either α- or β- secretase, but not both. In the non-amyloidogenic pathway, ADAM10 produces sAPPα and the C83 intermediate, which is then cleaved by γ secretase to form p3 and AICD. The amyloidogenic pathway involves BACE-promoted flAPP cleavage, forming sAPPβ and C99. C99 is further cleaved into Aβ (1-40 or 1-42, commonly), and AICD. AICD is a common byproduct of either pathway. C83, C99, and p3 are all named for the number of amino acids they contain.

indicating some cleavage occurs intraneuronally (Koo and Squazzo, 1994), but both BACE1 and γ-secretase are also located at the plasma membrane (Kinoshita et al., 2003). BACE1-directed cleavage releases sAPPβ, a substantially less (<100x) potent neurotrophic factor than sAPPα about which little is known (Turner et al., 2003). However, γ-cleavage of the remaining flAPP fragment generates Aβ peptides of 38-43 amino acids including Aβ 1-40 and Aβ1-42, proteins that tend to aggregate into the insoluble masses many believe to be responsible for AD pathogenesis. Whether these aggregate-capable proteins, termed amyloidogenic, and other flAPP cleavage products have a physiological role remains to be determined, but some functions have been discovered.

1.2.1.1 sAPPα

sAPPα is the most common byproduct of flAPP processing, making up as much as 90% of all flAPP processing (Hiltunen et al., 2009). The length of sAPPα is dependent on the isoform of flAPP cleaved; both APP750 and 751 generate sAPPα containing a kunitz protease inhibitor (KPI) domain that protects the peptide from degradation, while APP695 does not (Turner et al., 2003). sAPPα formation prevents Aβ production, as the α cleavage site delineates the C-terminus of sAPP lies 16 amino acids within the Aβ1-42 sequence. Thus, the α-secretase pathway is non-amyloidogenic, as demonstrated when overexpression of the α-secretase enzyme ADAM10 promoted sAPPα secretion while preventing plaque formation in the brains of AD transgenic mice (Postina et al., 2004). In addition, flAPP localization to the plasma membrane also promotes sAPPα formation, as the α-secretase enzymes are often found there (Parvathy et al., 1998, , 1999).

 -secretase-directed flAPP cleavage is normally promoted by neuronal activity at the synapse. Activation of protein kinase C (PKC) contributes to α-secretase activity both *in vitro* and *in vivo* (McLaughlin and Breen, 1999; Rossner et al., 2000), possibly by a mechanism involving phosphorylation of the flAPP C-terminus (Turner et al., 2003). Also, neurotransmitter activation of metabotropic glutamate (mGluR1) and acetylcholine receptors (mACh2,4) as well as NMDA ion channels promotes sAPPα release (Nitsch et al., 1992; Fazeli et al., 1994; Nitsch et al., 1997). The insulin signaling pathway has demonstrated the capacity to increase sAPPα secretion as well, with insulin-receptor mediated activation of protein kinase B (AKT), the primary pro-survival protein in the insulin signaling pathway, promoting sAPPα formation (Solano et al., 2000; Adlerz et al., 2007). Upon cleavage, sAPPα is often secreted into the extracellular environment to direct many signaling events.

Physiological roles for sAPPα remain poorly understood, but several possible functions have been uncovered. Inhibition of platelet coagulation factor 1a by sAPPα can alter blood coagulation (Smith et al., 1990). sAPPα protects neurons from Aβ-induced cell death by preventing an excessive cytoplasmic calcium level increase (Mattson et al., 1993; Goodman and Mattson, 1994). In addition, adding exogenous sAPPα to cortical neuronal cultures promotes neurite outgrowth in a dose-dependent manner (Araki et al., 1991). In rat hippocampal slices, sAPPα can also facilitate long term potentiation (LTP), a strengthening of synaptic activity believed to be a key component of memory formation (Dunwiddie and Lynch, 1978; Dunwiddie et al., 1978; Ishida et al., 1997). Beyond neuronal stimulation, astrocyte stimulation by sAPPα induces secretion of glutamate (Barger and Basile, 2001) and interleukin-1β

(Li et al., 2000). Most functions ascribed to sAPPα are neurotrophic in nature and imply an importance in memory development.

sAPPα activity in the brain promotes several facets of memory processing, and reduced sAPPalpha level may also be linked to memory loss associated with AD. Antibodies to the APP peptide used in animal models reduces memory formation and retention (Doyle et al., 1990; Huber et al., 1993), but this evidence is indirect due to a lack of specificity for sAPPα itself. Yet intracerebroventricular injection of the neurotrophic RERMS peptide sequence normally found within sAPP increased spatial memory acquisition in mice (Roch et al., 1994; Meziane et al., 1998), and sAPPα injection prevented synaptic plasticity and memory deficits normally observed in sAPP-null mice (Ring et al., 2007). In addition, poor performance in spatial memory tasks correlates with reduced cerebrospinal fluid (CSF) sAPP levels in aged rats, indicating a role for the protein in memory formation (Anderson et al., 1999). Low sAPP CSF levels observed in both FAD (Almkvist et al., 1997) and sporadic AD patients (Sennvik et al., 2000; Colciaghi et al., 2002; Tyler et al., 2002) also imply a possible link between reduced sAPPα availability and AD severity. While neurotrophic properties attributed to sAPPα signaling seem clear, proteins that may mediate sAPPα functions remain unknown.

Several unique protein interactions involving sAPPα have been reported, despite an unknown dedicated receptor. Apolipoprotein E (ApoE) binds sAPPα in serum, possibly as part of a removal mechanism for sAPP (Barger and Mattson, 1997). This interaction, in addition to sAPPα binding to the ApoE regulated LDL-receptor related protein (LRP) at the membrane (Kounnas et al., 1995; Knauer et al., 1996), indicates that sAPPα activity could moderate cholesterol metabolism as well

(Turner et al., 2003). Immunolabeling revealed that sAPPα at the plasma membrane was associated with the protein guanylate cyclase, but whether this is a receptor-mediated event is unclear (Barger and Mattson, 1995). Interactions between both flAPP and sAPPα indicate a signaling pathway involving the membrane-bound integrinβ1 protein that promotes neurodevelopment and neurite outgrowth (Young-Pearse et al., 2008), but this association may not fulfill the requirement for other sAPPα-promoted neurotrophic activities. Thus while several binding partners have been identified none can encapsulate the majority of sAPPα's neurotrophic functions.

1.2.1.2 Aβ

Aβ proteins are amyloidogenic, defined as the ability to form insoluble fibrous aggregates, and are generated from flAPP by cleavage at both β and γ sites. Intracellular Aβ accumulation results from flAPP and cleavage enzymes targeted to organelle membranes, at the ER, Golgi apparatus, and mitochondria (Mizuguchi et al., 1992; Kinoshita et al., 2003), as opposed to extracellular Aβ production due to the enzymes localized to the plasma membrane (Kinoshita et al., 2003). While the β cleavage occurs precisely at amino acid 672 (of APP695), the γ site can vary by 2-4 amino acids to generate species of varying length, due to the promiscuous nature of the γ-secretase complex (Selkoe and Wolfe, 2007). The most common forms are the Aβ1-42 and Aβ1-40, differing only by two C-terminal amino acids. Normally Aβ1-42 production makes up only ~10% of all Aβ peptide cleavage with the remaining 90% comprised of Aβ1-40 formation. However, a mutation in APP or PS1 genes increases Aβ1-42 formation to 40%, with a parallel decrease in Aβ1-40 levels (Selkoe and Podlisny, 2002). Amyloidogenicity is much higher in Aβ1-42 due to the last two C-

terminal hydrophobic amino acids, causing the longer peptide to form plaques quicker than the Aβ1-40 form (Selkoe and Podlisny, 2002). Thus, as plaques were widely considered to be the cause of AD formation, Aβ1-42 was considered more toxic than other isoforms with little to no physiological activity.

Some beneficial roles for Aβ1-42 have been suggested but may be hard to distinguish from pathological roles normally associated with AD. Like sAPPα, Aβ also binds ApoE (Barger and Mattson, 1997; Ohm et al., 2001), but whether this is part of a clearance mechanism or cholesterol maintenance function is uncertain (Ohm et al., 2001). Aβ1-42 also has a high affinity for nicotinic acetylcholine nAch7 receptors (Wang et al., 2000b; Wang et al., 2000a) and can block nicotinic ion channel currents (Pettit et al., 2001), but the physiological relevant event is debatable given the dramatic loss of cholinergic neurons in advanced AD (Zamani and Allen, 2001). Aβ can both increase and decrease LTP (Bishop and Robinson, 2004), where differences in LTP could be mediated by the aggregated state of the peptide. Aβ1-42 also has antioxidant properties, by preventing iron-induced neurotoxicity (Bishop and Robinson, 2003). In addition, Aβ monomers can protect neuronal cultures from a lack of trophic support, signaling through the PI3kinase/AKT prosurvival signaling pathway (Giuffrida et al., 2009). However, more research is required to determine the conditions under which Aβ may be beneficial, in contrast to its widely-reported harmful effects.

1.2.2 Presenilin-1

Presenilin-1 (PS1) is a transmembrane protein of 450-470 amino acids commonly found at the membrane of the endoplasmic reticulum (ER) within the cell

(Rogaev et al., 1995; Sherrington et al., 1995). It acts mainly as a component of the γ-secretase complex, a multimeric protein complex involved in cleavage of several proteins, including flAPP into Aβ (Steiner et al., 2008; Wolfe, 2009). Presenilin gene mutations are the most commonly seen in FAD (Bertram and Tanzi, 2004), leading to altered processing of the flAPP protein. γ-secretase activity can be imprecise, cleaving within a few amino acids of the designated cleavage site, which can generate Aβ peptides ranging in size from 39 to 42 amino acids. The promiscuity of γ-secretase, with its ability to cleave several different substrates, is the likely reason for generation of multiple amyloidogenic proteins

Unlike other transmembrane enzymes, the γ-secretase protease is unusual due to its specificity for multiple substrates, each with different functions necessary for normal physiology and neurodevelopment. The Notch receptor is the most well-studied of these and regulates cell differentiation upon cleavage of its C-terminus (De Strooper et al., 1999). Signaling of the neuregulin family through the ErbB receptor, another γ-secretase substrate, is responsible for axonal guidance and neuronal myelination (Mei and Xiong, 2008). Other cleavage targets include the low density lipoprotein receptor, and Notch ligands Delta and Jagged (Kopan and Ilagan, 2004). PS1 knockout mice often do not survive beyond early development (Shen et al., 1997; Wong et al., 1997), indicating the importance of the PS1 protein for animal survival.

In addition, the discovery that PS1 can act as an endoplasmic reticulum (ER) calcium leak channel (Tu et al., 2006) demonstrates a potential role in calcium homeostasis. The ER is the largest calcium store within the cell, partially responsible for calcium-induced signaling events, and for maintainance of calcium homeostasis (Berridge, 2002; Glazner and Fernyhough, 2002). The ER leak channel prevents

overaccumulation of ER luminal calcium, and the identification of PS1 as that channel indicates that the protein can aid in neuronal calcium regulation. Indeed, PS1 FAD mutations disrupt the channel resulting in elevated ER calcium levels (Tu et al., 2006). This could be especially important in AD, as loss of calcium homeostasis is an important feature in the disease. Thus, basal PS1 signaling lost through genetic mutations can have a profound effect on multiple systems, disrupting calcium homeostasis in addition to cell signaling pathways.

1.3 FAD mutations and mouse models

Unknown causes of sporadic AD often focus on signaling pathways involved with FAD mutations in an attempt to find correlations between the two forms of the disease. FAD onset occurs sooner than sporadic, as early as 30 years of age, and involves a mutation in one or more genes. The first mutation discovered affects the flAPP protein, resulting in increased Aβ accumulation by either increasing BACE-induced protein cleavage or translating more flAPP protein (Goate et al., 1991; Murrell et al., 1991; Rovelet-Lecrux et al., 2006), but most reported FAD cases have mutations in the PS1 gene (Sherrington et al., 1995). Other genetic risk factors involve the ApoE4 gene (Corder et al., 1993) which encodes the Aβ-binding protein apolipoprotein E (Saunders et al., 1993), and mutations of the MAPT gene resulting in elevated phosphorylation of tau protein that promotes formation of neurofibrillary tangles that are also a pathological feature of AD (Hutton et al., 1998; Poorkaj et al., 1998). Transgenic animal models containing one or more FAD mutations have been generated to allow both *in vitro* and *in vivo* analysis of signaling mechanisms that contribute to AD pathogenicity (Crews et al., 2010; Elder et al., 2010).

Few natural animal models exist for study of the sporadic AD, as the disease is primarily human. Some animals, such as aged canines, can develop a similar condition that correlates well due to insoluble plaque formation (Cotman and Head, 2008). However these models are not conducive to lab research due to the many years required for symptoms to develop. As common laboratory mice do not develop AD due to differences between mouse and human Aβ amino acid sequences, human FAD mutations are introduced into mice to study the disease (De Strooper et al., 1995; Jankowsky et al., 2007). As a result, transgenic FAD mice often have an overly-aggressive pathological development that accelerates disease progression while developing AD markers that coincide with the emergence of cognitive deficits, including poor memory formation and retention (Crews et al., 2010; Elder et al., 2010). However, few models demonstrate the neuronal loss and brain degeneration commonly attributed to advanced AD, making them imperfect models at best. Yet despite this discrepancy, AD-related neuropathologies are often studied using these animal models.

1.3.1 PS1 mutations and mPS1 mice

Despite the prevalence of PS1 mutations in FAD, AD pathology is subtle, if not absent, in many mPS1 mouse models. Clinically, PS1 FAD mutations promote generation of Aβ1-42 instead of 1-40, thus increasing the Aβ42/Aβ40 ratio (Selkoe and Wolfe, 2007). However, mPS1-only mice have no Aβ deposition despite having increased Aβ1-42 formation and intraneuronal accumulation (Duff et al., 1996; Chui et al., 1999). In addition, electrophysiology in mPS1 brain slices is mostly normal and behavioral testing of mutant animals reveals no significant deficits compared to wild-

type controls (Holcomb et al., 1999; Janus et al., 2000; Huang et al., 2003; Lalonde et al., 2003), leading many to question the usefulness of the mPS1 mouse as a model for FAD. However, some abnormal phenotypes leading to AD-type symptoms can occur in these mice. Brain analysis of 13 month old mPS1 H163R and L286V animals reveals increased apoptotic neurons despite the absence of plaques (Chui et al., 1999). The increased neurofilament phosphorylation results in neurofibrillary tangle formation is also observed in mPS1 mice (Lazarov et al., 2007; Yang et al., 2009). Other physiological problems in these mice include thinned blood vessels as often seen in AD brains and reduced acetylcholinesterase (AChE) activity (Silveyra et al., 2008; Gama Sosa et al., 2010). Assessment of cultured mPS1 hippocampal neurons demonstrates impaired synaptic activity and reduced glutamatergic ion currents (Priller et al., 2007). Increased sensitivity to excitotoxic insult and elevated calcium efflux from ER calcium stores has caused many researchers to focus on the calcium functions of PS1 mutations in AD (Guo et al., 1999; Grilli et al., 2000; Leissring et al., 2000; Tu et al., 2006). However, while PS1 mice are important for examining the role of PS1 in disease development, the amyloid hypothesis of AD is most often studied using mutant flAPP mice.

1.3.2 flAPP transgenic mice

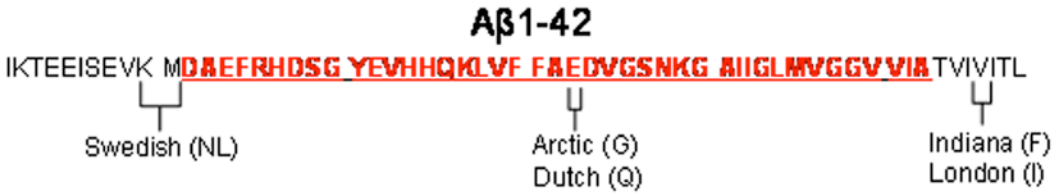
Unlike mPS1 mice, most flAPP transgenic mice develop both Aβ plaques and behavioral deficits. Although neuronal loss is normally absent, AD-like symptoms of neuronal impairment such as dystrophic neuritis, microglial inflammation and astrocytosis are also common (Games et al., 1995; Masliah et al., 1996). Cognitive impairment often coincides with first visual appearance of Aβ plaques as well (Chishti

et al., 2001), and many models also demonstrate intracellular Aβ accumulation which, some believe, is the more pathological Aβ pool in AD (Oddo et al., 2003; Wirths et al., 2009). Hyperphosphorylation of the tau protein, which is a precursor to tangle accumulation, is exhibited by several models as well (Andra et al., 1996; Oddo et al., 2003; Echeverria et al., 2004). Thus, these mAPP mouse models can more closely mimic Aβ-induced AD formation.

flAPP transgenic mice usually overexpress flAPP with FAD mutations named for the geographical location where the mutation pedigree was discovered. The most common missense mutations that enhance Aβ1-42 cleavage are the Swedish (K670N/M671L) and Indiana (V717F) mutations, with the Swedish mutation resulting in 10x more BACE-mediated cleavage than in normal flAPP (Yan et al., 1999). Dutch and Arctic mutations are targeted to amino acids within the Aβ sequence, as opposed to sites of cleavage (Figure 2). One or more of these mutations together will determine the aggressiveness of amyloid plaque formation, from 12 months to as quickly as 3 months. Tg2576 mice contain only the Swedish mutation and display brain deposition between 9 and 12 months of age, with some vascular deposition as well (Hsiao et al., 1996). With both Swedish and Indiana mutations, TgCRND8 mice develop plaques and associated dementia more quickly, often as early as 3-5 months of age (Chishti et al., 2001). Insertion of other FAD mutations in addition to mAPP can generate a greatly accelerated disease model, or models even more representative of the human condition. Addition of a M146L PS1 mutation to the Tg2576 mouse accelerates Aβ deposition from 9-12 months to 4 months of age (Borchelt et al., 1996). The popular AD 3xTg model, with APP (Swe), PS1(M146V) and Tau(P301L) FAD mutations, exhibits Aβ plaques at 9 months, tangle pathology in the hippocampus, and

behavioural deficits (Oddo et al., 2003). The multitude of various FAD models currently available allows for molecular analysis determining the manner by which Aβ is generated from flAPP holoprotein cleavage, how Aβ deposition may be affected, and mechanisms by which this deposition may result in neuronal or behavioural impairment.

Figure 2: Common FAD APP mutation sites used in generating mAPP transgenic mice



Part of the flAPP amino acid protein, with the sequence in red representing the 42 amino acid Aβ sequence. Labeled mutations are those commonly observed in FAD mutations. The Swedish (K670N/M671L) and Indiana (V717F) are the most often used in FAD mouse mutations. Some mutations also correlate strongly with cerebral amyloid angiopathy (CAA) (Ghiso and Wisniewski, 2004).

1.4 Aβ generation

1.4.1 Aggregation of Aβ1-42

Aβ1-42-mediated neurotoxicity is largely related due to its amyloidogenic nature and its ability to form complex aggregate structures. Protofibril and fibril complexes are the major component of Aβ dense-core plaque deposits (Walsh et al., 1999). Protofibrils consist of either Aβ1-42 or Aβ1-40 folded in a U-shape from amino acids 11-40, followed by stacking of this conformation to form a β-pleated sheet. Insoluble plaque formation occurs due to accumulation of fibrils consisting of numerous stacks of Aβ1-42 or 1-40 (Ahmed et al., 2010), but Aβ1-42 is the primary species found in plaques (Murphy and LeVine, 2010). Aβ1-42 plaques were once believed to be the underlying cause of AD, but recent studies have revealed that soluble Aβ oligomer could be more toxic (Klein et al., 2001). The C-terminus of Aβ1-42 allows an alternate multimeric non-β pleated conformation of up to 10 peptides (decamer), with each C-terminus projecting into the centre of the oligomer (Bernstein et al., 2009; Ahmed et al., 2010). The two amino acids terminating Aβ1-42 contribute to oligomer formation, explaining Aβ1-40's inability to form these soluble aggregates (Bitan et al., 2003). AD onset has been correlated to a higher Aβ1-42/Aβ1-40 ratio (Duering et al., 2005), and may correspond to a high soluble oligomer/plaque as well. Thus soluble oligomeric structure could be the primary component of Aβ-directed neuronal dysfunction in AD.

Determining oligomeric Aβ pathogenicity has revealed it to be more toxic than either the insoluble fibril or monomeric form. Aβ1-42 monomer injection into the brain did not alter neuronal electrophysiology, but soluble oligomers inhibited LTP in hippocampal neurons and induced cell death (Lambert et al., 1998; Hartley et al.,

1999; McLean et al., 1999; Walsh et al., 2002). Reducing oligomeric Aβ levels via conversion to amyloid fibrils limits neuronal toxicity *in vitro*, and has been suggested as a potential AD therapy (Ahmed et al., 2010). For this reason, many groups are concentrating attention on soluble Aβ oligomer-induced neuronal dysfunction (Lambert et al., 1998; Klein, 2002; Deshpande et al., 2006; Townsend et al., 2007; Zhao et al., 2008), and how reduced formation, mainly by reducing levels of Aβ monomer available for aggregation, could be beneficial in AD prevention.

1.4.2 Aβ clearance

In addition to increased Aβ production, the rate of Aβ clearance from the brain will also determine formation of oligomeric species. One component of clearance includes enzymatic degradation of the monomeric peptide, initiated by proteases such as neprilysin (NEP) or insulin-degrading enzyme (IDE). NEP activity is largely confined to the extracellular environment, while IDE works both intra- and extracellularly (Murphy and LeVine, 2010). Reduced activity of these enzymes is observed in aging, indicating a reduced capacity to remove Aβ as one gets older (Caccamo et al., 2005). Also, decreased levels of NEP in the CSF of early AD patients is associated with pathology (Maruyama et al., 2005; Miners et al., 2006). Other Aβ proteases have been suggested, such as endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), and matrix metalloproteases (MMP), but clinical evidence in AD patients regarding their function is relatively scarce compared to NEP and IDE (Miners et al., 2008). While uncommon, Aβ can also be removed through lysosomal degradation(Sun et al., 2008), and mutations in the PS1 protein reduce autophagy of the peptide (Lee et al.). Thus, reduced Aβ1-42 monomer

availability will limit the possibility of oligomer formation regardless of clearance mechanism.

Another component of Aβ clearance includes transport across the blood brain barrier (BBB) as reduced transport increases accumulation and aggregation in the brain (Van Uden et al., 2002). Soluble Aβ is shuttled from the brain by low-density lipoprotein receptor-related protein (LRP), a process hindered by deficiencies in Aβ-binding protein ApoE (Shibata et al., 2000). The receptor for advanced glycation end products (RAGE) expressed on blood vessels also can bind Aβ and increase brain accumulation (Deane et al., 2003). Insulin-like growth factor 1 (IGF1) signaling contributes to Aβ transport as well (Tanzi et al., 2004), increasing movement at the choroid plexus (Carro et al., 2002). These binding events could manifest as mechanisms to enhance transport to and degradation in the liver, where high concentrations of IDE are found (Kuo et al., 1993). Ensuring functioning transport and degradation mechanisms promotes low Aβ aggregation and reduced risk of AD development.

1.5 Possible causes of cell death and dysfunction in AD

Disruption of neuronal function by Aβ has been demonstrated numerous times both clinically and in animal models. Neuroinflammatory markers commonly seen in AD patients may lead to neuronal impairment and death. Homeostatic mechanisms that carefully regulate intracellular calcium are also heavily influenced by Aβ signaling, with various calcium-related disturbances seen in AD. Finally, biochemical indicators of mitochondrial dysfunction have been observed in both AD and in

transgenic models. While not a complete list of AD neuronal dysfunction, these events may account for much of the cellular pathology associated with the disease.

1.5.1 Neuroinflammation

Chronic inflammation may be a central component of Aβ-induced cell death in AD, with inflammatory mediators observed in different brain regions of AD patients (McGeer and McGeer, 1995). Microglia are non-neuronal cells responsible for immune defense in the CNS by migrating to a target and initiating cytokine release following activation, leading to an inflammatory response that promotes phagocytosis of free radicals and cellular debris (Schwab and McGeer, 2008). Treatment of human microglial cultures with Aβ increases inflammatory cytokine release, indicating an induction of microglial activity (Walker et al., 2006). While inflammation is normally a necessary component of microglial repair mechanisms, Aβ limits phagocytosis , resulting in sustained activation, increased cytokine release and chronic inflammation (Walker et al., 2006; Schwab and McGeer, 2008). Prolonged microglial activation proves toxic, as treatment of co-cultures of microglial cells and hippocampal slices with Aβ results in an upregulation of pro-inflammatory molecules leading to increased neuronal death (Butovsky et al., 2005). Clinically, numerous inflammatory cytokines, the most prominent being tumor necrosis factor α (TNFα), are elevated in AD brain compared to controls (McGeer and Sibley, 2005). Using anti-cytokines such as the TNF-α receptor antagonist etanercept can prove beneficial by limiting inflammation to improve cognitive ability in AD patients (Tobinick et al., 2006). However, non-steroidal anti-inflammatory drug (NSAID) therapy designed for a similar purpose produced mixed results that did not necessarily benefit the patient (Launer, 2003;

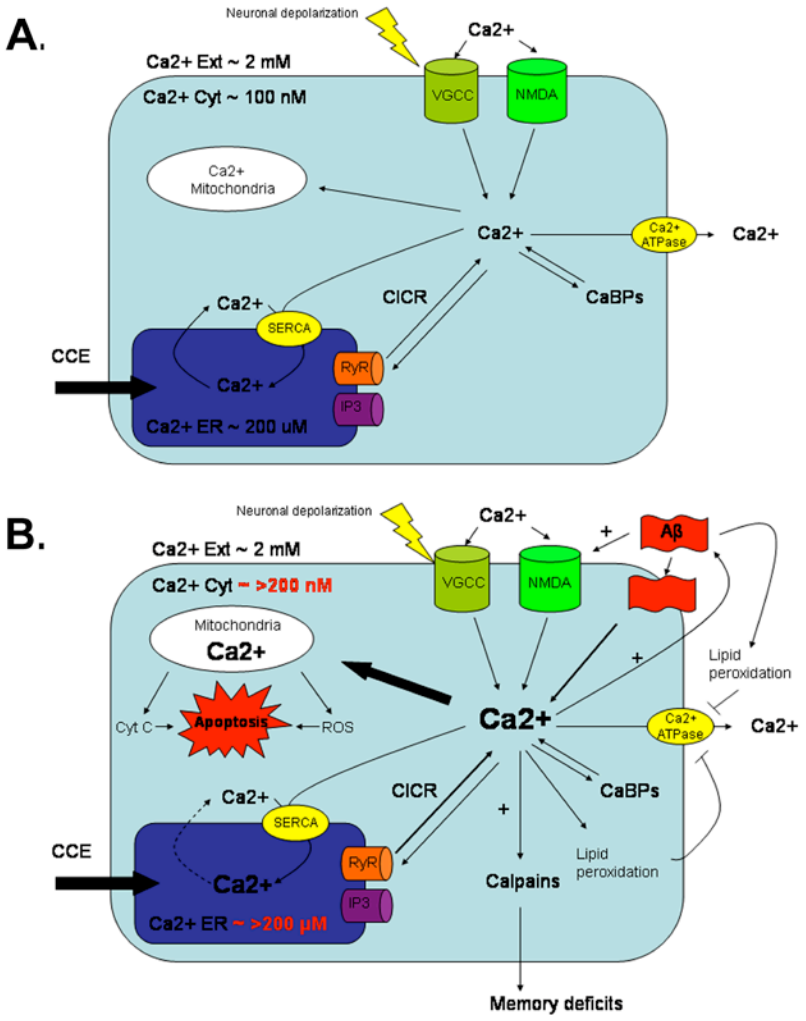
Szekely et al., 2007). Therefore, inflammation likely contibutes to only one component of AD pathology, with other neuronal disturbances, such as calcium dysfunction, contributing to AD-induced neuronal death.

1.5.2 Calcium signaling in AD

1.5.2.1 Neuronal calcium signaling

Calcium signaling regulates neuronal processes ranging from memory and synaptic plasticity to mitochondrial function (Berridge et al., 2000). Calcium influx towards the cytoplasm initiates many of these processes, which are mediated by calcium-binding proteins or by directly activating proteins (Berridge et al., 2000). Homeostatic mechanisms are heavily regulated to prevent calcium overload, and Figure 3 displays crucial areas for maintaining neuronal calcium balance. A large chemical and electrical gradient across the plasma membrane drives calcium ion influx, with a 20,000-fold difference from extracellular to cytoplasm (2 mM to 0.1 uM, respectively). However, the plasma membrane is relatively impervious to ion flow, and requires gated ion channels to shuttle calcium across the gradient. Neuronal depolarization opens voltage gated calcium channels (VGCC) on post-synaptic membranes to allow calcium influx (Berridge et al., 2000). Ligand-gated calcium channels such as the glutamate-activated NMDA receptor also requires depolarization, which removes Mg^{2+} ion blockage allowing calcium to move down the large gradient into the cell (Berridge, 1998). Increased cytoplasmic $[Ca^{2+}]_i$ initiates calcium-induced calcium release (CICR) (Berridge et al., 2000), a phenomenon by which increases in cytoplasmic calcium ions initiates calcium efflux from the endoplasmic reticulum (ER), the largest calcium store within the cell.

Figure 3: Calcium signaling in normal and AD neurons



Summary of calcium homeostatic mechanisms in neurons under normal conditions (A) and in AD (B) (for reviews, see (Berridge et al., 2000; Camandola and Mattson, 2010). (A) In normal neurons, depolarization activates VGCCs and NMDA channels to allow entry of calcium ions, which induces a CICR response that drives calcium efflux from the calcium storing endoplasmic reticulum (ER) organelle. Calcium binding proteins, Ca^{2+}-ATPases on both the plasma and ER membrane, and mitochondria sequestration all act to buffer excess cytoplasmic calcium and limit excitotoxicity. In AD (b), Aβ increase Ca^{2+} through NMDA receptors and through insertion of a calcium-permeable channel into the membrane. Also, mPS1-induced impairment of the ER leak channel increases CICR, further increasing cytoplasmic calcium. Lipid peroxidation via excess ROS from Ca, and Aβ-induced formation, inhibits calcium exchangers as well. Excessive cytoplasmic calcium overloads mitochondria to produce ROS, as well as release pro-apoptotic signals such as cytochrome c. Therefore neurons are more susceptible to Ca^{2+}-induced neurodegeneration in AD.

Basal ER calcium levels in the micromolar range also provide a strong impetus for calcium movement into the cytoplasm. A Ca^{2+} leak channel residing in the ER membrane allows a constant flow of ions from the ER lumen, and is balanced by the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, which shuttles Ca^{2+} back into the ER upon ATP hydrolysis (Berridge, 2002). This constant cycling of Ca^{2+} from ER to cytoplasm prevents Ca^{2+} overaccumulation in the ER lumen, maintaining a careful balance. Consequences of ER Ca^{2+} depletion can be catastrophic, as demonstrated when the SERCA inhibitor thapsigargin induced cell death in neuronal cultures (Verkhratsky and Toescu, 2003). Both CICR or ligand-driven ER Ca^{2+} release is mediated by ER membrane IP3 and ryanodine (RyR) receptors, stimulated by IP3 and Ca^{2+}, respectively (Clapper et al., 1987; Berridge et al., 2000). Upon receptor-mediated ER Ca^{2+} depletion, capacitative calcium entry (CCE) assists ER calcium replenishment using store-operated channels (SOCs) to shuttle extracellular calcium directly into the ER (Putney et al., 2001). Calcium currents fluctuate extremely rapidly, and levels are also carefully maintained by numerous Ca^{2+} binding proteins, mitochondrial sequestering, and Ca^{2+} exchangers on the plasma and ER membranes (Berridge, 2002). The flow of Ca^{2+} and increases in cytosolic Ca^{2+} will trigger multiple signaling pathways with long and short-term effects that result in transcriptional upregulation and phosphorylation events, but a poorly regulated system can be lethal.

Overactivation of Ca^{2+} channels or poor Ca^{2+} flux leads to excitotoxic events that can quickly result in neuronal dysfunction (Glazner and Fernyhough, 2002). Thus, prompt removal of excess Ca^{2+} across the plasma or ER membrane is normally required, and ATP-drive Ca^{2+} exchangers can serve in this capacity as long as energy

stores last (Berridge, 2002; Glazner and Fernyhough, 2002). Cytoplasmic Ca^{2+} can be sequestered by the mitochondria, but elevated levels in the organelle will initiate apoptosis (Rizzuto et al., 1993; Berridge et al., 2000). In addition, ER Ca^{2+} depletion initiates an ER stress response that limits protein translation while inducing activity of transcription factors with opposing functions. Some of these, such as Grp78, will protect neurons (Yu et al., 1999; Siman et al., 2001), while other proteins such as CHOP (Wang et al., 1996; Maytin et al., 2001) and caspase 12 (Nakagawa et al., 2000), promote apoptosis. Sustained cytoplasmic or mitochondria Ca^{2+} level elevations will induce formation of lipid peroxides and reactive oxygen species (ROS), both of which impair neuronal function (Lu et al., 2002). Thus, the severity of a Ca^{2+} insult will promote a life or death pathway, depending on energy stores in the cell. Dysregulation of Ca^{2+} handling is a common occurrence in neuropathologies such as stroke and AD.

1.5.2.2 Ca^{2+} dyshomeostasis in AD

The ability of neurons to maintain Ca^{2+} homeostatis is compromised by multiple mechanisms in AD, as shown in Figure 3. Aβ oligomers induce Ca^{2+}-mediated excitoxicity in neurons by forming a Ca^{2+}-permeable channel in plasma membranes (Arispe et al., 1993; Demuro et al., 2005), or by directly modulating NMDA receptors to increase their sensitivity and mediate Ca^{2+}-induced excitotoxicity (Shankar et al., 2007). Lipid peroxidation, a common consequence of Aβ aggregation, impairs Ca^{2+} exchangers and glutamate transporters resulting in Ca^{2+} overload in the cytoplasm (Mattson, 2004). These Aβ-driven events result in sustained increases in cytoplasmic calcium, as confirmed in FAD transgenic models; cultured cortical

neurons from adult 3xTg mice have cytoplasmic Ca^{2+} concentrations twice as high as seen in control mice (Lopez et al., 2008), and *in vivo* imaging of brains of hAPPSwe mice revealed elevated cytoplasmic Ca^{2+} in neurites and spines surrounding Aβ plaques (Kuchibhotla et al., 2008). Thus Aβ-induced cytoplasmic calcium elevations could be a prominent contributor to neuronal dysfunction in these models and cell death in AD.

Ca^{2+} levels of the ER are also affected by mutations in the PS1 protein, and in AD in general. Fibroblasts taken from a patient carrying the PS1 (A246E) mutation had increased Ca^{2+} release from IP3 stores, whereasmPS1 and PS2 mice display similar characteristics (Ito et al., 1994; Leissring et al., 1999a; Leissring et al., 2000). Cortical neurons cultured from PS1 M146V animals showed increased glutamate-induced ER Ca^{2+} release that caused excitotoxic cell death preventable with the ER Ca^{2+} blocker dantrolene (Guo et al., 1999). Enhanced ER Ca^{2+} release is likely caused by decreased function of the PS1-linked ER Ca^{2+} leak channel resulting in ER Ca^{2+} store overload (Tu et al., 2006; Nelson et al., 2007). Thus, individuals with PS1 FAD mutations would have an overreaction to normal ER Ca^{2+} channel stimulation resulting in greater ER efflux due to a larger ER luminal Ca^{2+} pool. In addition increased RyR expression has been demonstrated in FAD models such as the 3xTg and TgCRND8 (Smith et al., 2005; Supnet et al., 2006), which would also contribute to increased ER Ca^{2+} release from IP3 and RyR stores (Stutzmann et al., 2004; Stutzmann et al., 2006; Chakroborty et al., 2009). Thus, FAD mutations at the ER could contribute to elevated CICR that could result in Ca^{2+} dyshomeostasis and neuronal malfunctioning.

1.5.2.3 Consequences of calcium disruption in AD

Elevated cytosolic Ca^{2+} in AD often results in altered Aβ production and related AD pathology. Increased Aβ production was observed in an flAPP expressing cell line following treatment with the Ca^{2+} ionophore A23187, implying that increased cytosolic [Ca^{2+}], independent of ion channel activity, is enough for Aβ pathogenesis (Querfurth and Selkoe, 1994), although the physiological relevance for this is unclear. Ca^{2+} channel-mediated Aβ secretion has also been demonstrated, by ER Ca^{2+} efflux was stimulated from RyR receptors by caffeine (Querfurth et al., 1997), and by activation of voltage-gated Ca^{2+} channels at the plasma membrane (Pierrot et al., 2004). Conversely, reduced Ca^{2+} current across the plasma membrane increased Aβ secretion as well in cell lines, instead of decreasing levels as expected (Dreses-Werringloer et al., 2008). Yet most data support a mechanism by which increased Aβ secretion is driven by increases in either cytoplasmic Ca^{2+} or Ca^{2+} channel ion flow.

Neurotoxicity and synaptic impairment often occurs following Ca^{2+} disruption in AD and transgenic AD models. Ca^{2+}-induced mitochondrial disruption leads to reactive oxygen species (ROS) production, cytochrome C release, and neuronal death (Brustovetsky et al., 2005; Bernardi et al., 2006; Manczak et al., 2006), and markers of these events in AD brains imply that increased Ca^{2+} could partially explain neuronal loss in the disease (Maurer et al., 2000; Lin and Beal, 2006). ER stress-related anti-apoptotic protein Grp78 was reduced in the brains of FAD patients, indicating both ER calcium dysfunction and greater susceptibility to neurotoxicity (Katayama et al., 1999). Elsewhere in the neuron, calpain overactivation by excess cytoplasmic Ca^{2+} results in deficits in memory formation in AD models as well (Trinchese et al., 2008; Vosler et al., 2008). Interestingly, mPS1 FAD animals, which do exhibit enhanced

neuronal Ca^{2+}, do not show these deficits and often display enhanced LTP due to their increased CICR response (Parent et al., 1999; Barrow et al., 2000; Zaman et al., 2000; Odero et al.). However, this is likely an early indicator of AD pathophysiology, as older transgenic animals (13-14 months) exhibited impaired LTP (Auffret et al., 2009). In addition, PS1 mutations reduce expression of ER stress response proteins, possibly due to ER Ca^{2+} surplus (Katayama et al., 2001; Verkhratsky and Toescu, 2003). Thus, Ca^{2+} dysfunction observed in both sporadic and familial AD would seem to promote further AD pathogenesis as characterized by increased Aβ secretion, as well as neuronal dysfunction.

1.5.3 Mitochondrial dysfunction and oxidative stress in AD

Defects in both mitochondrial activity and oxidative stress coping mechanisms are indicated as significant contributors to AD pathology. Normally, mitochondrial enzymes including cytochrome C oxidase catalyze electron transfer to oxygen, which provides the impetus for an H+ ion gradient that drives production of ATP, as well as ROS by-products normally removed by antioxidants (Arredondo and Nunez, 2005). However, cytochrome C oxidase deficiencies and dysfunction are consistently observed in AD brain (Kish et al., 1992; Mutisya et al., 1994), increasing ROS levels and limiting ATP synthesis (Smith et al., 1996; Davis et al., 1997). Elevated levels of antioxidant enzymes such as manganese superoxide dismutase (MnSOD) in AD brains indicate that AD neurons may be trying to compensate for increased ROS production (Marcus et al., 2006). Metabolic activity is interrupted as well, with reduced glucose metabolism in prodrome, or early, AD patients (Santos et al., 2010), and impaired mitochondrial enzyme functioning in FAD models prior to the onset of AD-like

memory deficits (Hauptmann et al., 2009; Yao et al., 2009). Thus, compromised mitochondrial energy production could be an early event in AD pathogenesis. Intracellular oligomeric Aβ can induce mitochondrial release of cytochrome C to initiate apoptosis (Rodrigues et al., 2001), indicating another possible mechanism for AD-associated mitochondrial dysfunction. In addition, Aβ1-42 can bind and reduce Cu and Fe metal ions to produce lipid peroxidation products including 4-hydroxynonenal (4-HNE), which is found at high levels in the hippocampus of AD patients (Hensley et al., 1998; Butterfield et al., 2002). By-products of lipid peroxidation, such as 4-HNE, are especially dangerous due to their long half-life compared to ROS, leading to greater neuronal damage and death. Therefore, poor mitochondrial function may lead to neuronal deficits long before the appearance of established AD pathology including plaques and neurofibrillary tangles.

1.6 AD pathology conclusions

Summarizing observations regarding Aβ and Aβ-induced AD pathology is a daunting task, with thousands of articles available on different facets of the disease. Studies focusing on Aβ interactions could provide clues towards therapeutic targets that would limit these pathological events and therefore slow or halt the onset of the disease. Insulin signaling has recently been demonstrated to be affected by AD, and also specifically by amyloid peptides, indicating a physiological association between the two pathways that could contribute to AD pathology and also lead to new promising drug treatments.

2 Insulin signaling and AD

2.1 Insulin and IGF signaling in the body

The blood-borne hormone insulin is responsible for glucose management throughout most of the body. Insulin-induced glucose uptake results in anabolic mechanisms of cellular metabolism through lipogenesis and glycogen synthesis, while limiting proteolysis, gluconeogenesis, and lipolysis. Elevated blood glucose results in pancreatic insulin release, which travels to different tissues to activate its own receptor and initiate increased glucose uptake. Tissues with the highest insulin receptor concentrations include adipose tissue, liver and skeletal muscle (Bailyes et al., 1997), all of which have a heavy requirement for glucose. Both liver and adipose tissue are storage tissues that use sequestered glucose for production of glycogen and lipids, respectively. Unlike other tissues, however, the brain does not require insulin for glucose uptake, despite being the greatest consumer of glucose in the body. Yet most physiological glucose uptake in the body is mediated by insulin-regulated glucose transporters.

Glucose transporters (GLUT) are the proteins largely responsible for glucose uptake. There are four identified isoforms of class 1 transporters, with GLUT4 being the major isoform responsible for glucose uptake into adipose and muscle cells (Birnbaum, 1989). GLUT4 is mostly located within vesicles in the cytoplasm, with less than 5% found in the plasma membrane. Following insulin receptor stimulation, GLUT4 transporters are incorporated into the plasma membrane to allow glucose uptake, with up to 50% of all cellular GLUT4 transporters localizing to the membrane following insulin exposure (Calderhead et al., 1990; Marette et al., 1992; Czech et al., 1993; Voldstedlund et al., 1993). The primary glucose transporter in neurons,

GLUT3, works by a facilitative mechanism that is still unclear. However, the transporters low Km value for glucose compared to other transporters and its glucose-dependent expression (Simpson et al., 2008) likely contributes to its function in the brain.

Insulin signaling family members insulin-like growth factor 1 & 2 (IGF1, 2) have no effect on glucose uptake but are similar polypeptides to insulin that often utilize the same signaling pathway to different effect. While insulin promotes cellular metabolism, the IGF family usually increases cellular growth throughout the body (Messier and Teutenberg, 2005). While they have their own dedicated receptors, they are also capable of binding insulin receptor homo- and heterodimers, as IGF1R and insulin receptors share significant sequence homology (Belfiore et al., 2009). Like insulin, IGF1 is blood-borne, but it is found at nanomolar concentrations as opposed to the picomolar concentrations of insulin (Messier and Teutenberg, 2005). Most IGF1 is bound to the IGF binding proteins that prevent activity (Baxter et al., 1998). Unlike insulin, IGF1 production normally occurs in the liver (Messier and Teutenberg, 2005). Regardless of their differences, IGF and insulin ligands promote numerous neurotrophic functions in the brain.

2.2 Insulin signaling in the brain

2.2.1 Insulin/IGF levels in the brain

Insulin signaling was once believed to be absent from the brain, due to its largely insulin-independent glucose uptake mechanisms. However, the hormone was detected in the brain in the late 70s, and shortly thereafter was localized to specific areas including the hippocampus (Havrankova et al., 1979; Baskin et al., 1983). The

origin of brain insulin is uncertain, although most is transported across the blood brain barrier (BBB) from peripheral sources (Banks, 2004). However, insulin mRNA expression in the hypothalamus, cortex and hippocampus indicates the potential for local production as well (Steen et al., 2005). Regardless of the source, brain insulin levels fluctuate based on levels in the periphery. Diabetic animal models show no change in brain insulin concentration versus controls (Havrankova et al., 1979), but animals injected with streptozotocin (STZ), a compound that destroys pancreatic β cells to limit insulin production, have increased insulin transport across the BBB (Banks et al., 1997). An inverse relationship between peripheral and brain insulin levels was confirmed by studies showing hyperinsulinemic animals with lower brain insulin, an effect further compounded by age (Havrankova et al., 1979; Baskin et al., 1985; Gerozissis et al., 1993; Gerozissis et al., 2001). The presence of IGF1 in brain is more evident than insulin, however, as high levels are found throughout the brain (Bondy and Cheng, 2004), as well as with IGF1 mRNA expression in the hippocampus, cerebellum and hypothalamus (Rotwein and Hall, 1990). In addition, IGF1 crosses the BBB with relative ease compared to insulin (Reinhardt and Bondy, 1994), cementing its role in neuronal activity. Regardless of the origin of either insulin or IGF1, the insulin signaling pathway is an important factor in basal brain function.

2.2.2 Insulin receptor expression in the brain

The insulin receptor (IR) is a trans-membrane tyrosine kinase receptor consisting of two subunits connected by disulfide bonds. The N-terminal α chain is responsible for extracellular ligand binding, while the C-terminal β chain is predominantly intracellular and is a tyrosine kinase responsible for substrate

recruitment and phosphorylation (Lawrence et al., 2007). Functional receptors form as a dimer, either as a homodimer with another α and β-conjugated insulin receptor protein, or as a heterodimer with the structurally and functionally similar IGF1R (Belfiore et al., 2009). Receptors are expressed throughout the brain, including the hypothalamus, hippocampus, cerebral cortex and cerebellum (Plum et al., 2005; van der Heide et al., 2006), but different IR subtypes are tissue-dependent.

Insulin receptor splice variant subtypes IRa and IRb differ at the ligand binding site by 12 amino acids, which contributes to ligand specificity (Moller et al., 1989; Mosthaf et al., 1990). IRb is the predominant form in adipose tissue and liver, accounting for 80% of all insulin receptor subunits, while IRa is most commonly seen in fetal and developing tissue, as well as also being the primary form seen in the brain (Belfiore et al., 2009). Insulin receptor homodimer function is dependent on subtype composition, with IRa-homodimers initiating cell differentiation and anti-apoptotic mechanisms, and IRb homodimers promote insulin-induced metabolic pathways (Brogiolo et al., 2001; Drakas et al., 2004). Receptor responsiveness to insulin may be governed by subunit composition, as insulin insensitivity is observed in monkeys with a high IRa:IRb ratio (Huang et al., 1994; Huang et al., 1996). This is likely due to ligand selectivity, as the lack of the 12 amino acids in the IRa subunit promotes strong binding of the ligand IGF2, which can displace insulin from the active sight (Yamaguchi et al., 1993; Frasca et al., 1999; Benyoucef et al., 2007). Conversely, IRb homodimers bind insulin more strongly than either IGF1 or IGF2 ligands (Yamaguchi et al., 1993; Frasca et al., 1999; Benyoucef et al., 2007). Thus, receptor subtype can drastically alter sensitivity to a given ligand.

In addition to splice variants, different heterodimer subunit composition will also contribute to both ligand specificity and potential downstream consequences. IR:IGF1 heterodimers form randomly, but increased insulin receptor levels promote heterodimer formation (Moxham et al., 1989; Soos and Siddle, 1989; Soos et al., 1990; Soos et al., 1993). Indeed, heterodimers are often the predominant form of insulin receptor expression, with greater than 50 % of all functional insulin receptors being heterodimers in many tissues (Bailyes et al., 1997). Heterodimers have a higher affinity for IGF1 and IGF2 ligands than to insulin, implying tissue responsiveness can be graded depending on the subunits present (Soos et al., 1993). As well as ligand specificity, dimer composition lends itself to different functions, with insulin receptor homodimers affecting metabolism, IGF receptor homodimers promoting growth, and heterodimers performing a combination of the two (Belfiore et al., 2009). These receptor combinations allow maximal flexibility in the insulin signaling pathway in response to the requirements of the brain at a given time.

2.2.2 Insulin signaling and pathway functions in central neurons

Neuronal insulin signaling provides benefits that are largely independent of glucose metabolism. Neuronal glucose transporters are primarily of the GLUT3 variety and GLUT1 is present in some brain vessels and astrocytes (Simpson et al., 1999; Duelli and Kuschinsky, 2001). Yet neither of these transporters are insulin sensitive, indicating that glucose uptake works regardless of circulating insulin levels, and supports the function of GLUT3 as a facilitative transporter. Insulin-sensitive GLUT4 transporters are localized to the cerebellum and indicate an insulin-dependent mechanism of glucose control, which may provide a partial explanation for

psychomotor impairments observed in some type 1 diabetics (Ryan and Williams, 1993). Despite an apparent indifference to insulin signaling regarding metabolic activity, basal neuronal functioning still requires a healthy insulin signaling pathway.

Both insulin and IGF1 receptors respond in a similar manner upon ligand binding, undergoing autophosphorylation, and recruiting adapter proteins such as IRS1 and SHC to the membrane. Receptor conformational changes following binding of ligands to the α chain bring β chains of each subunit into close proximity, allowing for autophosphorylation at several key amino acid residues (Patti and Kahn, 1998). Phosphorylation of tyrosine residues will cause activation, but sustained receptor activation causes phosphorylation at serine or threonine amino acids which deactivates the receptor (Youngren, 2007). Early tyrosine phosphorylation events promote recruitment of docking proteins such as IRS-1 and SHC, which in turn are themselves phosphorylated (White, 1997; Taguchi and White, 2008). Once brought to the membrane, phosphorylated IRS-1 activates phosphoinositide 3-kinase (PI3K), which in turn phosphorylates the pro-survival AKT protein, a major mediator of insulin signaling (Belfiore et al., 2009).

Insulin-induced AKT activation promotes cell survival mechanisms in neurons. AKT prevents hypoxia-induced death in the hippocampus, and overexpression of the AKT protein protects neurons from apoptosis (Philpott et al., 1997; Matsuzaki et al., 1999; Yamaguchi et al., 2001; Rodgers and Theibert, 2002). Neuronal survival is mediated by suppression of several pro-apoptotic proteins, including cytoplasmic proteins such as the Bcl-2 associated death promoter (BAD), which binds anti-apoptotic Bcl-2 thus activating the pro-apoptotic Bax protein. Bax then relocalizes to the mitochondria where it encourages cytochrome c release (Datta

et al., 1997), which in time activates caspase-9, a mediator of cytochrome c-induced neuronal apoptosis (Cardone et al., 1998). Transcription factors in the forkhead box (FOXO) family are also key targets of insulin-induced AKT signaling, with phosphorylation of Foxo3 preventing its nuclear transport and thus inhibiting pro-apoptotic protein transcription (Van Der Heide et al., 2004). Inhibition of the tumour suppressor p53 has a strong anti-apoptotic effect as well (Yamaguchi et al., 2001). Deactivation of GSK3, a protein involved with glycogen synthesis and apoptosis, is another important mechanism of insulin-induced cell survival (Cross et al., 1995; Turenne and Price, 2001), and also has been implicated in AD pathology, most notably as the kinase that phosphorylates tau (Sperber et al., 1995; Baum et al., 1996). Numerous anti-apoptotic protein targets of AKT insure that insulin signaling via AKT can promote neuronal survival in multiple pathways.

Insulin signaling has a demonstrable efficacy in enhancing learning, memory and synaptic plasticity. In healthy human adults, memory formation is improved following insulin infusion (Craft et al., 1999; Craft et al., 2003), and rats have higher expression levels of brain insulin receptor mRNA and protein following performance in the Morris water maze, a test for spatial memory (Zhao et al., 1999). Insulin may affect this memory formation by increasing synaptic plasticity through changes in LTP and LTD at glutamatergic synapses. Most LTP/LTD response occur following Ca2+ influx through NMDA receptors, an event assisted by glutamate-induced AMPA receptor-mediated depolarization (Malenka, 2003). Through a mechanism that is not yet entirely understood insulin increases cycling of AMPA receptors, internalizing them from the plasma membrane to reduce synaptic excitability and enhance LTD (Man et al., 2003; Huang et al., 2004; van der Heide et al., 2005), but can also promote

AMPA receptor insertion into the membrane to enhance LTP (Man et al., 2003). Insulin's global effect on LTP enhancement, despite reports of increased LTD, was explained by an insulin-induced sensitization of neurons to lower frequencies through NMDA receptors (van der Heide et al., 2005). Insulin can enhance NMDA-mediated LTP signaling by potentiating NMDA receptor response to glutamate or by recruiting NMDA receptors to the membrane (Liu et al., 1995; Chen and Leonard, 1996; Liao and Leonard, 1999; Skeberdis et al., 2001). Thus, insulin signaling may be important to memory formation and retention, through manipulation of NMDA-related long-term potentiation processes. Clinical observations in memory deficits involved with diabetes, a disease involving poor insulin signaling, would seem to confirm this hypothesis.

2.3 Insulin signaling in diabetes

Altered insulin signaling and poor glucose metabolism are key features of the metabolic disease diabetes. Two forms of diabetes have been characterized, each with different origins but similar symptoms. Type 1, or juvenile, diabetes is termed insulin-dependent diabetes mellitus (IDDM) , and is caused by an autoimmune reaction that destroys insulin-producing pancreatic β cells induced by autoantigens such as glutamic acid decarboxylase (GAD) or insulin itself (Bach, 1995; Harrison et al., 1999). Type 2 diabetes, or non insulin-dependent diabetes mellitus (NIDDM), occurs as a result of unbalanced diet and obesity (Groop, 1997; Krentz and Bailey, 2001), inducing hyperinsulinemia that promotes insulin insensitivity (Krentz and Bailey, 2001). However, both Type 1 and Type 2 have similar consequences, with faulty insulin signaling that leads to reduced insulin-mediated glucose uptake and hyperglycemia.

Hypoinsulinemia can also cause increased glucose production, upregulated glycogen breakdown, and increased lipolysis, with consequences such as ROS generation, lipid peroxidation and apoptosis (Niedowicz and Daleke, 2005). Systemic difficulties of extended hyperglycemia can involve serious dysfunctions such as atherosclerosis, blindness and neuropathy (Brownlee, 2001). Thus an impaired insulin signaling pathway can begin a chain reaction of metabolic breakdown that can affect whole organ systems.

While reduced insulin signaling is common to both forms of diabetes, insulin receptor responsiveness to activation is the crucial difference. Type 1 diabetics require regular insulin therapy to maintain glucose metabolism, but their insulin receptors are responsive and active, and they can live relatively normal lives with regular insulin supplementation (Zimmet et al., 2001). Type 2 diabetics, however, are insulin resistant, with receptors insensitive to circulating insulin. Elevated blood glucose and poor insulin response leads to even greater insulin secretion, producing hyperinsulinemic levels which further desensitizes receptors (Kahn, 2003). Mechanisms than enhance receptor insensitivity remain uncertain, but speculation exists that IRS1 activation becomes impaired in NIDDM (Morino et al., 2006). Sustained insulin signaling induces Ser/Thr residue IRS1 phosphorylation which deactivates the substrate protein and promotes its dissociation from the insulin receptor, preventing activation of the PI3K/AKT axis (Morino et al., 2006). For this reason, NIDDM can be more dangerous than IDDM, as the body's physiological response to insulin is drastically changed, and maintaining normal function is difficult. However, lack of an effect of insulin in both cases results in cellular disturbances that could contribute to neuropathologies including dementia and AD.

2.4 Insulin and dementia/AD

Diabetes and reduced insulin signaling generates cognitive dysfunction in a manner independent of glucose metabolism. Type 2 diabetics were found to be at greater risk for developing dementia (Ott et al., 1996), but hyperinsulinemia, and not hyperglycemia, is the predominant risk factor, as shown in studies of a healthy, elderly population (Kalmijn et al., 1995; Stolk et al., 1997b; Stolk et al., 1997a). In addition, dementia in Type 1 & 2 diabetic animal models is linked to resulting elevated neuronal loss (Li et al., 2007c), but greater cell loss in Type 2 models (Li et al., 2007c) implies insulin receptor insensitivity may be more damaging than insulin depletion. These observations are further corroborated by studies in AD as well.

Both AD patients andAD-related transgenic animals have impairments in insulin signaling pathways. AD patients have both elevated blood insulin and hyperinsulinemic cerebrospinal fluid (CSF) levels, accompanied with insulin resistant (Bucht et al., 1983; Fujisawa et al., 1991; Razay and Wilcock, 1994). Gene expression and function of both insulin and IGF1 are reduced in the brains of sporadic AD patients (Frolich et al., 1999; Steen et al., 2005). In AD-related transgenic aged mice overexpressing mAPP have high blood insulin levels and hyperinsulinemia at 13 months of age (Pedersen and Flynn, 2004), but treatment with rosiglitazone, a diabetes drug that increases insulin receptor sensitivity, prevents hyperinsulinemia and diabetes-induced cognitive deficits (Pedersen et al., 2006). Conversely, inducing further insulin dysfunction in AD-related transgenic mice exacerbates progression of AD-like symptoms, as a diet of high sucrose led to hyperinsulinemia, increases Aβ deposition and accelerates cognitive impairment in an FAD APP/PS1 mouse (Cao et al., 2007). Induction of type 2 diabetes produces similar results in hAPPSwe mice

(Ho et al., 2004). Similarly, insulin resistance induced by intracerebroventricular STZ injection enhances Aβ deposition in hAPPSwe mice (Plaschke et al., 2009). As insulin pathway activation seems to correlate with the severity of AD, molecular associations between insulin and Aβ have been suggested.

2.5 Interactions between insulin signaling and amyloid peptides

2.5.1 Insulin degrading enzyme (IDE)

IDE is a metalloprotease of 110 kDa that degrades insulin and several other substrates, such as amylin and the IGF proteins (Kirschner and Goldberg, 1983; Misbin and Almira, 1989; Bennett et al., 2000). Expression is both ubiquitous, with highest levels in the liver, muscle and brain (Kuo et al., 1993), and age-dependent, with reduced levels in older animals (Runyan et al., 1979). Insulin removal is highly dependent on IDE levels, and IDE overexpression or removal via antibody sequestering results in increased or decreased insulin degradation, respectively (Shii and Roth, 1986; Kuo et al., 1994). However, IDE has other substrates including Aβ (Qiu et al., 1998), whose degradation limits formation of toxic oligomers (Chesneau et al., 2000). Apolipoprotein E facilitates the process by binding and transporting Aβ (Jiang et al., 2008), possibly bringing monomers to unsaturated IDE enzyme in the liver. Increased risk of developing AD associated with the ApoE4 allele may be due to difficulties with these Aβ degradation mechanisms.

IDE has a higher affinity for insulin than Aβ, and difficulties in IDE-mediated Aβ degradation arise when degrading time for insulin increases and prevents Aβ from associating with IDE. As a consequence, hyperinsulinemia commonly observed in AD patients would saturate the IDE enzyme, thus preventing Aβ1-42 removal and

allowing its accumulation (Luchsinger et al., 2004; Qiu and Folstein, 2006), as demonstrated *in vitro* when increasing insulin levels impaired IDE-directed Aβ degradation (Qiu et al., 1997). Reduced IDE levels in AD further exacerbates this removal with less available protease to cope with peptide degradation (Perez et al., 2000). The reason for an AD-related reduction in IDE levels is unknown, but as insulin signaling through PI3K normally induces IDE expression (Zhao et al., 2004) impaired insulin signaling due to insulin insensitivity could compromise IDE protein formation. Thus a combination of hyperinsulinemia-induced IDE saturation and reduced IDE expression due to insulin receptor insensitivity could drastically increase Aβ aggregation and enhance the development of AD.

2.5.2 Insulin-induced amyloid secretion

Different flAPP cleavage products are promoted via insulin signaling, depending upon which downstream effectors are upregulated. Insulin signaling can occur through PI3K-AKT or MAPK signaling pathway, by recruiting either IRS or SHC receptor binding proteins respectively (Belfiore et al., 2009). Insulin-induced activation of the PI3-K/AKT axis, but not ERK, increases secretion of sAPPα in SH-SY5Y cells (Solano et al., 2000), and IGF1 increases α-secretase activity and sAPPα secretion in a similar fashion (Adlerz et al., 2007). However, as overexpression of constitutively active AKT actually inhibits sAPPα formation (Shineman et al., 2009), a balanced AKT activation system could be required. In contrast, MAPK activation by insulin may produce different flAPP cleavage products, as increased Aβ1-42 secretion by insulin was mediated through MAP kinase, while simultaneously inhibiting IDE-promoted degradation in neuronal cultures (Gasparini et al., 2001).

Multiple signaling pathways may provide a sensitive mechanism for insulin/IGF1 to modulate amyloid peptide secretion depending upon as yet unknown requirements, implying physiological roles that extend beyond known pathological ones.

2.5.3 Aβ peptides acting upon the insulin pathway

Both monomeric and oligomeric forms of Aβ have demonstrated the capacity to act upon the insulin pathway, with opposing results. In a cell-free system, oligomers compete with insulin for insulin receptor binding and prevent autophosphorylation in the tyrosine kinase domain (Xie et al., 2002). In hippocampal neuronal cultures soluble Aβ oligomers can inhibit AKT and MAPK activation while binding to insulin receptors (Townsend et al., 2007), and resulting in synaptic damage (Zhao et al., 2008). This damage seems partially receptor-mediated, as insulin displaces Aβ oligomers from the receptor resulting in neuronal protection from Aβ-induced inhibition (Zhao et al., 2008; De Felice et al., 2009; Lee et al., 2009). Aβ oligomer-induced insulin receptor impairment has been suggested to cause insulin insensitivity and subsequent hyperinsulinemia observed in AD patients (Zhao et al., 2008). A recent study revealed oligomer inhibition of IRS1 via phosphorylation at Ser/Thr residues mimics a similar effect observed in Type 2 diabetics (Ma et al., 2009), possibly causing insulin resistance and increased cell death due to an absence of AKT-induced neuroprotection. While still preliminary, study of Aβ monomer signaling provides evidence of physiological effects that oppose actions observed with Aβ oligomers. Treatment of insulin-deprived hippocampal cultures with Aβ monomers provides a neuroprotective effect through activation of PI3K and AKT proteins (Giuffrida et al., 2009). Thus, oligomeric and monomeric Aβ interactivity

with the insulin pathway could moderate insulin-promoted pro-survival functions. A relation between healthy synaptic activity and moderate Aβ levels has been suggested, with significant changes in either of these two factors causing neuronal pathology contributing to AD (Palop and Mucke, 2010).

2.5.4 GSK3 and AD

The pro-apoptotic GSK3 normally requires suppression through increased insulin signaling, with AKT phosphorylating Ser/Thr residues of GSK3 to inactivate the protein. Reduced insulin signaling in AD seems to contribute to a corresponding increase in GSK3, as GSK3 activity and expression is increased in the frontal cortex and hippocampus, respectively, of brains from AD patients, (Blalock et al., 2004; Leroy et al., 2007). This could lead to increased production of AD-associated pathologies such as hyperphosphorylated tau and increased Aβ accumulation. For example, GSK3 can enhance AD-type neurofibrillary tangle formation by increasing tau hyperphosphorylation both *in vitro* and *in vivo* (Hanger et al., 1992; Lucas et al., 2001; Asuni et al., 2006). Indeed, increased tau phosphorylation is also associated with FAD mutations in presenilin-1, whereas wildtype presenilin-1 aids in basal GSK3 suppression (Takashima et al., 1998; Baki et al., 2004). In addition, altered Aβ production is observed following lithium-induced GSK3 inhibition, reducing Aβ secretion in cell lines expressing flAPP (Sun et al., 2002). Inhibiting GSK3 also prevents Aβ1-42 production and deposition in mAPP/PS1 mice (Phiel et al., 2003). Thus, elevated GSK3 activation due to insulin resistance and AD-associated pathology could promote a potential feed-forward loop towards greater Aβ deposition and tangle

formation resulting in neuronal dysfunction. However, the degree to which GSK3 activity affects AD pathology remains uncertain.

2.5.5 IGF1 and Aβ

While both insulin and IGF1 can contribute to flAPP cleavage, IGF1 has a specific Aβ clearance system independent of the insulin ligand. Tg2576 animals have reduced serum IGF1 levels compared to controls, and IGF1 injection reduces cerebral Aβ levels and increases Aβ in the CSF due to an Aβ transport mechanism that shuttles the peptide across the blood brain barrier at the choroid plexus (Carro et al., 2002). IGF1R blockade prevents this transport and could also exacerbate AD-type symptoms (Carro et al., 2002). This mechanism may or may not be related to IGF1s ability to assist ApoE-mediated Aβ clearance via the low-density lipoprotein receptor-related protein (Tanzi et al., 2004; Freude et al., 2009). Regardless of mechanism, increased Aβ shuttled to the liver could aid IDE-directed degradation (Kuo et al., 1993). As IGF1 signaling may be reduced in AD patients (Steen et al., 2005), an Aβ transport impairment could also contribute to Aβ accumulation in the brain.

2.5.6 Summary of insulin and AD

Increasing evidence supports an association between AD development and impairments in insulin signaling, but the chronology of these events is difficult to ascertain. Aβ oligomer-mediated inhibition of the insulin receptor would indicate that insulin dysfunction observed in AD may occur as a result of Aβ production. This is supported by data regarding certain animal models of FAD. Younger Tg2576 mice have lower insulin levels than control animals but older transgenic are hyperinsulinemic, implying that Aβ produced by these animals could induce insulin

resistance (Pedersen and Flynn, 2004). However, as insulin signaling impacts both clearance and secretion of Aβ, an impaired insulin pathway may accelerate AD progression. The actual situation observed may likely be a combination of the two, with their reciprocal relation feeding into dysfunction on either side of the equation. Continued study will be required to determine if a therapeutic advantage exists in manipulating the insulin signaling system for treatment of AD.

3 AD therapies and possible future targets

As no effective therapies exist for treatment or prevention of AD, new pharmaceuticals are constantly being developed to provide relief for AD patients. Perhaps surprisingly, current accepted therapies have little relation to established AD markers or their prevention. Many current studied drugs target Aβ formation and aggregation, while others attempt to alleviate symptoms or improve quality of life through other means, as discussed below. However, even these have questionable efficacy, making continued development of newer therapeutic targets a necessity.

3.1 Current therapies

Only two classes of drugs are currently approved by the Food and Drug Administration (FDA) for treatment of AD. The first class are acetylcholinesterase inhibitors (AChEI) which prevent the degradation of acetylcholine, a neurotransmitter commonly used by cholinergic neurons in the forebrain. Cholinergic activity is often deficient in advanced AD, with significant cholinergic neuronal loss in the forebrain (Hardy, 2006). AChEIs prevent degradation of the neurotransmitter acetylcholine to prolong activity at the synapse of cholinergic neurons. Three AChEI drugs (donepezil, galantamine and rivastigmine) are currently used and can temporarily (ie.

~6-9 months) improve cognition in AD patients at multiple stages of the disease (Rogers et al., 1998; Rosler et al., 1999; Tariot et al., 2000). The second class of drugs inhibit NMDA function, such as memantine, and are designed to decrease intracellular calcium levels and limit excitoxicity, and has demonstrated an ability to improve cognitive ability in patients with moderate to severe AD (Tariot et al., 2004; van Dyck et al., 2007). Combination therapy of AChEIs and memantine increases treatment efficacy, and may have long term effects (Atri et al., 2008; Lopez et al., 2009). However, these therapies seem to address AD symptoms only and are often prescribed in late-stage sporadic AD, with little to no effect on disease reversal. Other therapies that target prodrome AD could be more advantageous.

3.2 Potential therapies for disease reduction

3.2.1 Altered secretase activity

Promotion of α-secretase activity, or limiting activity of either β or γ-secretase, would prevent Aβ formation, thus inhibiting Aβ-induced neuronal impairments. Few specific α secretase activators are available, but activation of muscarinic acetylcholine receptors (mAChR) increases α-secretase protease activity while simultaneously inhibiting the γ-secretase enzyme (Fisher et al., 2003; Caccamo et al., 2006). Clinical studies using specific M1 receptor agonists to enhance α-secretase cleavage of flAPP result in reduced CSF Aβ levels (Nitsch et al., 2000). In addition, retinoic acid receptors (RAR) activators retinoic acid and acitretin also increase α-secretase activation to reduce Aβ levels in both mAPP/mPS1 and mAPP(Swe) mice (Tippmann et al., 2009; Jarvis et al., 2010). While α-secretase can preclude cleavage at the β site, direct inhibition of BACE or presenilins may also have the desired effect on Aβ

production. BACE knockout animals are viable and contain lower brain Aβ levels than controls, with few side effects (Luo et al., 2001; Roberds et al., 2001), but most current BACE inhibitors are too large to cross the blood brain barrier, with only a few small enough to be possible drug candidates (Ghosh et al., 2006; Ghosh et al., 2008). Presenilin inhibition, however, is more difficult due its necessary role in cleavage of other substrates, including the Notch receptor (Golde and Kukar, 2009), but γ-secretase inhibition has proven effective in lowering CSF Aβ in AD clinical trials (Bateman et al., 2009). NSAID therapy could be an effective alternative by binding to APP and preventing γ-secretase cleavage, thus maintaining basal γ-secretase activity required for Notch pathway activation (Kukar et al., 2008). While some of these treatments could be effective in preventing Aβ production, their validity in preventing AD development remains to be demonstrated.

3.2.2 Aβ removal and aggregation prevention

Aβ removal, either by increased clearance or degradation, could prove as effective as inhibition of Aβ production. Virally-directed overexpression of neprilysin, one of the most common Aβ removal enzymes, resulted in lower Aβ levels in some AD models (Marr et al., 2003; Iwata et al., 2004). However, overexpressing neprilysin in hAPP-J20 mice had no effect on Aβ oligomer levels or cognitive ability versus mAPP mouse controls, despite a reduction in total Aβ (Meilandt et al., 2009). Immunologically directed studies have had some success, with animal vaccination trials using the full length Aβ peptide effectively reducing Aβ levels and cognitive decline (Schenk et al., 1999; Morgan et al., 2000), and preliminary results suggesting a similar benefit in humans as well (Hock et al., 2003). However, the clinical trial for

the Aβ vaccination AN1792, involving immunization with the peptide, was terminated due to cases of immune-mediated meningoencephalitis (Orgogozo et al., 2003). Passive antibody therapy involving direct Aβ antibody injection may be as effective while preventing T-cell induced toxicity, and early trials with Tg2576 mice were promising in this regard, with injections increasing microglia activation and reducing Aβ levels (Wilcock et al., 2004). Two monoclonal antibody-based Phase III trials are currently underway to test treatment efficacy, but whether removal of Aβ in this manner will impact symptoms of the disease is not certain. Further clinical trials will be needed to determine if Aβ removal is an effective strategy for AD treatment.

As soluble Aβ oligomers are notably neurotoxic, pharmacological agents designed to inhibit aggregation may be as effective as removing Aβ entirely. Anti-aggregate cyclohexanehexol prevents Aβ-induced LTP and cognitive impairments *in vitro*, and has been safely tested in humans (McLaurin et al., 1998; McLaurin et al., 2006; Townsend et al., 2006). However, AD patients in a Phase III trial for the similar drug tramiprosate demonstrated little improvement (Sabbagh, 2009). Nutritional agents, such as polyphenols, omega-3 fatty acids, and curcumin are currently being studied as potential anti-oligomeric agents (Ono et al., 2008; Ma et al., 2009), as diets composed of these compounds reduce plaque burden in the brains of transgenic animals (Lim et al., 2005; Wang et al., 2006; Green et al., 2007). Epidemiological studies of populations that consume large amounts of these substances in their diet indicate a possible reduced risk for AD (Morris et al., 2003; Schaefer et al., 2006). As dietary supplements, with few if any side effects, these could be attractive treatments in disease prevention, if proven effective.

3.2.3 Anti-oxidant therapy

Dietary anti-oxidants and mitochondrial drugs have demonstrated a positive effect against AD symptoms. In addition to an anti-amyloidogenic effect, polyphenols such as resveratrol and curcumin have both anti-oxidant and anti-inflammatory qualities (Cole et al., 2005; Vingtdeux et al., 2008). Cultures that cook with curry, a spice containing high amounts of curcumin, have less incidences of AD than other cultures (Ganguli et al., 2000). Research using curry demonstrated a neuroprotective effect *in vitro*, and reduced plaque load in flAPP transgenic mice (Shishodia et al., 2005; Yang et al., 2005). Metal chelators, designed to remove metal ions that can lead to oxygen reduction and ROS formation, have also been tested. The metal ion chelator PBT2 has demonstrated an efficacy in reducing CSF Aβ levels, although its mechanism is unclear (Lannfelt et al., 2008; Cahoon, 2009). The drug dimebolin is designed to stabilize the mitochondrial membrane and improve the organelles' function, and could possibly have a use in AD therapy (Doody et al., 2008). However, these therapies may again only address AD symptoms more than AD development, which could limit their usefulness in AD prevention.

3.2.4 Insulin-related therapies

Insulin-related therapies have been a popular choice of late, given the well-studied AD-insulin association. Like neprilysin, reduced IDE expression is observed in AD patients (Perez et al., 2000), and supplementing patients with the enzyme to remove Aβ could prove beneficial. Overexpression of neprilysin and IDE together have a profound impact on AD pathology in APP(Swe/Ind) mice, preventing plaque burden and significantly reducing dystrophic neurites (Leissring et al., 2003), and IDE

activators are currently being tested as a pharmacological intervention (Cabrol et al., 2009). Direct insulin therapy has proven beneficial, with both intravenous or intranasal insulin application reducing cognitive impairments in AD patients (Craft et al., 1996; Reger et al., 2008). Intranasal administration also localizes the peptide mostly to the CNS, limiting peripheral metabolic response. Rosiglitazone, a PPARγ agonist used in diabetes to sensitize insulin receptors, has also been suggested as another possible treatment, but the efficacy of this treatment is still unknown (Risner et al., 2006). Whether insulin-focused therapies will receive mainstream use remains to be determined.

4 Neuregulin – another therapeutic target?

Insufficient effective AD therapies have driven the search for new and novel therapeutic targets. Numerous drugs based upon established Aβ formation and secretion pathways are currently being tested, and are at various stages of development. Other therapies designed to enhance neuronal survival or limit synaptic inhibition could be effective in combating AD symptoms, or, preferably, inhibit pathology before it starts. Studies assessing the neuronal effects of the protein neuregulin (NRG) indicate potential therapeutic roles, with molecular associations to both amyloid pathogenesis and insulin signaling.

4.1 Neuregulin (NRG) & the ErbB receptor – structure

The NRG protein family is composed of six different transmembrane proteins that act upon receptors at synaptic membranes. All members contain an epidermal growth factor (EGF) site, a distinctive N-terminal extracellular region that determines protein function and a C-terminal intracellular region. Type III NRG is the only

exception, with two membrane spanning regions and no exposed N-terminus. Different isoforms determine protein localization and function in the brain (Carraway et al., 1997; Meyer et al., 1997), but most NRG proteins are cleaved to shed their EGF domain and stimulate NRG receptors from the ErbB family post-synaptically.

ErbB receptors form homo- or heterodimers that bind the EGF domain of NRG proteins to initiate downstream signaling. Much like the insulin/IGF1 receptor, they are tyrosine kinase receptors that undergo autophosphorylation upon ligand binding (Bublil and Yarden, 2007). ErbB1 through 4 have been discovered, but ErbB4 is the only receptor capable of being bound by NRG and acting as a tyrosine kinase following binding. Neither ErbB1 nor ErbB2 have binding affinity for NRG, but mediate kinase function by forming ErbB4 heterodimers (Bublil and Yarden, 2007), and ErB3 binds NRG, yet has no kinase function and therefore requires dimerization with an active kinase subunit (Bublil and Yarden, 2007). ErbB4 subunits seem to be the most clinically relevant for neurological activity, as ErbB4 mutant mice have schizophrenia-like positive symptoms, while ErbB2 and ErbB3 mutant mice are phenotypically normal (Gerlai et al., 2000). Analysis of the consequences of NRG/ErbB signaling has revealed a potential role in both neurodegeneration and neurodevelopment.

4.2 NRG/ErbB signaling

Both the NRG ligand and its ErbB receptor are acted upon by proteases to form multiple cleavage products capable of forward and backward signaling (Figure 4). In traditional forward signaling, the N-terminus and EGF domain of NRG is released from the membrane upon enzymatic cleavage by TNFα converting enzyme

(TACE) (Loeb et al., 1998) and BACE, the β-secretase enzyme partially responsible for Aβ release from flAPP (Hu et al., 2006; Willem et al., 2006). Following release, NRG stimulation of ErbB receptors results in autophosphorylation and recruitment of second messengers such as PI3K and ERK, also targets of the insulin pathway (Sawyer et al., 1998; Junttila et al., 2000). However, in backward signaling, ErbB4 receptors act as a ligand, with TACE cleavage at the plasma membrane resulting in secretion of its N-terminus (Mei and Xiong, 2008). This product can bind the EGF domain of the membrane-bound Type III member of the NRG family, although the consequences of this interaction remain unclear. In addition, both NRG and ErbB receptors can be cleaved by γ-secretase at the C-terminus to release the intracellular domain (ICD) of NRG and ErbB, both of which relocalize to the nucleus to mediate transcription (Bao et al., 2003; Sardi et al., 2006). Thus, both forward and backward signaling represent multiple regulatory points that maintain NRG functioning.

Figure 4 – NRG-ErbB signaling

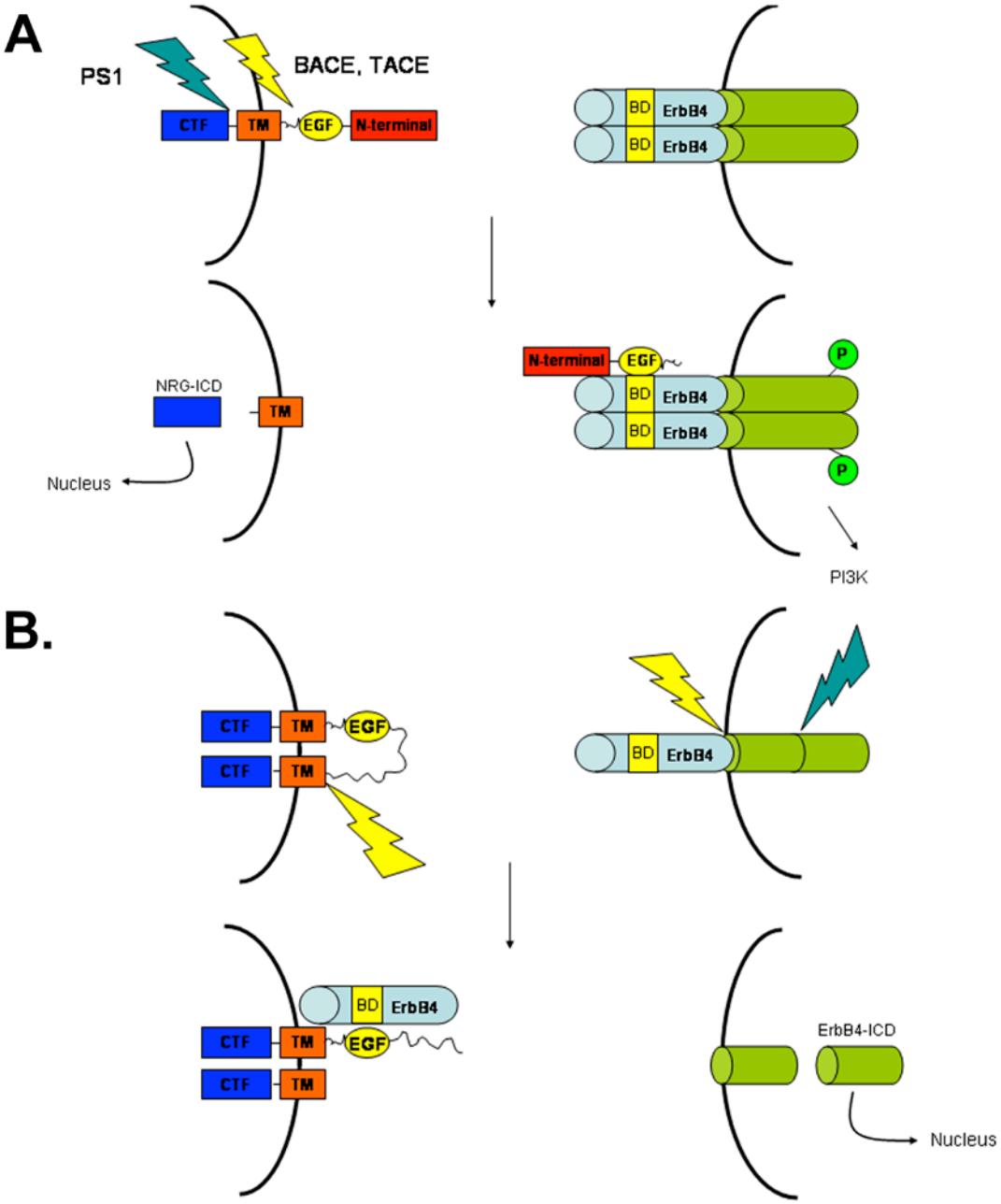


Diagram of potential NRG signaling. A) Traditional NRG signaling involves TACE or BACE cleavage at the membrane, releasing the EGF-containing fragment that binds to a cystein rich binding domain (BD) of an ErbB receptor at the post-synaptic membrane. Further cleavage of the NRG C-terminus by PS1 generates an NRG-ICD that translocates to the nucleus. In non-traditional signaling (B), TACE cleaves the ErbB4 receptor to shed an ecto-ErbB4 fragment, which can then bind the EGF domain of the membrane-bound type III NRG protein, initiating backward signaling. (CTF, C-terminal fragment, TM=transmembrane)

4.3 Function of NRG in neurons

While most NRG signaling can affect neuronal development, cell migration and myelination, the modification of synaptic plasticity and neuronal survival suggest a role for NRGs in neurodegeneration, possibly by regulating excitatory and inhibitory synaptic transmission. For example, in hippocampal tissue, ErbB4 receptors associate with PSD95, a scaffolding protein involved with glutamatergic ion channels at the synaptic membrane (Garcia et al., 2000; Huang et al., 2000). ErbB4:PSD95 interactions allow NRG to attenuate or prevent LTP induction in an acute, if uncertain, manner (Kwon et al., 2005; Pitcher et al., 2008), although no direct effects on GABAergic transmission or NMDA receptor phosphorylation/activity has been detected in the hippocampus (Huang et al., 2000; Iyengar and Mott, 2008). NRG signaling does affect AMPA receptors and currents, albeit in a contradictory fashion; NRG1 decreases AMPA receptor currents and increases receptor internalization in hippocampal neuronal cultures (Kwon et al., 2005), but NRG-ErbB4 signaling also stabilizes AMPA receptors at the synapse, and pathway inhibition prevents glutamatergic signaling (Li et al., 2007a). Thus, NRG signaling regulates glutamate signaling under conditions that are not yet fully understood. *In vitro* analysis of NRG signaling on neuronal survival has yielded positive results, emphasizing its neurotrophic nature. Neuregulin protects cortical neurons from apoptosis due to reduced trophic support, by increasing PI3K activation (Li et al., 2003). Similarly, NRG family member glial growth factor 2 (GGF2) prevents apoptosis in cultured dopaminergic neurons following serum deprivation (Zhang et al., 2004). Thus, the ability of NRG signaling to modulate neurotrophic pathways makes it an attractive therapeutic target for disease.

4.4 Neuregulin signaling in disease

Neuregulin signaling is involved in neurological disorders such as multiple sclerosis and schizophrenia. Reduced NRG expression in Multiple Sclerosis (MS)-induced axonal lesions suggest a correlation between demyelination and low NRG levels (Viehover et al., 2001). Injection of glial growth factor 2 (GGF2), a member of the NRG family, in animal models of experimental autoimmune encephelamyelitis (EAE) can delay and reduce disease symptoms of MS while enhancing myelination (Cannella et al., 1998; Marchionni et al., 1999). However, the role of NRG signaling in schizophrenia pathogenesis has been more intimately studied, as neuregulin is a candidate susceptibility gene for the disease (Stefansson et al., 2003). Schizophrenia is partially characterized by NMDA receptor dysfunction (Olney et al., 1999) and improper NRG modulation of glutamatergic signaling could downregulate NMDA receptors causing schizophrenia. Increased NRG/ErbB4 activity is detected in the forebrain of schizophrenics causing substantial NMDA receptor inhibition (Hahn et al., 2006), implying the importance of NRG-modulated NMDA function in the development of the disease. Continued analysis in animal models of schizophrenia has been difficult, however, as they display positive symptoms, such as hallucinations, that are difficult to detect and interpret. Yet NRG and ErbB4 mutant animals display symptomatic behaviour characteristic of schizophrenia, such as hyperactivity (Gerlai et al., 2000; Golub et al., 2004). Treatment with the anti-psychotic clozapine, commonly prescribed to treat schizophrenia, attenuates the hyperactivity in these animals (Rimer et al., 2005). Thus, an impaired NRG-ErbB4 axis could result in the development of schizophrenia-type symptoms. A similar impairment is also suggested in AD, with both NRG and ErbB4 proteins associating with Aβ plaques in

mutant APP/PS1 transgenic mice (Chaudhury et al., 2003). Thus NRG dysfunction could account for neurodegenerative pathology in multiple diseases, including multiple sclerosis, schizophrenia, and AD.

Research on cerebral ischemia, however, might prove the most informative in terms of the use of NRG as a therapy for AD or for other neurodegenerative disorders. Elevated neuregulin levels at the site of a cerebral ischemic event suggest a potential compensatory mechanism for neuronal protection (Parker et al., 2002), and rats pre-treated with NRG1 either 30 minutes or immediately prior to inducing cerebral ischemia display reduced infarct size and neuroprotection (Shyu et al., 2004; Xu et al., 2004). NRG is also effective when injected up to 12 hours post-arterial occlusion, a surgical model for cerebral/infarct ischemia, with treated animals showing greater neuronal protection and functional recovery than controls (Xu et al., 2006). Amazingly, NRG family member GGF2 could provide substantial protection when administered 7 days following stroke, emphasizing the protein's potency as a neuroprotective treatment (Iaci et al., 2010). A mechanism of action is suggested by the observation that NRG treatment is protective in a rat ischemia model in a similar manner to the NMDA antagonist MK801, and a synergistic effect is observed when the two were combined (Li et al., 2007b). Thus, NRG signaling possibly reduces calcium influx through NMDA receptors and limits ischemia severity caused by excess calcium levels. As NRG can modify glutamate ion channel composition at the synapse and possibly prevent from excitoxicity in ischemia, a potential therapeutic role for limiting excitoxicity commonly associated with AD could be possible.

Chapter 2 - Theme of this thesis

Although FAD gene mutations and markers such as Aβ and tau have been established hallmarks of AD for decades, a definitive cause of the disease remains elusive. The original goal of this thesis project was to study cell signaling pathways associated with AD pathology, looking at downstream consequences of either gene mutations, or mechanisms of Aβ signaling that may contribute to neuronal dysfunction. This resulted in three separate studies involving AD-induced cellular pathology and possible therapeutical intervention for the disease.

The first project involved determining interactions between pro-/anti-apoptotic signaling mechanisms and cellular calcium in mPS1-containing neurons. Presenilin mutations are the most common genetic determinant of AD and determining how the mPS1 mutation changes NF-κB signaling and ER calcium release may provide an insight into how the disease progresses in many FAD patients. In our hands, mPS1 neurons were as resistant to Aβ toxicity as WT neurons, due to an additional protective measure induced by elevated stress-induced ER calcium release. While we believe this project is important in understanding neuronal pathology for many forms of FAD, a potential association to sporadic AD is questionable. Thus, once this pathway was elucidated, another project was to be started using TgCRND8 mice that overexpress mAPP. These mice develop Aβ deposition that correlates well with AD-like memory deficits (Chishti et al., 2001). As Aβ is still considered by many to be a trigger of sporadic AD pathogenesis, study of signaling pathways in the TgCRND8 mouse model of amyloidogenesis may provide more insight into how AD develops in relation to Aβ production.

However, preliminary data in our laboratory and from existing literature suggest the neurotrophin NRGβ1 could modify glutamatergic synapses to limit excitoxicity and promote cell survival, and a signaling pathway was assessed in wild-type neuronal cultures with the intention of continuing our assessment in TgCRND8 *in vitro* hippocampal cultures, hopefully to enhance survival and prevent Aβ-induced cell death. We were successful in discovering that NRGβ1 could enhance glutamate-induce calcium signaling in wild-type hippocampal cultures, through calcium efflux from ER stores. However, difficulties with the NRGβ1 project including limited commercial availability of the protein and the expense and difficulty of culturing neurons from the transgenic mice prevented us from continuing with the project in TgCRND8 hippocampal cultures. Therefore, in order to continue working on an Aβ-induced AD cellular pathology study as intended, an *in vivo* TgCRND8 project was initiated to evaluate the role that hypoinsulinemia has on development of AD-like pathology in the mice.

The original goal of our *in vivo* study was to determine if low insulin levels would exacerbate AD-like symptoms as drastically as hyperinsulinemia could. However, this project began to change when a substantial beneficial effect of hypoinsulinemia was observed in early diabetes of young TgCRND8 mice, and our follow-up to this observation revealed a potential role for sAPPα in insulin signaling. Thus, sAPPα could become a therapeutic agent both for diseases of insulin insufficiency and for AD as well.

These studies as they developed, and their objectives, are listed below in the chronology they were performed:

Chapter 4

mPS1(M146V) mice contain a FAD mutation in the PS1 gene that leads to increased Aβ production and early onset AD in humans. However, these animals do not demonstrate any AD phenotype and contradictory reports on stress-induced neuronal death in mPS1 neurons in culture are difficult to reconcile. Neurons from mPS1 mice have increased ER calcium release, which can have paradoxical effects, including activation of the anti-apoptotic protein NF-κB or the pro-apoptotic protein GADD153. Our hypothesis was that an interaction between the two proteins could explain this questionable neuronal vulnerability. The objectives for this study were:

- To analyze Aβ-induced calcium signaling response of neurons of mPS1 mice compared to neurons from C57Bl mice
- To determine the role of NF-κB and CHOP in neuronal survival and functioning

Chapter 5

Calcium dyshomeostasis contributes to the neuronal death in AD, leading to memory impairment and, eventually, death. The NMDA receptor antagonist drug memantine has had limited success in increasing cognitive ability in AD patients, by decreasing cytoplasmic calcium levels. However, severe side effects including psychoses upon receptor blockade (Domino and Luby, 1981) limit the use of the drug. For this reason, new pharmaceuticals that limit calcium-induced neuronal excitotoxicity resulting in neuronal death could provide relief from AD symptoms, or even prevent disease development in pre-symptomatic patients. The neurotrophic protein neuregulin activates ErB receptors at synaptic membranes, which results in modification of glutamatergic ion function. As NRG signaling decreases AMPA ion

currents, and can also generate NMDA hypofunction *in vivo*, NRGβ1 could reduce calcium influx in glutamatergic synapses to limit excitotoxicity. To establish this possibility, the objectives of this study were:

- To verify whether NRG signaling can change intracellular calcium levels
- To determine mechanism by which NRG may alter calcium levels

Chapter 6

Hypoinsulinemia has a significant impact on cognitive ability, including reduced spatial memory and memory formation. Despite numerous studies indicating that insulin dysfunction results in exacerbation of AD pathology, a lack of significant deficits was observed in pre-symptomatic mutant APP transgenic mice following insulin removal. Therefore, a moiety of the APP peptide could have a positive effect on insulin signaling that prevented deficits normally associated with insulin deficiency. flAPP can be cleaved into several different products, each of which has been demonstrated to have a different function. The most commonly studied product, Aβ, has been shown to interact with the insulin signaling pathway to both positive and negative effect, but whether secreted portion of flAPP released by alpha secretase cleavage (sAPPα) could also change insulin signaling was unknown. The objectives of this study were:

- To determine whether sAPPα can signal through the insulin pathway *in vitro,* in the absence of insulin
- To examine if sAPPα works the same pathway *in vivo*

Chapter 3 – Detailed Materials and Methods

Experimental reagents. Neurobasal media, B27 supplement, and calcium dye Fura-2AM were obtained from Invitrogen. Custom-made antisense oligonucleotides for CHOP/GADD153 and IkB mRNA (CHOP: GACTCAGCG CCATGA; IkB: GCGCTCGGCCGCCTGGAACATGGC) were also provided by Invitrogen. Aβ1-42 peptide was purchased from Bachem. Antibodies to CHOP, NF-κB consensus binding sequence, and ECL kit for Western Blot detection were purchased from Santa Cruz Biotechnology. Peroxidase-conjugated secondary antibodies were provided by Jackson Immunopharmaceuticals. DC protein assay kit was obtained from Bio-Rad.

For NRGβ1 experiments, recombinant full length neuregulin β1 was purchased from Medicorp. Caffeine, ATP and ruthenium red was purchased from Sigma-Aldrich. NMDA, AMPA, and t-ACPD was obtained from Tocris. Glutamic acid and KCl was purchased from Fisher Scientific.

For the sAPP□ study, streptozotocin (STZ) was obtained from Sigma-Aldrich. Antibodies were purchased as follows: pY972-IR (Stressgen); insulin receptor beta, β-tubulin isoform III, Alexa Fluor 568 (Santa Cruz Biotechnology); pAKT (Cell Signaling); 6E10 (Cedarlane Labs). Protein A/G conjugated agarose and bovine serum albumin (BSA) was obtained from Fisher. sAPPα was provided by Dr. S. Barger (University of Washington, US). Neurobasal media and B27 supplement was purchased from Invitrogen. DC protein assay kit was purchased from Bio-Rad. ECL Advanced luminal kit was purchased from GE Healthcare. Blocking reagent was purchased from Roche.

Animals. The mPS1 M146V mice (Guo et al., 1999) were maintained in the RO Burrell Lab Animal Facility (Winnipeg, MB). C57Bl6 mice, the background strain used to generate the mPS1 mouse, were used as a control. CD1 mice (ATCC) were used for sAPP□ cell culture experiments.

Primary cortical/hippocampal cultures. Brains of E16 embryonic mPS1 M146V mice were removed following decapitation by dorsally bisecting the head from the base of the neck up the midline towards to the forebrain. Hindbrain tissue was discarded, and brains separated sagitally. The midbrain and meninges were removed prior to isolation of desired neural tissue. For pure (~95%) cortical cultures, the hippocampus was separated and discarded prior to cortical dissociation. For pure hippocampal cultures, the opposite was performed.

Once all embryos were dissected, tissue was mechanically dissociated gently 8-10X via trituration in 4-8 ml of Ca^{2+}/Mg^{2+}-free Hank's Buffered Saline Solution (HBSS) [5.4 mM KCl, 0.44 mM KH_2PO_4, 137mM NaCl, 0.338 mM Na_2HPO_4, 25 mM HEPES,5.6 mM D-glucose] using a flame-polished Pasteur pipette. Supernatant was removed from dissociated tissue, and the trituration step was repeated. Cell viability was assessed by Trypan blue staining, cells were seeded in poly-D-lysine coated 6 well dishes (for cortical cultures, 800,000 neurons/well; for hippocampal neurons, 200,000 neurons/well), in Neurobasal media containing B27 supplement and 10% fetal bovine serum (FBS). The following day, media was removed and NB media with B27 supplement was added (no FBS). Astrocyte growth was limited by adding cytosine arabinoside (CA, 500 nM) to cultures 3 days post-plating, using a

half-media change containing Neurobasal and 1 μM CA. Experiments were carried out 7-8 days following plating.

For the sAPP□ study, CD1 mice were used, with experimental procedures performed at 7-10 days *in vitro*.

*Cell survival assay based on neuronal morphology***.** Neuronal viability was assessed using a Zeiss light microscope with a 20x objective. Hatch marks were etched onto the bottom of each well with a scalpel blade, and four pictures were taken at random vertices locations using a Sony digital camera. Following experimental treatment, pictures were taken at designated time points, at previously recorded locations where baseline photos were taken. Neurons with intact and smooth membranes were considered live, while those with disrupted membranes, dystrophic/damaged neurites or vacuolated cell body were considered non-viable. Percentage of surviving cells was determined by comparing viable neuronal numbers at time-points post-treatment versus numbers at baseline time point prior to experimental treatment.

*Immunoblotting***.** Following experimental treatment, neurons were washed 1x in ice-cold PBS, and then lysed in Laemmli buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS). Samples were boiled for 15 minutes, triturated with a 27-gauge needle and 1 ml syringe, and centrifuged at 25,000 g for 15 minutes. Supernatants were kept and assayed from protein levels using a DC protein assay kit. Proteins were separated on an acrylamide gel (from 6% to 12 % depending on protein, 10-20 μg/lane) for 90 minutes at 150 V and transferred to nitrocellulose or PVDF membranes using a semi-dry apparatus for 15 minutes at 30 V, or a wet-transfer at 100 V for 90 minutes.

Transferred membranes were blocked with casein-based blocking buffer (Roche), 5 % BSA or 5 % skim milk powder and incubated with primary antibody overnight at 4 ^{o}C. Following four washes (15 minutes each) in Tris buffered saline containing 0.1 % Tween-20 (TBST), membranes were incubated with secondary antibody specific to primary antibody species for 1 hour at room temperature, in buffer containing 1 or 5% blocking buffer and TBST. An additional four washes were carried out and membranes were exposed to luminal substrate for 5 minutes. Horseradish peroxidase-induced signal was collected using a Fluor-S Max CCD camera system.

Electrophoretic Mobility Shift Assay (EMSA). Neurons (7-12 days *in vitro)* grown in 6-well plates were scraped in buffer containing 20 mM HEPES pH 7.9, 350 mM NaCl, 20 % glycerol, 1% IGEPAL, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, and 50 μM DTT. Upon transfer to 1.7 ml centrifuge tubes, cells were allowed to lyse for 30 minutes on ice, followed by centrifugation at 25,000 g for 15 minutes to remove cellular debris. Protein levels of supernatants were determined by Biorad protein assay kit. EMSA binding reactions then composed of the following: 2-10 μg protein, 100 mM Tris, pH7.6, 250 mM KCl, 50 mM EDTA, 5 % glycerol, 1 mM DTT, 1 μg/μl poly (dI-dC) and 2x10^{5} cpm of ^{32}P-ATP. Once made, reactions were incubated at room temperature to allow protein: DNA binding. Samples were loaded on a native 6% acrylamide gel and separated by electrophoresis for 2 hours at 200V. Each gel was then placed on two pieces of thick blot paper and dried in a gel drying system (Bio-Rad). Dried gels were placed in a cassette with autoradiography film (Kodak, Fisher Scientific) overnight at -80 ^{o}C before developing.

*Calcium imaging***.** Cytosolic calcium levels were measured at various treatment intervals. Hippocampal neurons (7 days *in vitro*) were incubated with Locke's buffer (154 mM NaCl, 3.6 mM $NaHCO_3$, 5.6 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, 2.3 mM $CaCl_2$) containing BSA and 2 uM Fura-2AM for 45 minutes. Cells were washed 3x in Locke's buffer without BSA and incubated for an additional 15 minutes at 37 ^{o}C. Cells were imaged on a Zeiss Axiovert inverted microscope with an attached CCD camera (Roper Scientific), using a 40x oil immersion objective. Intracellular calcium levels of 10-14 neurons were quantified following addition of experimental treatments to the culture (3-4 cultures/treatment).

For neuregulinβ1 experiments, baseline calcium was determined microscopically for 1-2 minutes prior to adding the experimental treatment directly to the culture with no perfusion/washout. Traces were recorded for 5-10 minutes post-treatment.

Immunoprecipitation. Lysis/IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 2 mM sodium orthovanadate, 10 mM NaF, 1x protease inhibitor cocktail) was used to lyse both neuronal cultures and tissue. For cortical neuronal cultures, media was removed and cells were scraped in 100 µl of lysis buffer. Samples were kept on ice for 20 minutes to lyse cells, followed by centrifugation at 25,000 g to remove cellular debris. Supernatant was assessed for protein concentration, and samples containing 500 µg protein and 2 µg antibody (either Insulin receptor β or 6E10) were made and incubated overnight with shaking at 4 ^{o}C. The following day, 20 µl of protein A/G slurry was added to each sample and incubated with shaking at 4 ^{o}C for 4 hours. IP buffer was added (500 µl) to each

sample and centrifuged at 2500 g for 3 minutes. Supernatant was removed, and pelleted beads were washed again, 2x with IP buffer, and 1x with PBS. Protein was eluted from the beads with 1x sample buffer containing 4%SDS by heating to 95 degrees at 5 minutes followed by another 2500 g spin to remove the beads. Eluted protein was electrophoretically resolved by SDS-PAGE, and immunoblotted as previously written.

For cortical tissue samples, the above protocol was used, with the following exceptions. Tissue was homogenized mechanically with an electric mortar and pestle system, using 10x tissue volumes.

Neurite outgrowth analysis. CD1 hippocampal cultures were seeded on a 96-well plate treated with 100 nM sAPPα at time of plating and incubated at 37 degrees C/5% CO2. After 48 hours, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by a wash in phosphate buffered saline (PBS). Cells were permeabilized in PBS containing 0.3% Triton X100 for 5 minutes, followed by 3 washes in PBS. Neurons were then blocked in blocking buffer (20% blocking buffer, 20 % FBS in PBS] for 1 hour at room temperature. The same buffer was used for primary antibody treatment (1:200 β-tubulin) overnight at 4 degrees on a rotary shaker. The following day, cells were washed 3x in PBS before incubating in secondary antibody (1:250 Alexa fluor 568 anti-rabbit) for 1 hour at room temperature. Cells were washed again 3x in PBS before storing in PBS at 4 degrees. Neurite outgrowth was assessed by taking pixel density from total fluorescence/well corresponding to FITC-tagged secondary antibodies binding to β-tubulin staining and substracting pixel density of FITC from the cell body.

STZ injection of animals. Animal colonies were maintained at the RO Burrell Lab Animal Facility (Winnipeg, MB). TgCRND8 mice overexpress the human amyloid precursor protein containing the Indiana and Swedish mutations (V717F; K670/M671) (Chishti et al., 2001). These mice were injected with 90 mg/kg STZ at 2 months of age on 3 successive days. Following the third day, blood glucose levels were determined; a value greater than 16 mmol/L indicated diabetes and animals were no longer injected. If less than 16, animals were injected again. This process was repeated every three days, until diabetic or animals had been injected 7 times. The injection immediately prior to the blood glucose determination was used as the start point for diabetes assessment. Once diabetic, animals were monitored for 6 weeks, at which point they were euthanised for experimental analysis.

Morris Water Maze. Prior to streptozotocin injection, animals to be used for the study were assessed for memory retention using a Morris water maze, as described previously (Odero et al., 2010). A circular plastic tub (1 m diameter) was filled with water at 22 ^{o}C and made opaque with skim milk powder. Colored spatial clues were placed at cardinal locations in the tub, and a platform was placed in a location between south and east locations just below the surface of the liquid. Animals were placed in the tub at a cardinal location, facing the wall, and time was recorded to find the platform, to a maximum time of 90 seconds. If animals did not find the platform, they were physically placed on the platform for 10 seconds to train them. All animals in a group ran the maze once before exposing the animals to three more trials, with each trail beginning at a different cardinal location. The process, termed a block, was

repeated twice more for a total of 3 days. Escape latency, the time to find the platform, and path length, the distance traveled to find the platform, was recorded for each animal and the average was determined for each experimental group, prior to statistic analysis with one-way ANOVA using Tukeys post-hoc multiple comparisons test.

[1]Chapter 4

NF-κB activated by ER calcium release inhibits Aβ-mediated expression of CHOP protein: Enhancement by AD-linked mutant presenilin 1

Mutations in presenilin which result in early-onset Alzheimer disease (AD) cause both increased calcium release from intracellular stores, primarily endoplasmic reticulum (ER), and changes in NF-κB activation. Some studies have also reported that neurons containing AD-linked mutant presenilins (mPS1) show increased vulnerability to various stresses, while others report no differences in neuronal death. The majority of these reports center on potential changes in ER stress, because of the enhanced ER calcium release seen in mPS1 neurons. One of the primary death effectors of ER stress is CHOP, also termed GADD153, which acts to transcriptionally inhibit protective cellular molecules such as Bcl-2 and glutathione. Because both CHOP and NF-κB are activated by increased intracellular calcium and stress, yet have diametrically opposite effects on neuronal vulnerability, we sought to examine this interaction in greater detail.

We observed that IP3-mediated calcium release from ER, stimulated by Aβ exposure, mediated both CHOP expression and NF-κB DNA binding activity. Further, specific inhibition of NF-κB resulted in greater expression of CHOP, while activation of NF-κB inhibited CHOP expression. The enhanced release of calcium from IP3-mediated ER stores in mPS1 neurons stimulated increased NF-κB compared to normal neurons, which inhibited CHOP expression. Upon blockage of NF-κB, exposure to Aβ caused significantly greater Aβ-mediated CHOP expression

[1] Schapansky J, et al (2007) NF-κB activated by ER calcium release inhibits Aβ-mediated expression of CHOP protein: Enhancement by AD-linked mutant presenilin-1. Exp Neurol 208: 169-176.

and death in mPS1 neurons compared to normal neurons. Thus, AD-linked PS1 mutations disrupt the balance between stress-induced NF-κB and CHOP, resulting in greater dependence on stress-induced NF-κB activation in mPS1 neurons.

Introduction

Neurons exposed to amyloid beta peptide 1–42 (Aβ1-42) exhibit disrupted calcium regulation resulting in increased cytoplasmic calcium, which is a hallmark of neurodegenerative pathways (LaFerla, 2002; Mattson and Chan, 2003; Canevari et al., 2004; Katayama et al., 2004). A portion of this calcium originates from intracellular stores, primarily endoplasmic reticulum (ER) (Mattson et al., 2001; Mattson and Chan, 2003; Verkhratsky and Toescu, 2003). Disruption of ER calcium, such as that resulting from Aβ1-42, can lead to a phenomenon termed ER stress, which may in turn modulate responses which can either stimulate or inhibit cellular death pathways (Berridge, 2002; Lehotsky et al., 2003; Verkhratsky and Toescu, 2003; Katayama et al., 2004). Among the pro-death responses is expression of the death effecter C/EBP homologous protein (CHOP; Oyadomari and Mori, 2004) also called GADD153, a transcription factor which acts to inhibit transcription of protective proteins such as Bcl-2 and to decrease glutathione (DeGracia et al., 2002). Inhibition of calcium release from ER protects neurons from Aβ-mediated death (McCullough et al., 2001; Suen et al., 2003; Ferreiro et al., 2004), as does inhibition of CHOP expression (Mattson et al., 2000), thus both ER-mediated calcium release and CHOP expression play critical roles in Aβ-mediated neuronal death.

Somewhat paradoxically, excess calcium release and ER stress also activate the anti-apoptotic transcription factor NF-κB (Pahl and Baeuerle, 1995, 1996, 1997).

Mobilization of calcium from internal (Pahl and Baeuerle, 1996; Quinlan et al., 1999; Suen et al., 2003) or extracellular (Kanno and Siebenlist, 1996) sources activates NF-κB, which in turn protects against excitotoxicity. Conversely, buffering calcium (Shatrov et al., 1997) or preventing calcium influx (Kanno and Siebenlist, 1996; Lazaar et al., 1998) decreases NF-κB activity. NF-κB-mediated transcription can protect neurons from a variety of insults relevant to neurodegenerative disorders, including Aβ1-42, the excitotoxin NMDA, and a number of oxidative insults (Barger et al., 1995; Mattson et al., 1997; Taglialatela et al., 1997; Glazner et al., 2000; Glazner and Mattson, 2000; Cardoso and Oliveira, 2003). NF-κB protects cells by inducing the expression of genes that promote cell survival such as those encoding for anti-oxidant (manganese superoxide dismutase (MnSOD); (Mattson et al., 1997) calcium stabilizing (calbindin D28K) (Cheng et al., 1994) and anti-apoptotic proteins (Bcl-2, Bcl-XL) (Mattson et al., 1997; Tamatani et al., 1999, 2000).

Though a minority of Alzheimer disease (AD) cases, familial AD (FAD) represents a very early onset, rapidly progressing form of the disease. The mutations causing FAD are 100% penetrant, and all persons carrying one of the mutant genes will develop the disease. FAD is most commonly caused by mutations in presenilin (PS)1 and PS2 (Levy-Lahad et al., 1995; Sherrington et al., 1996), which are integral membrane proteins found primarily in the plasma membrane and ER. Mutations in PS1 lead to increased production of Aβ1-42 and enhanced plaque accretion (Annaert et al., 2000; Fraser et al., 2000). In addition, cultured cells containing human AD-linked presenilin mutations (mPS1) exhibit increased calcium release from intracellular stores in response to stress (Leissring et al., 1999a,b, 2001; Schneider et

al., 2001). Presenilin mutants in neurons cause increased calcium release from both ER-resident IP3 receptors (Stutzmann et al., 2004; 2007 and ryanodine receptors (Smith et al., 2005; Lee et al., 2006; Stutzmann et al., 2007). In normal and mPS1 neurons, specific inhibition of ER calcium release protects neurons from death caused by Aβ1–42 (Mattson et al., 2000). The presence of mutant presenilin in a cell line shows aberrant NF-κB DNA binding activity following exposure to Aβ1-42 (Guo et al., 1998), including an enhanced early NF-κB activation which may also result from the greater flux of calcium from ER in these cells. Of particular interest is a recent report that NF-κB activity directly suppresses CHOP expression in a cancer cell line (Nozaki et al., 2000), though this has not been demonstrated in neurons.

Though calcium disruption by mPS1 is well accepted, there are conflicting reports regarding increased vulnerability of mPS1 neurons to stress. Cells containing mPS1 have been reported to be more easily killed by exposure to Aβ42 (Guo et al., 1998), while other studies show no increased vulnerability (Siman et al., 2000). The observation that NF-κB can suppress CHOP in tumors (Nozaki et al., 2001) raises the possibility of a similar mechanism at work in neurons, and may shed light on the role of mPS1 in neuronal vulnerability. Here we examined in greater detail the link between NF-κB and CHOP in mPS1 neurons exposed to Aβ1–42

Methods and materials

Antibodies against CHOP, as well as antisense oligonucleotides for CHOP (5′GAC TCA GCG CCA TGA C3′), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IκB antisense oligonucleotides (5′GCG CTC GGC CGC CTG GAA CAT GGC3′) and Neurobasal culture media was purchased from

Invitrogen/Gibco BRL (Grand Island, NY, USA). Anti-mouse IgG and anti-goat IgG antibodies conjugated with peroxidase were purchased from Biocan (Mississauga, ON, Canada). DC Protein assay kit was provided by Bio-Rad (Mississauga, ON, Canada). Chemi-Glow Western blot detection kits were ordered from Canberra Packard. Fura-2 was purchased from Molecular Probes (Eugene, OR, USA).

Cell culture. Brains were removed from embryonic 16-day-old C57B mice or mice containing the M146V mutation in PS1 (Guo et al., 1999), and cerebral cortices and hippocampal regions dissected and placed in separate dishes containing HBSS Ca^{+2}/ Mg^{+2}-free media. Tissues were mechanically dissociated by trituration and seeded in 6-well culture plates (treated with poly- D-lysine) in Neurobasal containing 10% fetal bovine serum (FBS) and incubated overnight, and media was changed to Neurobasal without FBS the next day. Experiments were performed on day 7–8 after plating. Cortical cells were used for Western blot experiments, while hippocampal cultures were used in survival experiments.

Quantification of neuronal survival. Neuronal survival was quantified by established methods (Mattson et al., 1995). In brief, viable neurons in pre-marked fields (20× objective) were counted before experimental treatment and at time points following treatment. Neurons that died in the intervals between examination points were usually absent, and the viability of the remaining neurons was assessed by morphological criteria. Neurons with membranes and soma with a smooth round appearance were considered viable, whereas neurons with fragmented or distended membranes and vacuolated soma were considered nonviable. Neurons were counted in four random fields per culture, and the percentage of surviving neurons per culture was calculated.

Western blotting. Immunoblotting was performed using methods previously described (Glazner et al., 2001). Briefly, after experimental treatment, cultures were washed once with PBS and scraped with Laemmli buffer (0.125 M Tris–HCl pH 6.8, 4% SDS). Samples were boiled and pushed through a 27 gauge needle to fully denature proteins and remove biological activity. A Bradford assay was used to determine protein concentration. Proteins (10-50 μg/lane) were separated in a 12% acrylamide gel by electrophoresis and transferred to a nitrocellulose membrane. Membranes were blotted with 1:1000 dilution of antibody and detected with peroxidase-conjugated secondary antibody. Detected bands were quantified using Image software (Scion Corp., Frederick, MD).

Electrophoretic mobility shift assay (EMSA). Cell extracts from cultures were obtained by scraping with Totex buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1% igepal, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 5 μg/ml aprotinin, 50 μM DTT), followed by cell lysis on ice for 30 min, centrifugation at 14,000 rpm for 15 min at 4 °C, and retention of the supernatant. Protein levels were determined by the Bradford method (Biorad) and samples stored at −80 °C. Equal amounts of protein were incubated in a 20-μl reaction mixture containing 20 μg of bovine serum albumin; 1 μg of poly (dI–dC); 2 μl of buffer containing 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.9; 4 μl of buffer containing 20% Ficoll 400, 300 mM KCl, 10 mM dithiothreitol, 0.1% phenylmethylsulfonyl fluoride, and 100 mM HEPES, pH 7.9; and 20,000–50,000 cpm of ^{32}P-labeled oligonucleotide (S) corresponding to an NF-κB-binding site (5'- AGT

TGA GGG GAC TTT CCC AGG C – 3'). After 20 min at room temperature, reaction products were separated on a 12% non-denaturing polyacrylamide gel. Radioactivity of dried gels was detected by exposure to Kodak X-Omat film, and images on the developed film were scanned into a computer using a UMAX 1200s scanner. Densitometry was performed using Image software (Scion Corp., Frederick, MD). Paint Shop Pro software (Jasc Inc., Minneapolis, MN) was used for preparation of the final figures.

Calcium imaging. Rat hippocampal cells were imaged as described previously (Mattson et al., 1995). Briefly, at designated time points after experimental treatments, cells were incubated for 40 min in the presence of the 2 μM acetoxymethylester form of fura-2 (Molecular Probes). Immediately before imaging, dishes were washed twice in Locke's buffer. Cells were imaged on a Zeiss Axiovert microscope (40× oil immersion objective) coupled to an Attofluor imaging system. The average intracellular calcium $[Ca^{2+}]_i$ in 10–14 neuronal cell bodies per microscope field was quantified in three or four separate cultures per treatment condition. Experimental treatments were added to the bathing medium by dilution from 100–500× stocks.

Statistical analysis. Where appropriate, data were subjected to one-way analysis of variance (ANOVA) using the Statistical Package SPSS/PC+(SPSS, Chicago). Where the F ratio gave p b 0.05, comparisons between individual group means were made by Scheffe's multiple-range test at significance levels of $p = 0.05$. Where non-homogeneity of variance was apparent, single comparisons were performed using Student's t-test at significance levels of $p = 0.05$ or greater.

Results

Studies investigating the relative vulnerability of mPS1 neurons to various stressors have given disparate results. In our hands, a time course with three doses (0.1 μM, 1 μM and 10 μM) of Aβ1-42 showed no significant difference in survival between cultured embryonic neurons from wild-type and mPS1 mice (Fig. 5A) at any dose. Exposure to Aβ1-42 results in activation of the pro-death transcription factor CHOP (Ghribi et al., 2001, 2004), as well as in the anti-apoptotic transcription factor NF-κB (Kaltschmidt et al., 1999). In this study, levels of CHOP protein were equally low in both normal and mPS1 prior to Aβ1-42 exposure, and rose to equivalent levels, peaking within 6 h (Fig. 5B). Exposure of neurons to 1 μM Aβ1-42 but not the less toxic peptide Aβ1-40 induced greater activation of NF-κB DNA binding in mPS1 compared to normal neurons (Fig. 5C). Levels of NF-κB binding activity peaked in both cell types within 2 h, approximately 6-fold in mPS1 neurons and 4-fold in normal neurons. Thus, NF-κB levels were increased in mPS1 neurons relative to normal neurons following Aβ exposure, but neither Aβ-induced cell death nor CHOP protein expression were significantly different between the two cell types.

Mutations in presenilin induce increased calcium release from endoplasmic reticulum, through IP3- and caffeine- mediated calcium channels (Kaltschmidt et al., 1999; Leissring et al., 1999b; Smith et al., 2005; Lee et al., 2006; Stutzmann et al., 2004, 2007). This calcium release phenomenon is linked to both NF-κB activation (Pahl and Baeuerle, 1995; Glazner et al., 2001) and CHOP expression (Fornace et al., 1989; Carlson et al., 1993; Wang et al., 1996). In this study, inhibition of IP3-mediated calcium release from ER, using the specific inhibitor xestospongin C, spared both wild-type and mPS1 neurons from Aβ1-42 mediated death (Fig. 6A).

Neurons treated with XeC prior to Aβ1-42 exposure displayed approximately 20% death, compared to approximately 45% death in those treated with 1 μM Aβ1-42 alone. This result positively correlated to decreased levels of CHOP (Fig. 6B), in which pre- treatment with XeC inhibited Aβ-induced CHOP expression by approximately 40% in both normal and mPS1 neurons 6 h after exposure to 1 μM Aβ1-42. Pretreatment with XeC also significantly decreased NF-κB binding activity in both mPS1 and normal neurons following exposure to 1 μM Aβ1-42 (Fig. 6C). However, inhibition of ER calcium release resulted in a greater decrease in NF-κB activation in mPS1 neurons (47%) than in normal neurons (31%) (Fig. 6C). Thus, IP3-mediated calcium release stimulated by Aβ exposure is critical for both NF-κB and CHOP activity, though the two proteins work to opposite ends.

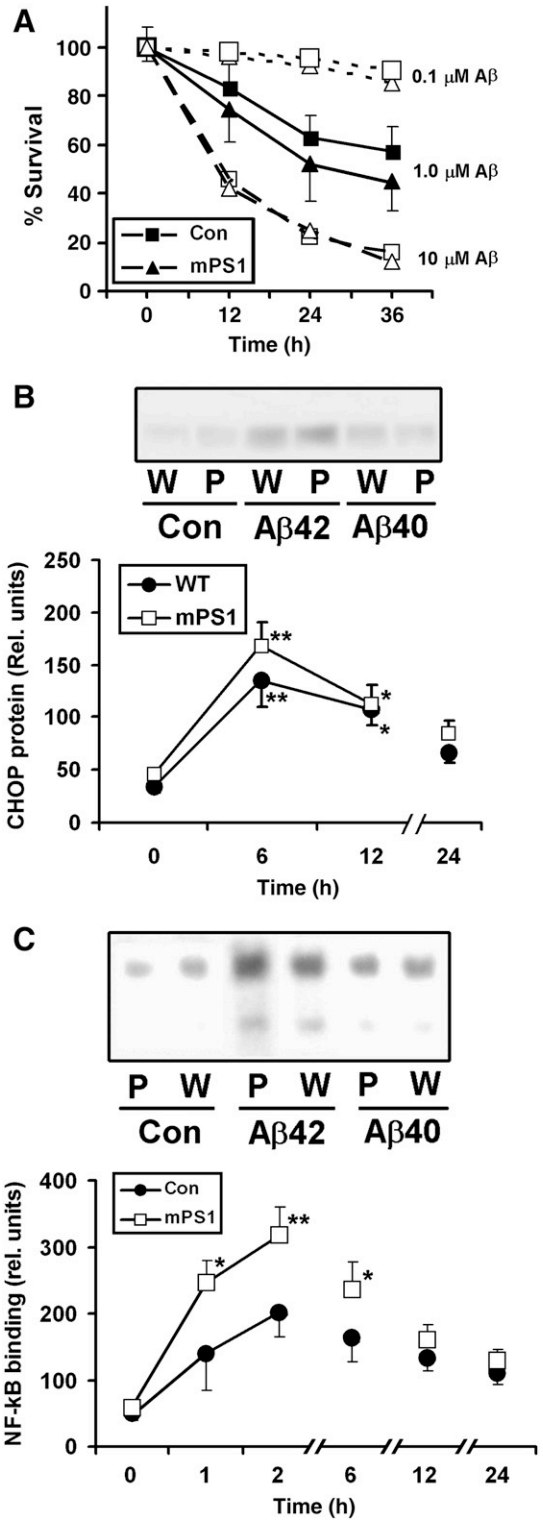
To investigate the calcium release properties, cultured hippocampal neurons were treated with Aβ1-42 and intracellular calcium levels measured. Initial calcium levels were equivalent between normal and mPS1 neurons (Fig. 7A). Following addition of Aβ1-42, intracellular calcium increased, reaching peak levels within 2–3 h, with calcium levels in mPS1 neurons significantly greater than those in normal neurons. This difference was lost within 24 h of initial treatment (Fig. 7A). Addition of 10 μM XeC immediately prior to 1 μM Aβ1-42 significantly reduced calcium levels both 3 h and 24 h after Aβ1-42 exposure compared to those treated with Aβ1-42 alone in both normal and mPS1 neurons (Fig. 7B). The reduction of calcium after 3 h was greater in mPS1 neurons (33%) than in normal neurons (19%), indicating a greater proportion of the rise in intracellular calcium induced by Aβ1-42 is derived from ER source in mPS1 compared to normal neurons. In fact, peak calcium levels between mPS1 and normal neurons were not significantly different in

those cells pretreated with XeC prior to Aβ1-42 exposure (Fig. 7B). Therefore, the increased intracellular calcium seen following Aβ exposure in PS1 relative to WT neurons is caused by increased IP3-mediated ER calcium release.

To determine if there was an interaction between NF-κB and CHOP activation in central neurons other than activation by ER calcium release, NF-κB binding activity was inhibited using the peptide SN50, which binds to the nuclear translocation site of activated NF-κB, or activated using IκB antisense. When NF- κB was inhibited by addition of SN50 for 12 h, exposure to 1 μM Aβ caused a significantly greater rise in intracellular calcium at both 3 h and 24 h than in those neurons treated with Aβ1-42 alone. The increase after 24 h was greater in mPS1 neurons than in normal neurons, at which point there were significantly increased levels of calcium in mPS1 than normal neurons (Fig. 8A). In contrast, activation of NF-κB by addition of IκB antisense for 12 h significantly inhibited the increase in calcium induced by 1 μM Aβ1-42 exposure (Fig. 8A) in both mPS1 and normal neurons at both 3 h and 24 h. Inhibition of NF-κB by SN50 treatment 24 h prior to exposure to Aβ1-42 resulted in significantly increased CHOP levels in both normal and mPS1 neurons after 6 h, demonstrating that NF-κB plays a major role in inhibition of Aβ-mediated CHOP expression. Following NF-κB inhibition, activation of CHOP was increased to a significantly greater degree in mPS1 neurons than in normal neurons (Fig. 8B). In addition, inhibition of NF-κB resulted in enhanced neuronal death in both normal and mPS1 mice. Again, this effect was significantly greater in mPS1 than in normal neurons (Fig. 8C). Therefore, the greater calcium release found in mPS1 neurons renders these cells more dependent on enhanced stress-activated NF-κB. Cells treated with CHOP antisense prior to addition of Aβ1-42 displayed significantly

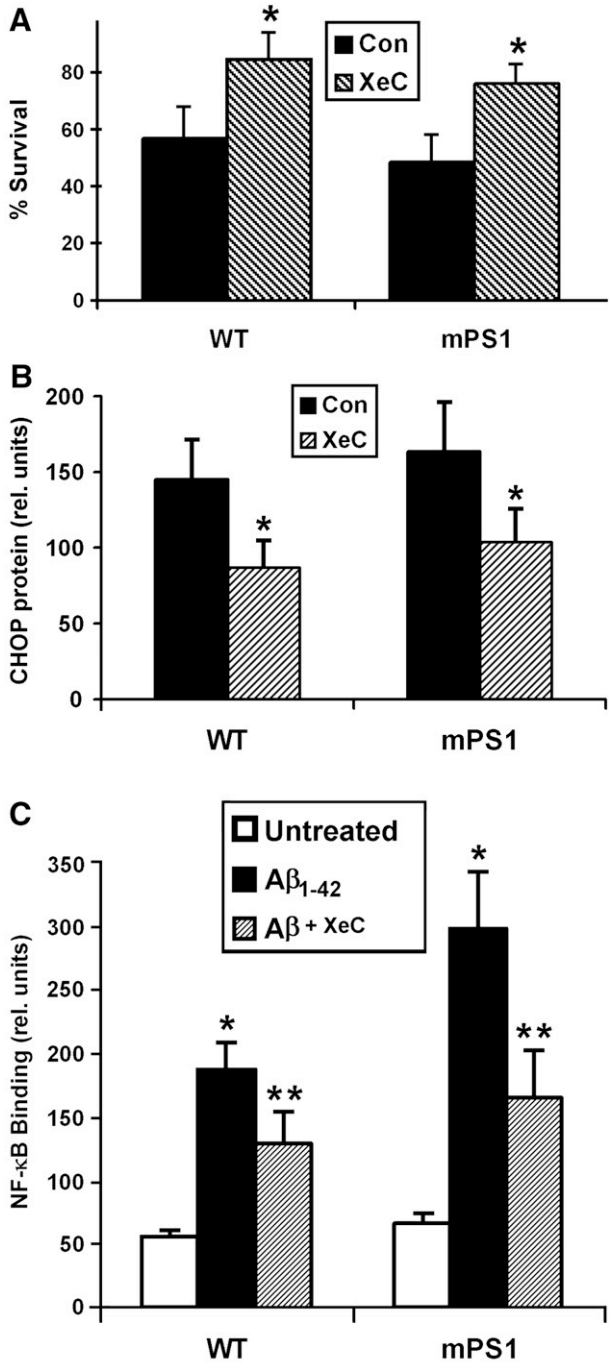
greater survival than those treated with Aβ1-42 alone, demonstrating the role of CHOP in Aβ-mediated cell death.

Figure 5. Aβ1-42-induced NF-κB activation is greater in mPS1 neurons than in wild-type neurons



Embryonic day 18 (E18) cortices were removed from either mPS1 or normal mice and cultured as described for 7 days. (A) Microscope fields with at least 15 neurons each were marked and photographed, and then cultures were treated with vehicle or Aβ1-42 (0.1, 1, or 10 μM). Survival was determined morphologically every 12 h following Aβ1-42 exposure. At the last time point (36 h) morphological survival was confirmed by trypan blue exclusion. Data are means from 4 separate experiments ± S.E., with 4 separate wells per experiment. (B) The upper panel shows a typical Western blot probed for CHOP protein 6h after exposure to either vehicle (Con), 1 μM Aβ1-42 or 1 μM Aβ1-40. W is wild type; P is mPS1. The lower graph shows quantitation by densitometry at 6, 12, and 24 h after exposure to 1 μM Aβ1-42. Data are means from 3 separate experiments ± S.E. *$p<0.05$; ** $p<0.01$ vs. control. (C) At the time points indicated, cells were harvested and nuclear proteins collected. Electrophoretic mobility shift assays were performed on the nuclear extracts for determination of NF-κB DNA binding activity. The upper panel shows a typical autoradiogram of extracts from cell treated with 1 μM Aβ1-42 or Aβ1-40 for 2 h. The graph shows results of four separate EMSA assays 1, 2, 6, 12 and 24 h following exposure to Aβ1-42. Data are mean densities ± S.E. * $p<0.05$; ** $p<0.01$ vs. WT.

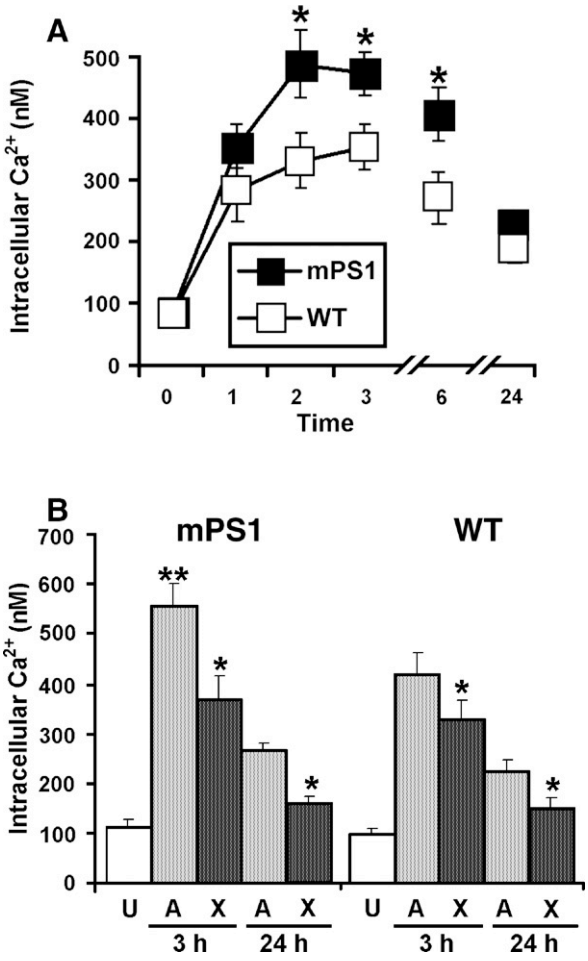
Figure 6. Inhibition of ER calcium release protects neurons from death.



Cultured wild type (WT) or mutant presenilin 1 (mPS1) cortical neurons were exposed to vehicle (Con) or 10 uM XeC followed immediately by 1 uM Aβ1-42. (A) Neuronal survival was determined morphologically 24 hr after exposure to Aβ1-42. Data are mean =/- S.E., *p<0.05 vs control. (B) After 2 h of exposure to Aβ1-42, levels of

CHOP protein were determined by Western blot analysis. Data are mean +/- S.E., *$p<0.05$ vs. control. (C) After 2 h exposure to Aβ1-42, cells were scraped and nuclear extracts prepared. Levels of NF-κB DNA binding activity was determined by EMSA analysis. Data are mean ± S.E., * $p<0.01$ vs. untreated; **$p<0.05$ vs. both untreated and those treated with Aβ1-42 alone.

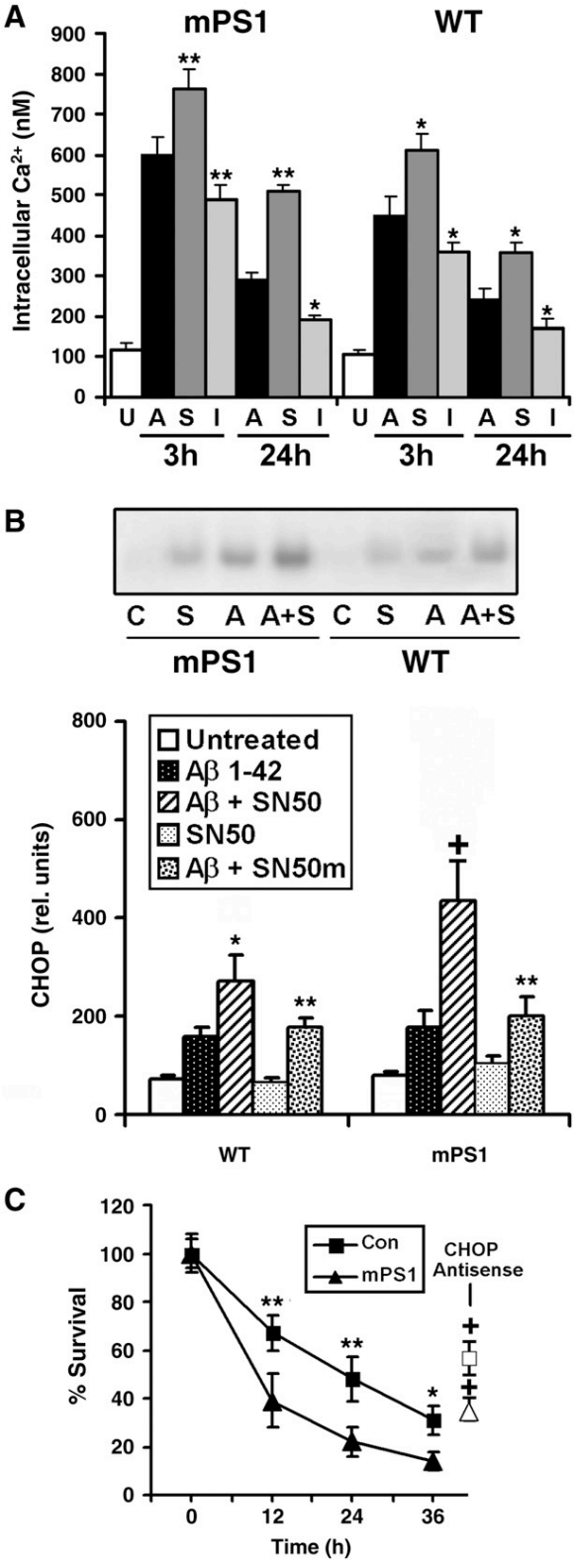
Figure 7: Aβ-induced ER calcium release is greater in mPS1 neurons versus WT neurons



Cultured normal and mPS1 hippocampal neurons were loaded with the calcium dye Fura-2, and calcium levels were determined prior to and after addition of 1 μM Aβ1-42. (A) Times indicated are 1–24 h following exposure to Aβ1-42. Data are mean ± S.E., * $p < 0.05$ vs. WT at the same time point. (B) Cultured normal and mPS1 hippocampal neurons were loaded with Fura-2 and left untreated (u) or treated with 1 μM Aβ1-42 only (A) or 10 μM XeC (X) immediately prior to addition of 1 μM Aβ1-42. Calcium levels were determined 3 h or 24 h after Aβ1-42 exposure. Data are

mean ± S.E., *$p < 0.05$ vs. those treated with Aβ alone; **$p<0.05$ vs. identically treated WT.

Figure 8: NF-κB inhibition in mPS1 neurons increases CHOP expression and neuronal cell death



Cultured normal and mPS1 hippocampal neurons were loaded with Fura-2 and left untreated (U), exposed to 1 μM Aβ1-42 only (A), or pretreated with either 10 μM SN50 to inhibit NF-κB (S), or 10 μM IκB antisense, to activate NF-κB binding (I) 12 h prior to Aβ1-42 exposure. Calcium levels were determined 3 h or 24 h after Aβ1-42 exposure. Data are mean ± S.E., * p < 0 . 0 5 vs. group A; **p<0.05 vs. identically treated WT. (B) Cultured normal and mPS1 cortical neurons were exposed to vehicle or 10 μM SN50, an NF-κB inhibitor, followed 2 h later by vehicle or 1 μM Aβ1-42. After 2 h, levels of CHOP protein were determined by Western blot analysis. Data are mean ± S.E., * p < 0.05 vs. those treated with Aβ1-42 only, * p<0.05 vs Aβ + SN50. (C) Cultured normal and mPS1 cortical neurons were treated with 10 μM SN50 and either vehicle or 10 μM CHOP antisense followed 2 h later by 1 μM Aβ1-42. Neuronal survival was determined morphologically at the time points indicated. Offset slightly in the figure, but also at 36 h, were cells pretreated with 10 μM CHOP antisense prior to Aβ1-42 exposure. Data are mean ± S.E., *p<0.05; **p<0.01 vs. mPS1; + p<0.05 vs. those not treated with CHOP antisense at 36 h.

Discussion

Exposure to Aβ1-42 results in a number of pathological changes to neurons, including increased reactive oxygen species generation, increased mitochondrial membrane permeability, and increased cytoplasmic calcium levels (Mattson, 2002; Eckert et al., 2003; Abramov et al., 2004). A portion of the increased calcium originates in release from intracellular stores, primarily ER (Mattson et al., 2001; Mattson and Chan, 2003; Verkhratsky and Toescu, 2003). Calcium release and reuptake from ER is a normal phenomenon, however excess calcium release is associated with cell death demonstrated by the observation that inhibitors of ER calcium release protect neurons from Aβ- mediated neuronal death (Guo et al., 1998). The ER stress that results from exposure to Aβ1-42 sets in motion a number of pathways which stimulate cell death, including activation of the transcription mediator CHOP[9]. CHOP levels rise upon exposure to Aβ1-42 (Ghribi et al., 2001, 2004), and inhibition of CHOP activity protects cells from Aβ-mediated death (Ghribi et al., 2004). Somewhat paradoxically, ER stress also stimulates activity of the neuroprotective transcription factor NF-κB. While the actions of CHOP result in mitochondrial dysfunction, including increased ROS and inhibition of Bcl-2 (McCullough et al., 2001), the action of NF-κB results in reduced formation of ROS, increased levels of Bcl-2 and stabilization of intracellular calcium. In addition, increased NF-κB activity also inhibits release of ER calcium (Camandola et al., 2005). Thus, though both CHOP and NF-κB are stimulated by Aβ1-42 exposure and ER stress, they seem to work to opposite ends. Here we show that IP3-mediated ER calcium release is critical to both activation of NF-κB and expression of CHOP under the same conditions, and that the interaction between ER calcium release, NF-κB

activation, and NF-κB inhibition of CHOP is altered in neurons containing AD-linked mutant presenilin 1.

Though the function of PS1 is not entirely clear, neurons containing this mutation have an enhanced release of calcium from ER stores. This might be expected to result in increased ER stress, activation of CHOP and increased neuronal toxicity upon exposure to Aβ1-42. Indeed, some labs have reported the neurons containing mPS1 are more vulnerable to stress, while others see no increased susceptibility. In our hands, mPS1 neurons exposed to various concentrations of Aβ1-42 exhibited neither increased cell death nor increased CHOP expression at any time point tested. However, we did observe an early increase in NF-κB activation mediated by enhanced ER calcium release. The increased activation of NF-κB in mPS1 neurons is at least partially responsible for the lack of difference in CHOP activation of neuronal death upon exposure to Aβ1-42. Indeed, when NF-κB was inhibited, both CHOP levels and neuronal death increased to a significantly greater degree in mPS1 than in wild-type neurons, while activation of NF-κB resulted in decreased CHOP expression and decreased neuronal death. Therefore, part of the protective response initiated by NF-κB in response to Aβ is inhibition of CHOP expression. This is a critical action, since inhibition of CHOP by antisense decreased neuronal death in both mPS1 and wild-type neurons, demonstrating the role this protein plays in Aβ- mediated death.

At relatively high concentrations of Aβ1-42, inhibition of ER calcium release protected neurons from Aβ-mediated death, and decreased both CHOP expression and NF-κB binding, thus at this level of insult, the pro-death effects of enhanced ER calcium release predominated over the protective pathways. Activation of NF-κB

can inhibit calcium release from ER by down-regulating the activity of IP3-mediated calcium channels localized to the ER membrane (Camandola et al., 2005). In the present study, prior activation of NF-κB reduced xestonspongin-inducible calcium release in both normal and mPS1 neurons. Since ER calcium release is central to Aβ-mediated CHOP expression, this supports a hypothesis that NF-κB activation may inhibit CHOP expression by reducing ER calcium release and thus ER stress. An alternative hypothesis is that NF-κB may act as a transcription inhibitor for CHOP, as has been reported for certain tumour cells.

Since IP3-mediated ER calcium release is increased in mPS1 neurons, and is central to Aβ-induced NF-κB binding activity, it is reasonable to hypothesize that the increased NF-κB binding observed upon exposure to Aβ in mPS1 neurons is due to increased ER calcium release. Indeed, we observed greater levels of IP3-mediated calcium release upon initial exposure to Aβ1-42 in mPS1 neurons than in normal neurons. However, within 24 h of exposure, intracellular calcium levels, though elevated, were equivalent in both mPS1 and normal neurons. Since NF-κB can decrease stress-induced calcium elevation through multiple pathways, including increased calcium binding proteins and decreased ER-resident IP3-mediated calcium channels, calcium levels in mPS1 neurons may return to control levels within 24 h because of the greater enhancement of NF-κB in these neurons compared to control neurons. Indeed, when NF-κB was inhibited prior to Aβ exposure, both normal and mPS1 neurons displayed greater elevation in calcium levels and greater death in response to Aβ1-42, with both parameters increasing to a significantly greater degree in mPS1 than normal neurons. This observation in particular supports the hypothesis

that the increased ER calcium release in mPS1 neurons results in greater early NF-κB activation, which in turn inhibits ER calcium release, ER stress, and expression of CHOP. Thus, though stress-induced NF-κB activation protects both normal and mPS1 neurons, in part through inhibition of CHOP, the mutation renders the latter cell type more dependent on this pathway. Inhibition of stress- induced NF-κB activation reveals this vulnerability, resulting in both greater CHOP expression and greater neuronal death. Any reduction in the ability of the cell to elevate NF-κB in response to stress would tilt the balance towards greater CHOP expression, and thus greater neuronal death. Embryonic brains, such as those used in this study, demonstrate a greater level of NF-κB activation than do adult brains, and thus NF-κB activation in response to neuronal stress may also be decreased with age, and in those with AD-linked mutations to PS1, this may increase neuronal vulnerability to Aβ relative to normal neurons.

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[2]Chapter 5

Neuregulin β1 enhances peak glutamate-induced intracellular calcium levels through endoplasmic reticulum calcium release in cultured hippocampal neurons

Modulation of intracellular free calcium levels is the primary second messenger system of the neuronal glutamatergic system, playing a role in regulation of all major cellular processes. The protein neuregulin (NRG) β1 acts as an extracellular signaling ligand in neurons, rapidly regulating currents through ionotropic glutamate receptors. The effect NRG may have on glutamate-induced changes in intracellular free calcium concentrations has not been examined, however. In this study, cultured embryonic rat hippocampal neurons were treated with NRGβ1 to determine a possible effect on glutamate-induced intracellular calcium levels. Long-term (24 h), but not short-term (1 h), incubation with NRGN resulted in a significantly greater glutamate-mediated acute peak elevation of intracellular calcium levels than occurred in vehicle-treated neurons. Long-term NRGβ1 incubation significantly enhanced calcium increase induced by specific stimulation of metabotropic glutamate receptors, but did not significantly alter the N-methyl D-aspartate (NMDA)- or KC1-induced calcium increase and paradoxically decreased the effect of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) treatment on intracellular calcium. Metabotropic glutamate receptors cause increased intracellular free calcium via release of calcium from intracellular stores; thus this system

[2] Schapansky J et al. (2009). Neuregulin β1 enhances peak glutamate-induced intracellular calcium levels through endoplasmic reticulum calcium release in cultured hippocampal neurons. Can J Physiol Pharmacol 87: 883-891.

was examined in more detail. NRGβ1 treatment significantly (greater than 2-fold) enhanced calcium release from endoplasmic reticulum stores after stimulation of ryanodine receptors with caffeine, but did not significantly increase calcium release from endoplasmic reticulum mediated by inositol trisphosphate ($1P_3$) receptors. In addition, ryanodine receptor inhibition with ruthenium red prevented the glutamate-induced increase in intracellular calcium levels in NRGβ1-treated neurons. These data show that long-term NRGB1 treatment can enhance glutamate-induced peak intracellular calcium levels through metabotropic glutamate receptor activation by increasing endoplasmic reticulum calcium release through ryanodine receptors.

Introduction

Intracellular free calcium level is an important regulator of cellular function in many cells of the body, including those of the central and peripheral nervous system (Berridge et al. 2000, 2003; Glazner and Fernyhough 2002). Basal intracellular levels are kept much lower than levels in the extracellular environment and lumen of the endoplasmic reticulum (ER), generating a large chemical gradient that can drive numerous processes with even slight shifts in calcium movement. Calcium influx can contribute to neuronal functioning through different plasma membrane ion channels, including glutamate ionotropic receptors, as well as second-messenger linked metabotropic glutamate receptors (mGluRs).

One protein recently found to modulate glutamate ionotropic receptors on the plasma membrane is the ligand neuregulin (NRG) β1. The NRG family consists of signaling proteins in the central and peripheral nervous system that regulate neuronal development, with functions including neuronal migration (Anton et al. 1997; Rio et al.

1997; Steiner et al. 1999; Yau et al. 2003), oligodendrocyte development, and myelin formation (Vartanian et al. 1999; Calaora et al. 2001). The *NRG* gene produces splice variants that contain an epidermal growth factor (EGF)-like domain for binding the NRG receptors ErbB 1, ErbB3, and ErbB4, which form homo- or heterodimers (Falls 2003). Another splice variant, ErbB2, has no binding site for the NRG ligand, but dimerizes with the others to facilitate intracellular signaling. These receptors have tyrosine kinase activity that, once bound to NRGβ1, can activate second messenger systems, including the protein kinase AKT (Liu et al. 1999; Li et al. 2003; Chae et al. 2005) and mitogen-activated protein (MAP) kinase pathways (Marte et al. 1995; Vaskovsky et al. 2000; Krainock and Murphy 2001). The ErbB4 receptor has been found localized at postsynaptic densities with the protein PSD-95, implying that NRGβ1 is important in synaptic plasticity (Garcia et al. 2000; Huang et al. 2000).

Many researchers have shown that NRGβ1 can modulate glutamate receptor activity, but the mechanism by which it does this is not known for certain. NRGβ1 can reduce a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor ion current, caused by an increase in receptor internalization, but does not change N-methyl D-aspartate (NMDA) activation (Kwon et al. 2005). Depression of NMDA ion currents occurs, however, in neurons of the prefrontal cortex, the area of the brain most commonly affected in schizophrenia, with increased NMDA receptor trafficking into the cell (Gu et al. 2005). NRGβ1 can also interrupt long-term potentiation (LTP) induction with no noticeable effect on NMDA currents (Huang et al. 2000). With a functional link to glutamate receptor activity and corresponding synaptic plasticity studies, NRG could be an important target for neuronal diseases such as schizophrenia, which has also been linked to glutamate receptor activity. For example, recent data show impaired NRGβ1-

ErbB4 signaling in the prefrontal cortex of schizophrenics, leading to NMDA hypofunction (Hahn et al. 2006). Further study is required to elucidate the NRGβ1-glutamate receptor interaction and any correlative role in synaptic plasticity.

Although numerous studies have been performed on the alteration of glutamate receptor ion current caused by NRGβ1, the effect on intracellular free calcium levels has not been addressed. Since calcium acts as the primary immediate second messenger system of the glutamatergic system leading to most of the long-term changes caused by glutamate signaling, we examined the effects of NRGβ1 specifically on glutamate-induced peak intracellular free calcium.

Materials and methods

Cell culture. Fetal brains from embryonic day 18 Sprague-Dawley rats were dissected and mechanically dissociated, separating cortical and hippocampal regions. Cells were seeded on poly-lysine-treated 6-well dishes and incubated at 37 °C in a 5% CO_2 atmosphere overnight to allow cell attachment and recovery. Cells were plated in neurobasal media (Gibco) with 10% fetal bovine serum (FBS) (VWR) and B27 supplement (Gibco) and changed to neurobasal media alone with supplement the next day. Cytosine arabinoside (500 nmol/L) was added to cultures 4 days postculturing to limit astrocyte proliferation. Experiments were conducted between 7-14 days postplating. In most experiments, NRG (recombinant human heregulin (31, 30 kDa extracellular domain, Medicorp, Canada) was added 24 h before analysis.

Calcium imaging. Rat hippocampal neurons were seeded on cover slips and experiments were performed at 9-11 days in vitro (DIV). After treatments, cells

were incubated in Locke's buffer (in mmol/L: 154 NaCl, 3.6 $NaHCO_3$, 5.6 KC1, 1.0 $MgCl_2$, 5 glucose, 5 Hepes, 2.3 $CaCl_2$) containing 0.1% BSA and 2 μmol/L fura-2AM (Molecular Probes). Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 45 min, and then washed 3 x in Locke's and incubated in Locke's with-out BSA for 10-15 min, to allow de-esterification of the dye and trap it within the neuron. Media were changed to fresh Locke's buffer (without Mg^{t+}) for NMDA studies. Cells were then imaged at 340/380 nm on an Axiovert 200 inverted microscope (Zeiss) with Metafluor imaging software (Molecular Devices). Agonists used were purchased from the following: NMDA, AMPA, and 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) (Tocris), glutamate/KC1 (VWR), caffeine (Sigma), and ruthenium red (Calbiochem). Once a steady baseline was observed, the agonist was added and images recorded. Changes in ratio were transformed to Excel for quantification, followed by conversion to percent-age of controls.

Statistics. Data are presented as means ± SE of individual neurons. Full time courses were analyzed with ANOVA, while peak values (the 20 s period immediately after addition of agonist) were analyzed by comparisons of NRG-treated, untreated, and baseline values by one-way ANOVA and Tukey's post hoc test.

Results

Glutamate-induced elevation in cytoplasmic calcium levels in cultured hippocampal neurons were increased with 24 h, but not 1 h, incubation of NRGβ1.

NRGβ1directly modulates glutamate receptor activity (Gu et al. 2005; Kwon et al. 2005; Hahn et al. 2006), but NRGβ1 signaling effects on cytoplasmic calcium levels have not been reported. To determine whether treatment of hippocampal neurons

with NRGβ1 could alter glutamate-mediated intracellular calcium concentrations, cultured neurons were treated with NRGβ1 for either 1 h or 24 h followed by imaging with the ratiometric calcium dye fura-2 AM. Upon addition of 100 umol/L glutamate, neurons treated with NRG for 24 h demonstrated significantly increased intracellular calcium levels over the entire time course (Fig. 9A) and a significantly greater acute peak increase in intracellular calcium (Fig. 9B). Incubating cells for a shorter time (1 h) did not increase overall (Fig. 9C) or peak (Fig. 9D) cytoplasmic calcium after glutamate receptor activation. In all remaining experiments, neurons were treated with NRG for 24 h before analysis.

NRGβ1 treatment did not increase AMPA-, NMDA-, or KC1-induced peak acute cytoplasmic calcium levels. To determine whether the increased glutamate-induced cytoplasmic calcium caused by NRG treatment was from an extracellular source, neurons were pretreated with NRGβ1 for 24 h followed by calcium imaging using AMPA, NMDA, or KCl. Treatment with KC1, which depolarizes neurons and activates voltage-dependent calcium channels, did not demonstrate enhanced cytoplasmic calcium levels after 24 h incubation with NRG (Fig. 10A), and peak values were also nonsignificant between treatments (Fig. 10B). Addition of NMDA to neurons in Mg^{+2}-free media to activate NMDA-type ionotropic glutamate receptors demonstrated a trend to increase cytoplasmic calcium in the first 10 s in NRGβ1-treated neurons versus control, followed by an opposite trend thereafter (Fig. 10C). NRG pretreatment did significantly increase cytoplasmic calcium levels induced by NMDA when the entire time course was analyzed (Fig. 10C), but there was no significant effect on acute peak values of cytoplasmic calcium (Fig. 10D). Paradoxically, NRG pretreatment significantly inhibited the AMPA-

mediated rise in cytoplasmic calcium, both over the time course (Fig. 11A) and during the initial peak (Fig. 11B).

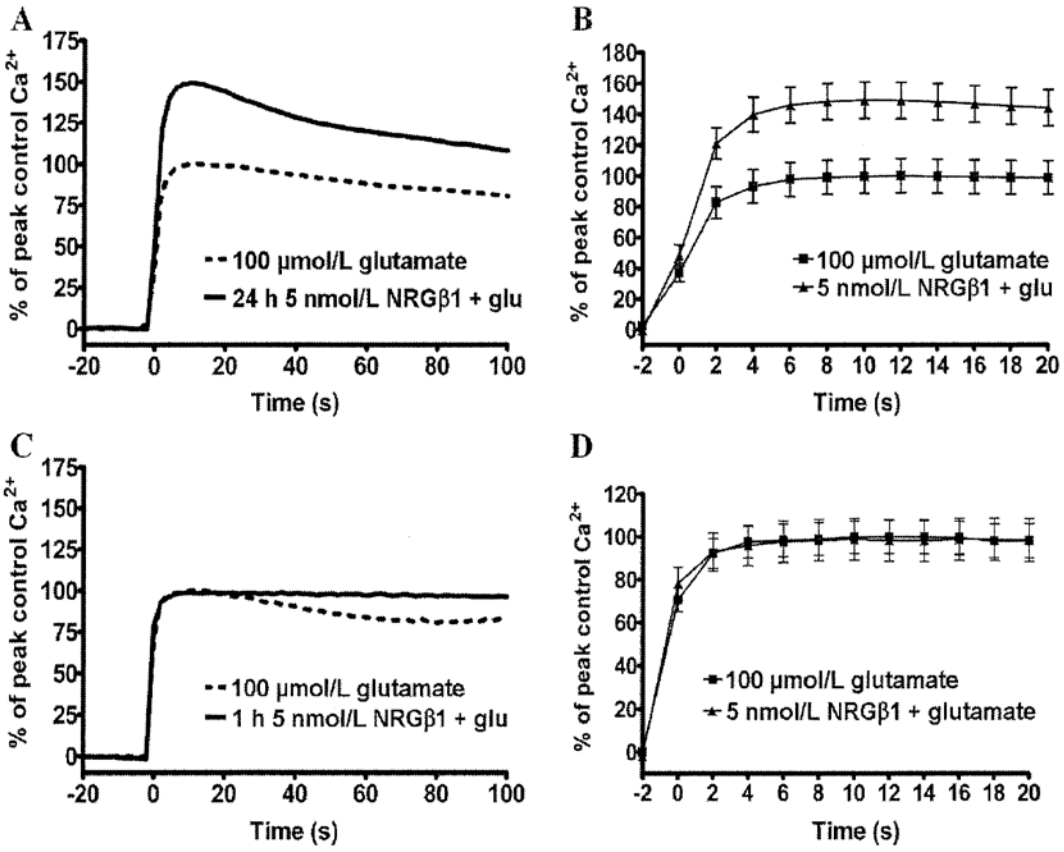
Enhanced mGluR-mediated intracellular calcium was observed after NRGβl treatment. Neither NMDA nor AMPA mimicked the NRG-stimulated intracellular calcium enhancement seen with glutamate exposure; thus we examined the possibility that response to mGluR was enhanced. Because mGluRs stimulate ER calcium release through second messenger activation, the effect of NRG incubation on mGluR calcium release was determined by treating neurons with the mGluR agonist ACPD. Pretreatment with NRG stimulated a rise in the acute peak cytoplasmic calcium level stimulated by ACPD (Fig. 12A), followed by a decrease at longer time points. Peak calcium levels occurred rapidly after addition, as has been characterized elsewhere (Yoshida et al. 2003). NRG treatment increased peak mGluR-stimulated intracellular calcium levels by almost 100%, a highly significant effect ($p < 0.001$) (Fig. 12B). This peak effect was rapid, as NRG-treated intracellular calcium concentrations quickly dropped to levels observed in untreated neurons. Figure 4B shows this initial peak from Fig. 12A, after which Ca^{2+} levels rose higher in control than in NRGβ1-treated neurons.

NRG treatment increased calcium release from ryanodine-mediated, but not IP_3-mediated endoplasmic reticulum calcium pools. Activation of mGluR causes release of ER calcium through both ryanodine-mediated and inositol trisphosphate (IP_3)-mediated calcium pools; therefore we tested calcium release from each pool. Hippocampal neurons were pre-treated with NRGβ1 for 24 h, then imaged for

calcium using 10 mmol/L caffeine to stimulate release from ER-resident ryanodine receptors (RyR) or 100 μmol/L ATP to stimulate calcium release from ER-resident IP_3 receptors (IP_3R) (Verkhratsky and Shmigol 1996; Svichar et al. 1997). Neurons treated with NRG for 24 h followed by caffeine displayed a significant and dramatic increase in both overall cytoplasmic calcium levels (Fig. 13A) and in acute peak calcium (Fig. 13B). In contrast, there was no significant difference between NRG-treated and untreated neurons in ATP-stimulated cytoplasmic calcium levels, either overall (Fig. 13C) or in acute peak levels (Fig. 13D).

NRGβ1-induced increase in enhanced glutamate-mediated calcium was prevented by inhibition of ryanodine receptors. To determine whether NRG increased the glutamate-induced rise in cytoplasmic calcium primarily through increased ER-resident RyR activation, NRG-treated neurons were exposed to ruthenium red, a RyR blocker, before glutamate exposure (Fig. 14). RyR blockade in NRGβ1-treated neurons resulted in prevention of enhanced glutamate-induced intracellular free calcium levels, with levels similar to that of glutamate and ruthenium red treatment alone (Fig. 14A). NRG-treated neurons displayed a greater than 40% increase in glutamate-induced peak acute cytoplasmic calcium concentration relative to controls. However, after pretreatment with ruthenium red, NRG-treated neuronal cytoplasmic calcium was only 10% greater than control neurons (Fig. 14B). This result indicates that calcium release from ER-resident RyR channels was the primary source of the NRG effect on enhancement of glutamate-induced rise in cytoplasmic calcium.

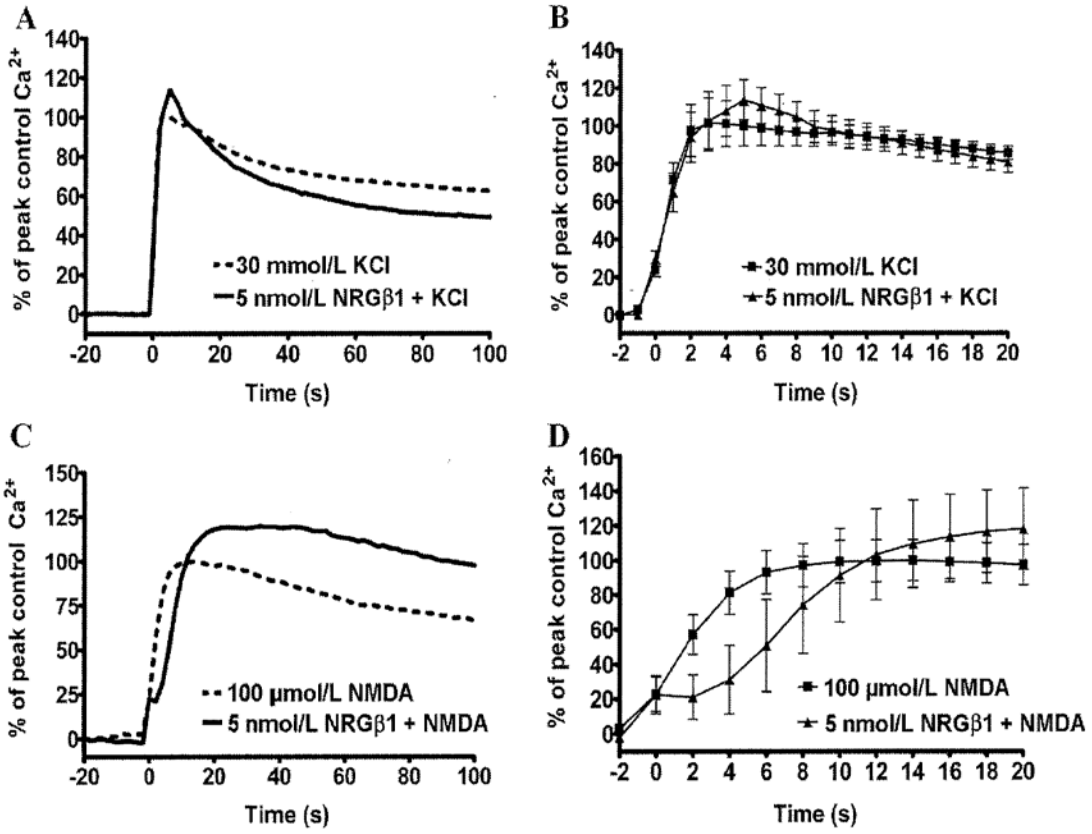
Figure 9: Long-term NRGβ1 treatment increases glutamate-induced cytoplasmic calcium in hippocampal neurons



Long-term incubation (24 h) of neurons with neuregulin (NRGβ1) (panels A and B) increased glutamate-induced cytoplasmic calcium, while short-term (1 h) treatment (panels C and D) did not. Rat hippocampal neurons were pretreated with 5 nmol/L NRGβ1 for 24 h or 1 h before loading with the calcium dye fura-2 AM and adding 100 µmol/L glutamate. After addition of agonist, readings were taken for 100 s. Traces were averaged from all cells; time courses shown in A and C reflect percentage response in relation to peak control values, which were given a value of 100%. B and D are the corresponding peak responses in the first 20 s after glutamate addition. One-way ANOVAs were performed on peak values compared with

baseline taken before glutamate addition, using a Tukey's post hoc test. For B and D, $p < 0.001$ and $p > 0.05$, respectively (n value of at least 60 neurons for 24 h treatment, 30 neurons for 1 h treatment).

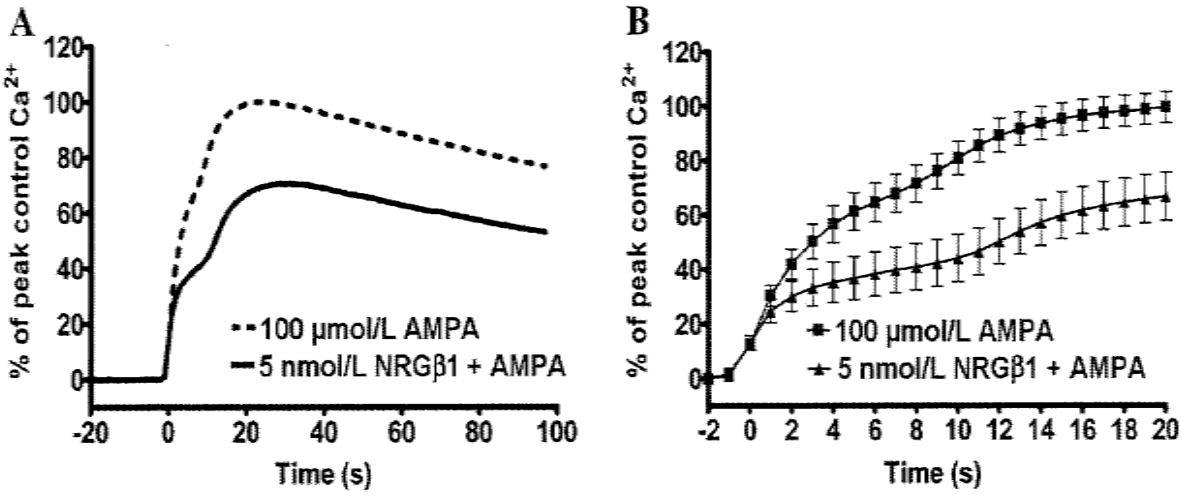
Figure 10: NRGβ1 treatment did not affect NMDA or voltage-gated calcium channels



Long-term NRGβ1 treatment did not affect NMDA- or KCI-induced peak cytoplasmic calcium levels. Rat hippocampal neurons were pretreated with vehicle or 5 nmol/L NRGβ1 for 24 h before loading with fura-2 AM and adding either 30 mmol/L KC1 to activate voltage-gated calcium channels (A) or NMDA in Mg^{t+}-free media to stimulate NMDA receptors (C). Traces were averaged from all cells, and time courses A and C reflect percentage response compared with peak untreated control values, which were given a value of 100%. B and D are the corresponding peak values in the first 20 s after addition of agonist to cells. One-way ANOVAs on peak graphs using a Tukey's posthoc test gave values of $p > 0.05$ for both treatments

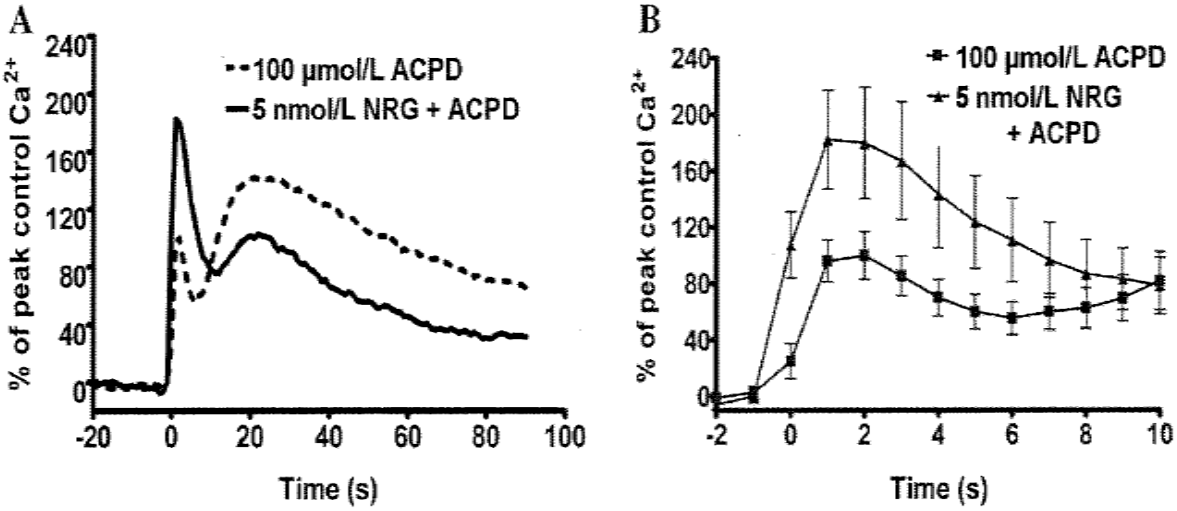
(for NMDA treatments, n >15 neurons; for KCI, n = 9 neurons). NMDA, N-methyl D-aspartate.

Figure 11: NRGβ1 treatment reduces AMPA receptor activation.



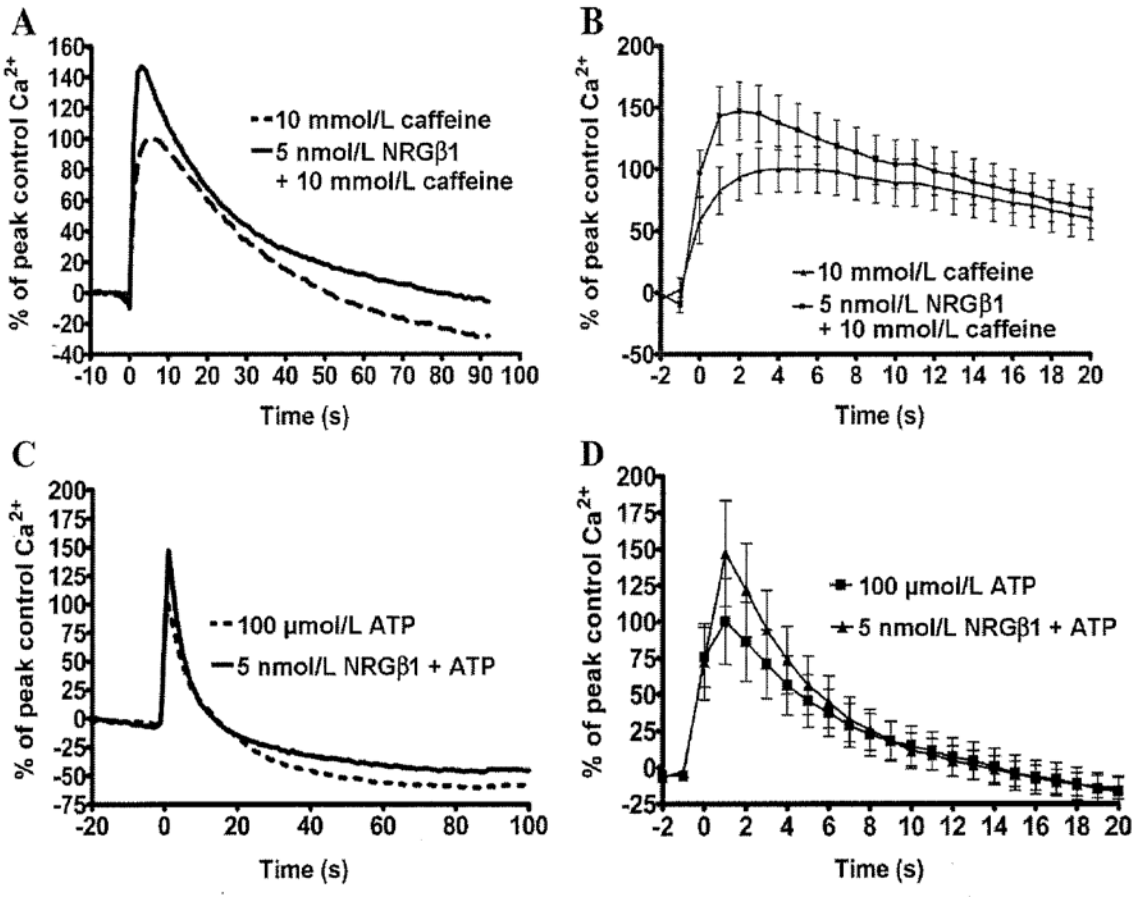
Long-term incubation (24 h) with NRGβ1 decreased AMPA-induced cytoplasmic calcium increase. Rat hippocampal neurons were pretreated with vehicle or 5 nmol/L NRGβ1 for 24 h before loading with fura-2 AM and adding 100 µmol/L AMPA. Traces were averaged from all cells, and time course A reflects percentage response compared with the peak untreated control value, which was given a value of 100%. B displays the acute peak values (first 20 s) after addition of AMPA. One-way ANOVAs on peak values after agonist addition using a Tukey's post hoc test gave a value of $p < 0.001$ *(*n = 24 neurons). AMPA, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

Figure 12: NRGβ1 increases mGluR1-induced cytoplasmic calcium



NRGβ1 incubation increased acute peak cytoplasmic calcium levels after activation of metabotropic glutamate receptors. Rat hippocampal neurons were pretreated with vehicle or 5 nmol/L NRGβ1 for 24 h before loading with fura-2 AM and adding 100 µmol/L ACPD, a general metabotropic glutamate receptor agonist. Traces were averaged from all cells, and time course A reflects percentage response compared with peak untreated control value, which was given a value of 100%. B displays the acute peak values (first 10 s) after addition of ACPD. One-way ANOVAs performed on peak values after ACPD addition using a Tukey's post hoc test gave a value of $p < 0.001$ ($n = 40$-50 neurons). ACPD, 1-aminocyclopentane-1, 3-dicarboxylic acid.

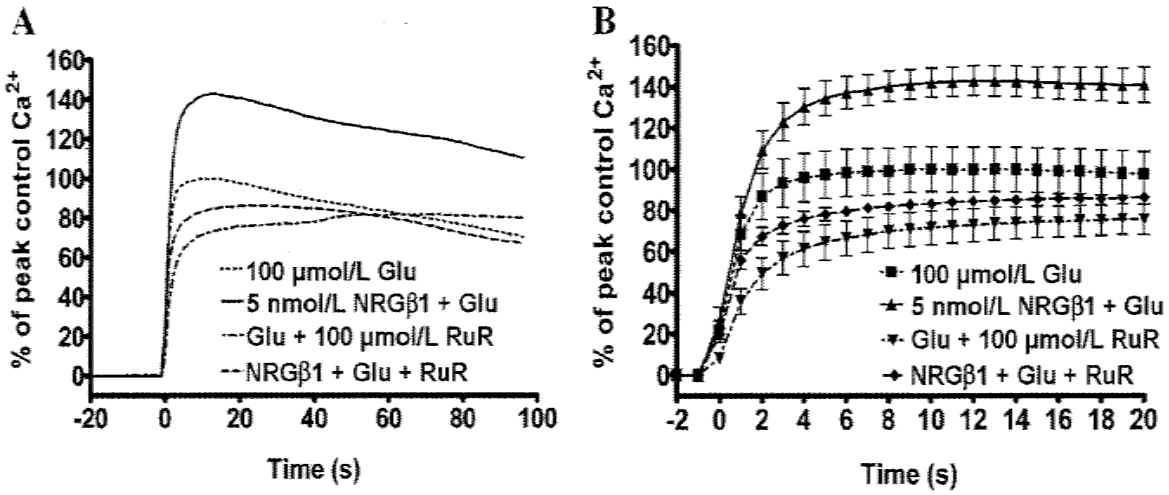
Figure 13: NRGβ1 increases ER calcium release from ryanodine receptors, but not IP3 receptors



NRGβ1 increased caffeine-induced, but not IP_3 receptor-mediated, ER calcium release. Rat hippocampal neurons were pretreated with vehicle or 5 nmol/L NRGβ1 for 24 h before loading with fura-2 AM and adding either 10 mmol/L caffeine to activate ryanodine receptors (A) or 100 µmol/L ATP to activate IP_3 receptors (C). Traces were averaged from all cells, and time courses A and C reflect percentage response compared with peak untreated control values, which were given a value of 100%. B and D are the corresponding acute peak values (first 20 s) after addition of caffeine (n = 30-35 neurons) or ATP (n = 24-26 neurons). One-way ANOVAs were performed on peak values after agonist addition compared with baseline taken

before glutamate addition, using a Tukey's post hoc test. Values of $p < 0.001$ for B and $p > 0.05$ for D were calculated. IP_3, inositol trisphosphate; ER, endoplasmic reticulum.

Figure 14: Inhibition of ER calcium release attenuates NRG enhancement of glutamate-mediated cytoplasmic calcium elevations



NRGβ1-induced glutamate-enhanced cytoplasmic calcium levels were prevented by pretreatment with a ryanodine receptor inhibitor. Rat hippocampal neurons were pretreated with vehicle or 5 nmol/L NRGβ1 for 24 h before loading with fura-2AM. Immediately before glutamate (Glu, 3 min) treatment, ruthenium red (RuR, 100 µmol/L) was added to block ryanodine receptor activation. Traces were averaged from all cells, and time course A reflects percentage response compared with peak untreated control value, which was given a value of 100%. B displays peak values after addition of Glu. One-way ANOVAs were performed on peak values using a Tukey's post hoc test. $p < 0.001$ for NRGβ1 + Glu compared with Glu alone, and $p < 0.001$ for NRGβ1 + Glu compared with NRG + Glu + RuR treatment *(n* = 12-20 neurons/treatment).

Discussion

Regulation of cytoplasmic calcium is the primary glutamate-mediated second messenger system in central neurons, controlling phenomena as diverse as neurite outgrowth, neurotransmitter release, and apoptosis. Because glutamate is the most prevalent excitatory neurotransmitter, understanding the glutamate/calcium system has been a central goal of neuroscience for decades. There are genetic and functional links between glutamate and NRG signaling families and the neurodevelopmental disorder schizophrenia (Hakak et al. 2001; Stefansson et al. 2002), and therefore the ErbB receptor ligand NRGβ1 has become an increasingly studied target for glutamate receptor activation. Although NRGβ1 has been shown to regulate glutamate receptor ion current in different systems, its effect on glutamate-mediated intra cellular calcium levels has not been studied. In this manuscript we have demonstrated for the first time that pretreatment with NRGβ1 can enhance peak glutamate-mediated intracellular calcium levels. In addition, we have provided evidence that the primary source of this calcium is mGluR-mediated release from ryanodine pools.

Most research has concentrated on early (<1 h) effects of NRGβ1 signaling; however, in the present study, calcium enhancement required long-term exposure (24 h) to NRG and was not replicable with 1 h pretreatment, which was further demonstrated by comparison of peak calcium levels between groups (Fig. 9). In either case, because we did not observe an effect with short-term NRG treatment, our results are likely to be due to a NRG-mediated pathway distinct from that which alters glutamate receptor currents.

Voltage-gated calcium channels are a primary contributor to glutamate-induced elevations in cytoplasmic calcium, triggered by glutamate-mediated membrane depolarization. Exposure to KC1 causes neuronal membrane depolarization but does not activate glutamate receptors; thus any enhanced activity of voltage-gated ion channels would be revealed. However, KC1-induced intracellular calcium levels were unchanged in NRGβ1-treated neurons versus untreated cells with no significant difference in peak calcium levels between treatment groups (Figs. 10A and 10B). NMDA receptors are the primary plasma membrane glutamate-mediated calcium channel, and account for the majority of rapid calcium influx after glutamate exposure. Acute (first 20 s) peak calcium levels did not differ significantly in untreated versus NRGβ1-treated cells after NMDA incubation (Fig. 10); however, calcium levels did rise later to levels significantly greater than control in NRG-treated neurons exposed to NMDA. Although NMDA-induced calcium levels took longer to peak than those of other treatments, other studies have shown similar reaction times under similar conditions (Barger 1999; Yoshida et al. 2003). It is suspected that this may be partially due to residual Mg^{t+} ions in the extracellular environment that delay NMDA channel opening, in addition to an internal Mg^{t+} block that would have been in place due to a lack of neuronal depolarization.

Although not the focus of this paper, our observation that NMDA-induced calcium elevation in NRGβ1-stimulated cells is significantly higher beyond our initial peak is an interesting one. Current evidence linking NRGβ1 signaling and NMDA function is ambiguous at best. Most studies have focused on short-term (5-60 min) treatment of neurons with NRG, studying glutamate channel current and

LTP (Huang et al. 2000; Gu et al. 2005; Kwon et al. 2005). Whereas some have shown little or decreased effect on NMDA receptors (Huang et al. 2000), others have shown in-creased subunit expression (Ozaki et al. 1997) and decreased NMDA activation in those with less NRGβ1 signaling (Hahn et al. 2006). Our observation of a sustained calcium elevation in hippocampal cells would indicate an enhancement of LTP, as calcium through NMDA receptors is a primary source of that phenomenon. Follow-up studies on NMDA signaling will be required to analyze whether this calcium elevation has significant long-term effects.

AMPA receptors are the primary excitatory glutamate receptor, leading to neuronal depolarization and thus both triggering voltage-gated calcium channels and allowing NMDA receptors to open under normal circumstances. Paradoxically, long-term incubation with NRG resulted in a significantly depressed AMPA-induced increase in cytoplasmic calcium levels (Fig. 11). This result is clearly not the source of the glutamate effect we were examining, but it is consistent with previous reports that NRG treatment significantly reduces AMPA current. Ionic current and the rise in intracellular free calcium are not equivalent, but they are related by the fact that reduced current may serve to limit AMPA effect on depolarization. This result indicates that the positive effect of NRG on glutamate-induced cytoplasmic calcium is occurring despite the significantly decreased contribution of AMPA relative to control neurons. Therefore, the increased acute peak glutamate-mediated calcium caused by NRG treatment is not caused by enhanced NMDA or voltage-dependent channels, and in fact is occur-ring despite decreased AMPA-mediated cytoplasmic calcium, leaving release of intracellular stores as the primary remaining option. Compared with other treatments, calcium response to AMPA was quite slow. As

AMPA receptors are usually responsible for sodium, not calcium, ion influx, it is possible that the existing subunit composition partially consisted of GluR2 subunits normally required for calcium ion influx. Although we cannot fully explain the result, it was not pursued, as it was obvious that this response was not responsible for our initial NRGβ1-glutamate effect.

Release of calcium from intracellular stores is induced by glutamate in 2 major ways: stimulation of mGluR, which activates ER-resident calcium channels through second messenger systems, and calcium influx from the extracellular environment, which induces a process called calcium-induced calcium release (Verkhratsky and Shmigol 1996). Unlike NMDA and AMPA, NRG treatment resulted in a significantly greater cytoplasmic peak acute calcium level after stimulation with the metabotropic agonist ACPD (Fig. 12). Thus, mGluR, which stimulates ER calcium release, was the only class of glutamate receptors tested that demonstrated significant NRG-mediated enhancement of peak cytoplasmic calcium. The observation that neither NMDA nor KC1 induced a significant rise in the acute peak intracellular calcium indicates that the effect of NRG did not increase CICR and therefore is probably a more complex effect than, for example, simple upregulation of RyR, which would be expected to result in greater CICR.

mGluR activation stimulates release of calcium from both IP_3-mediated and RyR-mediated pools. In our hands, treatment of neurons with ATP, which stimulates ER calcium release from IP_3R, did not show NRG-mediated enhancement (Figs. 13C and 13D). However, caffeine treatment of hippocampal neurons caused a dramatic increase in cytoplasmic calcium in NRGβ1-treated cells versus controls, both in peak and sustained levels (Figs. 13A and 13B). NRGβ1 also increased the number of cells

in which caffeine-induced calcium elevations were observed from 59% to 79% in control and NRG-treated, respectively (data not shown). Thus, peak cytoplasmic calcium values have been increased with glutamate, mGluR agonist, and RyR agonist, leading to the hypothesis that our initial observation, enhancement of glutamate-induced cytoplasmic calcium, is mediated through mGluR and ER-resident RyR. To test this hypothesis, NRGβ1-treated neurons were exposed to the RyR blocker ruthenium red followed by addition of glutamate. NRG-treated neurons showed greater than 40% elevation of glutamate-mediated cytoplasmic peak calcium in the absence of ruthenium red compared with control neurons, but only 10% enhancement in the presence of ruthenium red. Thus, at least 75% of the NRG effect on glutamate-mediated cytoplasmic calcium was due to greater release of calcium from ER-resident RyR.

There are a number of observations in this study that permit speculation about the pathway through which NRG has the observed effect, although this is not a definitive study. It is apparent that the primary means of increasing acute peak cytoplasmic calcium was not through extracellular calcium currents, since both NMDA and KC1 showed no enhancement, and AMPA actually displayed significant inhibition. However, while the source of the primary effect was from intracellular stores, it will require more work to delineate exactly what molecular system is being affected. Since NRG pretreatment so dramatically enhanced caffeine-induced calcium release, and ruthenium red blocked over 75% of the enhancement of glutamate-mediated calcium, we can be fairly confident that changes to the RyR account for the majority of the NRG effect. We are most likely not seeing increased calcium-induced calcium release or we would have observed enhancement with NMDA and KCL. In

addition, the effect is probably not due simply to increased stimulation of RyR by neighbouring IP_3R-mediated calcium release because we did not observe a significant increase with ATP, which stimulates IP_3-mediated ER calcium release. The fact that mGluR did show enhancement, but ATP did not, means that something other than IP_3 generation alone is required, and this enhancement is supplied by mGluR activation. Thus, NRG treatment augments an mGluR - RyR pathway to enhance the peak glutamate-mediated cytoplasmic calcium concentration, an effect with important implications for neuronal modulation. This type of interaction, in which mGluR specifically targets RyR, has been increasingly studied (reviewed by Fagni et al. 2000). It should be noted that we also observed increased NMDA-stimulated intracellular calcium in NRG-treated neurons at times later than 30s, but not in the acute peak, and we observed the opposite pattern in ACPD-treated neurons, indicating that the acute peak concentration and the delayed intracellular concentration may be modulated differently by NRG. The functional importance of calcium as a signaling molecule in all cell types, including neurons, cannot be overemphasized (Berridge et al. 2000; Glazner and Fernyhough 2002; Berridge et al. 2003). Numerous channels on the plasma membrane and ER regulate Ca^{t+} movement, maintaining a spatial and temporal balance that is used to modulate processes such as neuronal excitability, gene expression and synaptic plasticity, and apoptosis. These processes are all extremely sensitive to changes in calcium levels. The effect of NRG on glutamate-mediated peak increase in cytoplasmic calcium could lead to the regulation of numerous processes in the neuron and could be an initiating factor in LTP formation as well as other phenomenon. Previous studies indicate that NRGri1 also affects LTP

through changes in glutamate receptor current, although the effect of NRG-induced cellular calcium enhancement on LTP has not been established.

The observations that acute application of NRG can change NMDA and AMPA currents, while long-term exposure enhances glutamate-mediated calcium signaling, demonstrate the complex and intimate regulation of glutamatergic processes regulated by NRG. Our results indicate that reduction in chronic NRG action would cause a concomitant decrease in glutamate-mediated signaling, possibly resulting in attenuation of postsynaptic transmission through a decrease in ER calcium release. Further analysis will be required to determine how NRGβ1-induced ER calcium efflux could affect processes in a healthy neuronal system or in a pathologically compromised one.

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[3]Chapter 6

Secreted Amyloid Precursor Protein α (APPα) Activates Neuronal Insulin Receptors and Insulin Signaling Inhibition in Hypoinsulinemic Brain

Secreted amyloid precursor protein alpha (sAPPα) is a potent neurotrophin in the CNS, but a dedicated receptor has not been found. However, protein interactions involving amyloid beta, a peptide cleaved from the same parent peptide as sAPP, indicate that neuronal insulin receptors could be a target of amyloid peptides. In this study *in vitro* analysis revealed that exogenous sAPP increased insulin receptor phosphorylation and insulin signalin pathway mediator pAKT in the absence of insulin. In an overexpressing human APP transgenic mouse model, sAPP bound insulin receptors in the cortex, with significantly greater binding in hypoinsulinemic animals. In addition, in contrast to wild-type mice, hypoinsulinemia did not result in either reduced insulin receptor or AKT phosphorylation in animals which overexpress APP, indicating that sAPP may have prevented diabetes-induced depression of the pathway through a direct interaction. Hypoinsulinemia also did not cause memory impairments in APP overexpressing mice as in wild type animals, implying a protective role of sAPP *in vivo* following insulin depletion. These data showed that sAPP bound and activated insulin receptors both *in vitro* and *in vivo*, and prevented diabetes-induced insulin pathway impairments through a direct activation of insulin receptors.

Introduction

Neurotrophic properties mediated by sAPPα, which is cleaved from full-length APP (flAPP), are mediated through an incompletely understood signaling pathway. sAPPα

[3] Schapansky J et al. (2010), manuscript in preparation.

induces neurite outgrowth in cortical neuronal cultures (Araki et al., 1991) and promotes neuronal survival by preventing hypoglycemic damaget to cells and glutamate-induced excitotoxicity (Mattson et al., 1993). sAPPα also enhances synaptic plasticity in hippocampal slices through promotion of long-term potentiation (LTP) (Ishida et al., 1997). However, although sAPPα has several potential binding partners, including apolipoprotein E (Barger and Mattson, 1997) and guanylate cyclase at the plasma membrane (Barger and Mattson, 1995), a dedicated neuronal receptor has not been found.

A physiological role for amyloid peptides cleaved from flAPP in insulin signaling has been suggested by structural and functional interactions at the insulin receptor (Xie et al., 2002; Giuffrida et al., 2009). Amyloid beta (Aβ) peptide competes with insulin for insulin receptor binding (Xie et al., 2002) and can be neuroprotective through the insulin/IGF1 pathway in neuronal cultures (Giuffrida et al., 2009). As differential cleavage by secretases imparts a common 16 amino acid sequence to sAPP (at the C terminus) and Aβ (at the N terminus), sAPPα may also activate insulin/IGF1 receptors.

With similar consequences to sAPPα, the insulin/IGF1 signaling promotes neuronal health in the brain and peripheral nervous system. Dimerized insulin and IGF1 receptor complexes are activated at the plasma membrane following ligand binding at the active site, leading to recruitment of kinases including PI3K and AKT to initiate signal transduction. Insulin increases neuronal outgrowth *in vitro* in a dose-dependent manner (Recio-Pinto and Ishii, 1984; Mill et al., 1985; Fernyhough et al., 1989; Wang et al., 1992), and prevents cell death through PI3K-induced AKT activation, which prevents activation of pro-apoptotic proteins (Cross et al., 1995; Datta et al., 1997; Cardone et al., 1998; Turenne and Price, 2001). Insulin also modulates synaptic plasticity *in vitro* (Liu et al., 1995; Man et al., 2003), while PI3K is required for memory retrieval and fear

conditioning in rats and mice (Lin et al., 2001; Chen et al., 2005), indicating a role for insulin in formation of memory. Clinically, intranasal administration of insulin in humans improves memory in healthy adults (Craft et al., 1999; Craft et al., 2003) and implies a potential therapeutic use for neurological diseases with a dementia component. A requirement for insulin signaling protein insulin response substrate 1 (IRS1) for sAPPα neurotrophic activity in PC12 cells (Wallace et al., 1997) also suggests an sAPPα/insulin signaling interaction. Because amyloid peptides can bind insulin receptors and sAPPα has neurotrophic effects similar to insulin, we chose to examine the possibility that sAPPα can bind and activate insulin receptors in neurons. In this study, sAPPα specifically activated insulin receptors *in vitro*, and could also bind insulin receptors in mice overexpressing APP , indicating that sAPPα is a potential binding agonist for the insulin/IGF pathway.

Experimental Procedures

Preparation of sAPP. Recombinant human sAPPα was purified from serum-free culture medium (50/50 MEM/F12) conditioned by a HEK293 line stably transfected with an APP695 cDNA into which a stop codon was inserted after amino acid 612 (the sixteenth amino acid in the Aβ domain). Conditioned medium from these lines was passed through a DE-52 anion exchange column, which was then washed with phosphate-buffered saline (PBS) and step eluted with PBS containing 0.75 M NaCl. The sAPP-containing fractions were pooled and loaded onto a fast-preparative liquid chromatography (FPLC) system equipped with a Hi-Trap™ heparin-Sepharose column (GE Life Sciences), which was then washed with PBS and eluted with a linear gradient of PBS to PBS + 1M NaCl. The sAPP-containing fractions from the heparin column were pooled and resolved by FPLC

using a MonoQ anion-exchange column (GE Life Sciences), which was washed with Buffer A (20 mM triethanolamine-HCl, 100 mM NaCl, pH 7.4) and then eluted with a linear gradient of Buffer A to Buffer B (20 mM triethanolamine-HCl, 1 M NaCl, pH 7.4). The sAPP- containing fractions were pooled and dialyzed against Buffer C (124 mM NaCl, 26 mM NaHCO3, 3 mM KCl, 2 mM CaCl2 1.4 mM MgCl2 1.25 KH2PO4, pH 7.4) before storage at -80 °C.

Embryonic cortical neuronal culture. Brain tissue was isolated from day 16 embryonic CD1 mice. Cortices in HBSS buffer were triturated with fire-polished glass pipettes and dissociated cells seeded in 25 mm plastic dishes coated with poly-D-lysine (Sigma), at $8x10^5$ neurons/well. Neurobasal media containing B27 supplement (Invitrogen) and 10% fetal bovine serum (FBS; Hyclone) was used at time of plating. Media was changed to Neurobasal+B27 supplement without FBS on the following day. Three days post-plating, half the volume of each dish was replaced with fresh Neurobasal+B27 containing 1 μM cytosine arabinoside (500 nM final), to limit astrocyte growth.

sAPPα dose-response experiment. Experimental treatments were carried out 7 days in vitro (DIV), in Neurobasal media alone. Cultures were incubated with 10, 30, or 100 nM sAPPα 695 for 15 minutes at 37 degrees/5% CO2. Following incubation, media was removed by aspiration, and cells were lysed in 4%SDS/50 mM Tris-HCl, followed by dilution with 4x sample buffer for immunoblotting analysis.

Immunoblotting/Immunoprecipitation. Cortical tissue from injected mice and controls were isolated, frozen on dry ice and stored at -80^{o}C until processed. RNA and protein was extracted using an All-prep kit (Qiagen), or by homogenization in 4% SDS buffer. Protein concentration was determined and samples were made up to 0.5 mg/ml in a 4x sample buffer. Either cell culture or cortical tissue samples (10-20 ug) were separated by

SDS-PAGE, transferred to PVDF membrane, and blocked with either 5% milk or 5% bovine serum albumin (BSA) in 0.1 TBST. The same solution was used for incubating with one of the following antibodies overnight at 4 degrees: pAKT (Cell Signaling), p-insulin receptor β [Tyr972] (Millipore), Aβ1-16 (6E10, Signet), insulin receptor β (Santa Cruz Biotechnology), β-actin (Sigma-Aldrich). The following day, membranes were incubated with an appropriate species-specific secondary antibody, washed in TBST, and exposed to a FluorSMax imaging system (Biorad).

For immunoprecipitation, either neuronal culture or brain tissue was homogenized in non-denaturing lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 % Triton, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate and protease inhibitors [Roche Diagnostics]). Homogenates were incubated at 4 degrees for 20 minutes to assist cell lysis, followed by a centrifugation at 14,000xg for 10 minutes to remove cellular debris. Following protein quantification, 500 μg of protein was incubated with 2 μg of antibodies to insulin receptor β (Santa Cruz Biotechnology) or the human amyloid beta 1-16 sequence (6E10; Signet) overnight at 4 degrees. Protein A/G sepharose beads (Pierce) were added and samples were mixed vigorously for 4 hours. Mixtures were washed twice in lysis buffer and once in ice-cold PBS before eluting with 4% SDS for immunoblotting.

Neurite outgrowth analysis. CD1 hippocampal neuronal cultures were seeded in 96-well plates in Neurobasal media containing B27 supplement without insulin (Gibco). At time of plating, treatments including 100 nM sAPPα and 20μM PI3K inhibitor (LY294002) were added to wells. Cultures were incubated at 37 ^{o}C/5% CO_2 for 48 hours prior to fixation with 2% paraformaldehyde. Cells were made permeable by 0.3% Triton X-100 (Sigma-Aldrich) prior to incubation with neuron specific β-tubulin isoform III antibody (rabbit, 1:200; Santa Cruz Biotechnology) overnight at 4^{o}C. Cultures were washed and

incubated with anti-rabbit Alexa Fluor 568 antibody (1:250; Molecular Probes) for 1 hour and washed again. Cells were imaged using LSM510 confocal microscope (Zeiss), fitted with a 40x air-immersion objective. Mean pixel area indicating total neurite outgrowth was measured per well, subtracting cell body contribution.

Animals and experimental treatments. TgCRND8 is a transgenic mouse overexpressing a modified human APP695 protein, the primary form of flAPP found in neurons. This modification includes two FAD APP mutations designed to promote Aβ cleavage (Swedish, K670N & M671L; Indiana, V717F). Gene transcription was driven by a cos Tet vector containing a Syrian hamster prion protein promoter that focalizes peptide expression to neurons, with minor contributions to astrocytes. Mice were developed on a C57 background that maximizes survival despite elevated APP expression (Chishti et al., 2001). These animals gestate and are born normally, but develop Aβ plaque deposition and cognitive impairments by 3 months of age (Chishti et al., 2001). Although cortical sAPP levels drop from >90% of all flAPP cleavage to 50-60% due to the mutation (Simons et al., 2001; Chauhan et al., 2004), gene overexpression ensures as much as 4x more APP is produced than in controls, maintaining high human sAPP expression. In this study, 8-week old animals were injected with the drug streptozotocin, a compound that specifically targets and destroys insulin-producing β-cells in the pancreas. This leads to a severe reduction in insulin response, causing Type 1 diabetes and hyperglycemia. A dose of 90 mg/kg was used on three successive days, with a 12 hour fast prior to injection. Blood glucose was determined prior to a fourth injection; if levels were higher than 20.0 mmol/l, the animal was considered diabetic and not reinjected. Otherwise, animals were re-injected once every three days until diabetic. Animals were housed for 6

weeks prior to euthanasia for tissue collection. Only animals diabetic for the same amount of time and same age were used for assaying purposes.

Morris Water Maze (MWM). Animals were tested using the MWM as previously reported (Odero et al., 2010), with some modifications. Briefly, a circular tub (3 feet in diameter) was filled with lukewarm water (24-26 ^{o}C), and made opaque with skim milk powder. Spatial cues were placed at cardinal locations just above the surface of the water. A platform was placed just below the surface of the liquid, invisible to the animal. Test subjects were placed in the tub in front of a spatial cue, and time to find the platform (escape latency) was measured. Longer escape latency times over the course of the experiment are indicative of memory impairment. If the platform was not found in 90 seconds, animals were trained by being manually placed on the platform. Trials were repeated three more times, using a different cardinal location each time as a starting point. A day consisting of 4 trials/animal, termed one block, was repeated twice more for a total of 3 days. MWM was performed prior to animal injection to establish baseline memory retention, before repeating in the same animals 4 weeks following hypoinsulinemia, using the same platform location as used previously.

Quantitative real-time RT-PCR (qRT-PCR). Total RNA was extracted from hippocampal tissue of STZ-injected animals and controls (6 weeks following injection) using the RNAeasy Kit (Qiagen), and 200ng of RNA was used for first-strand cDNA synthesis with random hexamer primers using the iScript cDNA Synthesis Kit (BIORAD, Mississauga, ON). A total volume of 1 µl of cDNA was used as the template in each 25 µl PCR reaction with iQSYBR Green (BIORAD). Cycling conditions were: 94°C for 10 min, followed by 40 cycles at 94°C for 15 s, T_m for primers (see Table 1) for 30 s, and 72°C for 30 s, 72°C for 7 min and held at 4°C. PCR assays were performed using the iCycler

Thermal Cycler (BIORAD, Mississauga, ON). Threshold cycle (C_t) values for each sample were used to calculate the initial quantity of cDNA template by the standard curve method. Data was normalized to GAPDH cDNA to correct for variability in individual samples. Negative control reactions were also performed without template cDNA.

Results

sAPPα increases insulin receptor phosphorylation in neuronal cultures. To assess exogenous sAPP-induced activation of the insulin/IGF1 pathway independent of insulin ligand, neurons were incubated in insulin-free media for 2.5 hours prior to treatment to limit insulin signaling. Cortical neuronal cultures were treated with sAPP (10, 30 & 100 nM). Neurons were lysed following treatment (15 min) and assessed for insulin receptor activation by immunoblotting for phosphorylated insulin receptor. The pIR [pTyr972] antibody detects phosphorylation of the insulin receptor at tyrosine 972 residue, required for insulin-mediated signaling. sAPPα increased Tyr972 phosphorylation, with a significant increase over controls at 100 nM (Figure 15A).

The pTyr972 antibody used for immunoblotting phospho-insulin receptors can cross-react with the IGF1 receptor (IGF1R), which shares a high level of sequence homology with the insulin receptor in the autophosphorylation region required for pathway activation. Therefore, insulin receptors were specifically isolated from neuronal lysates by immunoprecipitation, to determine if sAPP directly activated insulin, and not IGF1, receptors. Insulin was removed from cultures before sAPP addition, as previously described. A specific insulin receptor β antibody that does not bind IGF1Rs or interfere with ligand binding was used for immunoprecipitation from sAPP-treated and untreated neuronal cultures, followed by immunoblot with pTyr972 antibody. Untreated samples

had low levels of insulin receptor phosphorylation (Figure 15B), while sAPP showed increased insulin receptor phosphorylation compared to controls.

sAPP activates the insulin signaling pathway in vitro. Downstream of the insulin receptor, AKT is a major mediator of insulin pathway activation, responsible for anti-apoptotic effects and synaptic plasticity (de la Monte and Wands, 2005; van der Heide et al., 2006). One function of insulin signaling includes increased neurite outgrowth, a process also promoted by sAPP through activated IRS1 (Mill et al., 1985; Araki et al., 1991; Wang et al., 1992; Wallace et al., 1997). Neuronal insulin pathway activation by sAPP was tested by analyzing phosphorylated AKT levels and sAPP-induced neurite outgrowth. Without insulin, sAPP increased AKT phosphorylation compared to controls, as observed with phosphorylated insulin receptor, with a significant increase at 100 nM sAPP treatment (Fig. 16A). Neurite outgrowth analysis was performed on neurons plated with 100 nM sAPP, in the absence and presence of 20 μM LY294002, a selective PI3K inhibitor that prevents AKT activation. sAPP significantly increased neurite outgrowth as determined by neuronal β-tubulin staining in neuronal cultures versus controls (Figure 16B). PI3K inhibition with LY294002 prevented this increase, reducing outgrowth to control levels. Thus sAPP promoted neurite outgrowth through increased pAKT activity, possibly through insulin receptor activation.

sAPP physically interacts with insulin receptors in APPTg animals. Increased protein phosphorylation *in vitro* indicated sAPP could activate insulin signaling proteins in the absence of insulin. Lowering brain insulin *in vivo* would require reduced peripheral insulin production, as most brain insulin is produced peripherally and transported across the blood brain barrier (Banks, 2004). To mimic low insulin conditions under which *in vitro* experiments were performed, overexpressing APP

TgCRND8 transgenic mice were injected with streptozotocin (STZ). Four experimental groups were created, including wild-type animals injected with saline (Wt), wild-type animals injected with STZ (WtSTZ), transgenic animals injected with saline (APPTg), and transgenic animals injected with STZ (APPTgSTZ). Following the trial, blood glucose measurements at tissue collection confirmed that both STZ-injected TgCRND8 mice (APPTgSTZ) and injected wild type animals (WtSTZ) were Type 1 diabetic (>20 mmol/L blood glucose), with no difference between transgenic and wild-type animals (Table 2).

Potential physical interaction between the peptides and insulin receptor *in vivo* was assessed by co-immunoprecipitation (co-IP). Co-IP of insulin receptors in APPTg and APPTgSTZ brain homogenates was followed by immunoblot with an anti-amyloid 1-16 antibody (6E10) capable of binding full length amyloid precursor protein (flAPP), sAPP, and Aβ1-42. Using the 6E10 antibody insured specific binding to human flAPP products, as it does not cross-react with mouse APP proteins. Human sAPPα was detected in both APPTg and APPTgSTZ pulldown samples (Figure 17B), but APPTgSTZ animals had significantly greater sAPP levels than APPTg animals alone. Neither Aβ monomers (ABm) nor oligomers (ABo), two amyloid peptides that alter insulin signaling, were found in any insulin receptor co-IP samples (Figure 17B). Duplicating co-IP with 6E10 antibody followed by immunoblotting with insulin receptor antibody verified a physical interaction association between insulin receptor and sAPP in both APPTg and APPTgSTZ (Figure 17A).

Insulin depletion reduces insulin receptor activation in wild type but not transgenic animals. Insulin pathway activation in STZ-injected and control animals was assessed by immunoblotting with IR-[pTyr972] and pAKT antibodies, as performed *in vitro*. Cortical

pIR levels of hypoinsulinemic wild type animals (WtSTZ) were decreased versus wild-type (Wt) controls (Figure 18A). However, levels in insulin-deficient transgenic animals were unchanged compared to transgenic controls. A comparison of injected groups as percentage of their controls revealed a significant difference between the two (Figure 18B). As previously, phosphorylated AKT levels were used to analyze pathway activation downstream of the insulin receptor. Hypoinsulinemic wild type animals had less pAKT than controls, while hypoinsulinemic transgenics (APPTgSTZ) displayed no pAKT reduction versus transgenics alone (Figure 18B). Therefore STZ-injected transgenic animals did not have an insulin pathway signaling impairment, despite being hypoinsulinemic.

mRNA expression for insulin/IGF related protein differs between treatment groups. Insulin/IGF1 signaling can be initiated by multiple ligands and receptor dimer combinations (Belfiore et al., 2009). Members of the insulin signaling pathway, including the insulin receptor, the structurally-similar insulin like growth factor 1 (IGF1) receptor or the distinct insulin-like growth factor-2 receptor (IGF2R), can form hetero- or homodimers that have different ligand binding specificities. In addition, while peripheral insulin is the major source of brain insulin, insulin mRNA is expressed in different areas of the brain, including hippocampus and cortex (Steen et al., 2005). Thus, insulin pathway gene expression was examined to determine if the prevention of diabetes-induced insulin signaling depression in hypoinsulinemic transgenics was a result of increased expression of insulin, IGF1, or IGF2, or their respective receptor.

A significant reduction in IGF2 mRNA expression was observed in insulin-deficient wild-type animals versus wild-type animals (Figure 19E). However, IGF2 levels in insulin-deficient transgenic animals (APPTgSTZ) were not different from transgenic

controls (Tg) (Figure 19E). Insulin mRNA was detected, as expected (Steen et al., 2005), but there was no difference in either insulin or its receptor between any of the treatment groups (Figure 19 A, B). Also, no changes were seen in IGF1, IGF1R and IGF2R mRNA expression.

Hypoinsulinemia decreases memory retention in wild-type, but not transgenic, animals. It was important to establish whether hypoinsulinemic transgenic animals, which did not have impaired insulin signaling, showed memory impairments normally characteristic of hypoinsulinemic animals (Biessels et al., 1998; Tuzcu and Baydas, 2006; Reisi et al., 2009). Cognitive deficits were determined with the Morris water maze, a behavioural test designed to assess hippocampal-dependent spatial memory. In this study, baseline spatial memory prior to STZ injection was assessed in 8-week old asymptomatic TgCRND8 animals with the Morris water maze. No difference in escape latency was observed between the two groups (Figure 20A). Animals were injected with STZ following this initial water maze, and cognitive performance was assessed again with the water maze, four weeks following diabetes induction (>20 mmol/L glucose). Average escape latency of hypoinsulinemic wild type animals (WtSTZ) was significantly higher than wild-type controls (Wt) (Figure 20B). Distance traveled to the platform, or path length, was also significantly longer in insulin-deficient wild-type animals. However, hypoinsulinemic transgenic animals (APPTgSTZ) displayed no significant impairment in either escape latency or path length compared to APPTg animals (Figure 20B). These data imply insulin-deficient transgenic animals did not have the cognitive impairments that insulin-deficient wild type animals did.

Gene	Sequence (5' to 3')	Position (mRNA)	Size (bp)	T_m (°C)	Primer Conc. (uM)
Ins	TTC TAC ACA CCC AAG TCC CGT C (Forward)	145	135		2.0
	ATC CAC AAT GCC ACG CTT CTG C (Reverse)	279		62	
InsR	TGA CAA TGA GGA ATG TGG GGA C (Forward)	875	129		1.0
	GGG CAA ACT TTC TGA CAA TGA CTG (Reverse)	1003			
IGF1	GAC CAA GGG GCT TTT ACT TCA AC (Forward)	65	127		0.75
	TTT GTA GGC TTC AGC GGA GCA C (Reverse)	191			
IGF1R	GAA GTC TGC GGT GGT GAT AAA GG (Forward)	2138	113		0.75
	TCT GGG CAC AAA GAT GGA GTT G (Reverse)	2250			
IGF2	CCA AGA AGA AAG GAA GGG GAC C (Forward)	763	95		0.5
	GGC GGC TAT TGT TGT TCA CAG C (Reverse)	857			
IGF2R	TTG CTA TTG ACC TTA GTC CCT TGG (Forward)	1066	91		0.5
	AGA GTG AGA CCT TTG TGT CCC CAC (Reverse)	1156			
IGF2R	CTG GTT CCT ACC CCC AAT G (Forward)	1066	91		0.5
	CTC AGA TGC CTG CTT CAC CAC CTT C (Reverse)	1156			
GAPDH	CGT GTT CCT ACC CCC AAT GTG TCC (Forward)	766	186	69.4	1.0
	GAA GGT GGT GAA GCA GGC ATC TGA G (Reverse)	843			

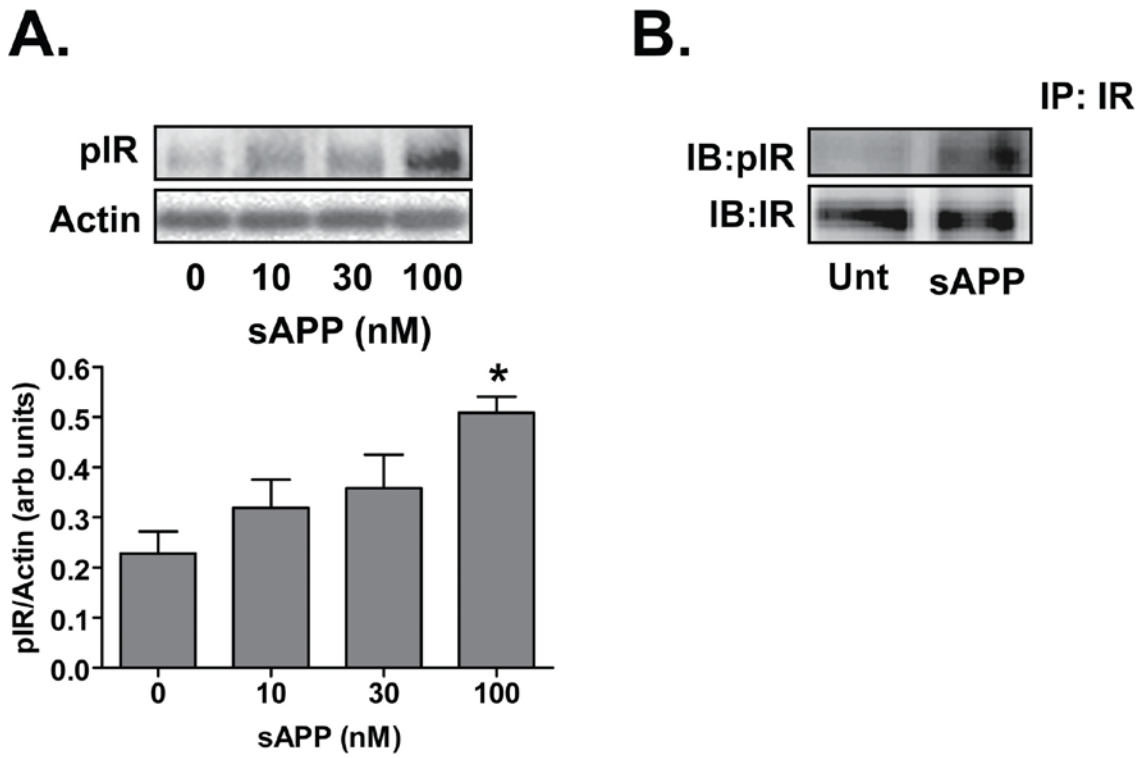
Table 1. Primer Sequences and qRT-PCR Conditions.

Table 2. Blood glucose levels and brain weights following STZ injections

	Blood glucose (mmol/L)	**Brain Weight (mg)**
Wt	13.1 +/- 0.54	449.7 +/- 6.8
Tg	12.3 +/- 0.67	431.6 +/- 3.5
WtSTZ	31.9 +/- 0.77	403.3 +/- 6.7
TgSTZ	30.8 +/- 1.24	407.5 +/- 9.5

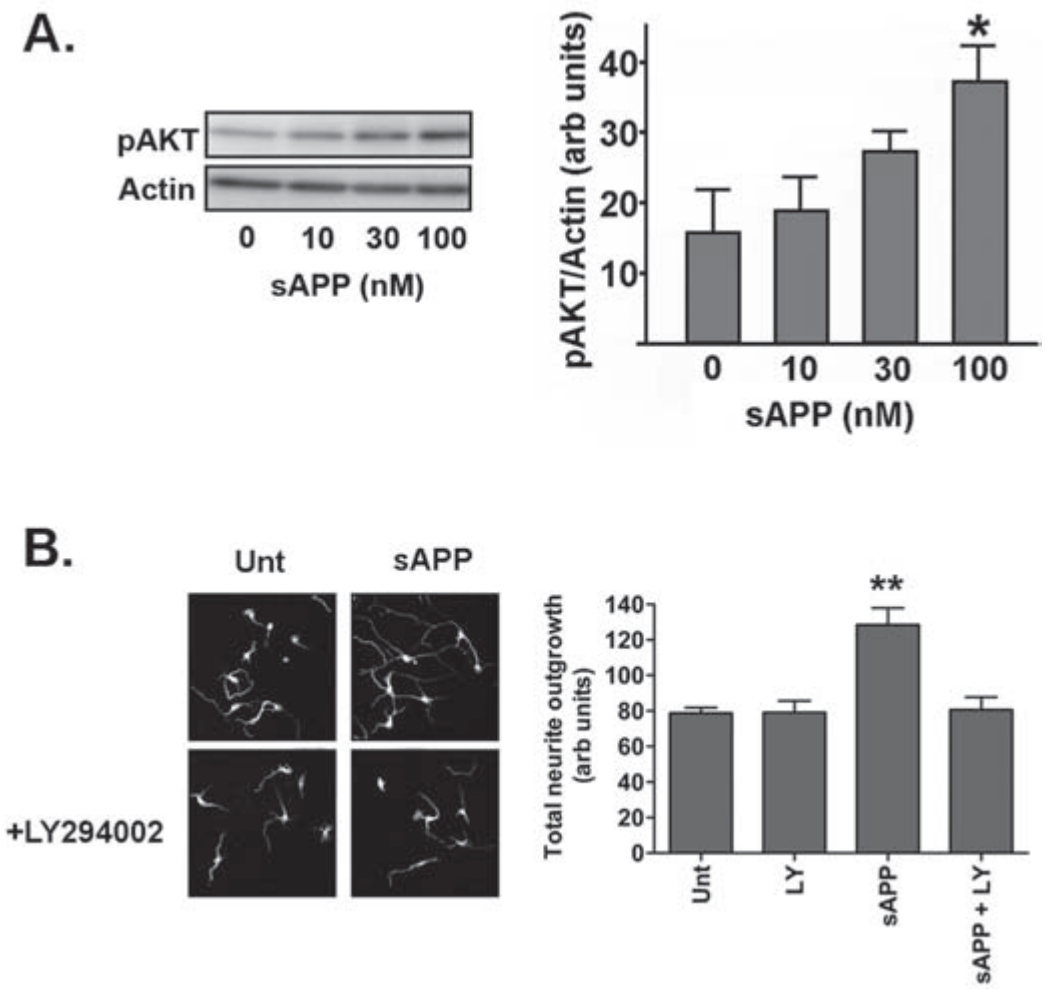
Blood glucose and brain weights were unchanged between injected animals. Levels were in the diabetic range for all injected animals at tissue collection (>20 mmol/L). Based on blood glucose, both control groups were not considered to be diabetic. Whole brain weight was taken following euthanasia. (n=5-11/group)

Figure 15: sAPP increases insulin receptor phosphorylation *in vitro*.



Seven day old cortical neurons were treated with sAPP (10 nM, 30 nM, and 100 nM) following insulin removal (2.5 hours in Neurobasal media without insulin) (A), for 15 minutes prior to collecting cell lysates. Immunoblotting with phosphorylated insulin receptor [(pTyr972, pIR)] antibody revealed a dose-related increase, with a significant rise in 100 nM APP treated neurons versus controls (Mean +/- SEM, One-way ANOVA, Tukey post-hoc, $p<0.05$, $n=3$). Immunoprecipitation with insulin receptor β antibody of sAPP-treated cultures followed by pIR immunoblot indicates specific activation of insulin receptors (B). (IB = immunoblot, IP=immunoprecipitation)

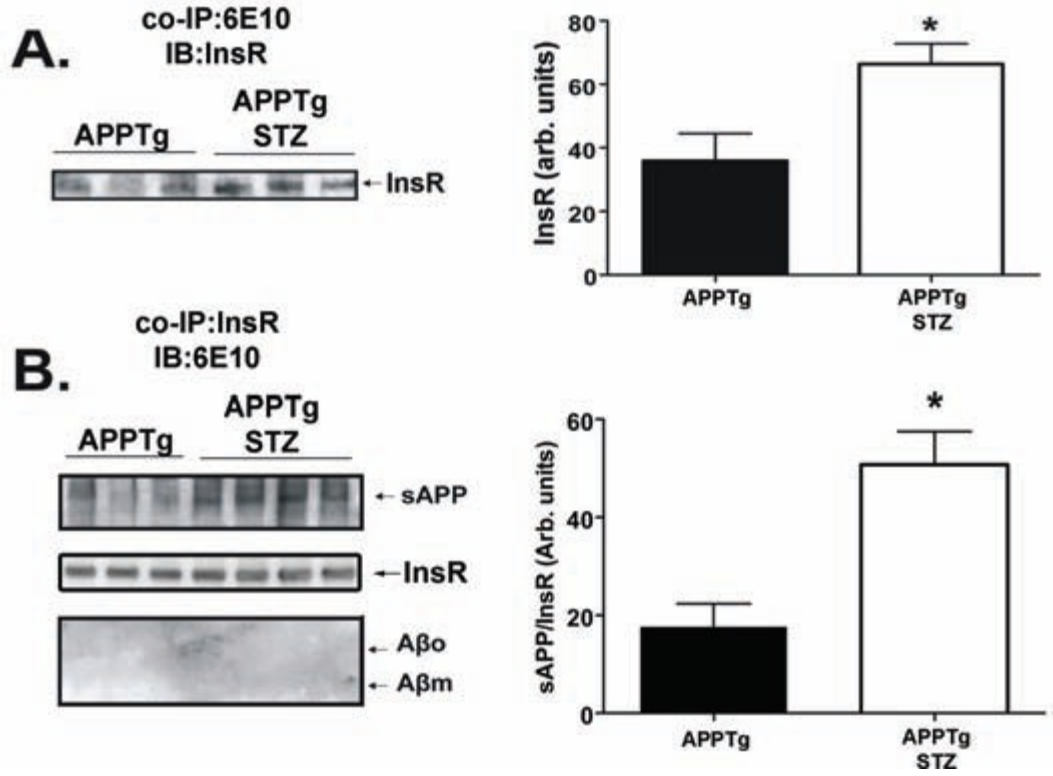
Figure 16: sAPPα activates the insulin signaling pathway in vitro.



Cortical neurons (7 days *in vitro*) treated with sAPPα were immunoblotted for pAKT, a kinase activated through insulin signaling (A). sAPPα increased AKT phosphorylation over the dose response, significantly at the 100 nM dose (15 minute treatment, Mean +/- SEM, One-way ANOVA, Tukey post-hoc analysis, $p<0.05$, n=3). CD-1 primary hippocampal neurons were seeded without treatment, 100 nM sAPPα, sAPPα + 20 μM LY294002 (PI3K inhibitor) or LY294002 alone, and neurite outgrowth was analyzed by immunofluorescence of neuronal-specific β-tubulin III after 48 hours (B). sAPPα significantly increase neurite outgrowth compared to untreated controls, while co-

treatment with LY294002 prevented this increase (Total pixel area/well without cell bodies, n=8-10 per treatment, One-way ANOVA with Tukey's post hoc, ** p=0.001).

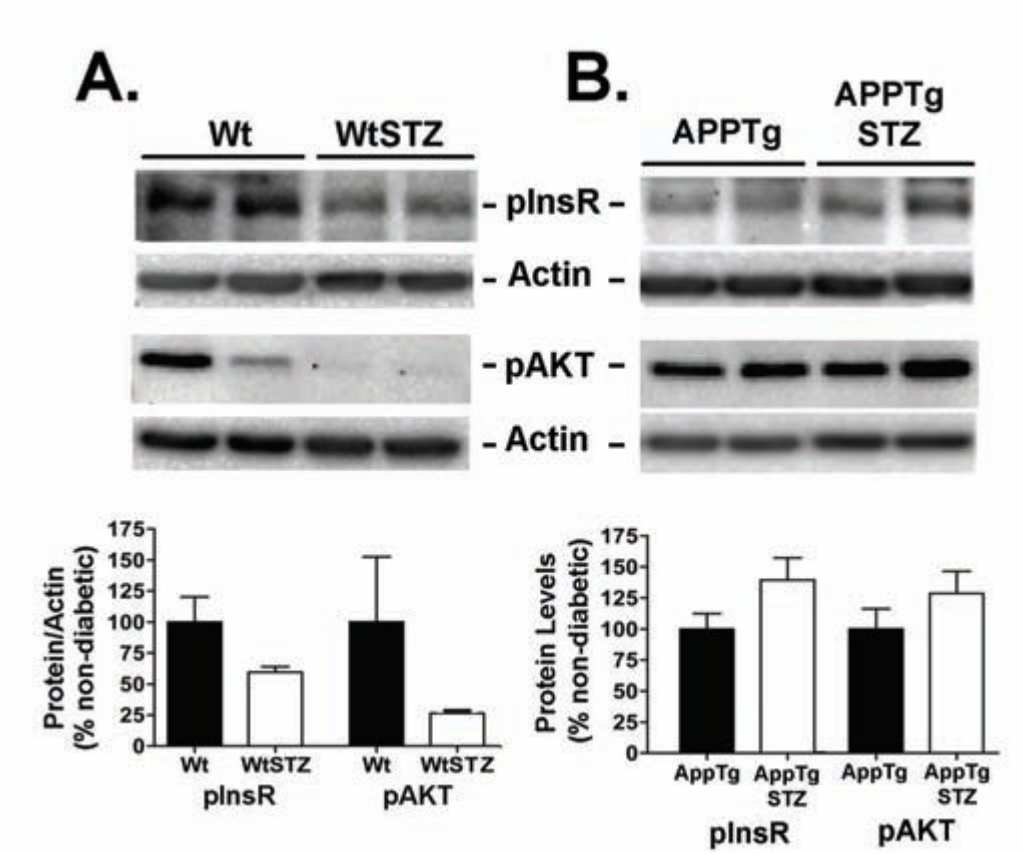
Figure 17: Physical interaction between sAPP and insulin receptor is greater in insulin-depleted animals.



Co-immunoprecipitation (coIP) of cortical tissue with a sAPP-recognizing antibody (6E10, A) or with specific insulin receptor antibody (InsR, B) was used assess amyloid protein/receptor interaction. (A) sAPP co-IP followed by immunoblot (IB) with the InsR antibody indicates that sAPP binds to insulin receptor in both transgenic (APPTg) and hypoinsulinemic transgenic (APPTgSTZ) cortical samples, but significantly higher in APPTgSTZ animals (Mean +/- SEM, t-test, n=3,4/group, * $p<0.05$). (B) InsR immunoprecipitation followed by immunoblot of amyloid beta 1-16 (6E10) antibody revealed the presence of sAPP protein, but not amyloid beta monomers or oligomers (Aβm or Aβo, respectively). Amount of sAPP protein bound to hypoinsulinemic

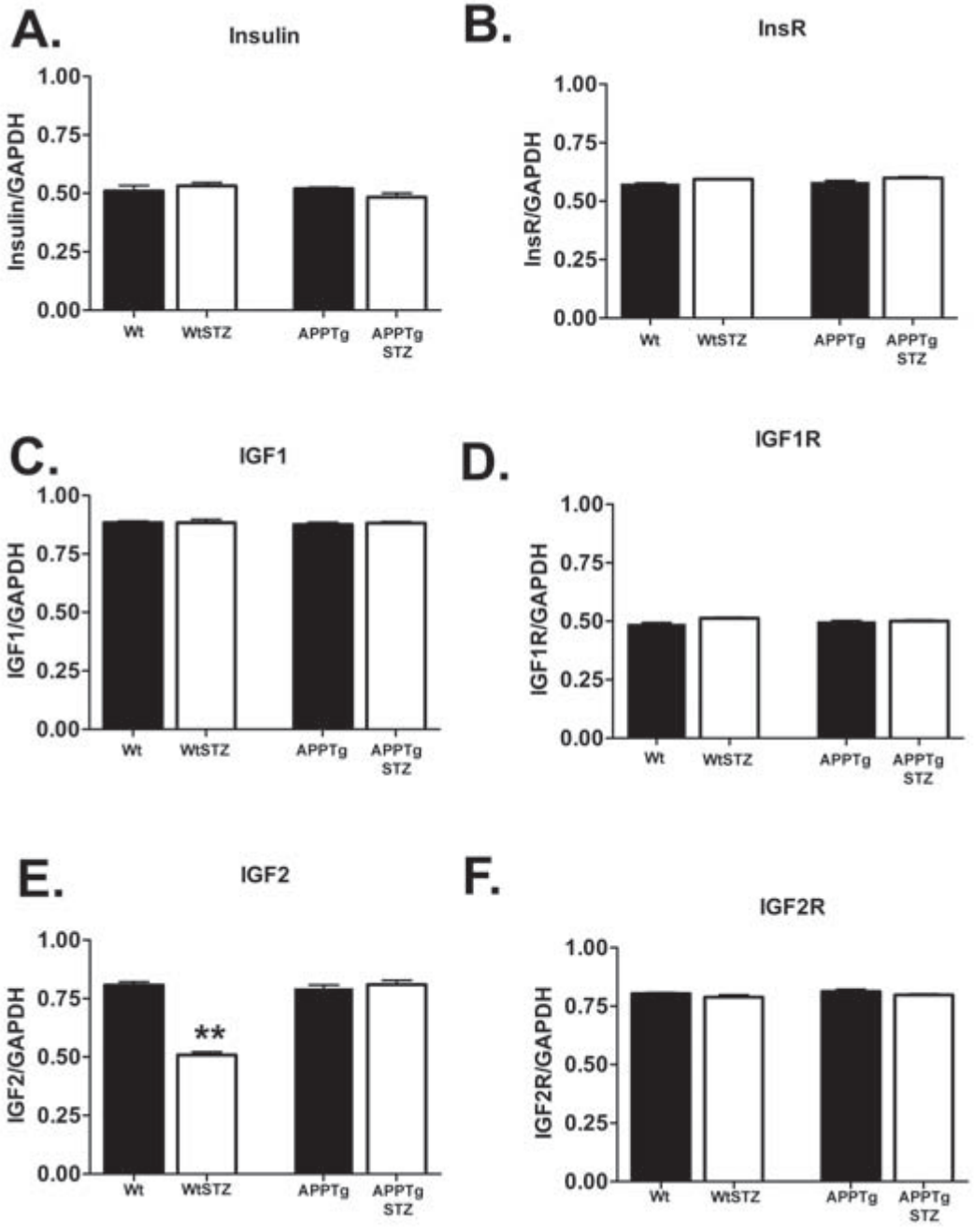
transgenic InsRs was significantly higher to that in transgenic cortical samples (Mean +/- SEM, t-test, n=3,4/group, * $p<0.05$)

Figure 18: Insulin pathway signaling decreases in insulin-deficient wild type animals but not in insulin-deficient transgenic animals.



Cortical tissue from control and insulin-deficient animals was probed for phosphorylated proteins in the insulin signaling pathway using immunoblot. Representative lanes are shown of blots using antibodies to (A) the phosphorylated insulin receptor at Tyr972 (pIR), the site required for docking of adapter protein IRS1, with insulin receptor and (B) the phosphorylated Ser473 of AKT (pAKT).

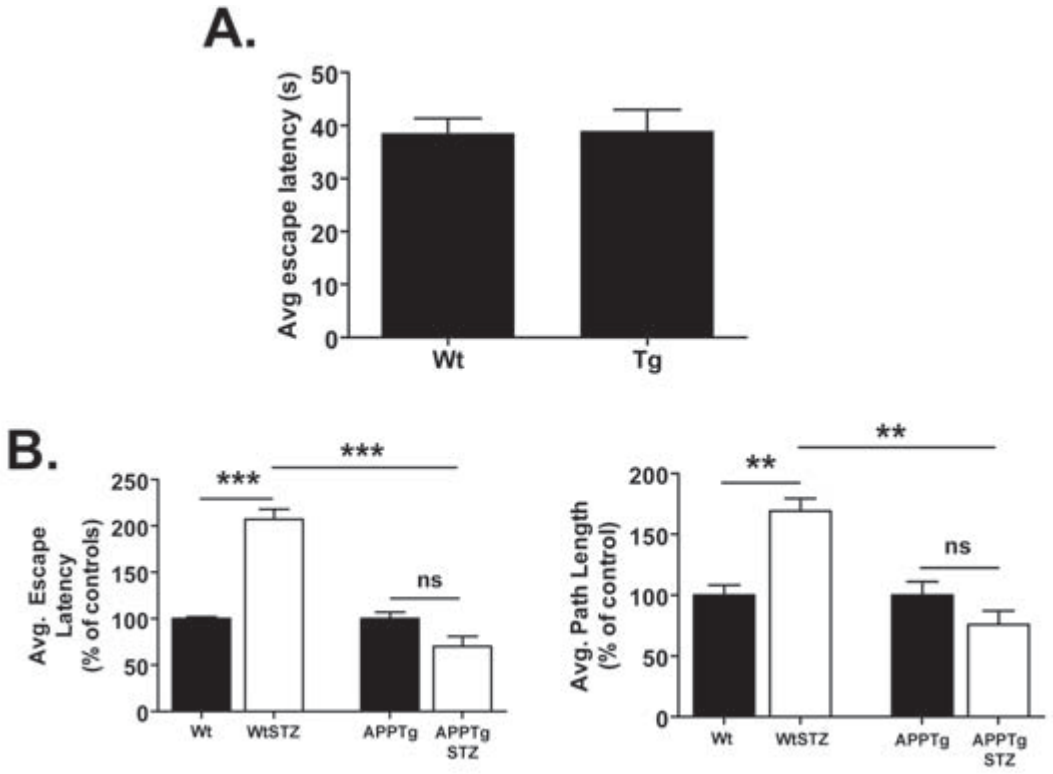
Figure 19: mRNA expression of insulin/IGF signalling proteins in brain.



Real time qRT-PCR was used to analyze gene expression in hippocampal tissue from experimental groups. No differences in expression were observed in insulin (A), insulin receptor (InsR, B), IGF1 (C), IGF1R (D) or IGF2R (F) in any groups. IGF2R mRNA dropped significantly in insulin depleted wild type animals (WtSTZ) versus wild type

controls (E). However, levels were unaffected by hypoinsulinemia in transgenic animals compared to transgenic alone. (Mean +/- SEM, one way ANOVA, Tukey's post hoc, n=4-7/group, * $p<0.05$, ** $p<0.001$)

Figure 20: Hypoinsulinemia decreases cognition in wild type, but not transgenic, animals.



Memory retention was determined by Morris water maze (MWM). Pre-injection MWM escape latency times were unchanged between the two groups at 8 weeks of age (t-test, $p>0.05$) (A). Four weeks after diabetes induction, hypoinsulinemic wild type animals (WtSTZ) were significantly impaired in MWM performance as measured by average escape latency and average path length over 3 days compared to wild-type animals (Wt). However, hypoinsulinemic transgenics (APPTgSTZ) were unchanged compared to transgenic controls (APPTg) in terms of performance (B). (t-test used to compare Wt and WtSTZ, APPTg and APPTgSTZ, average of three blocks (n=3) with n=4-6/experimental group, ** $p<0.01$, *** $p<0.001$)

Discussion

In CNS neurons, sAPP promotes both neuronal activity and survival through an unknown mechanism. In this study, we discovered that sAPP activated insulin receptors and the insulin signaling pathway, both *in vitro* and *in vivo*. In neuronal cultures, sAPP increased insulin receptor phosphorylation in a dose-dependent manner, as well as stimulated neurite outgrowth through PI3K and AKT. We also found a physical interaction between insulin receptors and sAPP that coincided with insulin receptor phosphorylation in APP overexpressing animals, supporting the hypothesis that sAPP is a ligand for the insulin/IGF pathway. STZ-induced insulin deficiency in wild-type animals caused reduced insulin receptor phosphorylation, which may have prevented by sAPP in transgenic animals. Additionally, memory impairments due to insulin depletion were prevented by APP overexpression, potentially due to a sAPP: insulin receptor interaction. Therefore, this study indicates that sAPP stimulates the insulin/IGF pathway *in vivo*, promoting neurotrophic actions.

Insulin/IGF receptor stimulation is mediated by multiple ligands, including insulin, IGF1 and IGF2 (Belfiore et al., 2009), and Aβ interactions with the insulin receptor indicate sAPP may directly stimulate insulin signaling as well (Xie et al., 2002; Zhao et al., 2008; Giuffrida et al., 2009). The similar effect that sAPP and insulin has on synaptic plasticity and LTP also suggests a commonality of function (Liu et al., 1995; Chen and Leonard, 1996; Ishida et al., 1997; Liao and Leonard, 1999; Skeberdis et al., 2001; Man et al., 2003). In the current study, sAPP stimulated insulin receptor phosphorylation without insulin, suggesting effects of sAPP independent of ligand. This hypothesis is also supported by the short time course of experimental treatment (15 min), as phosphorylation was unlikely to be due to activation of an alternate signaling pathway. In

addition, insulin receptors, and not other receptors in the insulin/IGF pathway, were specific targets of sAPP activation, as determined via immunoprecipitation with a specific insulin receptor antibody. Increased AKT phosphorylation downstream and neurite outgrowth entirely mediated by AKT suggests that sAPP/insulin receptor interaction was functional. This also supports previous reports that sAPP activity requires IRS1, an insulin signaling adapter protein (Wallace et al., 1997; Luo et al., 2001). Thus, here sAPP acted as a functional agonist of the insulin receptor in neuronal cultures.

Co-immunoprecipitation (co-IP) of sAPP:insulin receptor complexes with separate antibodies to either insulin receptor or APP revealed a physical interaction between the proteins in both normoinsulinemic and hypoinsulinemic transgenic mouse cortex. However higher amounts of sAPP bound to insulin receptors in insulin-deficient mice imply a competitive binding mechanism between sAPP and insulin for insulin receptor activation, and supports the hypothesis that sAPP and insulin share a binding site on the insulin receptor.

Aβ peptides are also capable of binding and interacting with the insulin receptor, resulting in modulation of insulin pathway activation (Xie et al., 2002; Zhao et al., 2008). Because the TgCRND8 mouse produces Aβ peptides, it was important to determine if Aβ may also bind to insulin receptor in immunoprecipitation experiments, since the 6E10 antibody used in these experiments interacts with a 16-mer sequence found in human sAPP, flAPP and Aβ. However, neither Aβ monomers nor oligomers were detected in any insulin receptor co-IP samples (Fig. 3). This is likely due to the young age (8 weeks) at which transgenic animals were injected, because Aβ binding would be expected to be very light at this age.

Insulin depletion had substantially different effects on insulin pathway activation in wild-type compared to transgenic animals. Following STZ injection, wild-type animals had less insulin receptor and AKT phosphorylation than uninjected animals (Figure 4), in agreement with previous studies (Sugimoto et al., 2008; Jolivalt et al., 2009). Therefore, simulation of the insulin signaling pathway was not possible following peripheral insulin depletion, with no ligand able to compensate for a reduction in insulin levels. However a similar situation did not arise in hypoinsulinemic transgenic animals (APPTgSTZ), suggesting that reduced insulin signaling due to insulin depletion was completely prevented through sAPP-induced insulin receptor activation. Thus, sAPP may have been able to replace insulin as an insulin receptor ligand in hypoinsulinemic transgenic animals, a mechanism suggested by insulin-free *in vitro* experiments.

Insulin-like growth factor-2 (IGF2) activates the insulin signaling pathway through insulin receptor homodimers, mediating neurodevelopment and neuroprotection (Cheng and Mattson, 1992; Belfiore et al., 2009). In type 1 diabetic rats, a drop in IGF2 levels in the hippocampus precedes the onset of memory deficits in Morris water maze tests (Li et al., 2002). Insulin replacement therapy, however, can reverse reduced IGF2 mRNA levels in insulin deficient animals (Wuarin et al., 1996). Therefore, decreased IGF2 is a marker for loss of insulin action. In agreement with previous studies (Wuarin et al., 1996; Li et al., 2002), diabetic wild-type animals in this study had significantly less IGF2 expression than wild-type controls (Figure 5). However despite being as insulin deficient as hypoinsulinemic wild type animals (Table 2), IGF2 mRNA levels did not decrease in TgAPPSTZ animals. Thus, sAPP stimulation of insulin receptors may have restored IGF2 expression, much as insulin replacement therapy does in wild-type insulin-deficient animals. Restored expression of IGF2, in addition to sAPP-induced insulin pathway

activation, may have contributed to stimulation of insulin/IGF1 signaling in hypoinsulinemic transgenics.

Clinical studies have revealed that reduced insulin signaling increases the risk of developing dementia (Kalmijn et al., 1995; Ott et al., 1996; Stolk et al., 1997b; Stolk et al., 1997a). Insulin depletion following streptozotocin injection causes spatial memory impairments in rodents assessed by MWM (Biessels et al., 1998; Tuzcu and Baydas, 2006; Reisi et al., 2009). Our insulin deficient wild type animals also had significant memory impairments compared to wild-type controls, with longer average escape latency time and total path length to platform. However, hypoinsulinemic transgenics displayed no memory impairments compared to untreated transgenics. Lower insulin signaling due to insulin deprivation may have been replaced by both sAPP and IGF2, protecting transgenic animals from cognitive deficits associated with insulin insufficiency. Both brain weights and blood glucose levels were similar for wild-type and transgenic injected groups (Table 2), indicating that increased performance in hypoinsulinemic transgenics was not due to reduced brain wasting or altered STZ response, respectively, compared to injected wild-type animals. Hence, APP overexpression could entirely overcome insulin depletion during this study (6 weeks), indicating again that sAPP could be more than adequate in replacing insulin signaling.

Reduced insulin signaling can often result in AD-related pathology exacerbation in APP overexpressing animals, especially in older animals (Ho et al., 2004; Burdo et al., 2009; Jolivalt et al., 2009). However, the use of young animals (8 weeks) and a short time diabetic time course (6 weeks) in this study ensured activation of insulin receptors by sAPP while reducing complications due to increased Aβ and insulin deficiency. We expect that an extended time course in our model would lead to a full reversal of

neurotrophic sAPP effect, since Aβ accumulation and poor metabolic health due to long term diabetes would enhance cognitive difficulties in injected animals compared to transgenics alone, resulting in the situation observed in older hypoinsulinemic TgCRND8 animals of the Jolivalt et al 2009 study. Therefore, the aim of this study was to introduce insulin deficiency in asymptomatic animals, prior to formation of detectable Aβ, and terminate the study just as TgCRND8 animals began to display Aβ-induced impairments. Thus, we maximized exposure of insulin receptors to sAPP and reduce any contribution of Aβ peptides to insulin signaling, a situation confirmed by high sAPP:insulin receptor ratios and absent Aβ:insulin receptor complexes in hypoinsulinemic transgenic co-IP experiments (Figure 3). As a result, sAPP activation of insulin receptors may have delayed onset of diabetes-induced memory impairments, over the short time course of this study.

Traditionally, activation of the insulin/IGF1 pathway was initiated by only three ligands: insulin, IGF1 and IGF2. With sAPP able to completely compensate for lost insulin signaling in transgenic diabetics, sAPP could be as potent a ligand of the pathway as the aforementioned conventional agonists. In fact, this study may introduce the possibility that sAPP is a normal contributor to basal insulin signaling in the brain, through the insulin/IGF1 pathway, and should be categorized as an insulin receptor activator. Further analysis will be required to understand the conditions under which sAPP normally contributes to neurotrophic signaling via the insulin/IGF1 pathway.

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Chapter 7 – Conclusions

AD is a complex disorder characterized by interruption of metabolic activity, neuronal dysfunction, and eventually neuronal death. Little can be definitively stated about disease etiology, despite identification of pathological markers such as Aβ, and their associative signaling pathways. The projects described within this thesis were originally designed to elucidate components normally affected in AD using *in vitro* and *in vivo* models, or to increase the understanding of how neurons behave under conditions of AD-induced neuronal stress. In doing so, several cell signaling pathways have been partially elucidated , in the hope that future research may provide methods to manipulate these pathways to alter disease pathology.

Altered presenilin gene sequences are the most commonly observed FAD mutation (Sherrington et al., 1995) and molecular clarification of neurons from mutant PS1 transgenic mice could be important for understanding disease progression in the human condition. Survivability of mPS1 neurons following excitotoxic insult is questionable, with opposing reports of either enhanced cell death or no change upon Aβ exposure (Schapansky et al., 2007). In our hands, a combination of both situations was discovered. mPS1 neuronal death was no different than C57Bl cultures when exposed to Aβ, despite elevated ER calcium release as others have reported (Leissring et al., 1999a; Leissring et al., 1999b; Leissring et al., 2001; Schneider et al., 2001). However, mPS1 neurons also demonstrated higher than normal NF-κB activation that prevented Aβ-induced cell death. NF-κB activity was critical for mPS1 neuronal survival, as attenuation of NF-κB function resulted in greater Aβ-mediated cell death, making NF-κB a vital neurotrophic protein in neurons with this PS1 mutation. Thus the lack of neuronal

deficits commonly associated with mPS1 FAD mice (Holcomb et al., 1999; Janus et al., 2000; Huang et al., 2003; Lalonde et al., 2003) could be a result of reduced neuronal pathology due to mPS1-enhanced NF-κB DNA binding activity. Indeed, enhanced LTP observed in younger mPS1 mice (Parent et al., 1999; Barrow et al., 2000; Zaman et al., 2000; Odero et al., 2010) could partially be explained as a result of enhanced NF-κB activity, as NF-κB can alter synaptic plasticity in hippocampal neurons (Albensi and Mattson, 2000). Yet early disease onset in mPS1-containing humans indicates additional significant neuronal impairment/death that increased NF-κB cannot compensate for. A follow-up study on older mPS1 animals would be important to establish how aging and chronically-impaired ER calcium dyshomeostasis could compromise ER calcium enhanced NF-κB activity and resultant inhibition of pro-apoptotic CHOP.

The therapeutic value of manipulating PS1 function in AD is still undecided due to the many roles the protein appears to have, both on its own and as part of the γ secretase complex. As many substrates of the complex are required for basal neuronal function and/or development (De Strooper et al., 1999; Kopan and Ilagan, 2004; Mei and Xiong, 2008), total PS1 inhibition to prevent Aβ production is not a viable option. In addition, PS1s role as an ER calcium leak channel (Tu et al., 2006) would indicate the protein is crucial for maintaining ER calcium homeostasis, and PS1 inhibition would likely result in an ER stress response that would to neurotoxic (Berridge, 2002; Verkhratsky and Toescu, 2003). However, a moderate reduction in PS1 activity might prove beneficial, limiting γ secretase activity to decrease Aβ production while simultaneously increasing NF-κB activity via enhanced ER calcium efflux in response to cellular stress. Yet PS1 inhibition would require a subtlety likely beyond our current knowledge of pharmacology and genomic manipulation, to prevent an ER stress response

and excitotoxicity. Nevertheless, this project demonstrated that neurons with compromised PS1 function may only exhibit increased death when the NF-κB system is compromised or overwhelmed, and alludes to the importance of a healthy neurotrophic balance for those that suffer from familial AD.

Calcium dyshomeostasis is a significant component of Aβ-induced cell death *in vitro*, and may be a major contributor to neuronal death in AD. Indeed, elevated calcium could be a starting point for AD cellular dysfunction, as many death cascades can originate with excitoxicity. The NRG-ErbB4 axis is a potential regulator of glutamatergic synapse formation, and can reduce ion currents through the AMPA receptor (Garcia et al., 2000; Huang et al., 2000; Li et al., 2007a). Calcium influx from NMDA receptors and resultant CICR from the ER is a major source of neuronal excitoxicity. Thus, NRG-modulation of glutamatergic synapses in AD patients by reducing Aβ-induced neuronal excitoxicity and resulting cell death. However, our published results revealed an opposite effect on intracellular calcium levels, with chronic neuregulin treatment increasing glutamate-mediated intracellular calcium (Schapansky et al., 2009). The effect was specific to glutamate receptor activation, as voltage-gated calcium channel activation did not replicate the effect. Furthermore, inhibition of ER calcium efflux prevented the NRG-glutamate effect. This study represents the first time that NRG has been shown to increase cytoplasmic calcium through glutamate receptor activation, and that the source of this elevated calcium was due to ER calcium release.

Based upon our data, manipulation of NRG signaling as therapy for AD is questionable but could still be possible. The original goal of establishing a functional signaling pathway for neuregulin β1 in neurons was to reduce high intracellular calcium observed in TgCRND8 neuronal cultures (Olson and Glazner, unpublished results), as it

was hypothesized that reduced AMPA currents would subsequently reduce NMDA receptor activity and thus excitotoxicity. NRG-induced increases in cytoplasmic calcium makes treatment of TgCRND8 cultures with an ErbB4 receptor agonist to prevent excitotoxic cell death seem counterintuitive. Yet, NRG treatment has been used to reduce severity of cerebral ischemia (Shyu et al., 2004; Xu et al., 2004; Xu et al., 2006), a disorder associated with chronic calcium excitoxicity. However, preliminary evidence would indicate NRGs effect in reducing infarct volume is related to inhibition of inflammation (Xu et al., 2004), and not related to regulating calcium homeostasis. Inhibition of ErbB receptor activity may be more practical in limiting Aβ-induced calcium dyshomeostasis, but this would affect other important physiological pathways regulated by NRG/ErbB signaling, such as myelination. While this calcium function needs to be characterized further to determine a valid physiological response, the use of NRG as a therapeutic agent to reduce ischemic neurodegeneration suggests that the pathway could be manipulated in a similar manner for AD prevention, perhaps in a manner unrelated to its ability to manipulate neuronal calcium dynamics.

A link between diabetes and AD has been well established at both a clinical and basic research level. Among symptoms of diabetes, a stronger correlation exists between AD and poor insulin signaling than with hyperglycemia, and thus increased attention is being given to how reduced neuronal insulin signaling can provoke dementia. In transgenic AD mice, insulin insensitivity and hyperinsulinemia results in exacerbation of AD-like symptoms including increased Aβ production and memory impairments (Ho et al., 2004; Cao et al., 2007). However, hypoinsulinemia can accelerate disease progression as well, as shown in our own animal model, the TgCRND8 (Jolivalt et al., 2009). Yet these studies commonly address acceleration of an existing AD-type

condition, where symptoms are often already present. Thus, our original focus was to establish if hypoinsulinemia could affect AD *onset*; hence STZ injections were done prior to Aβ deposition and any resulting AD symptoms. When preliminary data indicated that hypoinsulinemic TgCRND8 mice had no significant deficits, either in insulin signaling or spatial memory tests, the question was posed as to what agent associated with the hAPP mutation could compensate for reduced insulin in TgCRND8 animals. Aβ monomer can increase insulin signaling in cultured neurons (Giuffrida et al., 2009), and was initially believed to be the contributing factor in our *in vivo* model. Yet corroborating data from *in vitro* analysis indicated that secreted amyloid precursor protein alpha was the more likely neurotrophin, preventing deficits in the insulin pathway due to insulin scarcity.

In establishing potential therapies within each of the projects listed within this thesis, the strongest possible candidate is likely sAPPα with its interaction with the insulin pathway. The data displayed here is the first indication that sAPPα could act as a prospective biological ligand, possibly extending beyond the brain and into the entire body. In addition, further enhancement of insulin pathway activation beyond what we observed could still be possible, based on other variables that have not yet been tested. Isoforms containing a KPI domain to protect the protein from degradation (Turner et al., 2003) could keep the neurotrophin at the receptor longer for a prolonged insulin-like response. Also, APP signaling at theIGF1 receptor signaling has not been fully tested, and as ligand specificity is heavily dependent on receptor subunit composition (Belfiore et al., 2009), the strength of sAPP-insulin pathway interactions could be further modulated by different receptor combinations. However, the most promising characteristic of a sAPP-insulin signaling pathway is the higher receptor binding in the

absence of insulin *in vivo,* indicating that sAPP or derivatives of the peptide could be a strong therapy for insulin-dependent conditions such as diabetes.

Hyperinsulinemia and insulin insensitivity, both symptoms of type 2 diabetes and AD, compromises insulin degradation by saturating existing IDE protease and limits production of new IDE protein, respectively (Luchsinger et al., 2004; Zhao et al., 2004; Qiu and Folstein, 2006), forming a feed-forward cycle to increase insulin dysfunction. As a result, reduced IDE activity would thus contribute to further insulin insensitivity in both type 2 diabetes and AD, and also contribute to further Aβ aggregation. However, IDE production would likely have no effect on sAPP degradation, as the IDEs Aβ substrate recognizing sequence is not found within the sAPPα protein (Kurochkin, 1998). Thus, with no IDE-targeted degradation to limit its function, sAPP would remain longer at insulin receptor-containing synapses to enhance insulin signaling, increasing neurotrophic activity and, possibly more importantly, producing more IDE protein to catalyze excess insulin and/or Aβ removal. Further characterization of the sAPP/insulin axis in both type 2 diabetes and AD would provide invaluable information as to whether sAPPα to activate the insulin receptor in an insulin-resistant system as a treatment for those diseases. While entirely speculative, the potential benefit sAPPα activation of the insulin receptor could provide relief for numerous conditions involving insulin impairment.

Obviously, any discoveries made from these studies are only a small component of the research required to deliver a safe pharmacological product into the hands of physicians and patients. Establishing new therapies for diseases such as AD requires years of research, to go from the basic research level to clinical trials to test efficacy and safety in humans. In the case of both neuregulin, as a potential calcium homeostatic agent, and sAPPα, an insulin receptor ligand, the pathways studied here will need to be

characterized much more to begin even a discussion of therapeutic value. However, at the very least, we've partially established three separate signaling pathways that may contribute to a better understanding of AD pathogenesis.

Abbreviations

4-HNE – 4-hydroxynonenal

ACPD - 1-aminocyclopentane-1,3-dicarboxylic acid

AChE, I – Acetylcholinesterase, inhibitor

AD – Alzheimer's disease

Aβ – amyloid beta

AMPA - a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

ApoE – Apolipoprotein E

ATP – adenosine triphosphate

BBB – blood brain barrier

BSA – bovine serum albumin

CA – cytosine arabinoside

CCE – capacitative calcium entry

CHOP – C/EBP homologous protein

CICR – calcium-induced calcium release

CSF – cerebrospinal fluid

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

EGTA – ethyleneglycoltetraacetic acid

EMSA – electrophoretic mobility shift assay

ER – endoplasmic reticulum

FBS – fetal bovine serum

FAD – familial Alzheimer's disease

flAPP – full length amyloid precursor protein

GGF2 – glial growth factor 2

GLU - glutamate

GLUT – glucose transporter

HBSS – Hank's buffered saline solution

IDDM – insulin-dependent diabetes mellitus

IDE – insulin degrading enzyme

IGF – insulin-like growth factor

IP - immunoprecipitation

IP3 – inositol 1,4,5-triphosphate

IR – insulin receptor

IRS-1 – insulin receptor substrate 1

KPI – Kunitz protease inhibitor

LTP/LTD – long-term potentiation/depression

mGluR – metabotropic glutamate receptor

MMSE- mini-mental state exam

MnSOD – manganese superoxide dismutase

MRI – magnetic resonance imaging

MS – multiple sclerosis

NEP – neprilysin

NF-κB – nuclear factor κ B

NIDDM – non insulin-dependent diabetes mellitus

NMDA – N-methyl-D-aspartate

NRG (β1) – neuregulin (β1)

PBS(T) – phosphate buffered saline (with Tween)

PET – positron emission tomography

PI3K – phosphoinositide 3-kinase

PMSF - phenylmethylsulfonylfluoride

PS1,2 – presenilin-1,-2

qRT-PCR – quantitative real time polymerase chain reaction

ROS – reactive oxygen species

RuR – ruthenium red

RyR – ryanodine receptor

sAPPα – secreted amyloid precursor protein α

SDS-PAGE – sodium dodecyl sulfate poly acrylamide gel electrophoresis

SERCA – sarco-endoplasmic reticulum calcium ATPase

STZ – streptozotocin

TBS(T) – Tris buffered saline (with Tween)

TNF-α – tumor necrosis factor α

VGCC – voltage-gated calcium channel

Wt – wild-type

XeC – xestospongin C

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