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Stress Induced Protein 1 (STI1) protects neurons against Beta-Amyloid (Abeta) neurotoxicity

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology

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Abstract:

The glycosylphosphatidylinositol (GPI)-anchored Prion Protein (PrP^C) is known for mediating neurotrophic actions after binding to the Stress Induced Protein 1 (STI1). STI1 induces neuronal survival through Ca^{2+} influx via the PrP^C-alpha 7 nicotinic acetylcholine receptor (α7nAChR) complex. Recently, PrP^C was found to be a receptor for beta-amyloid oligomers (Aβ) and a mediator of Aβ neurotoxicity. We hypothesized that STI1 promotes neuronal survival against the neurotoxicity of Aβ. A Ca^{2+} signaling assay was used to test the STI1 and Aβ dependence on the PrP^C-α7nAChR complex for Ca^{2+} influx. Cell death assays were performed to assess the STI1 ability to protect against Aβ-induced neurotoxicity on embryonic hippocampal neurons. Aβ induced sustained Ca^{2+} influx in a PrP^C and α7nAChR dependent way. Aβ-induced neuronal death was dependent on the presence of PrP^C. STI1 rescued neurons against Aβ-induced neurotoxicity. Results indicate that STI1 can protect against Aβ, possibly through the PrP^C-α7nAChR complex.

Keywords:

Neuronal survival, hippocampus, Aβ neurotoxicity, STI1 neuroprotection, PrP^C functions, α7nAChR functions, Ca^{2+} signaling, cell death assay, therapeutic target

Co-authorship

The following people aided in my thesis work in the following ways:

Dr. F. Beraldo: in giving advice and guiding throughout my experiments

Anu Thomas: in helping me prepare the cultures

Dr. V. Ostapchenko: in providing multiple preparations of Aβ oligomers

Sanda Raulic: in preparing the recombinant STI1

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Also, I want to thank my family, who despite being so far, almost deserved to share co-authorship in this study!

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List of Abbreviations

AD - Alzheimer's Disease

STI1 – Stress Induced Protein 1

STI1 KO/WT – Heterozygous STI1 knockout

PrP^{C} – Cellular Prion Protein

SPs – Senile Plaques

NFTs – Neurofibrillary tangles

AC – Amyloid Cascade

Ca^{2+} - Calcium

Aβ – Amyloid Beta

APP – Amyloid Precursor Protein

α7nAChR – α7nicotinic Acetylcholine receptor

ACh – Acetylcholine

AChE - Acetylcholinesterase

ERK – Extracellular-signalling kinases

LTP – Long-term potentiation

MAPK - Mitogen-activated protein kinase

nAChR – Nicotinic acetylcholine receptor

NMDAR - N-methyl-D-aspartic acid receptor

PSD – Postsynaptic density

DIV – Days in vitro

GPI – Glycosylphosphatidylinositol

M129V – Methionine/Valine polymorphism

PRNP – Prion Protein gene

HSP 70 – Heat Shock Protein 70

HSP 90 – Heat Shock Protein 90

PKA – Protein Kinase A

COS-7 – CV1 Origin SV40 -7

1. INTRODUCTION

The number of Alzheimer's disease (AD) patients worldwide is increasing every year with projections reaching 65 million AD patients worldwide by 2030 (www.alzforum.org). The risk of developing AD increases with age and is of concern in Canada since the population of Canada is aging. Canada's senior population, individuals over the age of 65, has grown to 2.1% by 2011 and is projected to reach 14.1% of the overall Canadian population (www.hc-sc.gc.ca). Although age is the strongest risk factor for the most common type of dementia, AD, the natural aging process does not include the pathology observed in AD. With the continuous aging of a population, especially with increasing life expectancy, a solution needs to be found for AD. Currently, there is no way to prevent AD. As a result, there is considerable hope that research in the field of AD will produce preventative measures that cut the incidence rate of AD and alleviate the economic and social burdens of AD in Canada.

1 Background

1.1 Alzheimer's disease

AD is a progressive terminal disease that results in the degeneration of brain neurons. The two types of AD are i) early-onset AD, also known as Familial AD, with symptoms appearing before the age of 60, and ii) late-onset AD, also known as Sporadic AD, with symptoms appearing after the age of 60. Symptoms of AD include; lack of short-term memory, compromised decision-making, and communication difficulties (Braak, Del

Tredici, Schultz, & Braak, 2000; Braak, Rub, Schultz, & Del Tredici, 2006; Minati, Edginton, Bruzzone, & Giaccone, 2009; Morris & Price, 2001; Selkoe, 2001; Walsh & Selkoe, 2004). AD postmortem brains are characterized by general brain atrophy, ventricular enlargement, and accumulation of different protein aggregations (DeLegge & Smoke, 2008; Zipp & Aktas, 2006).

1.1.1 *Current Treatment Approach*

The majority of medications and most AD clinical research is focused on delaying or preventing the symptoms of AD, but not on preventing the disease. Drugs currently used to treat AD are either acetylcholinesterase (AChE) inhibitors or N-methyl-D-aspartate receptor (NMDAR) antagonists. Cholinergic tone is disrupted in AD patients due to a decrease in the level of acetylcholine (Ach) at the synapse (Lahiri, Rogers, Greig, & Sambamurti, 2004). The first line of drugs used to treat AD patients is the AChE inhibitors. AChE inhibitors increase the level of Ach at the synapse resulting in enhancement of cholinergic tone (Whitehouse, 1998). AChE inhibitors are widely used in treating the symptoms of AD.

Excessive release of the excitatory neurotransmitter glutamate can result in neuronal death, due to NMDA receptor mediated excitotoxicity. Memantine, an NMDAR antagonist, is administered for the treatment of moderate-to-severe AD since glutamate excitotoxicity and neuronal degeneration are characteristic of later stages of AD

(Hyman, 2011). However, neither AChE inhibitors nor Memantine can prevent the progression of AD (Schmitt, Bernhardt, Moeller, Heuser, & Frolich, 2004). Therefore, current treatment is focused on alleviating the symptoms of AD, but not on dealing with the proposed causes of AD, such as the accumulation of amyloid-β (Aβ).

1.1.2 *Amyloid Cascade Hypothesis*

Research conducted on AD is mainly driven by clinical observations made in the AD field. Some of the original discoveries in AD include the identification of insoluble aggregates of Aβ (Lesne et al., 2006), found in senile plaques (SPs); and the discovery of intracellular filaments of hyperphosphorylated tau protein, named neurofibrilary tangles (NFTs) (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995). Although SPs and NFTs were found to be reliable as diagnostic tools for AD in postmortem brains (Newell, Hyman, Growdon, & Hedley-Whyte, 1999), SPs and NFTs do not accurately predict the severity of AD (Katzman et al., 1988). Alternatively, SPs and NFTs are considered final molecular reservoirs of the active toxic species, the quantity of which could be more predictive of the progress of AD.

Genetic variations in the genes of the Amyloid Precursor Protein (APP) (Goate et al., 1991; Mullan et al., 1992) and enzymes involved in the cleavage of APP (γ-secretase complex) may be involved in the pathophysiology of AD. Mutations in these genes can

result in accumulation of Aβ peptide (Goate et al., 1991; Levy-Lahad et al., 1995) and increasing the occurrence of familial AD (Levy-Lahad et al., 1995). In addition, certain alleles of Apolipoprotein E (Castellano et al., 2011; Deane et al., 2008), a protein involved in the clearance of Aβ peptides, have been correlated to the incidence of AD (Mahley, Weisgraber, & Huang, 2006; Strittmatter et al., 1993). The discoveries of the insoluble SPs and the NFTs from analysis of postmortem brains, and the genetic tests that identified the factors involved in the production and accumulation of Aβ reinforce the original Amyloid Cascade (AC) hypothesis (J. A. Hardy & Higgins, 1992; Selkoe, 1991).

According to the AC hypothesis, the origin of AD pathogenesis is Aβ production, Aβ accumulation (Goate et al., 1991; Levy-Lahad et al., 1995) and intracellular deposition of NFT (Bondareff, Mountjoy, Roth, & Hauser, 1989; Grundke-Iqbal et al., 1986; Santacruz et al., 2005). The etiology of dementia according to the AC hypothesis is Aβ-induced neuronal dysfunction and death. Nonetheless, the original AC hypothesis, proposed by Drs. Hardy and Higgins in 1992 (J. A. Hardy & Higgins, 1992), attributes the symptoms of AD to the pathology caused by the insoluble depositions of Aβ peptides. Interestingly however, the accumulation of soluble Aβ oligomeric species correlates better with the progress of the disease than accumulation of insoluble plaques (McLean et al., 1999). Therefore, recent literature focuses on how Aβ is produced, why Aβ accumulates, and the molecular mechanisms utilized by the soluble mobile Aβ to induce neurotoxicity. Consequently, the reduction in Aβ generation and the enhancement of its clearance

have become one of the therapeutic approaches currently studied (Reitz, 2012). These trials rely on a body of evidence linking the neurotoxicity of soluble Aβ to deficits in synaptic plasticity. Indeed, impairment of synaptic plasticity and deformation of synaptic structure correlate better with the progress of AD (Terry et al., 1991) and occur prior to formation of Aβ deposits in AD transgenic mouse models (Hsia et al., 1999; Lambert et al., 1998). It is known that very small concentrations of Aβ oligomers can result in inhibition of long-term potentiation (LTP) (Cullen, Suh, Anwyl, & Rowan, 1997; Lambert et al., 1998), the cellular correlate of learning and memory. Furthermore, Aβ oligomers were found to induce major cognitive dysfunction when infused in the CNS (Cleary et al., 2005; Lesne et al., 2006). Together, these results emphasize the synaptotoxic effects of Aβ oligomers.

Although the AC hypothesis provides general links that aid in understanding the pathology of AD and the symptoms of dementia, it provides no molecular explanation of how Aβ can alter physiological signaling pathways leading to neuronal dysfunction and disruption of synaptic transmission. Thus, many hypotheses stemming from the AC hypothesis are aimed at understanding how soluble Aβ oligomers can disturb synaptic plasticity and cellular processes leading to neuronal dysfunction, stress, and death. There are many hypothesized Aβ-driven mechanisms that can be included in the cellular pathogenic cascade of the AC hypothesis. Some of the molecular mechanisms include involvement of NMDA receptors (J. Hardy & Selkoe, 2002; Hsia et al., 1999; Kamenetz et

al., 2003; Klein, 2002; Lambert et al., 1998; Walsh & Selkoe, 2004), AMPA receptors (Chang et al., 2006; Hsieh et al., 2006; Ikonomovic et al., 1997; Parameshwaran et al., 2007), mGlu receptors (Chin, Ma, MacTavish, & Jhamandas, 2007; Ikonomovic et al., 1997), and Post Synaptic Density (PSD) proteins (Kim & Sheng, 2004). Additionally and more importantly to the present study, Aβ has been shown to utilize the alpha7 nicotinic acetylcholine receptor (α7nAChR) and the cellular Prion Protein (PrP^C) on neurons as putative receptors to cause pathogenesis (Chen, Yadav, & Surewicz, 2010; Chung et al., 2010; Dineley, Bell, Bui, & Sweatt, 2002; Dineley et al., 2001; Lauren, Gimbel, Nygaard, Gilbert, & Strittmatter, 2009; Nygaard & Strittmatter, 2009; Wang, Lee, Davis, & Shank, 2000; Wang, Li, Benedetti, & Lee, 2003).

α7nAChRs and AD

1.1.3 *α7 Nicotinic Acetylcholine Receptor*

Nicotinic acetylcholine receptors consist of several classes. One of these classes is the homomeric α7. Nicotinic receptors consist of variable combinations of subunits arranged in a basic pentameric structure, which forms an ion pore (Sargent, 1993). The important roles of α7nAChRs in memory and synapse formation are due to the Ca^{2+} permeability of α7nAChRs (Broide & Leslie, 1999).

1.1.4 *Cholinergic Tone and Beta-Amyloid*

The α7nAChR is particularly abundant in the basal forebrain (Ikonomovic et al., 2009), where major axons feed into the hippocampus and neocortex from the cortex. In AD, these areas are considered vulnerable targets and are linked to the early manifestations observed in AD patients, such as impairment of hippocampus-based episodic memory and lack of attention (Doody et al., 2001; Minger et al., 2000). The pathological deterioration of areas with high α7nAChR expression causes cholinergic input to be impaired resulting in part of AD symptoms (Gray, Rajan, Radcliffe, Yakehiro, & Dani, 1996; Ji, Lape, & Dani, 2001; Rezvani, Bushnell, & Levin, 2002). Moreover, the symptomatic improvement of AD patients upon administration of cholinomimetics (Ach-like chemicals) (Lopez-Arrieta, Rodriguez, & Sanz, 2000, 2001; Quirion et al., 1995; Zamani & Allen, 2001) strengthened evidence in support of cholinergic malfunction being relevant to AD symptoms.

There is compelling evidence linking Aβ to alteration of glutamate release (Dougherty, Wu, & Nichols, 2003; Wu, Khan, & Nichols, 2007) and localization of NMDARs subunits modulated by α7nAChR through Ca^{2+} influx. α7nAChR modulates the ratio of NR2A- to NR2B- containing NMDA receptors on the synapse and extra-synapse (Snyder et al., 2005). Thus, by deregulating the function of α7nAChR, Aβ is able compromise the intricate ratio of NR2A- to NR2B- containing NMDA receptors. It has been shown that Aβ activates the mitogen-activated protein kinase (MAPK) pathway, which results in an

increase in AChE levels through activation of mGluR 1 and mGluR 5 (Small, Mok, & Bornstein, 2001). The subsequent increase in AChE activity increases the rate of Ach degradation and, consequently decreases cholinergic tone. As a compensatory strategy for the decreased synaptic activity in AD, postsynaptic receptors may increase in number (Davis & Goodman, 1998). Of the several nAChR subtypes localized in the brain, expression of α7nAChR was found to be elevated in transgenic mouse models engineered to produce human Aβ (Bednar et al., 2002; Dineley et al., 2001). In the same study, the increase in α7nAChR was observed before the formation of insoluble plaques in the AD mouse model, which emphasized the role of the soluble Aβ as the active species causing disruption of cellular homeostasis.

1.1.5 α7nAChRs *and Beta-Amyloid*

Aβ was found to bind to α7nAChR in picomolar affinity (Wang, Lee, D'Andrea, et al., 2000; Wang, Lee, Davis, et al., 2000) and to induce its activation (Dineley et al., 2002; Dineley et al., 2001). Interestingly, *Xenopus* oocytes expressing rat α7nAChR responded with small currents to nanomolar concentrations of Aβ (Dineley et al., 2002; Grassi et al., 2003). The small currents produced by α7nAChR show that α7nAChR can become inactive if continuously exposed to Aβ, and/or that *Xenopus* oocytes do not possess proteins needed for prolonged activation of α7nAChR. In another study, treatment with picomolar Aβ concentrations led to sustained increases in presynaptic Ca^{2+} via α7 and other nAChRs (Dougherty et al., 2003). Furthermore, Aβ treatment resulted in

intracellular Ca^{2+} influx increase in mouse cortical neurons derived from wild-type mice but not in neurons derived from the α7nAChR-knockout (Khan, Tong, Jhun, Arora, & Nichols, 2010; Mehta et al., 2009).

In contrast, other studies have shown that Aβ blocks α7nAChR (Liu, Kawai, & Berg, 2001; Pettit, Shao, & Yakel, 2001; Tozaki et al., 2002). Nonetheless, in these studies, a relatively high concentration of Aβ was needed completely block α7nAChR. Furthermore, blockage required pre-application of peptide, and inhibition was reversible and noncompetitive. The contradictory evidence for both stimulatory and inhibitory effects of Aβ on α7nAChR may reflect a complex biological interaction between the two proteins. The contrasting evidence also reflects different types of cells, different stoichiometry of Aβ preparations, or variable detection methods used in each laboratory.

Cellular Prion Protein (PrP^C): a New Player in AD

1.1.6 *Cellular Prion Protein*

The Cellular Prion Protein (PrP^C) is a glycosylphosphatidylinositol (GPI)–anchored protein present at the cell surface and in intracellular compartments (Madore et al., 1999). PrP^C is highly expressed in both the central and peripheral nervous systems from early development to adulthood. Moreover, PrP^C is important in signaling pathways that are responsible for neurotrophic activities (Chiarini et al., 2002). The role of PrP^C started

to emerge in experiments where PrP^C expression prevented neuronal cell death of PrP^C-null hippocampal-derived cells cultured on serum-free media (Kuwahara et al., 1999). These early results were reinforced by studies showing a possible neurotrophic role for PrP^C (Coitinho et al., 2007; Martins et al., 2010; Roffe et al., 2010). Some authors have proposed that PrP^C acts as a docking element on the cell membrane integrating multiple signaling pathways by scaffolding sets of extracellular and transmembrane molecules on lipid rafts (Martins et al., 2002).

1.1.7 *Cellular Prion Protein and Beta-Amyloid*

Recently, expression-cloning experiments were conducted on CV1 Origin SV40 -7 (COS-7) cells to identify receptors for Aβ oligomers. COS-7 cells were chosen for the expression cloning experiments because they showed low levels of binding of Aβ oligomers compared to hippocampal cells. These unbiased studies identified PrP^C as a high-affinity binding site for Aβ oligomers (Lauren et al., 2009). Aβ oligomers bind to PrP^C on amino acids 95-110 (Lauren et al., 2009) and 23-27 (Chen et al., 2010). The PrP^C 95-110 stretch of amino acids lies within an unstructured central domain of PrP^C involved in neuronal toxicity in vitro and in neurodegeneration in mice (Baumann et al., 2007).

On mice hippocampal slices, Aβ oligomer treatment resulted in a decrease in synaptic strength in a PrP^C-dependent fashion. Despite the fact that many subsequent studies confirmed the binding of Aβ oligomers to PrP^C (Chen et al., 2010; Chung et al., 2010; Lauren et al., 2009), not all studies found that the effects of Aβ are PrP^C –dependent (Calella et al., 2010; Kessels, Nguyen, Nabavi, & Malinow, 2010). This controversy may be related to variations in the methodology used to study synaptic plasticity, the source of Aβ peptides or the animal models used. Although binding of PrP^C to Aβ oligomers and the consequences of this binding to synaptic plasticity were described, the molecular mechanisms leading to pathogenesis are not understood.

1.1.8 *Cellular Prion Protein and AD*

The methionine/valine (M129V M129V) polymorphism of the PrP^C gene, PRNP, is suspected to have links with the sporadic form of AD. Homozygosity at this codon resulted in variations at the unstructured central domain of PrP^C, the domain where Aβ oligomers bind to PrP^C. The variations at the unstructured domain of PrP^C were linked to Creutzfeldt-Jakob disease, a neurodegenerative disease resulting from the improper folding of PrP^C (Palmer, Dryden, Hughes, & Collinge, 1991). One study showed that the (M129V) genetic variation is linked to increased risk of AD (Del Bo et al., 2006). However, there is no evidence indicating that the molecular basics of the link between

the M129V polymorphism and risk of AD is due to increased binding of Aβ oligomers to PrP^C.

Moreover, there is conflicting evidence regarding the cognitive deficits and behavioral abnormalities observed in AD mouse models. Lack of PrP^C rescued behavioral abnormalities and early death in a mouse model of AD (Gimbel et al., 2010). However, AD-related behavioral abnormalities were not PrP^C-dependent in another paper dealing with a different mouse model of AD (Cisse et al., 2011).

1.2 Stress Induced Protein 1: a PrP^C ligand

1.2.1 *Stress Induced Protein 1 (STI1)*

The STI1 protein is a conserved 66-kDa protein found in many distant species. STI1 was originally discovered in yeast where it was shown to interact with heat shock protein 70 and 90 (HSP 70 and HSP 90), and this interaction is important for the proper folding of proteins (Bukau, Weissman, & Horwich, 2006; Caplan, Mandal, & Theodoraki, 2007; Picard, 2006; Pratt & Toft, 1997, 2003; Wegele, Muller, & Buchner, 2004). STI1-null embryos did not survive past an early developmental embryonic phase, indicating an important role of STI1 in mouse embryogenesis (Beraldo et al., in preparation). Furthermore, mouse embryonic fibroblasts derived from STI1-null embryos could not survive in culture (Beraldo et al., in preparation). The fact that STI1 deficiency produces

a lethal phenotype provides evidence that STI1 may have a crucial role at the cellular level.

1.2.2 *Stress Induced Protein 1, PrP^C, and α7nAChR complex*

The interaction between STI1 and PrP^C was originally discovered in 1997. At that time, it was thought that STI1 acted as a PrP^C receptor (Martins et al., 1997). Regardless of the nature of the interaction, PrP^C and STI1 binding resulted in activation of signaling pathways such as the cAMP-Protein Kinase A and MAPK pathways leading to neurotrophic activities (Chiarini et al., 2002; Zanata et al., 2002). Later, it was shown that STI1 is neuroprotective and induces neuritogenesis in a PrP^C-dependent fashion when binding to neurons (Lopes et al., 2005). In 2007, a different theory was proposed for the interaction between STI1 and PrP^C. STI1 was described as a PrP^C ligand secreted by astrocytes that can protect against cell death (Lima et al., 2007). Moreover, infusion of the rat hippocampus with antibodies for PrP^C and STI1 resulted in cognitive behavioral deficits (Coitinho et al., 2007). Overall, these results suggest that the interaction of PrP^C and STI1 may be important for general neurotrophic actions and cognitive functioning.

In an attempt to elucidate the cellular functions and signaling pathways activated by STI1, recent studies demonstrated that Ca^{2+} signaling may be the trigger for the STI1-mediated and PrP^C-dependent physiological functions (Beraldo et al., 2010). Indeed,

recent results showed that the α7nAChR is required for transduction of signals by the STI1-PrP^C complex by allowing Ca^{2+} influx into neurons (Beraldo et al., 2010). Further, the same study showed that the STI1-induced neuroprotection is dependent on PrP^C and α7nAChR.

1.3 Aβ oligomers and Stress Induced Protein 1

There is evidence that STI1 and Aβ oligomers may need PrP^C to execute their effects (Beraldo et al., 2010; Chiarini et al., 2002; Lauren et al., 2009; Lopes et al., 2005; Resenberger, Winklhofer, & Tatzelt, 2012; Zanata et al., 2002). The binding sites of both STI1 and Aβ are adjacent on PrP^C (Chen et al., 2010; Lauren et al., 2009; Zanata et al., 2002). Also, there is evidence that STI1 and Aβ oligomers require the presence of α7nAChR to induce Ca^{2+} influx (Beraldo et al., 2010; Dougherty et al., 2003). Nonetheless, STI1 and Aβ oligomers have completely different effects on neurons; neurotrophic versus neurotoxic, respectively. Therefore, there may be competition between STI1 and Aβ oligomers for the PrP^C-α7nAChR complex.

Treatment of neurons with Aβ oligomers have been shown to result in Extracellular Regulated Kinases 1/2 (ERK1/2) phosphorylation (Chong et al., 2006; Ghribi, Prammonjago, Herman, Spaulding, & Savory, 2003; Ma et al., 2007). In a recent study it was shown that Aβ-induced ERK 1/2 phosphorylation may be PrP^C-dependent (Caetano

et al., 2011). Activation of ERK 1/2 is a common signaling pathway activated by Aβ peptides and at least one report suggests that this Aβ effect is dependent on α7nAChRs (Dineley et al., 2001). STI1 is also known to activate ERK1/2 phosphorylation after binding to PrP^C and induce neuritogenesis (Lopes et al., 2005). Although both ligands activate ERK1/2, each ligand can do so in different ways. The duration and the localization of the ERK1/2 activation can be different for both ligands.

1.3.1 *Evidence for Direct Competition on PrP^C*

The binding sites of Aβ oligomers and STI1 on PrP^C are adjacent. STI1 binds to amino acids 113-125 on PrP^C (Zanata et al., 2002); on the other hand, one of the binding sites of Aβ oligomers to PrP^C spans amino acids 90-105 (Lauren et al., 2009). Using Surface Plasmon Resonance, our laboratory has studied the interaction between PrP^C, STI1, and Aβ oligomers. These results show that STI1 and Aβ oligomers cannot bind to PrP^C concurrently, and that a competitive nature may exist between the two ligands upon binding to PrP^C (Beraldo et al., unpublished).

1.3.2 *Potential Roles for STI1 against Aβ oligomers and in AD*

In unpublished work conducted in collaboration with Dr. Vima Martin's laboratory, it was demonstrated that STI1 prevents the loss of synapses induced by Aβ oligomers on cultured neurons in a PrP^C-dependent fashion (Hajj et al unpublished). Furthermore, in order to study the effect of STI1 in a mouse model that reproduces neurodegenerative

diseases, our laboratory started by quantifying the level of STI1 in a mouse model of Alzheimer's disease (APPSwe/PS1dE9). The transgenic mouse model shows progressive accumulation of Aβ oligomers and increased plaque load (Ashe & Zahs, 2010; Duyckaerts, Potier, & Delatour, 2008). In this mouse model, levels of STI1 decreased by 50% during the initiation of amyloidosis (6 months), and this may be due to increased degradation of the protein. Interestingly, the levels of STI1 are restored back after an increase in STI1 mRNA levels at 9 months of age.

2 RATIONALE/HYPOTHESIS

2.1 Rationale

Overall, STI1 neurotrophic functions have been shown to be PrP^C- and α7nAChR-dependent (Beraldo et al., 2010), which suggested that STI1 utilizes the PrP^C-α7nAChR complex. As suggested by recent studies, STI1 neurotrophic functions may be important in protecting neurons against different neuronal insults (Chiarini et al., 2002; Lopes et al., 2005; Zanata et al., 2002). The reduced levels of STI1 in the APPSwe/PS1dE9 AD mouse model suggested that STI1 may be degraded. The consequences of reduced levels of STI1 in AD-related pathology are not known. On the other hand, the binding of Aβ oligomers to PrP^C (Chen et al., 2010; Lauren et al., 2009), the reported PrP^C-mediated Aβ neurotoxicity(Lauren et al., 2009; Nygaard & Strittmatter, 2009), and the involvement of this binding domain in neurodegenerative diseases (Palmer et al., 1991) strengthened the possibility for a PrP^C role in AD. However, PrP^C can also bind to STI1 and mediate its neurotrophic effects (Chiarini et al., 2002; Lopes et al., 2005; Zanata et al., 2002). Further, the involvement of α7nAChR in the pathology of AD (Dineley et al., 2002; Dineley et al., 2001; Dougherty et al., 2003; Dziewczapolski, Glogowski, Masliah, & Heinemann, 2009) and its necessity in mediating the neurotrophic actions of the STI1-PrP^C (Beraldo et al., 2010) strengthens the possibility for a PrP^C-α7nAChR complex role in the pathological aspects of AD and in normal neural physiology.

It is unknown if Aβ requires PrP^C and α7nAChR as a complex to mediate its neurotoxicity. Thus, STI1 might be able to protect against Aβ because Aβ may also utilize the PrP^C-

α7nAChR. Understanding how STI1 may be able to protect against Aβ through the PrP^C-α7nAChR complex may give us insights into a new potential therapeutic target, the PrP^C-α7nAChR complex.

2.2 Hypothesis

STI1 blocks the deleterious effects of Aβ oligomers on neurons via the PrP^C-α7nAChR complex.

Objective 1: Study the effect of Aβ oligomers on the PrP^C-α7nAChR complex with respect to Ca^{2+} influx and cell death

Objective 2: Assess the ability of STI1 in protecting against oligomeric Aβ-mediated toxicity via the PrP^C-α7nAChR complex

3. MATERIALS AND METHODS

3.1 Animals, Neuronal Preparation, and Cultures

Heterozygous STI1 knockout mice were generated as described by Beraldo et al., (in preparation). Briefly, non-conditional heterozygous STI1 knockout mice were generated by Ozgene (Australia) using standard homologous recombination techniques on C57BL/6jES embryonic stem cells. Firstly, chimeric mice were bred to C57BL/6j mice to result in germline transmission of the floxed STI1 allele. Subsequently, F1 mice were crossed to universal Cre mice to remove loxP flanked regions. The Cre recombined mice were then bred to C57BL/6j mice and the progeny was used to expand the mouse colony. PrP^{C}-knockout, generously donated by the Jirik laboratory, and α7nAChR-knockout, generously donated by the Jackson laboratory, mice were generated as described by Büeler et al., 1992 and Orr-Urtreger et al., 1997 (Manson et al., 1994; Orr-Urtreger et al., 1997), respectively. Primary cultured neurons were derived from E17 embryos by extracting the hippocampus from the embryonic brains. Subsequently, they were kept *in vitro* for 5 or 11 days before treatment. For cell death assays using neurons, $1.0X10^{5}$ cells were plated on 24-well plates or 4-well plates. Neurons were initially plated in wells coated with Poly-L-Lysine using neuronal plating medium. The media consisted of 10% heat inactivated Fetal Bovine Serum (Invitrogen, Grand Island, NY), 0.45% glucose (BioRad, Hercules, CA), 1% Sodium Pyruvate (Invitrogen), 1% Glutamine (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), and Minimum Essential Media (MEM) (Invitrogen). Two to four hours after plating, wells were gently shaken by

hand to remove any non-adherent cells and plating medium was replaced with neuronal maintenance medium. The maintenance media contained 2% B-27 supplement (Invitrogen), 1% Glutamine, 1% Penicillin/Streptomycin and Neurobasal medium (Invitrogen). Neurons were then cultured at 37 °C for 5 or 11 days *in vitro* (DIV).

3.2 Preparation of Aβ Oligomers and Recombinant STI1

Aβ peptide was purchased from *r-peptide* company, cat# A-1002-2 and oligomers were prepared in the laboratory according to Barghorn et al., 2005 (Barghorn et al., 2005). Briefly, Aβ peptide (1–42) was stored at −80°C prior to resuspension and was monomerized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma H-8508). After being stored at -80°C, anhydrous DMSO was added to the samples. The samples were then sonicated and left to oligomerize at 4°C for 24 hours. Samples were then left at -80°C until used for the studies. A scrambled peptide purchased from the same company (r-Peptide, Cat # A-1004-2) was prepared using the same procedures as for the non-scrambled form and was used as a negative control. All oligomeric preparations were verified using western blotting before using them to treat our cultures.

Recombinant STI1 was prepared according to Zanata et al., 2002 (Zanata et al., 2002) and kindly provided by Ms. Sanda Raulic. Briefly, protein expression was induced in *Escherichia coli* DH-5α by isopropyl-β-D-thiogalactopyranoside (IPTG). *E. coli* DH-5α

contains the expression vector His_6-STI1. Bacteria cells were then resuspended in lysis buffer and lysed in French press. Protein purification then followed.

The concentration of STI1 used for all experiments was 1μM. For the cell death assay, neurons were incubated with 1μM STI1 for 1 hour at 37 °C before treatment with Aβ oligomers. For Ca^{2+} signaling experiments, STI1 (1μM) was directly added to the Ca^{2+}-supplemented buffer.

3.3 Cell Death Assay

Primary hippocampal neurons ($1X10^4$) were treated with Aβ oligomers (1μM) for 48 hours 5 or 11 days after culture. For treatments involving STI1 or $STI1_{\Delta 230-245}$, neurons were preincubated with STI1 (1μM) or $STI1_{\Delta 230-245}$ (1μM) for 1 hour and then treated with Aβ oligomers (100nM, 500nM, 1μM, and 3μM) for 24 hours or 48 hours. The cell death assay used was the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) for mammalian cells. This kit contains Calcein-AM (Ex/Em 494nm/517nm - green) that labels live cells and Ethidium Homodimer-1 (517nm/617nm Ex/Em - red) that labels dead cells. Calcein-AM gets into live cells via the AM hydrophobic group and fluoresces after the AM group is cleaved off by the esterase activity of live cells. Ethidium homodimer 1 gets into dead cells with ruptured membranes and binds to DNA. The images of 5-8 fields were taken using a Zeiss LSM-510 confocal microscope (Carl Zeiss, Oberkochen, Germany). Image J software was used for counting cells following cell imaging. The formula [# of dead cells (red cells)/# of dead cells (red cells) + # of viable cells (green cells)] X 100 was used to calculate the % of cell death among neuronal

cultures. At least 3 independent experiments were conducted for testing PrP^C-dependent cell death. At least 10 independent experiments were conducted for testing the sensitivity of STI1 heterozygous-knockout neurons. One-way ANOVA and the Newman Keuls posttest were used for statistical analysis.

3.4 Ca^{2+} Imaging

For Ca^{2+} imaging, the UV excitable, ratiometric (ratio of double excitation wavelengths) Ca^{2+} indicator Fura-2 (Ex 345/380, EM 510) (Invitrogen) (Tsien, Rink, & Poenie, 1985) was used to quantify Ca^{2+} influx in neurons. Fura-2 has an AM group that allows Fura-2 to get passed the cell membrane into the cytosol of the cell. The AM gets cleaved off by the esterase activity of the cell and it gets excited at 380nm wavelength when its Ca^{2+}-free and excited at 340 nm when its Ca^{2+}-bound.

After plating neurons on gamma irradiated 35 mm uncoated glass bottom (14 mm microwell) culture dishes purchased from MatTek Corporation, neurons (4.1 X 10^4 cells) were kept 5 days or 11 days in vitro (DIV). Subsequently, cells were loaded with 10 µM Fura-2 diluted in neurobasal medium for 40 minutes. Cells were then washed three times with Ca^{2+}-free 1 X Kreb's buffer (1.3 M NaCl, 25 mM KCl, 250 nM $NaHCO_3$, 12 mM NaH_2PO_4, 12 mM $MgCl_2$) made in our laboratory. Subsequently, Kreb's buffer supplemented with Ca^{2+} to a final concentration of 1mM was added to the cells on the dish. Cells were kept at 37°C for an extra 20 minutes for the AM group on Fura-2 to be

hydrolysed before imaging. Differential interference contrast (DIC) light was used to focus on cells. Cells were subsequently imaged using excitation detection wavelengths 340 nm and 380 nm, alternatively. Imaging of each dish took approximately 5 minutes from the point of adding either STI1 or Aβ oligomers. Approximately 15-30 neurons were present in every field taken for Ca^{2+} imaging.

For analysis of Ca^{2+} influx, the excitation ratio (F340/F380) of the ratiometric indicator Fura-2 and time were recorded in Excel. Each cell was analyzed individually and quantification of increase in Ca^{2+} influx was averaged for all responding cells on each dish. Cells that did not respond were excluded from the analysis. Each dish with neurons derived from a different set of mouse embryos was treated as an independent culture.

4. Results

4.1 Aβ oligomers require both PrP^C and α7nAChR for sustained increase in intracellular Ca^{2+} and PrP^C for cell death

Some studies suggested that Aβ oligomers might disrupt Ca^{2+} homeostasis in neurons by compromising the mitochondrial and endoplasmic reticulum Ca^{2+} buffering mechanisms (Guo et al., 1996), and disruption in Ca^{2+} homeostasis has been linked to cellular stress and neuronal death (Zundorf & Reiser, 2011). Both PrP^C and α7nAChRs have been shown to interact with Aβ oligomers (Lauren et al., 2009; Wang, Lee, D'Andrea, et al., 2000; Wang, Lee, Davis, et al., 2000) and to regulate intracellular Ca^{2+} levels. In order to understand how Aβ oligomers can induce neuronal death, we treated 11 DIV hippocampal neurons derived from wild-type, PrP^C-knockout, and α7nAChR-knockout mice with Aβ oligomers and performed Ca^{2+} signaling experiments using Fura-2 AM Ca^{2+} indicator. Primary neuronal cultures derived from wild-type mice showed a sustained increase in intracellular Ca^{2+} levels when treated with Aβ oligomers (Figure 1). However, the same treatment with Aβ oligomers resulted in a transient intracellular Ca^{2+} increase in neuronal cultures derived from PrP^C-knockout and *α7nAChR*-knockout mice (Figure 1). These results suggest that Aβ oligomers might need both PrP^C and *α7nAChR* for at least part of the Ca^{2+} response in neurons. Neuronal death induced by Aβ oligomers is attenuated in PrP^C-null mice (Kudo et al., 2012). To determine if neurotoxicity induced by Aβ oligomers is dependent on the expression of PrP^C, we treated 11 DIV wild type and PrP^C-knockout hippocampal neurons with Aβ oligomers and measured cell death

using a Live/Dead staining assay. Primary hippocampal neurons obtained from wild type mice were significantly more sensitive to 500 nM and 1μM Aβ oligomers than their PrP^C-knockout counterparts (Figure 2 A&B). On the other hand, treatment with 3μM Aβ oligomers resulted in similar levels of neuronal death in both wild type and PrP^C-knockout neurons (Figure 2B). Overall, these results suggest that neuronal death induced by low concentrations of Aβ oligomers is dependent on PrP^C expression.

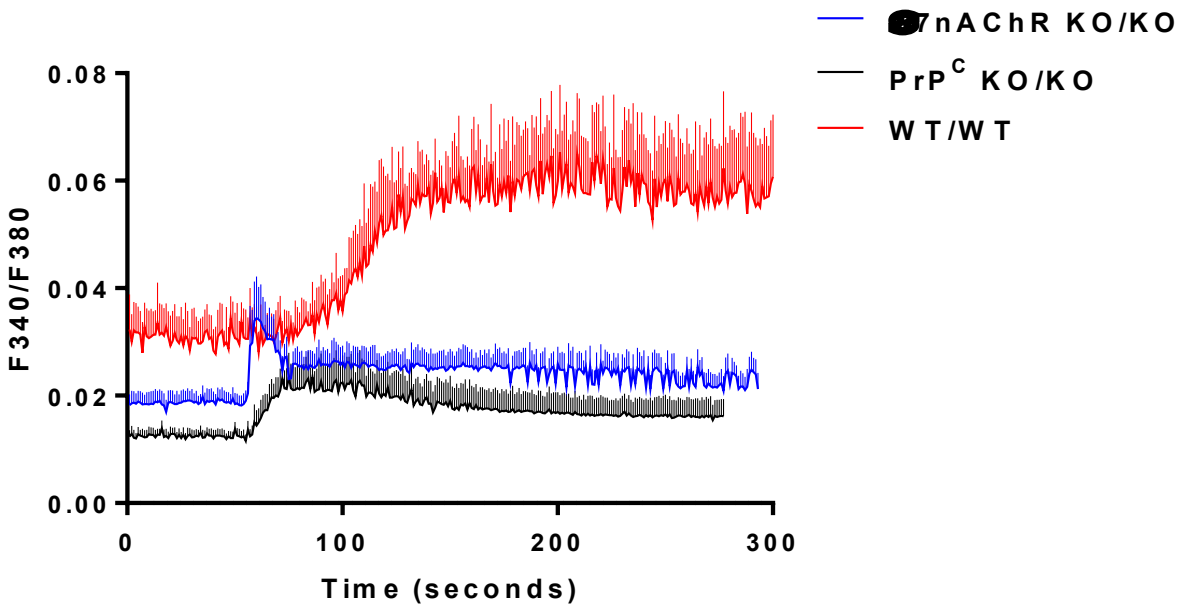
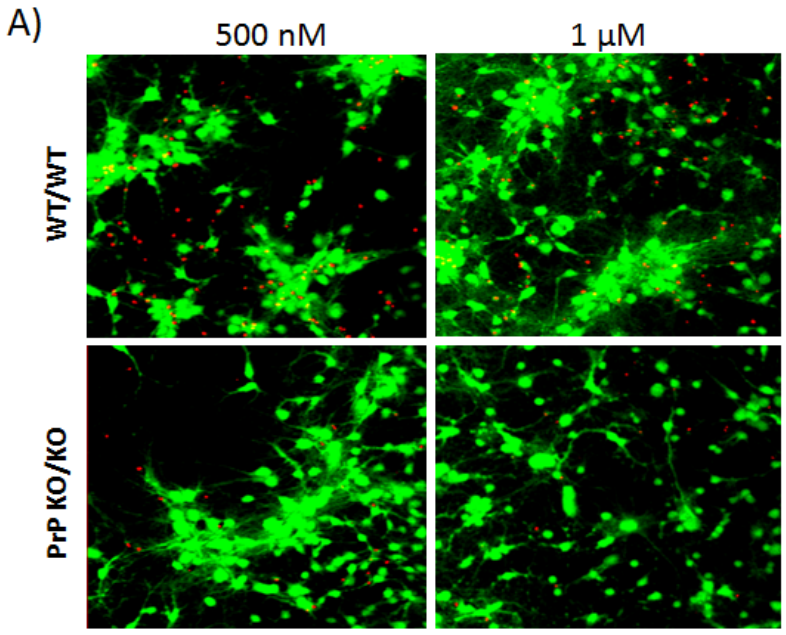
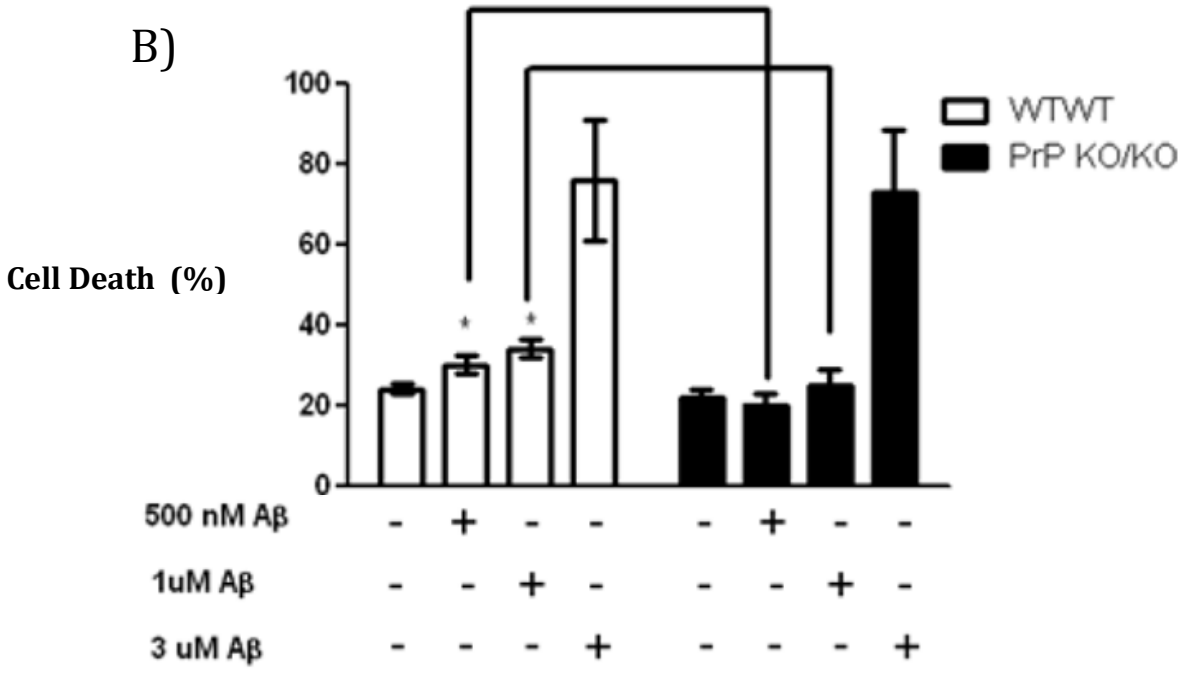


Figure 1. **Aβ oligomers require both PrP^{C} and α7nAChR for sustained Ca^{2+} influx** – Average Ca^{2+} influx curves of 11 DIV wild type (red line; 5 cells), α7nAChR-knockout (blue line; 11 cells), and PrP^{C}-knockout (black line; 14 cells) hippocampal neurons loaded with Fura-2 AM and treated with Aβ oligomers (1μM) in medium supplemented with Ca^{2+}.

Figure 2. **Aβ oligomers induce neurotoxicity in a PrP^C-dependent manner** – A, Representative images of live (green cells) and dead (red cells) 11 DIV primary wilt type (upper row) and PrP^C-knockout (lower row) hippocampal neurons treated with 500 nM (left column) and 1μM (right column) Aβ oligomers and loaded with LIVE/DEAD Viability Kit. B, quantification of relative wild type (white bars) and PrP^C-knockout (black bars) neuronal cell death after no treatment ($n_{wt/wt}$= 25, $n_{PrP^C KO/KO}$=4), treatment with Aβ oligomers (500nM; $n_{wt/wt}$=4, $n_{PrP^C KO/KO}$=3), treatment with Aβ oligomers (1μM; $n_{wt/wt}$=22, $n_{PrP^C KO/KO}$=4), treatment with Aβ oligomers (3μM; $n_{wt/wt}$=4, $n_{PrP^C KO/KO}$=3). Results represent the mean ±SE (error bars) of three independent experiments. Statistical analysis was conducted using two-way ANOVA, p=0.0137 between genotypes and p = 0.4598 between treatments for 500 nM, p= 0.0473 between genotypes and p= 0.0808 between treatments for 1μM.


A)
500 nM
1 µM
WT/WT
PrP KO/KO

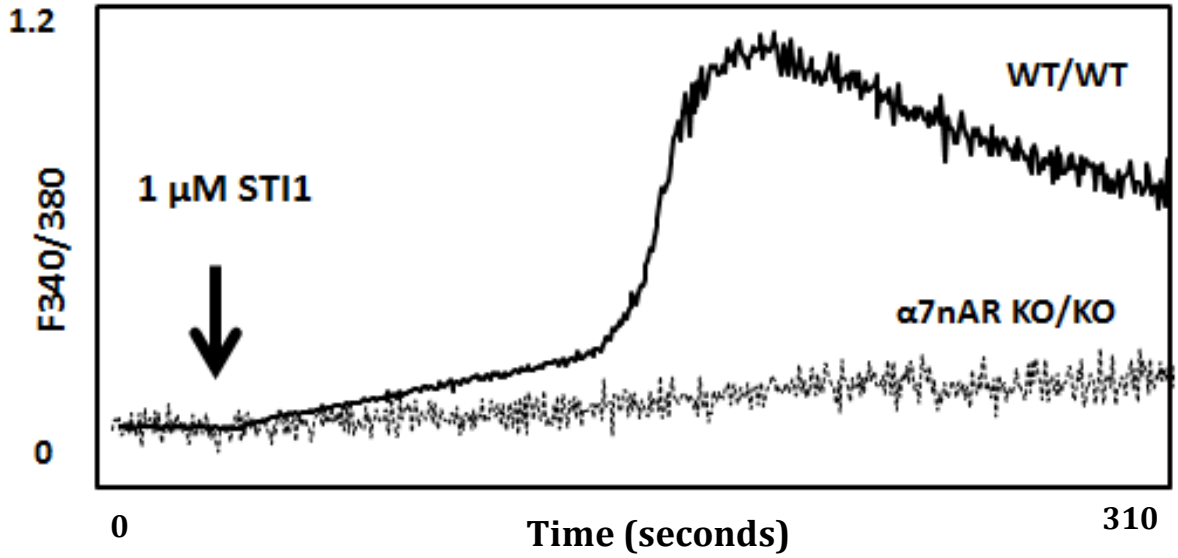

B)
Cell Death (%)
100
80
60
40
20
0
WTWT
PrP KO/KO
500 nM Aβ
1uM Aβ
3 uM Aβ

4.2 Action of STI1 is dependent on α7nAChR for elevation of intracellular Ca^{2+}

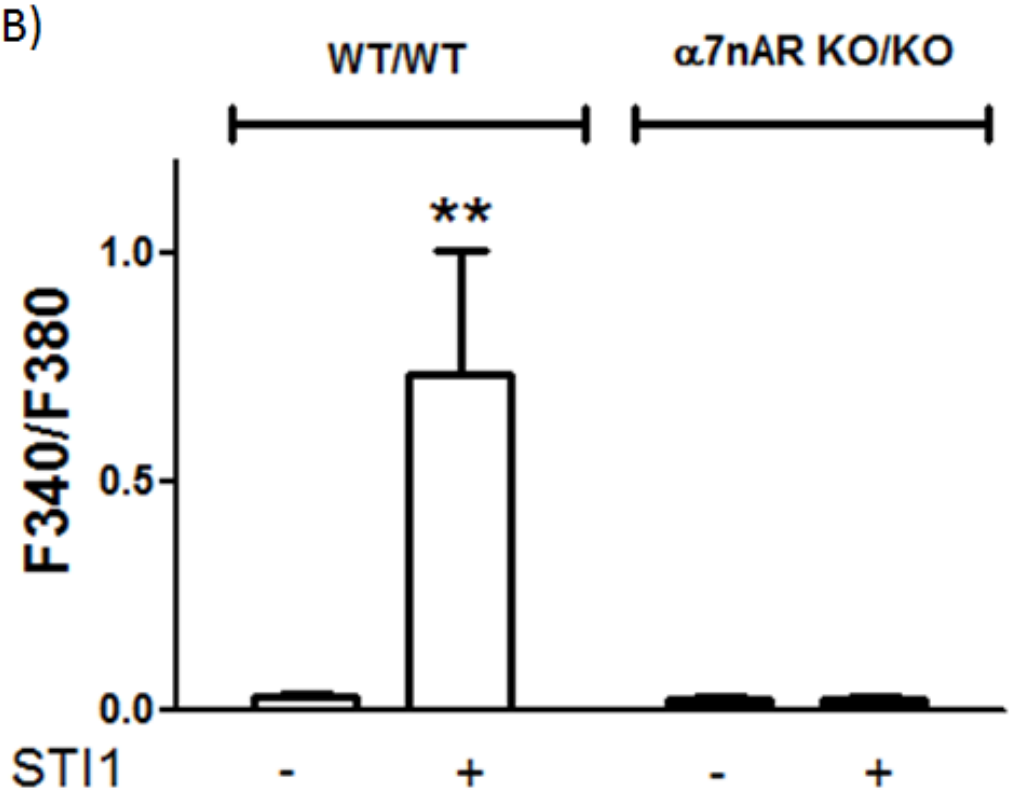
STI1 activates Ca^{2+} influx in neurons via α7nAChR. Recent experiments demonstrated that activation of PrP^{C} by STI1 in neurons increases intracellular Ca^{2+} level (Beraldo et al., 2010). Ca^{2+} influx activated a cascade of downstream signaling pathways, including ERK1/2 phosphorylation and PKA activation, which led to neuritogenesis and neuroprotection, respectively (Lopes et al., 2005). It was also demonstrated that the STI1-induced increase in intracellular Ca^{2+} on neurons was inhibited by alpha bungaratoxin (α-bgt), a selective α7nAChR-specific antagonist (Beraldo et al., 2010). Moreover, STI1-PrP^{C} signaling could be reconstituted in HEK-293 cells transfected with α7nAChRs (Beraldo et al., 2010). To further test if α7nAChRs have a role in STI1-mediated signaling, we used α7nAChR-KO hippocampal neurons. STI1 was able to induce increases of intracellular Ca^{2+} on 5 DIV hippocampal neurons derived from wild type mice, but not on hippocampal neurons derived from α7nAChR-knockout mice (Figure 3A&B). However, treatment of the same neurons with the Ca^{2+} ionophore Ionomycin resulted in an increase of intracellular Ca^{2+} in both wild type and α7nAChR-knockout neurons (data not shown). These results suggest that α7nAChR is needed on neurons for Ca^{2+} influx induced by STI1.

Figure 3. **STI1 promotes Intracellular Ca^{2+} Increase in an α7nAChR-dependent manner** – A, 5 DIV primary wild type (solid line) and α7nAChR-knockout (dashed line) hippocampal neurons loaded with Fura-2 AM and treated with STI1 (1μM) in medium supplemented with Ca^{2+} . B, quantification of relative Intracellular Ca^{2+} levels in wild type (white bars; n = 5) and α7nAChR-knockout (black bars; n =6) hippocampal neurons before and after treating with STI1 (1μM). Results represent the mean ±SE (error bars) of five independent experiments. Statistical analysis was conducted using one-way ANOVA and the Newman-Keuls post test, p = 0.0013.

A)


1.2
F340/380
0
1 µM STI1
WT/WT
α7nAR KO/KO
0
Time (seconds)
310

B)


WT/WT
α7nAR KO/KO
**
1.0
0.5
0.0
F340/F380
STI1
-
+
-
+

4.3 STI1-heterozygous knockout neurons show increased sensitivity to Aβ oligomers

Both STI1 and Aβ oligomers bind to PrP^C and they both seem to use PrP^C and α7nAChRs to induce an increase in neuronal Ca^{2+}. However, whereas STI1 promotes protection of neurons (Lopes et al., 2005; Zanata et al., 2002), Aβ oligomers are toxic (Yankner et al., 1989). To determine if endogenous STI1 could play a role in protecting neurons against Aβ-induced toxicity, we treated 11 DIV wild-type and STI1 heterozygous knockout hippocampal neurons with Aβ oligomers for 48 hours. Heterozygous knockout mice present a 50% decrease in STI1 levels (Beraldo et al., unpublished results). Neurons were treated with increasing concentrations of Aβ oligomers (100 nM, 500 nM, 1 μM, and 3 μM). While treatment of wild type neurons with 1 μM Aβ oligomers showed a tendency for increased cell death, there was no significant increase in cell death when wild type neurons were treated with 100 nM to 1 uM Aβ oligomers (Figure 4). In contrast, neurons derived from STI1 heterozygous knockout mice show significant cell death at 1 μM Aβ oligomer treatment (Figure 4). At a dose of 500 nM, STI1-deficient neurons also presented a tendency for increased cell death (Figure 4). Interestingly, a higher concentration of Aβ oligomers (3 μM) caused similar levels of neuronal death on neurons derived from both wild-type and heterozygous STI1-knockout mice. Together, these data suggest that STI1 deficiency increases sensitivity of these neurons to Aβ oligomers.

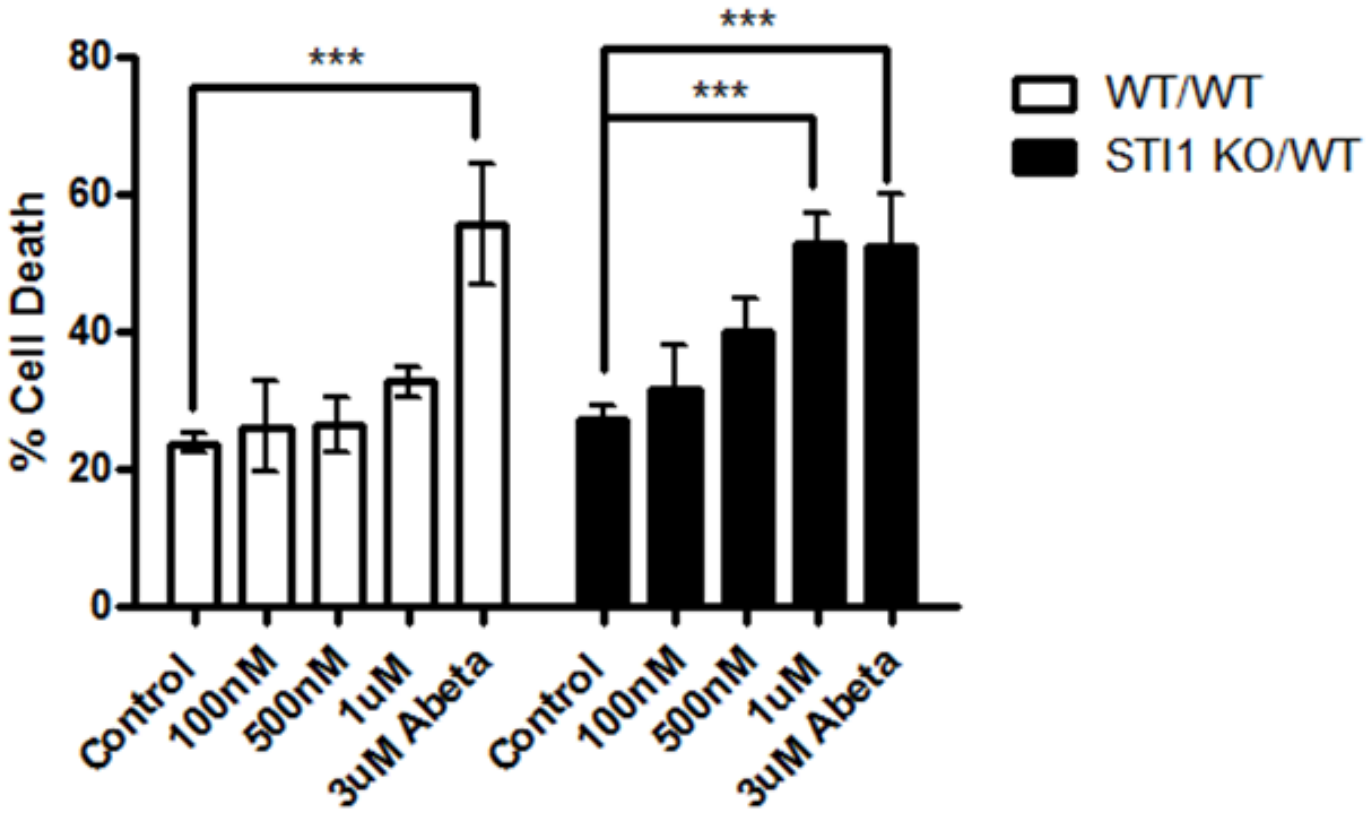


Figure 4. **STI1-heterozygous knockout neurons are more sensitive to Aβ oligomers than wild type neurons** – Quantification of cell death in a dose response curve of wild type (white bars) and STI1-heterozygous knockout (black bars) hippocampal neurons after no treatment ($n_{wt/wt}$= 19, $n_{STI1KO/WT}$= 19), treatment with Aβ oligomers (100 nM; $n_{wt/wt}$= 3, $n_{STI1KO/WT}$= 3), Aβ oligomers (500nM; $n_{wt/wt}$= 3, $n_{STI1KO/WT}$= 3), Aβ oligomers (1μM; $n_{wt/wt}$= 16, $n_{STI1KO/WT}$= 17), and Aβ oligomers (3μM; $n_{wt/wt}$= 9, $n_{STI1KO/WT}$= 11). Results represent the mean ±SE (error bars) of five independent experiments. Statistical analysis was conducted using one-way ANOVA and the Newman-Keuls post test, $p < 0.0001$ compared to controls.

4.4 Extracellular STI1 effect prevents Aβ oligomer-induced neuronal death

To further test for a protective role of STI1, we investigated whether recombinant STI1 protein could prevent cell death induced by Aβ oligomers. In these experiments we used 1 µM Aβ oligomers since we could see a small effect on wild-type neurons and a large effect on STI1 mutants (Fig. 5). Pre-incubation with 1 µM recombinant STI1 rescued neurons derived from both wild type and STI1 heterozygous knockout mice from the toxicity of synthetic Aβ oligomers (Figure 5&6A). Moreover, pre-incubation with 1 µM $STI1_{\Delta\ 230\text{-}245,}$ which lacks the PrP^{C} binding site, still resulted in protection against Aβ oligomers-induced cell death on heterozygous STI1-knockout neurons (Figure 6A). Treatment with synthetic 1 µM scrambled Aβ oligomers did not induce a significant increase in cell death in either genotype (Figure 6A). Treatment of neurons with 3 µM Aβ oligomers induced cell death in neurons derived from wild-type and heterozygous STI1 genotypes, and importantly, recombinant STI1 protected both wild type and STI1 heterozygous neurons against this concentration of Aβ oligomers as well (Figure 5&6B). Overall, these data suggest that extracellular STI1 can protect neurons against cell death induced by Aβ oligomers, and it can also rescue the increased sensitivity to neuronal death observed in STI1-deficient neurons treated with Aβ peptides.

Figure 5. **STI1 rescues neurons against Aβ oligomers-induced neurotoxicity** – Representative images of live (green cells) and dead (red dots) 11 DIV primary wild type (left panel) and STI1-heterozygous knockout (right panel) hippocampal neurons treated with Aβ oligomers (1μM) and loaded with LIVE/DEAD Viability Kit.

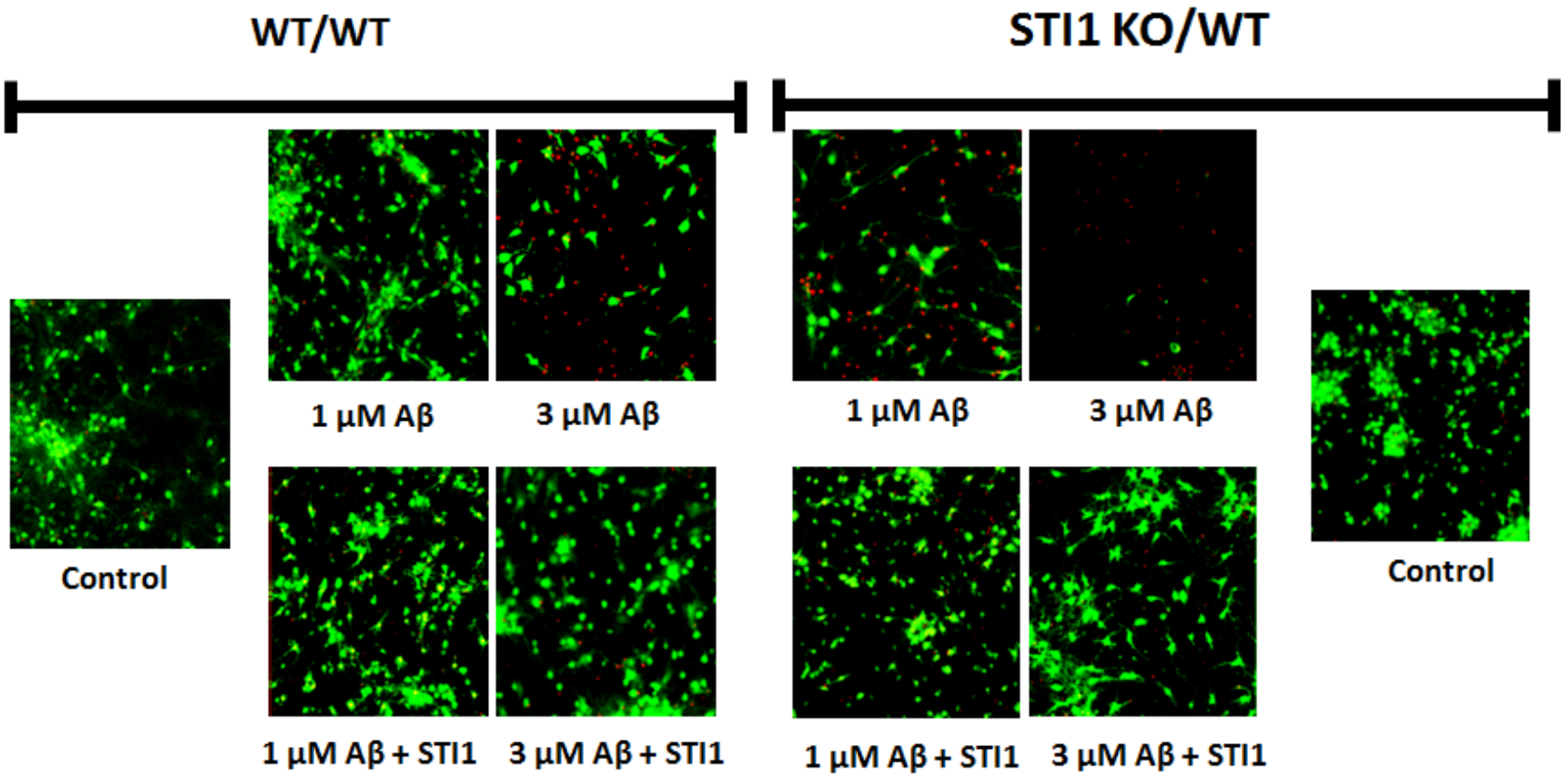
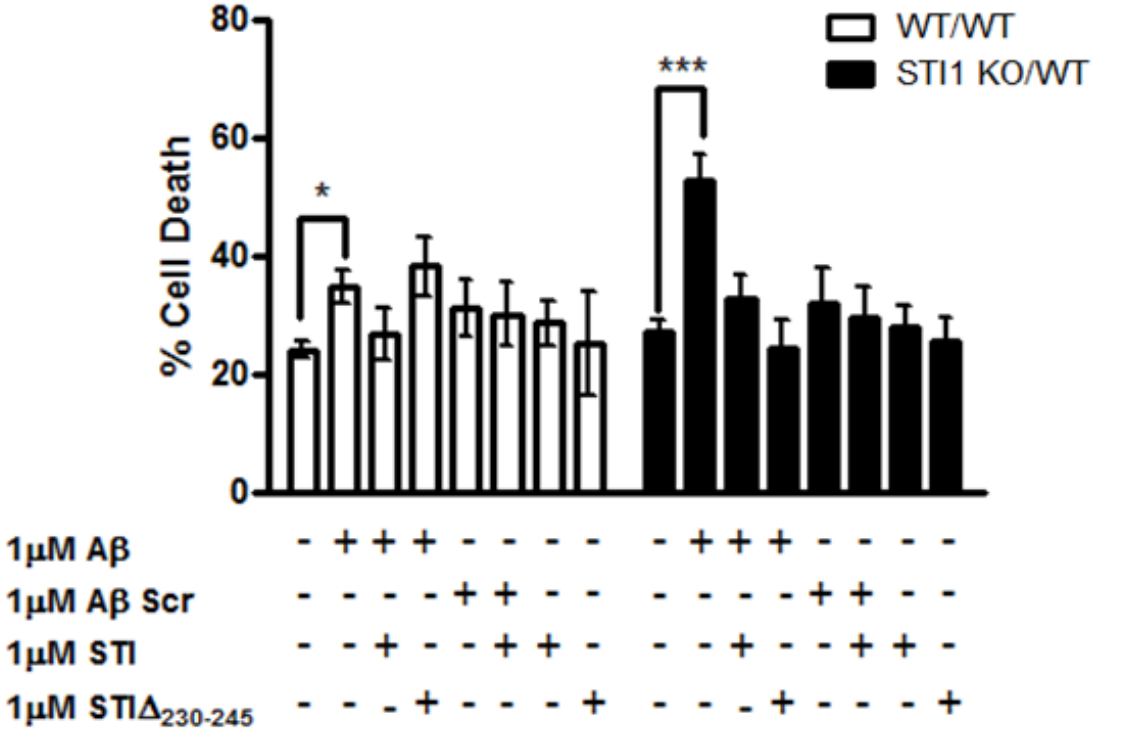
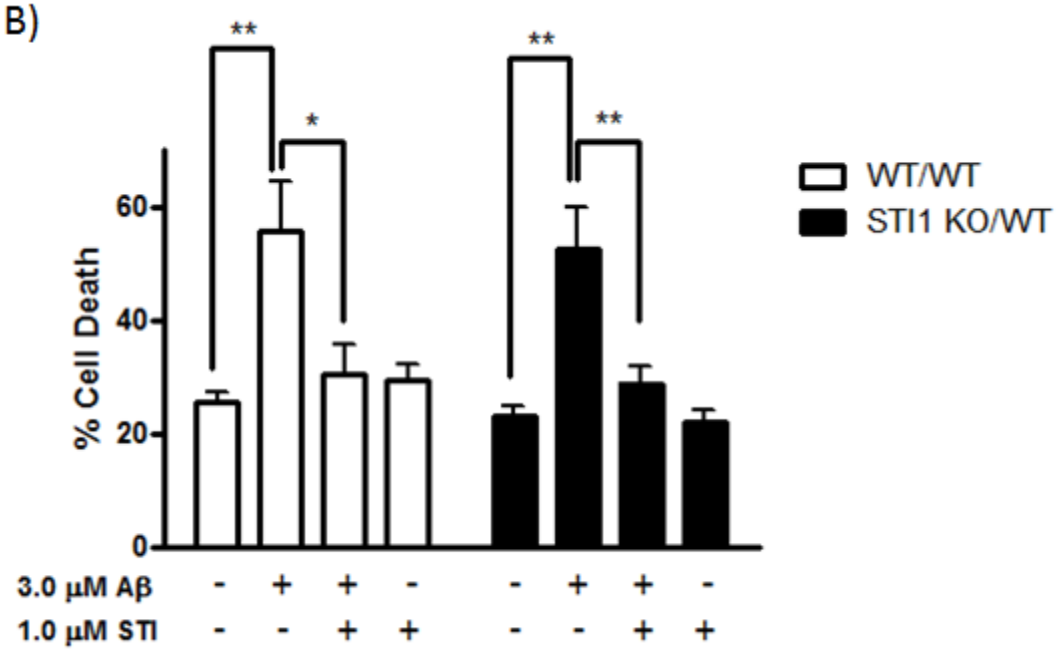

WT/WT
STI1 KO/WT
1 µM Aβ
3 µM Aβ
1 µM Aβ
3 µM Aβ
Control
Control
1 µM Aβ + STI1
3 µM Aβ + STI1
1 µM Aβ + STI1
3 µM Aβ + STI1

Figure 6. **STI1 rescues neurons against Aβ oligomers-induced neurotoxicity** –A, quantification of relative wild type (white bars) and STI1-heterozygous knockout (black bars) neuronal cell death after no treatment ($n_{wt/wt}$= 21, $n_{STI1KO/WT}$= 18), treatment with Aβ oligomers (1μM; $n_{wt/wt}$= 18, $n_{STI1KO/WT}$= 17), Aβ oligomers (1μM) and STI1 (1μM; $n_{wt/wt}$= 5, $n_{STI1KO/WT}$= 7), Aβ oligomers (1μM) and $STI1_{\Delta 230\text{-}245}$ (1μM; $n_{wt/wt}$= 3, $n_{STI1KO/WT}$= 5), Scrambled Aβ oligomers (1μM; $n_{wt/wt}$= 5, $n_{STI1KO/WT}$=… 5), Scrambled Aβ oligomers (1μM) and STI1 (1μM; $n_{wt/wt}$= 5, $n_{STI1KO/WT}$= 5), STI1 (1μM; $n_{wt/wt}$= 6, $n_{STI1KO/WT}$= 7), and $STI1_{\Delta 230\text{-}245}$ (1μM; $n_{wt/wt}$= 3, $n_{STI1KO/WT}$= 5). Results represent the mean ±SE (error bars) of ten independent experiments. Statistical analysis was conducted using one-way ANOVA and the Newman-Keuls post test, p =0.0945 (WT/WT), and p <0.0001 (STI1 KO/WT) compared to controls. B, quantification of relative wild type (white bars) and STI1-heterozygous knockout (black bars) neuronal cell death after no treatment ($n_{wt/wt}$= 10, $n_{STI1KO/WT}$= 11), treatment with Aβ oligomers (3μM; $n_{wt/wt}$= 9, $n_{STI1KO/WT}$= 11), Aβ oligomers (3μM) and STI1 (1μM; $n_{wt/wt}$= 3, $n_{STI1KO/WT}$= 7), and STI1 (1μM; $n_{wt/wt}$= 4, $n_{STI1KO/WT}$= 7). Results represent the mean ±SE (error bars) of four independent experiments. Statistical analysis was conducted using one-way ANOVA and the Newman-Keuls post test, p =0.0042 (WT/WT), and p <0.0001 (STI1 KO/WT) compared to controls.

A)


WT/WT
STI1 KO/WT
% Cell Death
80
60
40
20
0
*
***
1μM Aβ - + + + - - - - - + + + - - - -
1μM Aβ Scr - - - - + + - - - - - - + + - -
1μM STI - - + - - + + - - - + - - + + -
1μM STIΔ230-245 - - - + - - - + - - - + - - - +

B)


WT/WT
STI1 KO/WT
% Cell Death
60
40
20
0
**
*
**
**
3.0 μM Aβ - + + - - + + -
1.0 μM STI - - + + - - + +

5. Discussion

5.1 Neurotoxicity of Aβ Oligomers via the PrP^C-α7nAChR complex

5.1.1 Aβ-induced sustained Ca^{2+} influx: dual dependence

According to our results, the presence of PrP^C alone cannot initiate a cascade of events leading to sustained Ca^{2+} influx without α7nAChR following treatment with Aβ. Sustained Ca^{2+} influx might lead to disruption of Ca^{2+} homeostasis and ultimately to excitotoxicity and late cell death (Zundorf & Reiser, 2011). PrP^C was shown to require the activity of α7nAChR to induce Ca^{2+} influx and to induce signaling pathways (Beraldo et al., 2010). Thus, we propose that PrP^C might need to complex with α7nAChR to induce sustained Ca^{2+} influx.

Our results help to clarify the controversy concerning the role of α7nAChRs as an Aβ-binding partner. Some studies suggest that Aβ desensitizes (Dineley et al., 2002) or blocks (Liu et al., 2001; Pettit et al., 2001; Tozaki et al., 2002) the α7nAChR. On the contrary, other studies indicate that Aβ activates the α7nAChR (Dougherty et al., 2003; Wang et al., 2003). The Aβ/α7nAChR controversy might be due to unsuspected roles of other proteins involved in the interaction between Aβ and α7nAChR. As mentioned previously, Dineley et al., 2002 (Dineley et al., 2002) expressed rat α7nAChR alone in *Xenopus* oocytes. Aβ directly induced current through the α7nAChR, but the Aβ-induced currents were unsustainable and the α7nAChR rapidly desensitized. Our results together

with Dineley et al., 2002 (Dineley et al., 2002) might suggest that PrP^C and α7nAChR need to complex before mediating an Aβ-induced sustained Ca^{2+} signal. Indeed, it was not until Dougherty et al., 2003 (Dougherty et al., 2003) treated presynaptic terminals isolated from wild type mice with Aβ that a sustained agonist-like effect was observed. The Aβ-induced sustained Ca2+ influx on presynaptic terminals might suggest that all proteins needed for the Aβ-induced influx were present, including PrP^C.

Overall, these results suggest that Aβ might need PrP^C to complex with the α7nAChR in order to induce Ca^{2+} influx and excitotoxicity. Therefore, I propose that the Aβ dependence on PrP^C and α7nAChR to induce sustained Ca^{2+} influx to be partially the reason why Aβ is dependent on PrP^C (Kudo et al., 2012) and α7nAChR (Wang et al., 2003) for neurotoxicity. Understanding what Aβ requires to induce sustained Ca^{2+} influx may give us insights into how to mitigate the neurotoxic effects of Aβ oligomers, possibly by blocking interaction with PrP^C and α7nAChR. However, it is important to note that our results do not exclude other receptors or channels from being directly responsible for the sustained Ca^{2+} influx induced by Aβ.

5.1.2 The PrP^C-dependent Aβ-induced neuronal death

Our data indicate that neuronal death induced by 1μM Aβ was dependent on PrP^C. PrP^C was shown to be a putative receptor for Aβ (Chen et al., 2010; Lauren et al., 2009).

Other studies also supported the deleterious effects of the proposed Aβ/PrP^C interaction on neuronal survival (Kudo et al., 2012; Resenberger, Harmeier, et al., 2011; Resenberger, Winklhofer, & Tatzelt, 2011; Resenberger et al., 2012). These studies showed that overexpression of PrP^C resulted in increased sensitivity from neurotoxicity mediated by cell-derived Aβ oligomers (Resenberger et al., 2012). Kudo et al., 2012 (Kudo et al., 2012) demonstrated that Aβ induced neuronal death via Aβ both *in vitro* and *in vivo* using PrP^C-knockout mice. The mechanism by which Aβ induces neuronal death is not well understood. Nonetheless, Aβ/PrP^C interaction may compromise the PrP^C physiological functions. Thus, we need to have a good understanding of the PrP^C-dependent physiological mechanisms to distinguish the manipulations caused by the Aβ/PrP^C interaction.

As previously mentioned, PrP^C acts as a docking element on the cell membrane to mediate interactions between multiple proteins (Martins & Brentani, 2002; Martins et al., 2002). PrP^C mediates the effects of many ligands by initiating multiple signaling pathways downstream of the binding at the cell surface. Aβ/PrP^C interaction may compromise the regulatory functions of PrP^C by manipulating PrP^C-dependent physiological signaling pathways. Moreover, the binding of Aβ to PrP^C may also induce different PrP^C-dependent signaling pathways that are not initiated under normal physiological conditions.

Our data also indicate that neuronal death induced by 3μM Aβ was not dependent on PrP^C for inducing neuronal death. At higher concentrations, Aβ may be utilizing other mechanisms or proteins to induce neurotoxicity. Higher Aβ concentrations may result in over-activation of NMDARs, disruption of the lipid bilayer, or formation of reactive oxygen species (Fandrich, 2012) leading to neuronal death independently of PrP^C.

5.2 Synaptic protection and Neuroprotection of STI1 via the PrP^C-α7nAChR complex

5.2.1 STI1 promotes neuronal survival

We tested the dependence of STI1-induced Ca^{2+} influx on α7nAChR using α7nAChR-knockout hippocampal primary culture neurons. Our data provided evidence that STI1 required α7nAChR to induce Ca^{2+} influx in neurons. In Beraldo et al., 2010 (Beraldo et al., 2010), it was shown that STI1 required both the presence of PrP^C and the activity of the α7nAChR to induce protection against cellular insults like Staurosporine. Moreover, STI1 induced protection of neurons in a PrP^C-dependent fashion in other studies (Chiarini et al., 2002; Lopes et al., 2005; Zanata et al., 2002). Thus, the effect of STI1 seems to be dependent on the scaffolding property of PrP^C and on the transient Ca^{2+} permeability of the α7nAChR to induce protection of neurons.

5.2.2 Decreased levels of STI1 increase the sensitivity of neurons to Aβ

The dependence of STI1 and Aβ on the PrP^C-α7nAChR complex might result in direct competition between STI1 and Aβ for the PrP^C-α7nAChR complex. Indeed, our results suggested that lack of STI1 increased the sensitivity of neurons to Aβ. Also, it was already shown in unpublished results that recombinant STI1 competes with Aβ for binding to PrP^C (Beraldo et al., unpublished). The potential competition between STI1 and Aβ is also supported by the close proximity of the binding sites of STI1 (113-128) (Zanata et al., 2002) and Aβ (95-110) (Lauren et al., 2009) on PrP^C. The direct competition between STI1 and Aβ might mean that the PrP^C-α7nAChR complex may be used as a therapeutic target in AD.

As previously stated, our laboratory showed that there is a decrease in STI1 protein levels in 6-month-old APPSwe/PS1dE9 mice (unpublished observations). APPSwe/PS1dE9 mice, an AD animal model, show plaque formation, synapse loss and behavioral deficits (Games et al., 1995; Lesne et al., 2006; Mucke et al., 2000). Although the role of STI1 in AD is not known, I hypothesized that changed STI1 levels may contribute in part of the pathology observed on the APPSwe/PS1dE9 mice. Our data using neurons derived from heterozygous STI knockout mice supported this hypothesis. Lack of STI1 seems to increase sensitivity of neurons to Aβ oligomers. Therefore, it is

important to elucidate the mechanism by which lack of endogenous STI1 leads to increased sensitivity from Aβ.

5.2.3 STI1 protects against Aβ neurotoxicity

Interestingly, the addition of recombinant STI1 rescued neurons derived from both wild-type and heterozygous STI1-knockout mice against Aβ-induced neurotoxicity. Our findings suggested that STI1 might not protect neurons through a mechanism fully dependent on PrP^C, as treatment with $STI1_{\Delta 230\text{-}245}$ still resulted in protection. Nonetheless, unpublished data revealed that STI1 was able to rescue the synaptotoxic effects of Aβ in a PrP^C-dependent fashion by mainting the levels of synaptophysin protein (Hajj et al., unpublished). The PrP^C-dependence of the synapto-protective effect of STI1 against Aβ was tested on PrP^C-knockout neurons. Thus, the different ways of assessing PrP^C dependency may be the reason why STI1 was not fully dependent on PrP^C to protect neurons in our experiments. Furthermore, STI1 was able to rescue against Aβ-induced 50% drop in LTP (Beraldo et al., unpublished). Thus, there is mounting evidence linking the STI1-PrP^C complex to the synapse.

It is known that STI1 is involved in promoting neuritogenesis in a PrP^C-dependent fashion by inducing ERK 1/2 phosphorylation (Lopes et al., 2005). Beraldo et al., 2010 showed that the STI1-induced ERK 1l2 phosphorylation is dependent on PrP^C and the

activity of the α7nAChR. Further, α7nAChR is thought to be an important modulator of glutamatergic synapses (Lozada et al., 2012). Thus, the role of STI1 may be important in synapse formation by promoting neurite growth through the α7nAChR, and possibly in synaptic function by modulating the PrP^C-α7nAChR complex at the synapse.

Therefore, our results and previous data from others suggest that PrP^C and α7nAChR may be involved in the STI1-induced synapto-protective effects (Hajj et al., unpublished). Thus, I speculate that STI1 protection against the Aβ-induced disruption of synaptic plasticity may be the basis of the STI1 protection against the Aβ-induced neuronal death we observed. More specifically, I hypothesize that after formation of the STI1-PrP^C complex, which prevents the binding of Aβ to PrP^C (Figure 7, Model A), PrP^C recruits the α7nAChR. As a complex, I speculate that PrP^C and the α7nAChR regulate the synapse through controlled Ca^{2+} influx (Figure 7, Model B). The proper regulation of the synapse may prevent the PrP^C-dependent Aβ-induced synaptotoxic effects (Lauren et al., 2009) and the neuronal death we observed.

On the other hand, the binding of Aβ to PrP^C, in the presence of less STI1, may result in the recruitment of α7nAChR. Consequently, Aβ might change physiological Ca^{2+} influx through the α7nAChR (Figure 7, Model C). The potential PrP^C-α7nAChR complex regulation of the synapse may become compromised. Ultimately, the neuron will degenerate due to disruption of synaptic transmission leading to the neuronal degenerative phenotype observed in AD.

Confirmatory experiments can be done on mice produced from the breeding of heterozygous STI1 knockout and the APPSwe/PS1dE9 mice. The offspring will have less STI1 and develop amyloidosis and behavioral deficits concurrently. I would speculate that reduced levels of STI1 could affect the development of amyloidosis *in vivo.* Although the putative mechanism we proposed for the effects of STI1 involves a competition with Aβ oligomers, it is important to evaluate if changes in STI1 levels would affect the generation of Aβ peptides or plaque formation. Spatial memory, which is associated with hippocampal function, can also be evaluated in order to investigate if decreased STI1 levels can escalate the behavioral deficits in aged APPSwe/PS1dE9 mice. We would expect, based on our results, that the double transgenic mice would have greater behavioral deficits compared to APPSwe/PS1dE9 mice.

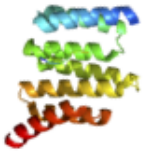
6. Future Directions

We hypothesized that STI1 can protect against Aβ-induced neurotoxicity via the PrP^C-α7nAChR. The dependence of STI1 on the PrP^C-α7nAChR for Ca^{2+} influx and for neurotrophic actions, and the PrP^C dependence of Aβ for neurotoxicity comprised rationale for this hypothesis. Although we showed that Aβ is dependent on PrP^C and α7nAChR for sustained Ca^{2+} influx, we did not test the dependence of Aβ on α7nAChR for inducing neuronal death. If Aβ was found to be dependent on α7nAChR for cell death, then the Aβ-PrP^C-α7nAChR complex could be linked to sustained Ca^{2+} influx and Aβ-induced cell death. If confirmed, the dependency of Aβ on α7nAChR would also strengthen our proposed link between sustained Ca^{2+} influx, as a source of excitotoxicity, and neuronal death. Also, we think that it is important to preincubate wild type neurons with STI1 and assess if STI1 can prevent the sustained Ca^{2+} influx after treatment with Aβ. If STI1 prevented the sustained Ca^{2+} influx, we can then use STI1 in combination with PrP^C antibodies and α7nAChR blockers to test if STI1 needs either PrP^C or α7nAChR for prevention of Aβ-induced sustained Ca^{2+} influx.

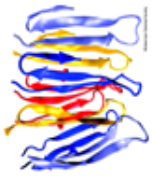
It would be interesting to test if STI1 can protect PrP^C-knockout and α7nAChR-knockout neurons against Aβ-induced neuronal death by using the same cell death assay used in this study. If STI1 did not protect PrP^C-knockout and α7nAChR-knockout neurons against Aβ-induced neuron death, then STI1 may require the presence of PrP^C and α7nAChR to protect against Aβ.

As mentioned previously, it was reported that knocking out PrP^C or α7nAChR from AD mouse models alleviated the behavioral and synaptic dysfunctions observed on these mice (Dziewczapolski et al., 2009; Gimbel et al., 2010). Thus, it would be very important and confirmatory to our studies to assess synaptic plasticity on neurons derived from APP/α7KO and APP/PrP^CKO mice after preincubation with STI1. It has already been shown that STI1 protects against Aβ synaptotoxicity in a PrP^C-dependent fashion (Hajj et al., unpublished). We can further assess synaptic structure by quantifying the synaptic marker, synaptophysin, on synapses derived from α7nAChR-knockout embryos after pre-incubation with STI1 and treatment with Aβ.

To test if NMDARs are directly responsible for the sustained Ca^{2+} influx observed after treatments with Aβ, we can assess Ca^{2+} influx in wild type, PrP^C knockout and α7nAChR-knockout neurons after pre-incubation with an NMDAR selective antagonist and treatment with Aβ. If the NMDAR antagonist prevented the Aβ-induced sustained Ca^{2+} influx then NMDARs might be responsible for the sustained Ca^{2+}influx. Also, we can use the NMDAR antagonist on with the cell death assays we used. We can pre-incubate PrP^C-knockout and α7nAChR-knockout neurons with the NMDAR antagonist and test for Aβ-induced cell death. Testing the effect of NMDAR antagonism on Aβ-induced sustained Ca^{2+} and neuron death can give us better understanding of the role of NMDARs in Aβ-induced excitotoxicity.



Stress Induced Protein 1 (STI1)



Beta - Amyloid (Aβ)

Alpha 7 nicotinic acetlcholine receptor (α7nAChR)

Cellular Prion Protein (PrP^C)

Legend of Figure 7

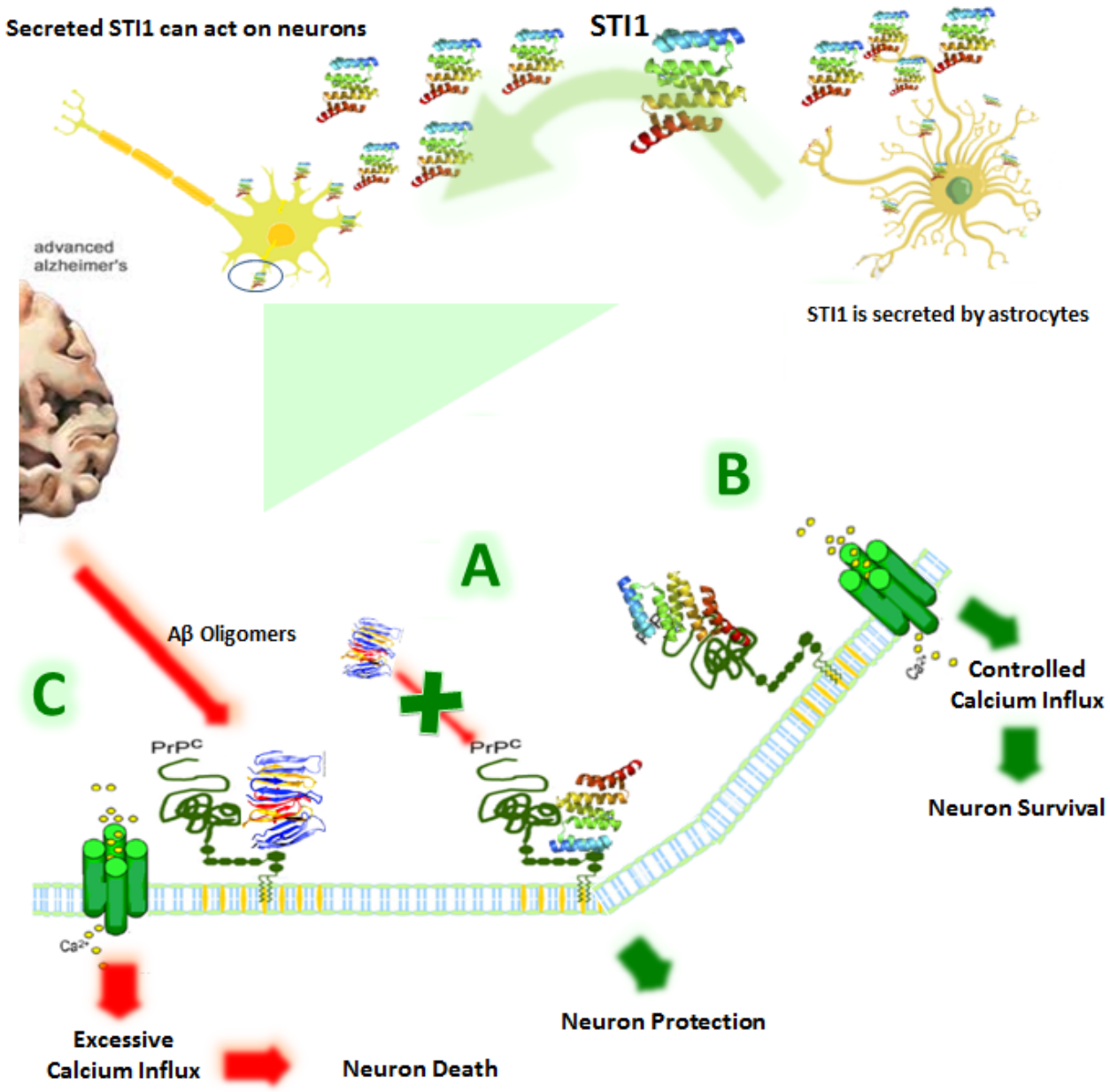


Figure 7. **The neurotoxic effects of Aβ and the neuroprotective effects of STI1 on neuron via the PrP^C-α7nAChR complex** – Model A, STI1 blocks the binding of Aβ to PrP^C and promotes neuron survival. Model B, STI1 binds to PrP^C. The binding of STI1 to PrP^C recruits the α7nAChR. The STI1-PrP^C-α7nAChR then promotes neuronal survival. Model C, Aβ binds to PrP^C and mediates neurotoxicity through PrP^C when no enough STI1 is present. After binding to Aβ, PrP^C recruits the α7nAChR and the Aβ-PrP^C-α7nAChR promotes neuronal death.

7. Conclusion

Our results suggest that Aβ may utilize the PrP^C-α7nAChR complex for sustained Ca^{2+} influx. The Aβ-mediated sustained Ca^{2+} influx might be the start of a pathological signaling cascade leading to our second observation, the Aβ-mediated and PrP^C-dependent neuronal cell death. In addition, we tested whether STI1, a PrP^C ligand proposed to utilize the PrP^C-α7nAChR complex for neuroprotection, requires α7nAChR for induction of Ca^{2+} influx. We tested the dependence of STI1 on α7nAChR for Ca^{2+} influx using α7nAChR-knockout hippocampal neurons and found that STI1 required the presence of α7nAChR to induce Ca^{2+} influx. Moreover, we tested if STI1 can protect against Aβ-mediated neuronal cell death. Neurons derived from a mouse model expressing half the level of STI1 (STI1 KO/WT) compared to wild type showed increased sensitivity to Aβ than their wild type counterparts. Furthermore, the treatment of STI1 KO/WT and wild type neurons with recombinant STI1 rescued STI1 KO/WT and wild type neurons against the Aβ-induced cell death.

In conclusion, our results suggest that the PrP^C-α7nAChR complex may be an important mediator of Aβ-induced pathology in AD. Therefore, this complex may be an important therapeutic target in AD. We also identified a protein that may be important in a physiological protective mechanism. STI1 may able to protect the synapse against Aβ and as such result in amelioration of AD-related behavioral deficits.

8. References

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***The* UNIVERSITY *of* WESTERN ONTARIO - COUNCIL ON ANIMAL CARE**

ANIMAL USE PROTOCOL

A. INVESTIGATOR/GRANT/PROJECT INFORMATION -

Mandatory Completion Required

Investigator Contact Information		
Investigator Name	**Department**	**Institution**
Office Address	**Lab Address**	**Email Address**
Office Telephone	**Lab Telephone**	

Protocol & Grant Titles/Support Documentation		
Application Type: *Pick One Only* ☒ **New** ☐ **Pilot** ☐ **Full Renewal**	**If Full Renewal, Previous** **Protocol #**	**Proposed Start Date:** *mm/dd/yy / /* **Proposed End Date:** *mm/dd/yy / /*
Project Title		
Grant Title *(if different from above)*		
Supporting Documentation Indicate 1 to 3 related publications supporting this proposed project. A website or attachment is acceptable.		

Funding Details

Funding Source Institutional **Name:**	**Source Grant #** to be determined *OR* R21 TW007800-01 (NIH) **Applied for** ☐	**Does this source conduct peer review?** *Pick One* ☒ Yes ☐ No ☐ Unsure	**Granting agency requires submission confirmation** ☐ Yes ☒ No **If *Yes*, Due Date:** *mm/dd/yy* / /

Purpose of Animal Use			
Describe the purpose of your use of animals using the following check boxes & drop-downs			
Purpose of Animal Use http://www.uwo.ca/animal/website/AUS/Content/Forms.htm	**Project Type** *Pick all that apply*	**Project Purpose** *Pick all that apply*	*Complete Related Section*
2-Medical or Veterinary Research	☒ **Acute** Non-recovery Surgery, Euthanasia for Tissue Collection	☒ **Research**	
CCAC Category of Invasiveness http://www.uwo.ca/animal/website/AUS/Content/Forms.htm		☒ **Breeding for Research**	F. 6
Choose maximum level within this study	☒ **Chronic** Long Term (i.e. nutrition), Recovery Study (i.e. post surgery)	☐ **Teaching**	H. Add. 1
D		☐ **Wildlife Study**	H. Add. 2

Check All Species Used **Within this Protocol**	☐ Bird ☐ Cat ☐ Dog ☐ Fish ☐ Frog ☐ Guinea Pig ☐ Hamster ☒ Mouse ☐ NHP ☐ Pig ☐ Rabbit ☐ Rat ☐ Squirrel ☐ Sheep ☐ Other, *explain*

What are the major events involving animals in this project?

In addition to all **mandatory** sections, complete all specified sections beside each checked element

Pick all that apply	*Complete Related Section*	*Pick all that apply*	*Complete Related Section*
☒ **Agent Use – Non Hazardous** ☒ Drugs ☐ Other	*F.2*	☒ **Euthanasia**	*F. 1*
☒ **Agent Use -** ☐ Hazardous ☒ Biological	*F.2 & H. Ad.3*	☐ **Fasting**	*F. 3 & 7*
☒ **Anaesthesia**	*F.2, 3 & 5*	☒ **Genetic Mutation,** *i.e. transgenic, knockout*	*F. 8*
☐ **Antibody Production**	*F. 2 – 5,* *H. Ad. 3 – Sched. 3*	☒ **Injections**	*F. 2*
☒ **Behavioural Study**	*F. 5*	☒ **Model Creation**	*F. 9*
☒ **Breeding**	*F. 6*	☐ **Observation / Monitoring**	*F. 5*
☐ **Capture**	*F. 5*	☐ **Radiation –**	*F.2& H*

		i.e. *CT, MRI, PET, X-ray*	*Ad.3*
☐ **Collection -** ☐ **Blood**	*F. 7*	☒ **Surgery – Recovery**	*F. 2 ,3,5*
☒ **Collection -** ☒ **Tissue** ☒ **Other,** *i.e. urine*	*F. 9*	☒ **Surgery – Non-recovery**	*F. 2 & 3*
☐ **Diagnostic Imaging**	*F. 9 & H. Ad. 3*	☒ **Treatment**	*F. 5*
☐ **Dietary Manipulation**	*F. 5*		

B. PROJECT LAY SUMMARY - *Mandatory Completion Required*

Describe your project concisely in lay terms at a Grade 9 level using 40 words or less per question **– Avoid technical and scientific terms –**
The Project Lay Summary is a CCAC required element to ensure committee lay member comprehension.
1. Project's Purpose
2. Expected Benefit
3. Reason for Using Animals
4. Reason for Using Species

C. RESEARCH STAFF & THEIR TRAINING REQUIREMENTS – *Mandatory Completion Required*

List all research staff & their related animal involvement within this specific project

TRAINING INFO - All personnel working with **live** animals require CCAC mandated training

– *Animal Care & Use* web-CT *lecture* and related ***hands-on workshops*** **-.**

Completion of the *Animal Care & Use* ethics web-CT *lecture* **once every 5 years** is **mandatory** for ALL personnel.

The **procedures** chosen per species per staff member **determine** hands-on **training requirements**. All research staff listed **below** will be contacted directly via their **email** address re. **auto-enrolment** in all **outstanding animal-based training**. Previous hands-on training obtained via another Canadian institution may be accepted; please submit previous non-ACVS Canadian animal training documentation with AUP Authorization pages. For additional **training requirement detail** and associated **costs**, go to http://www.uwo.ca/animal/website/VS/Content/Teaching_and_Courses.htm

FIRST NAME *Repeat If* ***Multiple Species*** *Used*	**LAST NAME** *Repeat If* ***Multiple Species*** *Used*	**ROLE** *Researcher* *Staff* *Student*	**EMAIL Address** ***Mandatory Field*** ***'…uwo.ca'*** ***Addresses Preferred***	**HANDS-ON Animal Work?** **YES** *or*	***NEW* SPECIES** ***Pick 1 Species To Be Used Per Row. Use extra rows***	***NEW* Expected *Species-Specific* START DATE** ***mm/dd/yy***	**PROCEDURES PER SPECIES** **1**=Basic Handling **2**=Health Monitoring **3**=Blood Collection **4**=Injections **5**=Anaesthesia **6**=Surgery-Recovery **7**=Surgery-Non-Recovery **8**=Euthanasia/Post Mortem **9**=Other, *Provide Detail Below*

				NO	***for persons Working with >1 species***		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
		Principal Investigator		Yes	Mouse		☒	☐	☐	☒	☒	☐	☒	☐	☐
		Staff		Yes	Mouse		☒	☒	☐	☒	☒	☒	☒	☒	☒
		Resea		Yes	Mouse		☒	☐	☐	☒	☐	☐	☐	☒	☒
		Resea	@yahoo.com.br	Yes	Mouse		☒	☐	☐	☐	☐	☐	☐	☒	☒
		Resea		Yes	Mouse		☒	☐	☐	☒	☐	☐	☐	☒	☒
		Studer		Yes	Mouse		☒	☒	☐	☒	☒	☒	☐	☒	☒
		Click H		Click	Click Here		☐	☐	☐	☐	☐	☐	☐	☐	☐

***NEW* EMERGENCY *AFTER HOURS* CONTACT NAMES & NUMBERS - NO LAB PHONE NUMBERS -**	**PRIMARY** EMERGENCY CONTACT LAST NAME & INITIAL : Prado VF	**PRIMARY** EMERGENCY CONTACT NUMBER: X36889; 519 6705109 (cel); 226 6630426 (home)
	SECONDARY EMERGENCY CONTACT LAST NAME & INITIAL : Martins-Silva C	**SECONDARY** EMERGENCY CONTACT NUMBER: 226 663 8203

OTHER STAFF DETAIL:	9 - Genotyping

D. PROJECT OVERVIEW - *Mandatory Completion Required*

Provide a BRIEF overview of your project.
Use Section **E.** for **Animal Number Justification**, and **F.** & **G.** for **Experimental Details**
1. Rationale
2. Hypothesis
3. Objective(s)
4. Approach/Research Plan – ***Brief*** *Summary only*

E. ANIMAL NUMBER JUSTIFICATION BY EXPERIMENTAL GROUP– *TWO OPTIONS* –

Mandatory Completion Required

i. Describe possible replacement, refinement and/or reduction alternatives to animal use, and offer justification if these are not to be employed, or a description of efforts to find such alternatives.	
ii. Indicate how you have determined your animal numbers by breaking their use down into experimental groups including controls – Label each group with a numeric identifier, i.e. 1, 2, 3	
Experimental	**←Use Experimental Group ID # (column directly left) to**

Group ID #	assign group to details in Sections F & G, right hand column
Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	212
Subgroup Animal Numbers	
Other Animal Number Related Detail	
Experimental Group ID #	←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column
Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	396
Subgroup Animal Numbers	
Other Animal Number Related Detail	
Experimental Group ID #	←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column

Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	444
Subgroup Animal Numbers	12
Other Animal Number Related Detail	
Experimental Group ID #	**←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	1352
Subgroup Animal Numbers	16
Other Animal Number Related Detail	
Experimental Group ID #	**←Use Experimental Group ID # (column directly left) to assign group to details in Sections F & G, right hand column**

Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	678
Subgroup Animal Numbers	
Other Animal Number Related Detail	
Experimental Group ID #	**←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	1017
Subgroup Animal Numbers	198
Other Animal Number Related Detail	
Experimental Group ID #	**←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	

Total Animal Numbers per Experimental Group	1356
Subgroup Animal Numbers	
Other Animal Number Related Detail	
Experimental Group ID #	**←Assign sequential Group ID # (column directly left). Use ID# in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	
Total Animal Numbers per Experimental Group	1356
Subgroup Animal Numbers	
Other Animal Number Related Detail	
Experimental Group ID #	**←Use Experimental Group ID # (column directly left) to assign group to details in Sections F & G, right hand column**
Name of Experimental Group &/or Species or Strain	

Total Animal Numbers per Experimental Group	1356
Subgroup Animal Numbers	264
Other Animal Number Related Detail	

F. EXPERIMENTAL DETAILS - *Mandatory Completion Required*

Using the groups you have indicated in Section E., provide details of all experiments, events & procedures involving animals in each of the groups.

Use the right-hand column to connect the 'event' with the specific group(s) identified in Section E.

F. 1. Animal Use Endpoints – *Mandatory Completion for All Projects*		**Identify Section E Experimental Group ID #s Involving This Element**
"*In experiments involving animals, any actual or potential pain, distress, or discomfort should be minimized or alleviated by choosing the earliest endpoint that is compatible with the scientific objectives of the research. Selection of this endpoint by the investigator should involve consultation with the lab animal vet and the animal care committee*." CCAC General Guideline		
For SOP detail go to http://www.uwo.ca/animal/website/VS/Content/SOPs.htm		
Experimental Endpoints	If mutant mice develop severe neurological symptoms and loss of weight they will be euthanized shortly after symptoms appear. Adult mice that have had stereotaxic surgery will be initially monitored according to Standard Operating Procedure # 321-02	☒ All *Or* ID #s

<table>
<tr><td></td><td colspan="5">(CRITERIA FOR EARLY EUTHANASIA / RODENTS). If any suspicious condition is observed the ACVS veterinary service is going to be called to examine the animal and assess the necessity for early euthanasia. After behavioral studies these mice are going to be euthanized with CO2 to obtain tissues for biochemistry and immunofluorescence analysis.</td><td></td></tr>
<tr><td rowspan="2">Euthanasia SOPs</td><td colspan="3">Pick all that will be followed

☒ #320 Euthanasia

☒ #321-Criteria for Early Euthanasia-Rodents</td><td colspan="2">☐ #322-Criteria for Early Euthanasia-Non-Rodents

☐ Other Criteria, please explain below</td><td>☒ All
Or
ID #s</td></tr>
<tr><td colspan="6">If ‘Other Criteria’, explain</td></tr>
<tr><td rowspan="3">Euthanasia Method</td><td colspan="6">Pick All Euthanasia Methods That Will Be Followed & Provide Euthanasia Related Agent Use Detail Below</td></tr>
<tr><td>☐ Animals Not Euthanized

☐ Barbiturate Overdose

☒ CO2

☐ Decapitation under Anaesthesia

☒ *Decapitation with No Anaesthesia*

☒ Cervical Dislocation with Anaesthesia

☐ *Cervical Dislocation with No Anaesthesia*

☐ Other Method(s), Anaesthetized - explain

☐ *Other Method(s), Awake* - explain</td><td>Agent Name</td><td>Dose</td><td>Route</td><td>Volume</td><td>☒ All
Or
ID #s</td></tr>
<tr><td colspan="4">If ‘Other Method(s)’, explain</td><td>☐ All
Or
ID #s</td></tr>
</table>

Scientific Justification	**If Method Type Has An Asterisk *, Provide Scientific Justification**	☐ All Or ID #s
Alternatives to Euthanasia	**Describe how animals will be 'disposed of' if not euthanized** Animals that are not going to be used in our experiments (extra mice) are going to be offered to other scientists for their use.	☐ All Or ID #s
Other Related Endpoint Detail	WT mice euthanized via CO2 will be made available to the Wild Life Rehabilitation Centre for feeding.	

F. 2. Agent Use

☐ **No Agent Use** *(Go to F 3.)*

NON HAZARDOUS AGENTS INCLUDING DRUGS

Agent Type	Agent Name	Species	Dose	Route	Volume	Frequency	Does this agent have potential to cause pain or pronounced debilitation?	Identify Section E. Experimental Group ID #s Involving This Agent
Analgesics	ketaprofen	Mouse	2.5-5mg/Kg	IP	1ml/kg	As needed	☐ Yes ☒ No	☒All *or* ID #s
		As Abov		Click Here				
		As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				

		As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				
Pre-Anaesthetic		As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				
General Anaesthetic		Mouse	87mg/kg + 13 mg/kg	IP	1 ml/kg	As needed	☐ Yes ☒ No	☒ All *or* ID #s
		As Abov		Click Here				
General Anaesthetic	Isoflurane inhalation	Mouse	1.5-3%	Gas anesthesia machine at appropriate settings for induction and maintenance (Harvard Apparatus)			☐ Yes ☒ No	☒ All *or* ID #s
Local Anaesthetic		As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				

Other **Non-Anaesthetic** **Agents** **i.e. Tranquilizers**		Mouse		IP			☐ Yes ☒ No	☐ All *or* ID #s
		As Abov		Click Here				
		Mouse		IP			☐ Yes ☒ No	☐ All *or* ID #s
		As Abov		Click Here				
		Mouse		IP			☐ Yes ☒ No	☐ All *or* ID #s
		As Abov		Click Here				
		Mouse		Other, det			☒ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				
HAZARDOUS AGENTS – Complete Below Section AND Section H, Addendum 3, Schedules 1-5							**Does this agent have potential to cause pain or pronounced debilitation?**	**Identify** **Section E.** **Experimental Group ID #s** **Involving** **This Agent**
Agent Type	**Agent Name**	**Species**	**Dose**	**Route**	**Volume**	**Frequency**		
Biological or	**Class** Animal Biosa **Category**	Mouse		Other, det			☒ Yes ☐ No	☐ All *Or* ID #s

Cell Line Agents **Go to Sect. H, Add. 3, Sch. 1-2**	Infectious **Name**							
	Has this cell line/biological agent been tested for murine pathogens? ☐ **Yes** ☐ **No** *If Yes, please send documentation along with this form.*							
	Class Click Here **Category** Click Here **Name**	As Abov		Click Here			☐ Yes ☐ No	☐ All *Or* ID #s
	Has this cell line/biological agent been tested for murine pathogens? ☐ **Yes** ☐ **No** *If Yes, please send documentation along with this form.*							
Chemical Or Radioisotopes **Go to Sect. H, Add. 3, Sch. 3-5**	**Class** Click Here **Category** Click Here **Name**	As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				
	Class Click Here **Category** Click Here **Name**	As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				
	Class Click Here **Category** Click Here	As Abov		Click Here			☐ Yes ☐ No	☐ All *or* ID #s
		As Abov		Click Here				

	Name							
Other Related Agent Use Detail	. Controled drugs such as cocaine are going to be stored in a 2 lock system. The cocaine will be in a locked box inside of a locked cabinet. Both keys will be kept in a separate room and will be unlabelled. We are going to keep a log sheet which will record date, amount taken, amount left, animal species and ID, and the signature of user. MSDS for all controled drugs will be kept in a binder close to the drug cabinet.							

F. 3. Anaesthesia or Surgery or Recovery Projects			**Identify Section E. Experimental Group ID #s Involving This Element**
☐ **No Surgery or Anaesthesia or Recovery Elements** *(Go to F 4.)*			
Fasting Involved?	☐ Yes ☒ No	If Yes, provide justification & duration detail	☐ All *or* ID #s
Post Op Care	**Care Person(s)**	**Contact #**	☒ All *or* ID #s
SOPs	**Pick all that will be followed**		

	☒ 330-Post-operative Care-Rodent ☐ 331-Post-operative/Post-Anaesthetic Care-Level 1 ☐ 332-Post-operative/Post-Anaesthetic Care-Level 2 ☐ 333-Post-operative/Post-Anaesthetic Care-Level 3		☐ 334-Post-operative/Post-Anaesthetic Care- Level 4 ☒ 343-Surgical Prep/Rodent/Recovery Surgery ☐ 350-Rodent Anaesthesia/Halothane/Induction Chamber ☐ Other, please explain	☐ All *or* ID #s
Surgery or Procedure Location	**Institution**	**Room #**	☐ Overnight Surgery/Recovery in non-approved area. If clicked, provide scientific justification:	☒ All *or* ID #s
Recovery Location	**Institution**	**Room #**	**When will animals be returned to the main facility?** Surgery will be performed early in the morning and mice are going to be monitored during the day according to SOP 321-02 (CRITERIA FOR EARLY EUTHANASIA / RODENTS) and SOP 330-03 (POST-OPERATIVE/POST ANAESTHETIC CARE-RODENTS). If everything is OK mice are going to be returned to the main facility at the end of the day	☒ All *or* ID #s
Other Related Detail				

F. 4. Antibody Production

☒ **No Antibody Production** *(Go to F.5.)*

F. 5. Monitoring or Chronic Projects – i.e. Post-op, Feed, Drug and/or Disease Induction Studies			**Identify Section E. Experimental Group ID #s Involving This Element**
☐ **No Monitoring or Chronic Elements** *(Go to F 6.)*			
Monitoring Criteria	*Pick all that will be followed* ☐ 100-Monitoring/Tumour Growth in Rodents ☒ 310-Holding Period Post Admission ☒ Other, *please explain*		☒ All *or* ID #s
Monitoring Type	☒ **Weight** ☒ **Food Intake** ☒ **Behaviour** ☐ **Urine Output** ☒ **Other**, *explain*		☒ All *or* ID #s
Monitoring Frequency	**Pick all that apply to this project:** ☒ Hourly ☒ Twice Daily ☒ Daily ☐ Weekly ☐ Other, *please explain*	**If other, or multiple frequencies chosen, please provide detail:** Weight, food and water intake, appearance and behavior are monitored post op (SOP 321-02 and SOP 330-03) or daily for experimental purposes.	☒ All *or* ID #s
Monitoring	**Care Person(s)**	**Contact #**	☒ All

Personnel			*or* ID #s
Monitoring Records	**Will animal monitoring records be kept with the animal?** ☒ **Yes** *or* ☐ **No**	**If 'No', identify monitoring record location**	☒ All *or* ID #s
Other Related Monitoring Detail			

F. 6 Breeding

☐ **No breeding elements** *(Go to F.7)*

I. Indicate Breeding Type	☒ Breeding for research within this protocol ☐ Breeding for research NOT within this protocol
Complete for All Breeding Elements	
II. Provide justification for maintaining a breeding colony	
III. Provide justification for breeding numbers *Quote animal numbers within Section G. Animal Requirements*	
IV. List procedures used in the breeding colony	
V. Number estimation and use of surplus animals *(those not required for experimental*	Surplus Number Estimate: Surplus Use: -Animals that are not going to be used in our experiments (extra mice) are going to be offered to

programs, or retired breeders)	other scientists for their use. - WT mice euthanized via CO2 will be made available to the Wild Life Rehabilitation Centre for feeding
VI. Breeding colony location, *if different from research housing.*	
VII. Research associates directly involved in the care of animals in this breeding colony	Name: Contact #: Email: Name: Contact #: Email:
Breeding for External Protocols Only	
VIII. List External Protocol Researcher(s)	
IX. List External Protocol Numbers	

F. 7. Blood Collection	**Identify Section E. Experimental Group ID #s Involving This Element**
☒ **No Blood Collection** *(Go to F.8.)*	

F. 8 Genetically Altered Animal Information					
☐ **No Genetically Altered Animals Used** *(Go to F. 9.)*					
i. Gene Name					

ii. Has this animal already been generated?		☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No
iii. If yes to ii., by which institution?						
iv. Is the animal commercially available?		☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No	☐ Yes ☒ No
v. If yes to *iv.* insert vendor strain information URL, then proceed to Sect. G						
vi. If no to *iv.* complete the following details	Strain ID					
	Gene(s) Symbol(s)					
	Background Strain					
	Type, *i.e. Tg, Ko*					
	Source, *i.e. Jax, CR*	/Duke/ UWO	/Duke/ UWO	/Duke/ UWO	/UWO	/Duke/ UWO
	Phenotype	*Complete details separately for hetero and homozygous animals* *Detail how this alteration affects the animal's quality of life, locomotion, eating, breeding, lifespan, tumor type & incidence, etc.*				
Heterozygote	Systems Affected					

	How systems are affected					
	Special Care Required					
Homozygote	Systems Affected					
	How systems are affected					
	Special Care Required					
Other Genetic Detail						

F. 8 Genetically Altered Animal Information

☐ **No Genetically Altered Animals Used** *(Go to F. 9.)*

i. Gene Name					
ii. Has this animal already been generated?	☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	☒ Yes ☐ No	☐ Yes ☐ No
iii. If yes to ii., by which institution?					
iv. Is the animal	☐ Yes ☒	☐ Yes ☒	☐ Yes ☒	☐ Yes	☐ Yes ☐

commercially available?		No	No	No	☒ No	No
v. If yes to *iv.* insert vendor strain information URL, then proceed to Sect. G						
vi. If no to *iv.* complete the following details	Strain ID					
	Gene(s) Symbol(s)					
	Background Strain					
	Type, *i.e. Tg, Ko*					
	Source, *i.e. Jax, CR*					
	Phenotype	*Complete details separately for hetero and homozygous animals* *Detail how this alteration affects the animal's quality of life, locomotion, eating, breeding, lifespan, tumor type & incidence, etc.*				
Heterozygote	Systems Affected					
	How systems are affected					
	Special Care Require					

	d					
Homozygote	Systems Affected					
	How systems are affected					
	Special Care Required					
Other Genetic Detail						

F. 9. Procedural Description - *Mandatory Completion for All Projects*

Minus the details presented in Sections 1 to 5,

provide a concise description of the procedural events experienced by the animals in each experiment.

The intent is to order, name and briefly describe the procedural events that animals of each experimental cohort will experience. The events should be presented numerically in chronological number.

Evaluate the potential to cause pain and indicate the experimental groups experiencing each procedural event.

Procedure	Name of Procedure	Short Description of the Experimental Procedure	Potential To Cause Pain	Experi-mental Group
1			☐ **Yes** ☒ **No**	☒ All *or*
2			☐ **Yes** ☒ **No**	☐ All *or*
3			☐ **Yes** ☒ **No**	☐ All *or*
4		Mice are anaesthetized and placed into a stereotaxic apparatus (Kopf). A single scalp incision is made. A burr hole (approximately 3 mm in diameter) is made in the skull and a glass micro pipette filled with 1 microliter of AAV-Cre is going to be stereotaxically microinjected into a specific brain area. Only one area of the brain will be injected in each animal. To minimize tissue injury,these injections are going to be performed using glass pipettes with a 10- to 20-micrometer diameter tip, and AAV-Cre is going to be slowly injected over 1 hr using a pressure-injection system. After an additional period of 5 minutes, the micropipette is removed and the scalp incision is closed with wound clips. Sham surgeries will be performed the same way,	☐ **Yes** ☒ **No**	☐ All *or*

		except that the pipette will be filled with vehicle, and no injection will be made. Animals are kept on heat pads or under heat lamps to maintain body temperature throughout the procedures. During recovery, animals are kept in the surgery room to allow frequent monitoring of breathing and general activity (according to SOP330-03 **POST-OPERATIVE/POST ANAESTHETIC CARE RODENTS)** before being transported back to the holding room. The procedure is going to be performed once in each animal and we will use adult mice (8-20wks).		
5			☐ **Yes** ☐ **No**	☐ All *or*

G. ANIMAL REQUIREMENTS - *Mandatory Completion Required*

Detail all animal requirements including, species, strain, age/weight, sex, housing, dietary and animal total details for the Upcoming Year Only
Use bottom row of this section to add more detail to any column *Researcher must provide scientific justification for overnight animal housing in non-approved areas, i.e. labs, & receive AUS approval:* Use Section F. 3 for surgery related housing & bottom of this section for all other housing justification

Species	Strain &/or Other Species Detail For Genetic Mutation Use F. 8 For Details	Age or Weight	Sex	Animal Housing *See Drop-downs for complete listing* *Abbreviations:* **CBM**=*Centre for Brain & Mind* **HSACF**=*Health Sciences Animal Care Facility* **LHRI**=*Lawson Health Research Institute, St. Joe's* LHSC-UC=*University Campus* **LHSC-SS**=*South Street* **LHSC-VRL**=*Victoria Research Lab* **LRCC**=*London Regional Cancer Centre* **RACF**= *Robarts Barrier Facility* **REB**= *Robarts Experimental Barrier* *WVB=West Valley Bldg*					Special Dietary Details *Complete* ***only*** *if Standard diet is not adequate*	1 Year Total Animal Numbers Required
				Housing Location	Use/Lab Location	Room #	If Specialized Housing Required, *i.e. barrier, lab,* ***Field Study, Other*** Provide Details	*Used in Breeding ?*		
Mouse			Male	HSACF	Same as H			Yes		15
Mouse			Femal	HSACF	Same as H			Yes		35
Mouse			M/F	HSACF	Same as H			No		
Mouse			M/F	HSACF	Same as H			No		
Mouse			Male	HSACF	Same as H			Yes		

Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			No		
Mouse			Femal	HSACF	Same as H			Yes		10
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		

Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			M/F	HSACF	Same as H			Yes		80
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			M/F	HSACF	Same as H			Yes		45
Mouse			M/F	HSACF	Same as H			Yes		45
Mouse			Male	HSACF	Same as H			No		60
Mouse			Femal	HSACF	Same as H			No		30
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		

Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			M/F	HSACF	Same as H			Yes		45
Mouse			M/F	HSACF	Same as H			Yes		45
Mouse			Male	HSACF	Same as H			No		
Mouse			Femal	HSACF	Same as H			No		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		

Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		

Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		

Mouse			Femal	HSACF	Same as H			Yes		
Mouse			Male	HSACF	Same as H			Yes		
Mouse			Femal	HSACF	Same as H			Yes		
Mouse			M/F	HSACF	Same as H			Yes		
Mouse	C57BL/6		Male	HSACF	Same as H			Yes		18
Mouse	129SvEv		Male	HSACF	Same as H			Yes		18
Mouse	Swiss	4-6 weeks	Male	HSACF	Same as Housing			No		4
Non-approved Housing Justification										
Other Animal Requirement Detail	We intend to house some of the breeding pairs at the Robarts Barrier Facility for precautionary measures, since the genetically modified animals are unique and we are the only researchers in the world that have them. Some of the mice kept at ACVS will be transferred to Robarts for behavioral studies. They will be transported in cages covered either with garbage bags or cloth covers, water bottles are going to be turned right side up to prevent flooding during transport and returned to its original down position once in the proper holding room									

H. ADDENDA

Addendum 1 –	☒ **No Teaching Elements**

Teaching ONLY	

Addendum 2 – Wildlife Study ONLY	☒ **No Wildlife Study Elements**

1.1.1

Addendum 3 - Hazardous &/or Biological Agent Use ONLY – Occupational Health and Safety	
☐ **No Hazardous &/or Biological Agent Use**	
University of Western Ontario **Animal Use Protocol Safety Form**	For further information, please contact J. Stanley - jstanle2@uwo.ca
1.1.2 Protocol Title:	Approved: June 10, 2003 ***OHS Use Only*** **Permit Number Issued**: We have submitted the application forms to the biosafety committee and are waiting for their approval.

Schedule I Work with Biological Organisms

1.1 List microorganisms that will be used in an animal (provide Material Safety Data Sheets or equivalent):

Organism	Route of Administration	Primary Host	Other Suseptible Species	Zoonotic	Route of Transmission
				☐ YES ☒NO	☐Handling of waste ☐Fomites ☐Body Excretions ☐Trauma ☐Aerosol ☐Other
				☐	☐Handling of waste

				YES ☐NO	☐Fomites ☐Body Excretions ☐Trauma ☐Aerosol ☐Other
				☐ YES ☐NO	☐Handling of waste ☐Fomites ☐Body Excretions ☐Trauma ☐Aerosol ☐Other
				☐ YES ☐NO	☐Handling of waste ☐Fomites ☐Body Excretions ☐Trauma ☐Aerosol ☐Other
1.2 Will any innoculated organism from question 1.1 remain viable in the animal model? If no, attach documentation for each.					☒YES ☐NO
If yes, how long will infection persist?					
1.3 Do infected animals require housing?					☒YES ☐NO
If Yes:	Are cage/animal generated aerosols a concern?				☐YES ☒NO
	Do bedding and dirty caging pose a hazard?				☐YES ☒NO
	Do bites or scratches pose a hazard?				☐YES ☒NO
	If YES, do you have a bite protocol?				☐YES ☐NO
1.4 How are the infected material(s) to be treated prior to disposal?					
1.5 How are the carcasses to be disposed of?					
1.6 Other?					

Schedule 2 Hazard Assessment for Use of Recombinant DNA

☒ **No Recombinant DNA Use *(Go to Schedule 3)***

2.1 Will proposed animal use involve genetically engineered	☐YES ☐NO

organisms or cells containing engineered molecules?	
If YES, indicate what changes have been affected	
2.2 Is this expected to increase the invasiveness, toxicity or tumorigenicity of the agent in the animal?	☐YES ☐NO
2.3 Describe steps taken to mitigate the risks.	

Schedule 3 Hazard Assessments for Work with Chemicals

No Chemical Use *(Go to Schedule 4)*		
3.1 Will potentially toxic chemicals be used with live animals?	YES NO	Chemical Name:
3.2 Will potentially carcinogenic chemicals be used with live animals?	YES NO	Chemical Name:
Please attach a Material Safety Data Sheet for any TOXIC or CARCINOGENIC chemical to be used in the protocol.		
3.3 How will the chemical(s) be administered into the animal?		
3.4 Will chemical or metabolite be excreted (faeces, urine, through skin, tears, sperm, etc.)?		YES NO
3.5 State preventative measure that must be taken to minimize the risk of exposure for research staff.		
3.6 State preventative measure that must be taken to minimize the risk of exposure for animal facility staff.		
3.7 Training Requirements	Personnel working with these products directly or indirectly require the following training – Go to the Website: http://www.uwo.ca/humanresources/facultystaff/h_and_s/training/training_idx.htm	* WHMIS * Laboratory Safety * Waste

		Manage ment

Schedule 4 Hazard Assessment and WHMIS Inventory for Work with Radioisotopes

No Radioisotope Use *(Go to Schedule 5)*			
4.1 Will radioisotopes be administered in live animals? *If No, please continue to* **Schedule 5**			☐YES ☐ NO
If YES	1. Laboratory Location		
	2. Radioisotope		Chemical Form
	3. Dose to the animal		☐ uCj/kg or ☐ kBg/kg
4.2 Will the radioisotope-contaminated animal be returning alive for housing in the Animal Quarters?			☐YES ☐ NO
If YES	1. Identify the primary route of excretion for the radioisotope		
	2. Animal quarters location	Building	Room #
	3. Duration of excretion		
	4. Storage locations of animal carcass	Building	Room #
If NO	Storage locations of animal carcass	Building	Room #
4.3 Internal Permit Holder		Name	Permit #
4.4 State preventative measures that must be taken to minimize the risk of			

exposure for staff.		
4.5 Training Requirements	Personnel named on the schedule require the following training, See Website http://www.uwo.ca/humanresources/facultystaff/h_and_s/training/training_idx.htm	* Radiation Safety

Schedule 5 Hazard Assessment and WHMIS Inventory for Work with Radiation

No Radiation Use *(Go to Schedule 6)*			
5.1 Will the animals undergo gamma irradiation at any time?			☐YES ☐NO
If YES	1. Name(s) of personnel who will perform animal irradiation		
	2. Has registration & training on the Gammacell-20 irradiator been completed?		☐YES ☐NO
5.2 Will the animals undergo x-rays, or CT Scan at any time?			☐YES ☐NO
If YES	1. Have personnel had training taking images with the X-ray machine?		☐YES ☐NO
	2. Location of X-ray machine	Building	Room
5.3 Other –Will animals undergo any other imaging MRI?			☐YES ☐NO
If YES	1. Have personnel received specific training (MRI, CT scan)?		☐YES

				☐NO
	2. Specify type and location.	Type	Building	Room
5.4 Is the machine in a human space?				☐YES ☐NO
If YES	Has permission been obtained?			☐YES ☐NO
5.5 ***NEW*** Will TLD badges be worn by all staff?				☐YES ☐NO
If NO	Please explain			
5.6 State preventative measures that must be taken to minimize the risk of exposure for staff.				
5.7 Training Requirements	Personnel working with these products directly or indirectly require the following training – Go to the Website: http://www.uwo.ca/humanresources/facultystaff/h_and_s/training/training_idx.htm			* Radiation Safety * Gamma Cell Training * X-ray Safety

Schedule 6 Approvals - Please Sign & Date Below

Principal Investigator	Protocol Title	Date
Print Name M. Prado **Signature**		*mm/dd/yy* 05/05/08
Occupational Health and Safety Approval *- Office Use Only*		
Signature of OHS Officer **Date** *mm/dd/yy* */*	**Institution** (UWO, RRI, LHRI, LHSC, SJHC, Other)	

Curriculum Vitae

Name: Amro Mohammad

Post-secondary Education and Degrees:

Western University Canada
London, Ontario, Canada
2006-2010 B.A.

Western University Canada
London, Ontario, Canada
2010-2012 M.A.

Honours and Awards:

Western University Canada
Alzheimer's Doctoral Award of the Alzheimer's Society of Canada
(declined due to change of laboratories)
2012

Western University Canada
Ontario Graduate Scholarship (OGS) –Waiting List
2012-2013

Western University Canada
Ontario Graduate Scholarship (OGS) awardee
2011-2012

Western University Canada

NSERC USRA awardee

2010

Western University Canada

Gamma Upsilon Scholarship awardee

2010

Western University Canada

UWO In-Course Scholarship Year IV

2009

Related Work Experience

Teaching Assistant

Western University Canada

2010-2012

Patents:

Application entitled: **A Novel Cell Protective Factor.** Issue date: March 6th, 2012. Serial Number: 61/607, 380. Gowling Inc. Dr. F. Beraldo, Dr. V. Ostapchenko, Daniela Fontes, **Amro Mohammad**, Dr. I. Soares.

Publications:

Quinn C., **Mohammad A**., Macfie SM. Accumulation of cadmium in near-isogenic lines of durum wheat (*Triticum turgidum* L. var durum): the role of transpiration. Physiology and Molecular Biology of Plants. Accepted on 21/09/2011