IN VITRO PATHOLOGY OF THE TGCRND8 MOUSE MODEL OF ALZHEIMER'S DISEASE

BY

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A Thesis
Submitted to the Faculty of Graduate Studies in
Partial Fulfillment of the Requirements
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ABSTRACT

The most common form of senile dementia is Alzheimer's disease (AD). Pathological characteristics include plaques of amyloid beta (Aβ) peptide, neurofibrillary tangles, and neuronal death.

The TgCRND8 mouse is an accelerated, early on-set familial representation of AD, encoding human APP695 housing both Swedish (K670N/M671L) and Indiana (V717F) mutations, leading to increased Aβ. Generally, overproduction of Aβ is associated with neurotoxicity, neurodegeneration, and cell death in vivo and in vitro, however this has not yet been fully established in TgCRND8. Calcium dyshomeostasis is also involved in neurodegenerative cascades.

The objectives of this thesis were to characterize in vitro biological properties (i.e. is Aβ being generated) and calcium handling, in TgCRND8. Primary cortical cells from TgCRND8 and Non-transgenic (Non-tg) littermates were dissociated from embryonic day-16 fetuses and seeded onto coated plates for 8-12 days in vitro (DIV) for all experiments. We report significantly greater production of amyloid precursor protein (APP), indicated by Western blot analysis, and secretion of Aβ (via sandwich ELISA) in TgCRND8 vs. Non-tg controls. The generation of reactive oxygen species (ROS) was significantly greater in TgCRND8 cortical cells and basal calcium concentration in this group, measured by Fura 2, was significantly greater 8- and 12-DIV compared to Non-tg controls. Furthermore, in vitro neuronal death was evident, significantly higher in TgCRND8 than in Non-tg, and more necrotic in nature. Intracellular calcium influx after 1mM glutamate application was parallel in both groups, but significantly increased in TgCRND8 primary neurons after treatment with NMDA. NMDA receptor subunit

protein expression was further enhanced in TgCRND8 vs. Non-tg controls. Additionally, increased endoplasmic reticulum (ER) calcium release after treatment with SERCA pump blocker thapsigargin was evident in TgCRND8 vs. Non-tg littermates. Moreover, intracellular calcium influx was enhanced after AMPA receptor inhibition in TgCRND8, but there was no difference between the two groups after NMDA receptor blockade.

This data indicate that cultured TgCRND8 neurons produce Aβ in vitro disrupting calcium and causing enhanced ROS resulting in cell death. This pathway is well characterized in studies using exogenous Aβ, but here we show a more physiologically relevant system that generates endogenous Aβ.

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DEDICATION

I share this thesis with so many amazing people, but none more deserving and incredible than my husband Scott and my mom, Karen. I have never had the privledge of knowing two more supportive, loving, selfless, intelligent individuals in my life. This thesis is as much their's as it is mine. Thank you both for walking with me and cheering me on as I took this journey, for picking me up when I stumbled, and for believing in me even when I didn't. I am blessed beyond measure to have you both in my life.

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CHAPTER 1 - Introduction and Review of Literature

1.0 Historical Review

Alzheimer's disease (AD) is the central and most common form of senile dementia, first documented by German physician, Dr. Alois Alzheimer in 1906. AD is manifested clinically by progressive, irreversible cognitive decline and disordered behaviours (Blass 1985). Currently, the only definitive means of diagnosing AD is through post mortem examination. Histopathologically, AD is characterized by an accumulation of plaques throughout the brain made up of an insoluble, aggregated form of a protein called amyloid beta (Aβ) (Glenner and Wong 1984); (Selkoe 1991). Another key lesion is the intracellular buildup of a microtubule-associated protein, tau, phosphorylated under normal cellular conditions but hyperphosphorylated in Alzheimer's disease (Jellinger and Bancher 1998). Finally, neuronal death is associated with the later stages of the disease (Selkoe 2001). Approximately 98% of all cases of AD are sporadic, occurring later in life, and have no known genetic cause, whereas the remaining 2% constitute early-onset familial autosomal dominant (FAD) cases, associated with mutations in the presenilin 1 or 2 (PS1, PS2) or the amyloid precursor protein (APP) genes, all of which result in elevated Aβ (Hutton, Perez-Tur et al. 1998); (Behl 2000). In Canada, approximately 300,000 individuals over the age of 65 have AD or related dementias (www.alzheimer.ca), at a cost to the health care system of $5.5 billon per year (Ostbye and Crosse 1994). Worldwide, AD and other related dementias afflict more than 24 million people with an expected rise in incidence to reach 81 million within the next 34 years (www.alz.co.uk/media/dementia.htm). Many brain regions are affected in AD

including hippocampus, cortex, and frontal, temporal, and parietal regions (Kosik, Joachim et al. 1986).

2.0 Amyloid Precursor Protein (APP) and Amyloid Beta (Aβ) Peptide

2.1 Location and Processing of APP

APP is a "transmembrane glycoprotein" encoded by a gene located in human chromosome 21 (St George-Hyslop, Tanzi et al. 1987). Differential protein cleavage results in various isoforms including APP695, APP751 and APP 770 (Kitaguchi, Takahashi et al. 1988); (Tanzi, McClatchey et al. 1988); (Tanaka, Shiojiri et al. 1989); (Golde, Estus et al. 1990), with APP695 being the most abundant isoform in the brain (Koo, Sisodia et al. 1990). There are two crucial different but convergent avenues by which APP is processed, the endosomal, and the lysosomal systems (Nixon, Cataldo et al. 2000). The endocytic pathway is responsible for continuous remodeling of the cell membrane, thus providing cells an opportunity to survey their extracellular environment (Nixon, Cataldo et al. 2000). It is in the early portion of this pathway that chief sorting receptors and other materials from the plasma membrane are directed to other destinations either within the endosomal pathway itself or elsewhere (Clague 1998). Furthermore, APP processing is generally accepted to occur in the early segment of the endosomal pathway (Nixon, Cataldo et al. 2000). Interestingly, the function of a number of the proteins linked to increasing the risk for Alzheimer's disease, like APP rely on the endocytic pathway (Hyman, Gomez-Isla et al. 1996); (Sabo, Lanier et al. 1999).

2.1.1 Location and Processing of Aβ

Throughout the process of APP trafficking, APP can be cleaved, resulting in varied lengths of the protein caused by a single or multiple sequential encounters from a group of proteolytic enzymes called secretases (Selkoe 2001). This processing results in engaging one of two known cleavage pathways, a non-toxic, "non-amyloidogenic" route (Lammich, Kojro et al. 1999) or a neurotoxic, "amyloidogenic" sequence of events (Vassar, Bennett et al. 1999), depending on which APP fragments are involved (Figure 1). APP sent to or near the plasma membrane is cleaved by α-secretase in the centre of the Aβ region liberating a C-terminal fragment called C83 (Esch, Keim et al. 1990); (Sisodia 1992); (Cao and Sudhof 2001). C83 can undergo additional cleavage by γ-secretase to generate two supplementary fragments, the APP intracellular domain (AICD) and P3 (Cao and Sudhof 2001). Alpha-secretase can also liberate a larger fragment called secreted-APP-α (sAPPα) (Stein and Johnson 2003). These cleavage actions are chiefly recognized as constituting the 'Non-Amyloidogenic' pathway (Lammich, Kojro et al. 1999). Secreted APP-α can provide neuroprotection by assisting in the maintenance of intracellular calcium homeostasis through the inhibition of glutamate and Aβ toxicity (Furukawa, Barger et al. 1996). Alternatively, APP can be incised via an additional pathway termed the 'Amyloidogenic' pathway, promoting the ultimate release of Aβ (Vassar, Bennett et al. 1999). Near the N-terminus of APP, beta-secretase (β-secretase) cleavage can liberate a peptide called secreted-APP-β (sAPPβ) (Seubert, Oltersdorf et al. 1993), as well as generating a C-terminus end fragment, CTFβ (Sankaranarayanan 2006). As a final step in the amyloidogenic pathway, gamma-secretase (γ-secretase) can cleave CTF-β to liberate 40- or 42-amino acid length fragments of amyloid beta (Aβ1-40 and

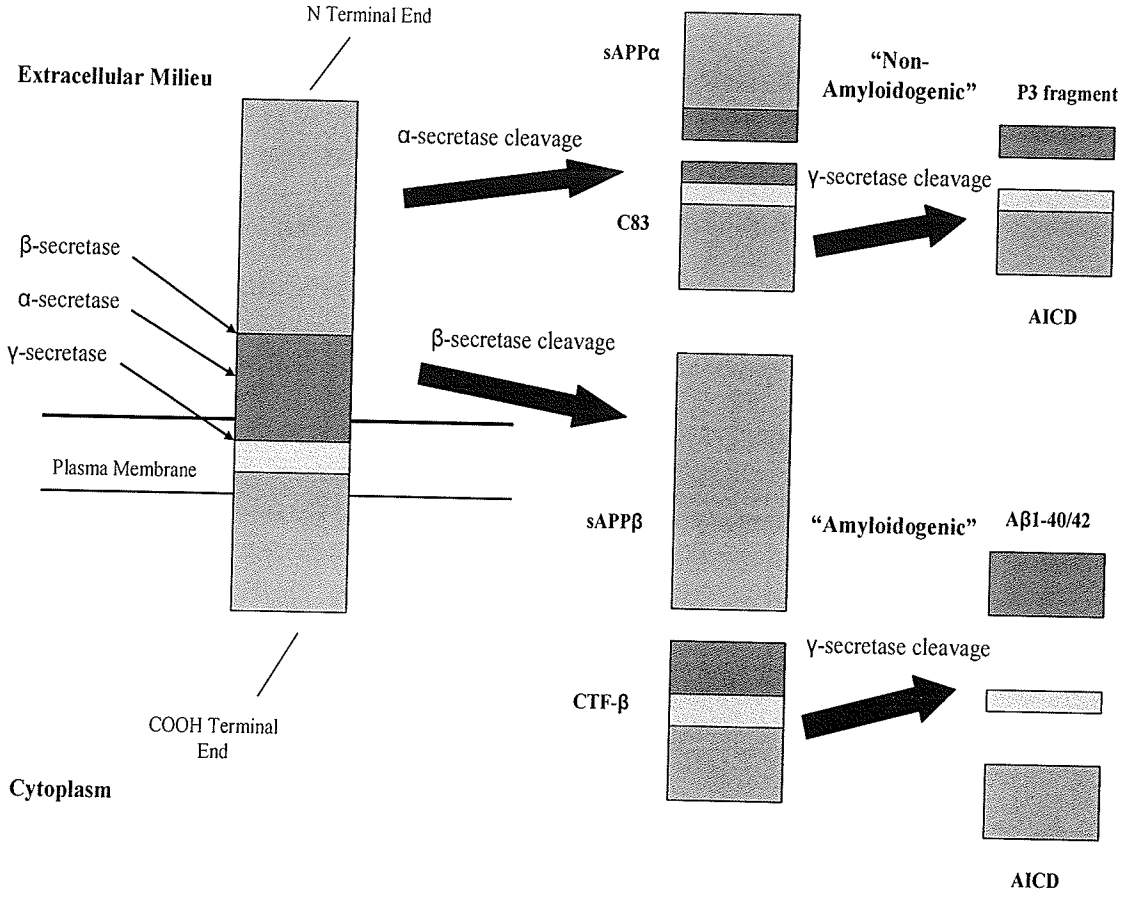
Aβ1-42, respectively) (Haass, Schlossmacher et al. 1992); (Shoji, Golde et al. 1992); (Seubert, Oltersdorf et al. 1993). The γ-secretase complex is made up of 4 proteins, nicastrin, presenilin-1 (PS1), presenilin enhancer-2 (Pen2), and Aph-1 (Mattson 1997) whose eventual production seems to be controlled by the cleavage activity of β-secretase (Sinha and Lieberburg 1999). The production of Aβ is dependent upon sequential secretase cleavage (Haass, Schlossmacher et al. 1992); (Shoji, Golde et al. 1992); (Seubert, Oltersdorf et al. 1993) thus, secretases and the genes that encode them have become strong potential contenders upon which to test possible therapeutic AD interventions (Tanzi and Bertram 2005)

Amyloid beta (Aβ) is a key factor in the neuropathogeneis of AD and is derived from APP (Glenner and Wong 1984). The chief location for Aβ generation and uptake from the cell surface is in the early endosomal pathway (Nixon, Cataldo et al. 2000), however evidence suggests that APP processing and subsequent Aβ generation can occur in the endocytic pathway (Haass, Schlossmacher et al. 1992); (Koo and Squazzo 1994), the Golgi apparatus (Peraus, Masters et al. 1997); (Tomita, Kirino et al. 1998); (Xu, Sweeney et al. 1997), and the endoplasmic reticulum (ER) (Chyung, Greenberg et al. 1997); (Cook, Forman et al. 1997); (Xia, Zhang et al. 1998), with the latter two compartments both from the secretory pathway. Aβ produced in the ER can remain in the cell, whereas Aβ from the Golgi apparatus is secreted (Skovronsky, Doms et al. 1998).

The lysosomal system contains lysosomal organelles, hydrolases wrapped in vesicles from the Golgi, and late endosomes (Nixon, Cataldo et al. 2000). This system is responsible for hydrolase synthesis (in the ER and Golgi) and cellular digestion known as autophagy, as well as endocytosis and phagocytosis (Nixon, Cataldo et al. 2000). Cell

size is controlled by managed lysosomal activity (Nixon and Cataldo 1995), which has been suggested to play a potential role in brain cell reduction observed in AD (Nixon, Cataldo et al. 2000). The development of increased activation of the lysosomal pathway occurs in nearly all cells influenced by AD, before the accumulation of Aβ (Cataldo, Peterhoff et al. 2000). As brain cells undergo surrender of normal metabolic function during disease like AD, continued increased noxious activation in the lysosomal system preside (Cataldo, Hamilton et al. 1994). Therefore, increased activation of the lysosomal system is not solely a product of natural aging (Nixon, Cataldo et al. 2000). Moreover, with advancing neurodegeneration as in AD, accumulating neurotoxic elements like Aβ1-42 may compromise lysosomal membrane integrity (Yang, Chandswangbhuvana et al. 1998); (Nixon, Cataldo et al. 2000).

Figure 1: Production of Aβ via Proteolysis of APP



APP undergoes two sequential enzymatic cleavage events that take place in one of two pathways that either elude or generate Aβ1-42. Alpha-secretase (α-secretase) cleavage takes place within the Aβ domain resulting in liberation of a carboxy terminal fragment (C83) which can be further cleaved by gamma-secretase (γ-secretase) to generate a non-amloidogenic fragment called P3. Additionally a second larger fragment consisting of a soluble extracellular amino-terminal segment (sAPPα) is further liberated from α-secretase cleavage. In the amyloidogenic pathway, beta secretase (β-secretase) must first cleave APP toward the N-terminus of the Aβ sequence. This liberates a soluble amino terminus (sAPPβ) fragment and a carboxy-terminal portion C99. Further cleavage of C99 by γ-secretase generates a protective segment known as APP intracellular domain (AICD). A final cleavage by γ-secretase is responsible for the liberation of Aβ1-40 or Aβ1-42.

Adapted from LaFerla (2002)

2.2 Isoforms and Mutations of APP

In early-onset AD, genetic mutations are identified as causing enhanced production of Aβ1-42 (Haass, Lemere et al. 1995); (Scheuner, Eckman et al. 1996). The first documented transgenic mutation in APP reportedly caused amyloidosis accompanied by genetically-linked cerebral hemorrhage in a Dutch (E693Q) ancestral lineage (Levy, Carman et al. 1990). The Indiana mutation (APP V717G) causes increased generation of the longer Aβ fragment, Aβ1-42 (Citron, Oltersdorf et al. 1992); (Suzuki, Cheung et al. 1994). Additionally the Swedish mutation (APP K670N, M671 L) whose liberation occurs at the N-terminal region of Aβ, increases both the development and generation of Aβ1-42 (Citron, Oltersdorf et al. 1992); (Suzuki, Cheung et al. 1994). Other mutations in APP include London (V717I) (Goate, Chartier-Harlin et al. 1991) and Iowa (D694N), that further augment Aβ1-42 generation (McGowan, Eriksen et al. 2006). After the discovery of genetically-linked mutations in APP, 2 additional genes were found to exhibit an association with early-onset AD (Sherrington, Rogaev et al. 1995); (Levy-Lahad, Wasco et al. 1995); (Rogaev, Sherrington et al. 1995). Presenilin 1 (PS1) and presenilin 2 (PS2) mutations are located on chromosomes 14 and 1, respectively (Levy-Lahad, Wasco et al. 1995); (Rogaev, Sherrington et al. 1995). PS1 is chiefly recognized as an early-onset familial AD gene (Tanzi and Bertram 2005) that is associated with γ-secretase (Sherrington, Rogaev et al. 1995). Both PS1 & PS2 can directly affect γ-secretase cleavage (De Strooper, Saftig et al. 1998); (Wolfe, Xia et al. 1999), thereby altering APP processing (Duff, Eckman et al. 1996); (Borchelt, Thinakaran et al. 1996); (Citron, Westaway et al. 1997) and production of Aβ1-42 (Mehta, Refolo et al. 1998); (Xia, Zhang et al. 1997). Therefore increased APP processing and generation of Aβ1-42

observed in mutations in APP are also common denominators in presenilin mutations (Sankaranarayanan 2006).

2.3 Role of APP

Although it is unclear as to the exact role(s) APP serves, it has been suggested to contribute to cell adhesion, synaptic plasticity, cell signaling and communication, transcription of genes and transport down axons (Kamal, Almenar-Queralt et al. 2001); (Turner, O'Connor et al. 2003). In addition, neurite outgrowth (Araki, Kitaguchi et al. 1991) and promotion of survival (Yamamoto, Miyoshi et al. 1994); (Mattson, Cheng et al. 1992) are also suggested to be affected by APP. Importantly, the production of amyloid beta is completely dependent on cleavage of APP (Haass, Schlossmacher et al. 1992); (Shoji, Golde et al. 1992); (Seubert, Oltersdorf et al. 1993). Therefore, mutations in APP ultimately lead to amyloid beta generation culminating in the toxic aggregation of amyloid-filled plaques, a key hallmark lesion in AD brains (Glenner and Wong 1984).

2.4 Amyloid Beta (Aβ) Accumulation and Secretion

Amyloid beta (Aβ) is generated from APP isoform 695, ninety-nine amino acids from the APP C-terminus (Shoji, Golde et al. 1992), liberated via sequential cleavage by β- and γ-secretases (Seubert, Oltersdorf et al. 1993). Aβ1-40 and Aβ1-42 can be produced and retained or secreted from endosomal and lysosomal systems, presenting in various ratio concentrations depending on location (Skovronsky, Doms et al. 1998); (Shoji, Golde et al. 1992); (Tamaoka, Fukushima et al. 1996). It is also important to note that the different processing pathways mentioned above also appear to be particular as to the fragment of Aβ (i.e. Aβ1-40 and Aβ1-42) that is liberated (Skovronsky, Doms et al.

1998); (Cook, Forman et al. 1997). Aβ accumulation within a cell can disrupt biochemical processes and initiate events that lead to neurotoxicity (LaFerla, Tinkle et al. 1995); (Zhang, McLaughlin et al. 2002); (Marques, Keil et al. 2003). Aβ is also capable of exerting toxic effects after secretion from cells in culture (Jarrett and Lansbury 1993). Furthermore, Aβ secretion increases over time in vivo, leading to the subsequent accumulation of the protein and eventual toxic plaque build-up (Games, Adams et al. 1995); (Hsiao, Chapman et al. 1996). Taken together, both intracellular and extracellular collections of Aβ seem to be correlated, with extracellular Aβ seemingly to some extent reliant on the collection of intraneuronal Aβ (Oddo, Caccamo et al. 2006).

2.5 Amyloid Beta Species and Aggregation States

There are a number of different fragments of Aβ but the two most prominent isoforms in AD are Aβ1-40 and Aβ1-42 (Tanzi and Bertram 2005). Amyloid beta is most generally accepted as being connected with neurodegenerative disorders, however individual roles for Aβ in disease progression have yet to be fully understood (Glabe and Kayed 2006). Genetic studies of familial AD have given credence to the toxic function of Aβ, especially the aggregated peptide, in the cascade of pathological events involved in this disease (Glabe and Kayed 2006). Although Aβ1-42 is the principle fragment found in plaques in the brains of patients with AD (Behl 2000), plaque volume does not equate well with the pathology of the disease (Terry 1996). Interestingly, this may be explained by virtue of the fact that Aβ can present in various forms that correspond to differences in toxicity (Glabe and Kayed 2006). For example, studies have shown that insoluble, fibrillar forms of Aβ do not correspond as well to the severity of AD as do soluble fragments (McLean, Cherny et al. 1999); (Lue, Kuo et al. 1999).

Protein misfolding encourages amyloid aggregation (Glabe and Kayed 2006). These aggregates vary tremendously in size (Burdick, Soreghan et al. 1992); (Soreghan, Kosmoski et al. 1994); (Walsh, Lomakin et al. 1997), and seem to be involved in the process toward development of fibril structures (Glabe and Kayed 2006). Fibrillar amyloid consists of an arrangement of hydrogen bonds creating a 'backbone' in corresponding formation to the fibrils (Kirschner, Abraham et al. 1986); (Kirschner, Inouye et al. 1987). Most commonly however, amyloid is found to be present in a beta-sheet assembly (Benzinger, Gregory et al. 1998); (Balbach, Petkova et al. 2002); (Antzutkin, Balbach et al. 2000). Soluble and insoluble amyloid oligomers represent two additional conformational arrangements. Amyloid oligomer assemblies have been suggested as being the most toxic form of amyloid to cells (Klein, Krafft et al. 2001); (Walsh, Klyubin et al. 2002); (Kirkitadze, Bitan et al. 2002), as well, they impart a common configuration and therefore may also partake in similar means towards achieving toxicity (Glabe and Kayed 2006). Noteworthy, amyloids do not appear in one cellular location only, rather they are found extracellularly, in the cytosol, as well as lumenally (Glabe and Kayed 2006). Aβ is generally accepted as presenting in oligomeric assemblies with the ability to disrupt the integrity of the plasma membrane (Caughey and Lansbury 2003), unlike amyloid fibrils that do not seem to be able to accomplish this (Bucciantini, Calloni et al. 2004); (Kayed, Sokolov et al. 2004).

2.6 Aβ Toxicity

Numerous studies have supported the role Aβ1-42 has in exerting toxic consequences to neurons in culture (Mattson 2004); (Koh, Yang et al. 1990); (Mattson 1997); (Yankner 1996); (Gabuzda, Busciglio et al. 1994). Early experiments reported the ability of

exogenous Aβ to cause cell death in vitro (Yankner, Duffy et al. 1990). These studies also presented a novel idea in that Aβ could be either neurotrophic in nature or neurotoxic, depending on concentration as well as age of neurons in vitro (Yankner, Duffy et al. 1990). For example, when Aβ1-40 was applied to cells during seeding until 2 days post-plating, neurotrophism was observed. But, when Aβ1-40 was applied 3 days post-plating, the neurons displayed toxic responses (Yankner, Duffy et al. 1990). Amyloid beta is postulated to initiate cognitive deficits and neuronal damage in AD through the production of reactive oxygen species (ROS), interruption of synaptic transmission, and promotion of inflammation in the brain (Mattson 2004); (Reddy and Beal 2005); (Tanzi and Bertram 2005). Programmed cell death (apoptosis) proteins (for example p53 and Bax) have been reported to be associated with Aβ found in plaques in brains of AD patients (Mattson 2000); (Eckert, Keil et al. 2003). Not only has Aβ been implicated in instigating oxidative stress via disruption of mitochondrial functions (i.e. impedance of the electron transport chain), it has also been a target for initiating intracellular calcium dyshomeostasis (Mattson 2004). Taken together, cells targeted in neurodegeneration exhibit increased cellular dyshomeostasis in energy metabolism, oxidative damage, and calcium handling due in part to the disharmonious activity of Aβ (Mattson 2004), although the extent of Aβ peptide participation is still not completely understood.

2.7 Aβ Clearance and Degradation

Strong evidence has lead to the hypothesis that Aβ clearance and degradation is impaired in AD (Tanzi and Bertram 2005). It has been suggested that the balance between Aβ production and clearance is mediated by a receptor involved in signaling and

scavenging called the 'low-density lipoprotein receptor-related protein' (LRP) (Tanzi, Moir et al. 2004); (Zlokovic 2004); (Herz 2003). Playing an important part in clearing Aβ from the brain, LRP substrates form assemblies with Aβ that are directed to late endosomes for internalization whereupon they are then either shuttled to lysosomes for subsequent decomposition or sent out to the extraneuronal environment through the blood brain barrier (Herz 2003). On the other hand unique to soluble Aβ only (Tanzi and Bertram 2005), this variety of Aβ can be driven out of the brain into plasma by immediate association with LRP (Deane, Wu et al. 2004). Previous in vivo investigations have further implicated neprilysin and insulin degrading enzyme (IDE) as having principal functions in the degradation process of Aβ (Guenette 2003); (Mukherjee and Hersh 2002). There is also evidence that Aβ can be degraded by the plasminogen system (Selkoe 2001), namely via PLG and PLAT genes, but their explicit connection with AD has not been confirmed (Tanzi and Bertram 2005).

2.8 Cell Death Mechanisms (Apoptosis and Necrosis)

Loss of neurons is one of the principle phenotypic cellular characteristics of AD (Terry, Peck et al. 1981); (Callahan, Vaules et al. 1999). In neurodegeneration, it is generally accepted that the two chief pathways implicated in cell death include necrosis and apoptosis (Jellinger 2001). Apoptosis or 'programmed cell death' relies on active, well-defined inherent molecular characteristics that the cell possesses to orderly dispose of ill or unnecessary cells initiated by various types of toxic stimuli (Wyllie, Kerr et al. 1980); (Majno and Joris 1995); (Yuan and Yankner 2000); (Reed 2000). Cell dissolution is a slow process featuring compartmentalization of the cell and its contents available for internalization by macrophages and adjacent cells, thus bypassing the initiation of

inflammation (Wyllie, Kerr et al. 1980); (Williams and Smith 1993). Some of the molecular contributors involved in apoptotic cascade of events are widely recognized as being part of a group of cysteine proteases collectively known as caspases (Behl 2000). These apoptotic death mediators execute their responsibilities by disabling pro-survival mitochondrial proteins that, in conjunction with other proteins, normally protect cells from insults (Behl 2000), such as Bcl-2 (Cheng, Kirsch et al. 1997); (Adams and Cory 1998), in a militant, efficient manner (Thornberry and Lazebnik 1998). Disruption of these pro-survival proteins can lead to the eventual liberation of cytochrome c from the mitochondria that can either directly lead to cell death (Behl 2000), or indirectly by further caspase activation leading to cell death (Yuan and Yankner 2000); (Reed 2000). Furthermore, it has been hypothesized that neurodegenerative factors such as Aβ may initiate analogous pathways leading to cell death (Yuan and Yankner 2000); (Behl 2000); (Wolozin and Behl 2000). Additionally, it has been suggested that caspases can alter the cellular structure of targeted cells by cleaving architecturally-associated mediator proteins, such as gelsolin (Kothakota, Azuma et al. 1997). Activation of a family of cysteine proteases called caspases is known to pathologically ensue the initiation of apoptosis (Behl 2000). This assembly of proteases constitutes a number of members each classified into two groups with varying actions. The first group is known as the inducer caspases, which include caspase 3, 8, and 9, that are in turn responsible for cleaving and thus activating the second group called effector caspases. This group includes caspase 3, 6, and 7, and are responsible for the cleavage of proteins involved in proliferating the apoptotic cascade (Thornberry and Lazebnik 1998). The combined result from caspase activation causes cell function to critically diminish through the disengagement of factors

and processes involved in ensuring cell viability (Glazner and Fernyhough 2002). Caspase 3 is involved in an established mechanistic association with APP processing where it is known to cleave proteins involved in apoptosis (Gervais, Xu et al. 1999). But perhaps the most critical of these proteases to ER stress is caspase 12 as the inactive form is located in the ER (Glazner and Fernyhough 2002). Interestingly, caspase 12 appears not to be influenced by the initiation of mitochondrial-associated apoptosis but instead seems intimately linked with ER stress activation, and reduced ER calcium (Nakagawa, Zhu et al. 2000). This dissociation of caspase 12 from mitochondrial-induced apoptosis allows the activated protease to work in concert with other caspases to complete its insults (Glazner and Fernyhough 2002). Furthermore, it has been reported that the elimination of caspase 12 in a murine system, exerted a protective effect over neurons assaulted with Aβ toxicity thus lending credence to the postulation that ER stress may be of particular importance in Aβ pathogenesis (Nakagawa, Zhu et al. 2000).

Although loss of neurons is a physiological characteristic of neurodegeneration in AD, there is much controversy in the literature as to the mode of this cell death. Some investigators have reported morphological features indicating apoptotic death in impaired and dying neurons (Behl 2000); (Hartmann, Hunot et al. 2000); (Jellinger and Stadelmann 2000); (Tatton 2000); (Su, Nichol et al. 2000). Conversely, others have reported small to non-existent indications of apoptotic cell death related to neurodegeneration (Selznick, Holtzman et al. 1999); (Banati, Daniel et al. 1998); (He and Strong 2000).

In AD, it is reported that an average of 30% of all neurodegenerating cells are situated in or close to aggregates of Aβ (Lassmann, Bancher et al. 1995). However, a minimal

number of neurons actually exhibited apoptotic morphology, thus providing support for the rare evidence of apoptosis in neurodegeneration (Jellinger 2001). Because mechanisms of apoptosis still require further characterization, the cascade of events responsible for this type of cell termination remain elusive (Behl 2000).

The second type of cell death mechanism is necrosis. This is a type of non-programmed cellular response to trauma (Levin, Bucci et al. 1999) whereupon release of toxic cellular contents into the extracellular environment initiates an enormous inflammatory response (Behl 2000).

The characteristics of apoptotic and necrotic methods of cell death, albeit different, may exhibit some cross-over during the process leading to cell fatality (Jellinger 2001). For example, a cell first encountering death by apoptosis may complete the termination in a necrotic-type fashion (Behl 2000). This cross-over effect between necrotic and apoptotic methods of cell death may begin and conclude via either route, however the relationship to the current project is perhaps best demonstrated by reports that in vitro Aβ may initiate a swift necrotic-like event, which may, over a period of time, stimulate apoptosis (Bonfoco, Krainc et al. 1995). Therefore, though separate pathological events, true classic apoptotic or necrotic death may not be the sole representative method for promoting cell death in the neurodegenerative cascade of events (Behl 2000); (Jellinger and Stadelmann 2000), as definitive mechanistic steps remain elusive (Jellinger 2001).

Table 1: Characteristics of Necrosis vs. Apoptosis

Necrosis	•Process **does not** require energy •Cells swell •Membranes dissipate •Cells lyse •Organelles dissolve • Ion dyshomeostasis •Irregular cell death •Inflammatory response initiated
Apoptosis	•Process **requires** energy •Cells shrink •Irregular membrane integrity •Cell fragmentation •Organelles remain whole •Caspase involvement •Regulated cell death •No initiation of inflammation

Adapted from Behl (2000)

Interestingly, Aβ is a known instigator of inflammation in cells, eventually leading to cell demise (Varadarajan, Yatin et al. 2000) increased concentrations of pro-inflammatory cytokines such as interleukin-1α (IL1A) and interleukin-1β (IL1B), chiefly generated through the activation of microglial cells, are present in the AD brain (Bamberger and Landreth 2001); (Tanzi and Bertram 2005). However, conflicting data seems to indicate that although these pro-inflammatory cytokines may be upregulated in the AD brain they may not contribute a great deal towards increasing risk for AD (Tanzi and Bertram 2005). Alternatively, another pro-inflammatory mediator, tumor necrosis factor-α (TNFα) has been identified as having a stronger relationship with AD (Bertram

and Tanzi 2004). It has been suggested that potential therapies intended to quell the inflammatory response, such as non-steroidal anti-inflammatory medication (NSAIDs), may be useful to those suffering with this debilitating disease (Rogers and Lahiri 2004).

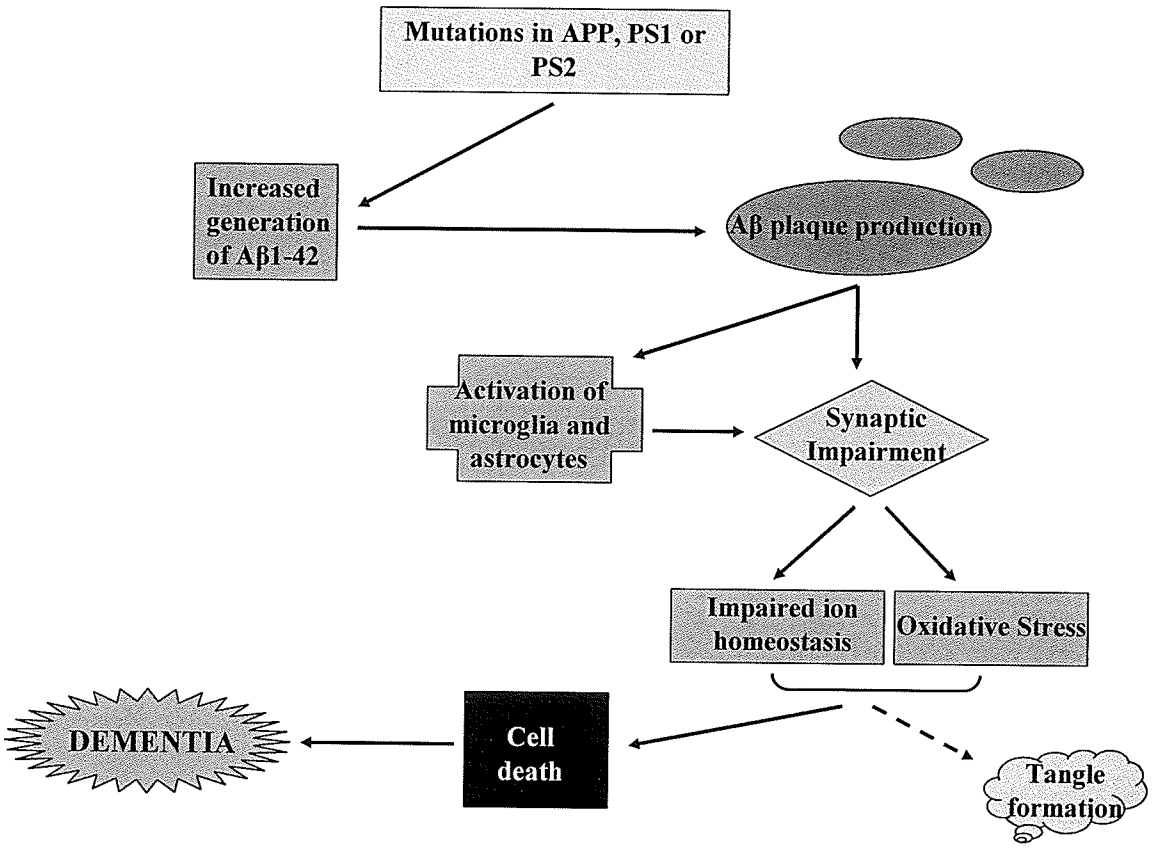
3.0 Neurodegenerative Hypotheses

3.1 Amyloid Cascade Hypothesis

For well over a decade the central premise to the 'Amyloid Cascade Hypothesis' has maintained that accumulated Aβ is the instigating molecular incident leading to both sporadic and familial AD (Hardy and Higgins 1992); (Hardy and Allsop 1991); (Selkoe 1991). Noticeably, this hypothesis has been updated to assert that rather than plaques being thought of as toxic entities themselves, they are now currently recognized as "sinks" containing neurotoxic amounts of Aβ (Hardy and Selkoe 2002), and Aβ1-42 specifically is accepted as the toxic determinant instead of total Aβ (Younkin 1995). Although intensely debated, this hypothesis is said to "not be proved but to be disproved" (Hardy 2006), because the preponderance of data supports this view (Hardy 2006); (Selkoe 1991); (Hardy and Selkoe 2002); (Hardy and Higgins 1992); (Citron, Oltersdorf et al. 1992). The strongest evidence supporting the 'Amyloid Cascade Hypothesis' has come from improved knowledge about the genes involved in initiation or risk enhancement for AD, as nearly every gene associated with this disease also regulates the formation or constancy of the Aβ protein (LaFerla 2002). For example, mutations in the APP gene normally occur at processing sites thus initiating a greater amount of Aβ1-42 to be generated (Hardy and Selkoe 2002). In addition changes in APP handling caused by mutations in presenilins further increase the amount of Aβ1-42 generated in the

system (Wilquet and De Strooper 2004). Taken together these data further support the 'Amyloid Cacade Hypothesis' and claim Aβ1-42 as the pathological instigator of AD onset (Hardy and Orr 2006). In accordance with these studies, earlier examinations suggested that the production of Aβ may be the introductory activity in the pathological cascade leading to AD (Hartmann, Bieger et al. 1997).

Figure 2: Cascade of Events in Alzheimer's Disease



A proposed cascade of pathological events directing steps toward dementia in AD. Mutations in APP, PS1 or PS2 lead to increased production of Aβ1-42 which accumulates and eventually forms insoluble plaques. Plaque production and consequent cellular stress leads to the activation of astrocyte and microglial cells and subsequent cytokine release, along with progressive synaptic destruction. Reactive oxygen species generation and ion dyshomeostasis develop, initiating tangle formation as well as cell death promotion. Neurodegeneration manifests as dementia observed in AD.

Adapted from Hardy, Selkoe (2002)

3.2 The Alternative Hypothesis

A number of investigators have questioned the 'Amyloid Cascade Hypothesis' as the prevailing mechanistic pathway for neurodegeneration citing grounds for an 'Alternative Hypothesis' to be contemplated (Perry, Nunomura et al. 2000); (Rottkamp, Atwood et al. 2002); (Lee, Casadesus et al. 2004). The premise for the 'Alternative Hypothesis' states oxidative stress produced by neurodegenerative factors contributes to enhanced Aβ production (Lee, Zhu et al. 2006). The two hypotheses do converge in that they acknowledge the same risk factors for AD such as the environment and mutant APP, but the 'Amyloid Cascade Hypothesis' states that these factors all enhance Aβ production leading explicitly to neurodegeneration (AD), whereas the 'Alternative Hypothesis' maintains oxidative stress produced by these identical AD risk factors triggers the independent enhancement of Aβ and the propensity of AD via separate routes (Lee, Zhu et al. 2006). The 'Alternative Hypothesis' also postulates that increased Aβ production by oxidative stress factors may fulfill an antioxidant-like role thereby tempering the enhanced stress (Lee, Zhu et al. 2006). It has been reported that although Aβ contributes to the generation of oxidative stress in vitro it is recognized that given the right conditions all antioxidants can develop oxidative tendencies (Lee, Zhu et al. 2006).

3.3 The Synaptic Aβ Hypothesis

The highlight and determining factor in the relatively newly stated 'Synaptic Aβ Hypothesis' is the increased emphasis on neurotoxic Aβ oligomers located around synapses (Tanzi 2005). Investigators have reported that damaged glutamatergic synaptic transmission, synaptic function and plasticity may all be attributed to a signaling mechanism that promotes enhanced Aβ (Snyder, Nong et al. 2005). Proponents of this

hypothesis maintain that the 'Amyloid Hypothesis' includes a contentious concept namely, Aβ-filled plaques do not correspond well to loss of cognition as observed in AD (Tanzi 2005), however, the Synaptic Hypothesis bridges this gap by stating that synaptic demise parallels level of dementia (Terry, Masliah et al. 1991). For example, using a transgenic mouse model of AD, disruptions in synaptic function were detected before the accumulation of Aβ (Larson, Lynch et al. 1999). Therefore, given additional data supporting the damaging role Aβ has on synaptic function, supporters of this hypothesis charge soluble Aβ oligomers with instigation of "synaptoxicity" followed by subsequent neurodegeneration, and not Aβ plaque load (Tanzi 2005). Interestingly, exogenous Aβ1-42 in cortical cultures was found to enhance internalization of a glutamatergic receptor called n-methyl-D aspartate (NMDAR), thereby decreasing receptor presence specifically at the synapse (Snyder, Nong et al. 2005). Corroborating evidence using transgenic mice that overexpress Aβ1-42 were also found to have decreased NMDAR on the plasma membrane (Snyder, Nong et al. 2005). However, it is conceded that perhaps other factors may be associated with NMDAR endocytosis brought on by Aβ (Tanzi 2005).

3.4 The Cholinergic Hypothesis

Cholinergic neuronal loss in the forebrain region and subsequent loss of cholinergic neurotransmission in the cortex and beyond significantly plays a role in the decline in learning and memory in people presenting with symptoms of AD (Bartus, Dean et al. 1982). Highlighting this hypothesis is the decline in choline acetyltransferase activity and the synthesis of acetylcholine, which are strongly related to the level of cognitive impairment observed in patients with AD (Francis, Sims et al. 1993). Interestingly, Aβ

may specifically target cholinergic neurotransmission in vitro as it can both decrease choline uptake and acetylcholine release (Auld, Kar et al. 1998; Kar, Issa et al. 1998).

3.5 The Calcium Hypothesis

First presented nearly two decades ago (Khachaturian 1989), the 'Calcium Hypothesis' maintains that the upstream source culminating into neurodegeneration in AD can be explained by previous prolonged aberrations in calcium homeostasis (LaFerla 2002). Support for this hypothesis focuses on the established knowledge that nearly every gene that yields increased risk for developing AD is also involved in some capacity in the regulation of molecular calcium signaling (LaFerla 2002). Because calcium signaling regulates a plethora of cellular processes, neurons possess an innate ability to promote calcium stability (LaFerla 2002). It has been suggested that in order for the Calcium Hypothesis to maintain posterity the following must be established; calcium imbalance must take place early in AD, and accumulation of Aβ and Tau hyperphosphorylation must be manipulated functionally by some form of calcium inconsistency (LaFerla 2002). It has been established that calcium dyshomeostasis does manifest early in AD disease progression in both human and AD models (Etcheberrigaray, Hirashima et al. 1998); (Larson, Lynch et al. 1999); (Guo, Fu et al. 1999); (Leissring, Akbari et al. 2000). Further, strong evidence supports the prerequisite that Aβ accumulation and Tau hyperphosphorylation are manipulated by calcium imbalance, in fact this dyshomeostasis is reported to increase these toxic processes (Mattson, Cheng et al. 1992); (Mattson, Tomaselli et al. 1993); (Mattson, Lovell et al. 1993); (Mattson 1990). Given supporting evidence for this hypothesis, it has been

concluded that an important proximal toxic event in AD may be the dysregulation of calcium homeostasis (LaFerla 2002).

4.0 Role of Calcium in Neurodegeneration

4.1 Calcium Homeostasis and Signaling

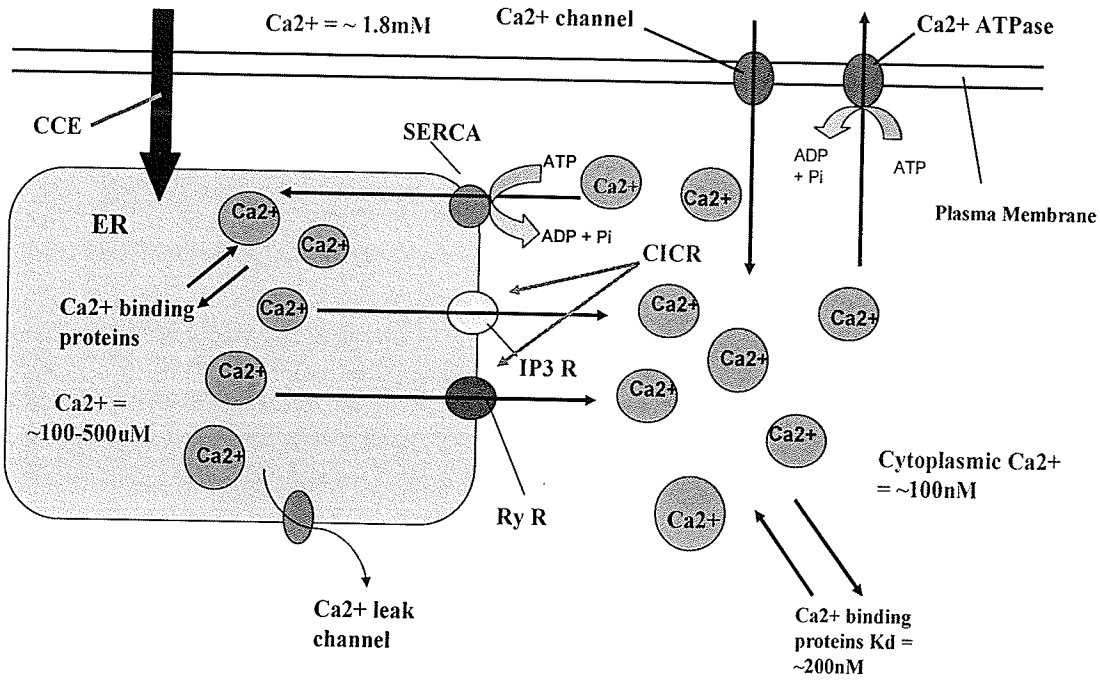
Calcium signaling regulates numerous cellular brain processes and is tightly regulated (LaFerla 2002), from both extracellularly and intracellularly derived sources (Brzyska and Elbaum 2003). Under non-diseased conditions the concentration of extracellular calcium ranges from 1-2mM, whereas cytoplasmic calcium levels vary between 50-200nM (Brzyska and Elbaum 2003). Consequently there is a strong concentration gradient maintained by the removal of cytoplasmic calcium via endoplasmic reticulum ATPases (i.e. sarco-endoplasmic reticulum calcium ATPase; SERCA) and plasma membrane exchangers (Brzyska and Elbaum 2003). Cytoplasmic calcium levels are maintained mainly by the ER, which provides source, storage and transport of calcium for cells, among cells, and their external milieu (Berridge, Bootman et al. 1998); (Marin, Encabo et al. 1999). Calcium concentrations in the ER range from 100-500μM (LaFerla 2002), yielding an additional concentration gradient, this one across the ER, regulated by membraneous channels and SERCA pumps, whereby calcium is taken up into the ER from the cytoplasm by energy driven SERCA pumps and passively expelled down the concentration gradient into the cytoplasm by leak channels and ligand-gated receptor channels termed inositol 1,4,5-triphosphate (IP3-R) and ryanodine (Ry-R) receptors (Brzyska and Elbaum 2003); (Ermak and Davies 2002). SERCA pump activity is upregulated during the depletion of ER calcium (Mogami, Tepikin et al. 1998);

(Solovyova, Veselovsky et al. 2002) and is decreased upon calcium refilling (Verkhratsky 2002). Ryanodine receptor (RyR) activity is modulated by ER and cytoplasmic calcium concentrations, and this activity is enhanced with the increase of ER calcium thus causing increased receptor sensitivity to a synthetic agonist caffeine and to cytosolic calcium (Shmigol, Svichar et al. 1996); (Koizumi, Bootman et al. 1999); (Koizumi, Lipp et al. 1999). Taken together, these two calcium transporters regulate calcium signaling via a process called Calcium-Induced Calcium Release (CICR) (Verkhratsky 2002). This calcium release and uptake via the ER may provide a homeostatic balance effected by the rate of calcium movement in either direction (Verkhratsky 2002); (Albrecht, Colegrove et al. 2002). Additionally, it was discovered that IP3 receptors (IP3R), activated by their agonist, IP3, may also induce calcium release from the ER (Khodakhah and Ogden 1995); (Khodakhah and Ogden 1993). This IP3-Induced Calcium Release (IICR) is further enhanced by intracellular calcium influx, thus translating this type of release into CICR (Berridge, Lipp et al. 2000). Moreover, calcium transmission regulation via IP3R may be critically important in modulation of synaptic plasticity (Svoboda and Mainen 1999); (Rose and Konnerth 2001). Intracellular calcium concentration is not only affected by release and uptake channels, it is also influenced by cytosolic and luminal buffering proteins (i.e. calbindin) that quickly bind free calcium (Berridge, Lipp et al. 2000) (Fierro and Llano 1996). Cytosolic buffering proteins are instrumental in forming calcium signals, namely location, duration and amplitude of transmission (Berridge, Lipp et al. 2000).

Collectively, these and other signaling pathways are affected by calcium release, uptake, and thus homeostasis. The information carried along these pathways seem to in

many cases interact with one another creating very different signaling combinations (Berridge, Lipp et al. 2000). Calcium assembly stimuli activate processes that lead to increased intracellular calcium, while conversely being attenuated by the removal of added intracellular calcium, thus maintaining homeostasis (Berridge, Lipp et al. 2000).

Figure 3: ER-dependant Calcium Homeostasis



Calcium balance is maintained by rapid calcium release through leak currents as well as extremely quick release through IP3 and Ry receptors. These release methods are balanced by calcium uptake into the endoplasmic reticulum (ER) by SERCA pumps on the ER membrane, as well as through CCE. These activities appear to be modulated by the binding capacity of resting calcium. CCE (capacitative calcium entry---influx of calcium in response to diminished ER calcium stores), SERCA (sarco/endoplasmic reticulum calcium ATPase---an ER membrane pump responsible for calcium entry into the ER), IP3 R (inositol-1, 4, 5-triphosphate receptor---ligand- and calcium-gated ER calcium release channels), Ry R (ryanodine receptor---ligand- and calcium-gated ER calcium release channel), CICR (calcium-induced calcium release---increased cytoplasmic Ca2+ causes ER Ca2+ release via activation of ER Ca2+ release channels).

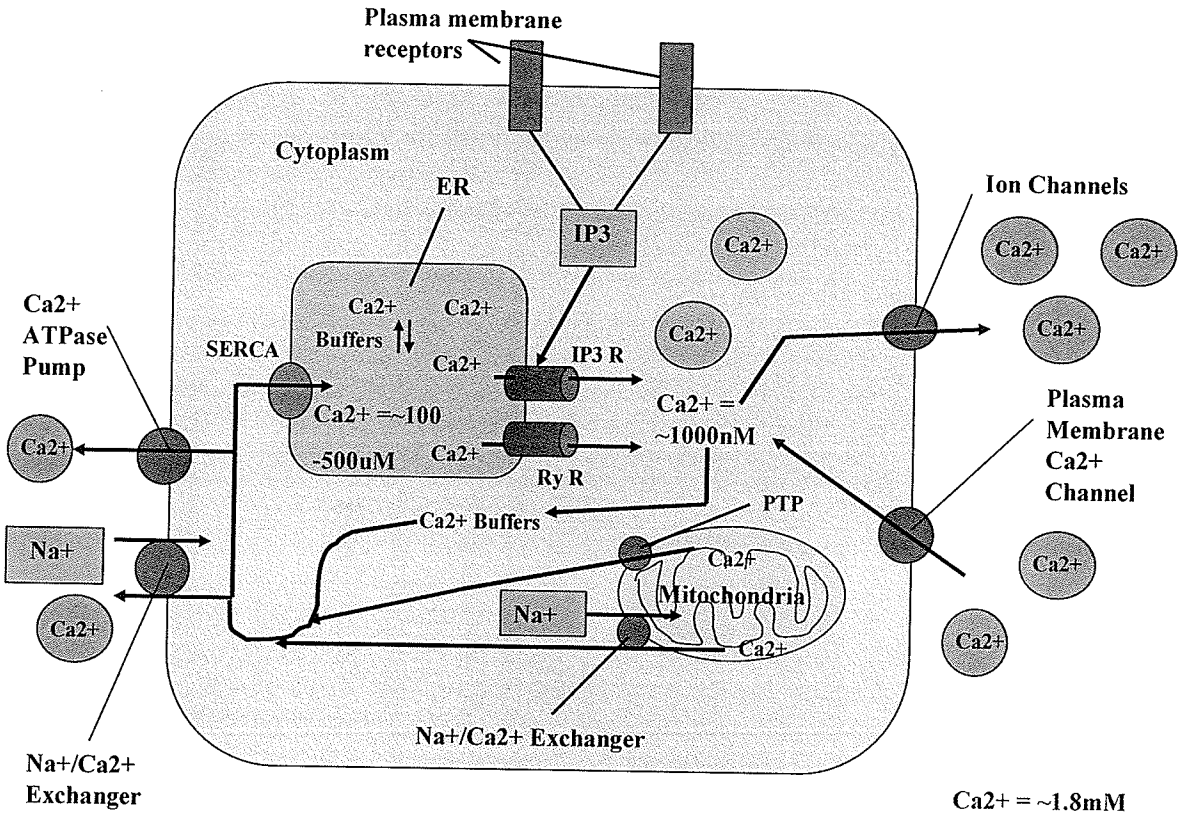
Adapted from Glazner, Fernyhough (2002) & LaFerla (2002)

4.2 Calcium Channels and Release Mechanisms

Calcium signals are produced using both intra- and extracellular calcium pools (Berridge, Lipp et al. 2000). As mentioned previously, calcium stored in the ER is released by IP3R and RyRs and taken up by SERCA pumps on the ER membrane (Brzyska and Elbaum 2003); (Ermak and Davies 2002). Extracellular calcium can bind to plasma membrane receptors (i.e. metabotropic receptors) and initiate G-protein coupled second messenger systems that determine ER receptor (i.e. inositol 1,4,5-triphosphate receptor; IP3R) activation status (Berridge, Lipp et al. 2000). In this example a G-protein coupled receptor at the plasma membrane is activated by metabotropic receptor stimulation causes phospholipase C to cleave phosphatidylinositol bisphosphate producing IP3 and diacylglycerol. IP3 in turn stimulates IP3R to release ER calcium (LaFerla 2002). The cyclic ADP ribose (cADPR) second messenger system regulates calcium capability to activate RyRs (Clapper, Walseth et al. 1987). Berridge and colleagues (2000) have suggested "ON" and "OFF" mechanisms for maintaining calcium entry and release (Berridge, Lipp et al. 2000). The function of "ON" mechanisms (or influx mechanisms) is to direct calcium to flow into the cytoplasm (Berridge, Lipp et al. 2000). Extracellular calcium influx through ligand-gated channels (via neurotransmitters such as glutamate) and voltage-gated channels on the plasma membrane, affect "ON" mechanisms (Berridge, Lipp et al. 2000). Also affected but much less understood, is Capacitative Calcium Entry (CCE) which is activated by empty internal calcium stores that cause channels to open on the plasma membrane allowing calcium influx (Berridge, Lipp et al. 2000). "ON" mechanisms stimulate a variety of calcium sensitive functions however "OFF" mechanisms (or efflux mechanisms; notably

Na+/Ca2+ plasma membrane exchangers and SERCA pumps on the ER) function to rid the cytoplasm of unnecessary calcium aimed at ensuring intracellular homeostasis (Berridge, Lipp et al. 2000). The calcium removal by various "OFF" mechanisms is further enhanced by uptake into the mitochondria during signaling, and slowly extruding it back into the cytoplasm upon recovery (Berridge, Lipp et al. 2000).

Figure 4: Cellular Calcium Signaling



Calcium signaling may occur through a number of different ways and evoke a number of different outcomes. Signals promoting the mobilization of calcium are brought on by stimulation of plasma membrane receptors, such as G-proteins. Stimulating G-protein receptors, for example, generated signals such as IP3 which then bind to IP3 receptors (IP3R) on the ER surface and promote calcium release into the cytoplasm. Ryanodine receptors (RyR) are activated in the same manner to release calcium from the ER. These "ON mechanisms" respond to membrane depolarizations and transmitter activity. The resultant levels of cytoplasmic calcium influences various calcium-sensitive activites. "OFF mechanisms" are responsible for removing calcium from the intracellular milieu. For example, Na+/Ca2+ exchangers and Ca2+ATPases on the plasma membrane remove intracellular calcium, while SERCA pumps on the ER surface deliver calcium back into the ER. Calcium from plasma membrane channels may enter the mitochondria through mitochondrial membrane receptors. After calcium has returned to resting levels, it is removed from the mitochondria through Na+/Ca2+ exchangers and taken up by the ER or removed through permeability transition pores (PTP). The mitochondria plays an important part in OFF mechanisms in that it quickly gathers calcium as the calcium signal is developing and then slowly lets it go during recovery.

Adapted from Berridge, Lipp, & Bootman (2000)

Additionally, as mentioned previously, voltage-gated channels are involved in the formation of calcium signaling. For example, gene activation directed by calcium signaling can be achieved through the depolarization of L-type voltage gated plasma membrane channels, whereas N- and P/Q-voltage-gated channels near synapses instigate neurotransmitter release (Berridge, Lipp et al. 2000). Furthermore, G-protein-coupled metabotropic plasma membrane glutamate receptors (GluR1-7), can act in concert with the voltage-gated channels to release calcium from internal stores (Nakamura, Barbara et al. 1999). Intracellularly, calcium transmission may also be initiated through IP3R and RyRs on the ER membrane, both chiefly regulated by calcium itself (Berridge, Lipp et al. 2000). Taken together this has served to touch briefly on the available information regarding calcium signaling. Much additional work is required to better comprehend the interplay between the function of calcium dyshomeostasis and AD pathology (LaFerla 2002). What is certain is that calcium signaling is a vital part of cellular functioning and any disruption may prove detrimental (Brzyska and Elbaum 2003); (Abramov, Canevari et al. 2004).

4.3 APP Processing and Calcium

There appears to be a detrimental feedback relationship between calcium and APP namely, dysregulation of calcium directs changes in APP processing, and these changes in APP processing tend to influence the balance of calcium (Brzyska and Elbaum 2003). For example upon delivery to the nucleus evidence suggests that the C-terminal domain of APP regulates calcium signaling (Leissring, Murphy et al. 2002); (Cao and Sudhof 2001). Two very important questions surrounding the interplay between calcium homeostasis and APP processing must be addressed to conceptualize their interactions

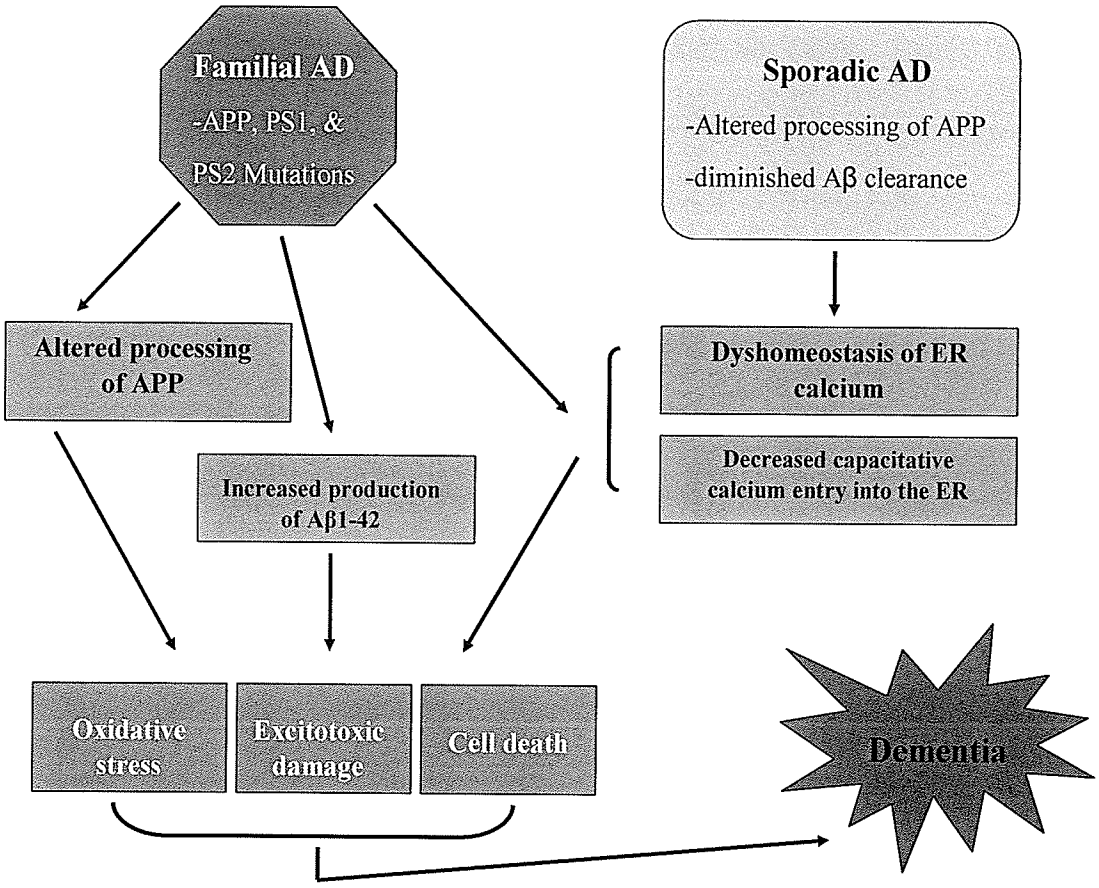
with each other. The first question is how does APP processing affect calcium dynamics and secondly, by what mechanism does calcium in turn modulate APP processing (LaFerla 2002)?. Nearly every APP fragment is known to stabilize or disrupt calcium signaling (LaFerla 2002). Further, Aβ-induced elevation of intracellular calcium and subsequent dyshomeostasis is a primary suspect in the progression of neurodegeneration (Mattson, Cheng et al. 1992); (Mattson, Tomaselli et al. 1993); (Goodman and Mattson 1994). The numerous consequences of calcium dysregulation include instigation of neuronal apoptosis (Mattson 1994), reactive oxygen species promotion (Mattson, Barger et al. 1995); (Mattson 1995), altered enzymatic processes (LaFerla 2002), and cytoskeletal alterations (Mattson, Engle et al. 1991).

Compelling evidence suggests that Aβ causes calcium imbalance by two primary mechanisms. The first is through the generation of reactive oxygen species (LaFerla 2002). Aβ deposition triggers reactive oxygen species which can then generate lipid peroxidation (Hensley, Carney et al. 1994) leading to the malfunction of ATPases and other calcium delivery systems thereby increasing cytoplasmic calcium (Mattson, Cheng et al. 1992); (Mark, Hensley et al. 1995); (LaFerla 2002). The second mechanism involves the suggested ability of Aβ to create plasma membrane cation channels (Kagan, Hirakura et al. 2002). In endothelial cells, Aβ1-42 forms calcium transport pores that instigated cell demise (Bhatia, Lin et al. 2000). Furthermore, others have suggested that oligomeric Aβ triggers calcium release from intra-organelles (Glabe and Kayed 2006). Although this is an Aβ-induced mechanism for increasing intracellular calcium, one has to be cognizant that other proteins have been implicated in the creation calcium channels

in the membrane (Lin, Mirzabekov et al. 1997); (Mirzabekov, Lin et al. 1996); (Kawahara, Kuroda et al. 2000).

It is possible that the modulation activity of APP by calcium may depend on the release location of calcium (i.e. intra-organelle release or influx from extracellular milieu) (LaFerla 2002). Both intra-organelle release of calcium and influx of external calcium are implicated as prominent mechanisms for increasing the production of Aβ (Querfurth, Jiang et al. 1997). Alternatively, others observed that when cytoplasmic calcium levels were enhanced, Aβ production subsided (Buxbaum, Ruefli et al. 1994). Despite support for either hypothesis, a clear definition of the role of calcium on APP processing remains unclear.

Figure 5: Calcium Regulation and Alzheimer's Disease



Two of the most common forms of AD are familial onset- and sporadic onset-AD. Familial onset AD accounts for ~5% of all cases, while sporadic onset AD makes up the remaining ~95%. Both forms are caused by mutations in APP, presenilin 1 (PS1), and presenilin 2 (PS2), which lead to altered APP processing and subsequent generation of Aβ1-42, however familial AD is caused by genetic deficits while origins for sporadic AD aren't completely known. Further, in sporadic onset AD, dyshomeostasis of ER calcium is a key contributor to cell death and eventual neurodegeneration. The combined effect of these pathological events leads to the generation of reactive oxygen species (oxidative stress), excitotoxicity, and cell death. These key features culminate in the manifestation of dementia in AD.

Adapted from LaFerla (2002)

5.0 Transgenic Models of Alzheimer's Disease

5.1 Design and Usage of Various Promoters

Murine models for AD do not naturally exist therefore the creation of transgenic representations have been of paramount importance (Chishti, Yang et al. 2001), and have proven invaluable to researchers (Mineur, McLoughlin et al. 2005). These animals provide a genetic and molecular foundation that can be manipulated depending on experimental requirements, and can be studied in ways that human subjects cannot (Mineur, McLoughlin et al. 2005). Suitable models for AD research must include a number of attributes namely, reliable stability of the phenotype (Crabbe, Wahlsten et al. 1999), the animal model must be similar to the pathology and major characteristics of AD (Mineur, McLoughlin et al. 2005), and finally the model must exhibit "predictive validity" meaning that it must provide a high degree of accuracy in representing safety and efficacy of therapeutic interventions (Mineur, McLoughlin et al. 2005).

There are a number of genetically altered mouse lines available. These include the following, separate or in combination with one another, 1) Knock-outs (K/O), whereby gene function is eliminated, 2) Transgenic animals, where the genome is manipulated with the insertion of a foreign gene, and 3) Knock-ins (K/I), which consist of specific gene mutations that trigger the ceasation of activity of proteins developed by that gene (Mineur, McLoughlin et al. 2005). The first genetically altered murine models used to study AD used the transgenic approach to gene manipulation (Mineur, McLoughlin et al. 2005); (McGowan, Eriksen et al. 2006). These models were created using the human APP gene that developed Aβ pathology (Mineur, McLoughlin et al. 2005). The first of these models was the PDAPP mouse that overexpressed a gene encoding APPV717F and

displayed Aβ plaques by 6-9 months (Games, Adams et al. 1995). Later, another group developed a similar model called Tg2576 that overexpressed human APP with a double mutation (K670M/N671L) (Hsiao, Chapman et al. 1996). Today, there are a number of human APP trangenic mouse models in use that include PDAPP, Tg2576, J20, APP23, and TgCRND8, each with varing backgrounds and mutations (Mineur, McLoughlin et al. 2005). The variety in characteristics in these animals leads to differences in gene expression and pathology (Mineur, McLoughlin et al. 2005). Not only have single, double and triple transgenics been created, but these animals have been crossed with other genetically altered mice to create additional genetically descript facets of modeling (McGowan, Eriksen et al. 2006). For example, mice that express mutant presenilin (i.e. PSAPP) have been crossed with human APP transgenic mice to create an animal model that displays increased Aβ production (Duff, Eckman et al. 1996); (Borchelt, Ratovitski et al. 1997) regulated by presenilin mutations, in this case (Borchelt, Thinakaran et al. 1996); (Citron, Westaway et al. 1997). Both mouse models (human APP and presenilin) represent the human condition of AD in that amyloid accumulation and plaque formation are evident which mimic human pathology (Games, Adams et al. 1995); (Hsiao, Chapman et al. 1996); (Borchelt, Ratovitski et al. 1997). Furthermore, a recent triple transgenic mouse has been developed which houses a double human APP mutation (K670M/N671L; Swedish) along with a tau (tangle) mutation (tauP301L) (Oddo, Caccamo et al. 2003); (Billings, Oddo et al. 2005); (LaFerla and Oddo 2005). Thus these mice present with plaques and neurofibrillary tangles that appear similar onset and arrangement to the human condition (Mineur, McLoughlin et al. 2005). The addition of the tau mutation (as observed in AD brains) (Sankaranarayanan 2006) is important as the

hyperphoshorylation of tau leads to microtubule disassembly (Alonso, Grundke-Iqbal et al. 1996). Although great strides have been made to encompass the classic hallmarks observed in the human condition of AD, no one model in particular completely satisfies every aspect of the disease (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006).

A number of molecular factors affect both the neurogenesis and the disease pathology in AD transgenic animals and the combination of these factors also affect the outcome of the model (Chishti, Yang et al. 2001). These factors include; background breeding lines, level of neonatal death in first generations, accurate physiologic activity of inserted gene, and type of promoter used (Chishti, Yang et al. 2001). For example, although the Tg2576 and APP23 mouse models of AD carry the same mutation (K670N/M671L; Swedish), they are under the control of different promoters (prion and Thy-1 promoters, respectively) (Games, Adams et al. 1995); (Hsiao, Chapman et al. 1996). The Tg2576 mice develop Aβ plaques at approximately 9 months (Hsiao, Chapman et al. 1996), whereas APP23 mice exhibit plaques at around 6 months of age (Sturchler-Pierrat, Abramowski et al. 1997).

One other important characteristic of AD that is crucial in creating appropriate models of AD is the presence of cognitive deficits and behavioural decline at or before the onset of pathophysiological indicators (Chishti, Yang et al. 2001). Behavioural changes and decline in cognition is observed in many mouse models of AD however, as with the pathology, the behavioural changes are also variable among the models (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006).

5.2 Specific Mouse Models of AD

The following transgenic mouse models listed are a snapshot of available animals that are employed to study AD. They have been examined both in vitro and in vivo with varying results and conclusions, thus emphasizing the importance of continued AD research using different strains, backgrounds, transgene involvement, as well as whole animal or cellular level investigation to garner a well-rounded view (albeit at the animal level) of the mechanisms contributing to AD (Sankaranarayanan 2006).

5.2.1 PDAPP

Among the first transgenic mouse models created to study AD (that developed Aβ plaques) was the PDAPP mouse, a model that overexpressed the APPV717F (Indiana) mutation that led to amyloid accumulation in approximately 6-9 months (Games, Adams et al. 1995). There is no cell loss or neurofibrilary tangle formation evident in this model however disruptions in synaptic function are present (McGowan, Eriksen et al. 2006); (Sankaranarayanan 2006).

5.2.2 Tg2576

Shortly after the development of the PDAPP mouse model of AD, the Tg2576 model was engineered (Sankaranarayanan 2006). This model houses the Swedish mutation (K670N/M671L) under the regulation of the prion promoter (Hsiao, Chapman et al. 1996). Tg2576 animals develop neuronal Aβ plaques 9 months after birth along with deficits in cognition, however no neurofibrillary tangles or cell death has been reported (McGowan, Eriksen et al. 2006).

5.2.3 APP23

Another single mutation mouse model of AD is the APP23. This animal was created to express the Swedish mutation (K670N/M671L) controled by the Thy-1 promoter, whereby amyloid accumulation is evident at 6 months, with some hippocampal cell death apparent in concert with Aβ plaque development (Sturchler-Pierrat, Abramowski et al. 1997); (Calhoun, Wiederhold et al. 1998); (Calhoun, Burgermeister et al. 1999).

5.2.4 J20

Efforts to increase amyloid accumulation and to decrease the time required for neurogenesis to occur, led to the creation of a double mutant mouse called the J20 (Sankaranarayanan 2006). Under the control of the platelet-derived growth factor (PDGF) promoter, this mouse overexpresses both Swedish (K670N/M671L) and Indiana (V717F) mutations, resulting in some synaptic demise over time (Sankaranarayanan 2006).

5.2.5 3X Transgenic

This triple transgenic AD mouse model was recently developed using the Swedish (K670N/M671L), tau (MAPT P301L) mutations backed on a knock-in presenilin 1 (PSEN1 M146V) strain (Oddo, Caccamo et al. 2003); (Oddo, Caccamo et al. 2003) under the Thy-1 promoter (Oddo, Caccamo et al. 2003). These animals exhibit increased Aβ generation over time, preceeding the accumulation of hyperphosphorylated tau (Oddo, Caccamo et al. 2003). Aβ plaques are evident at 6 months, whereas neurofibrillary tangles are present at 12 months (McGowan, Eriksen et al. 2006). Additionally, cognitive deficits were observed at around 4 months (Billings, Oddo et al. 2005).

5.2.6 Presenilin 1

This mouse model can be observed as housing either the PSEN1 (M146V) or PSEN1 (M146L) mutations however no plaque depositions have been reported in either line (Duff, Eckman et al. 1996). Animals crossed using PSEN1-A246E + APP (Swedish) (Borchelt, Ratovitski et al. 1997) however, did display increased rates of Aβ accumulation (McGowan, Eriksen et al. 2006).

5.2.7 TgCRND8 Mouse Model of AD

The TgCRND8 mouse model is an early on-set, familial representation of AD phenotypically defined by deposition of Aβ, with aggressive cognitive deficiences by 3 months of age (Chishti, Yang et al. 2001). This AD model does not produce neurofibrillary tangles (Chishti, Yang et al. 2001). Encoding the APP695 isoform, the TgCRND8 model houses a double mutation; Swedish (K670N/M671L) and Indiana (V717F), driven by the Syrian hamster prion promoter (Chishti, Yang et al. 2001). Using a cos.tet expression vector, the mutations were introduced into the cDNA cassette and microinjected into various background strains until a permissive strain was found using C3H X C57BL/6J oocytes, however the creation of the TgCRND8 strain was eventually produced in an identical outbred background (Chishti, Yang et al. 2001). The Cos-Tet expression vector allows rapid expression of the introduced transgene, as well as independent expression of the transgene regardless of where it inserts in the genome (Telling, Scott et al. 1995); (Moser, Colello et al. 1995); (DeArmond, Sanchez et al. 1997); (Irizarry, McNamara et al. 1997). The Swedish mutation initiates aberrant processesing of APP at the n-terminal cleavage area of amyloid beta, as well as enhances the production of Aβ1-42 (Qiu, Naten et al. 2001); (Citron, Oltersdorf et al. 1992);

(Suzuki, Cheung et al. 1994), while the Indiana mutation operates at the c-terminal end of the amyloid beta sequence on the APP holoprotein to increase the production of Aβ1-42 (Citron, Oltersdorf et al. 1992); (Suzuki, Cheung et al. 1994). The resultant overexpression of APP in the TgCRND8 mice was found to be approximately 5-fold greater than non-transgenic littermate controls which were well above the previously established threshold level of 4 times over the endogenous level needed for amyloid beta plaque accumulation by others (Hsiao, Chapman et al. 1996); (Hsiao, Borchelt et al. 1995). Furthermore, at just over 3 months of age, Aβ1-42 levels exceeded Aβ1-40 by a ratio of 5:1 (Chishti, Yang et al. 2001). This is an important feature as Aβ1-42 has been identified as the more toxic fragment (Selkoe 1991), as well as being the more prevalent of the two proteins in plaques found in the AD brain (Selkoe 1991). It was established that the TgCRND8 model presented with human Aβ1-40 and Aβ1-42 in their brains and this deposition was limited to the brain as no other organs were positive for the peptide (Chishti, Yang et al. 2001). Both Aβ1-40 and Aβ1-42 were detected in these animals by 4 weeks of age with a marked increase in Aβ1-42 by 10 weeks, with increases in both fragment levels with age (Chishti, Yang et al. 2001). At just over 3 months of age, Aβ plaques were evident in the cortex, hippocampus, and the olfactory regions of the brain (Chishti, Yang et al. 2001).

In an effort to eliminate influence from outside factors when engineering this model, developers retained a 90 amino acid sequence of 5'-untranslated APP mRNA, used a prion promoter with high expression to drive the transgene, incorporated to familial AD genetic mutations which promote overexpression of APP and subsequent production of

Aβ, and used a protective background with which to establish genetic mutations upon (Chishti, Yang et al. 2001).

In addition to phenotypic parallels with AD, the TgCRND8 mouse model also exhibit cognitive deficiencies and disordered behaviours. Using the Morris water maze, TgCRND8 animals exhibited longer search and escape latencies when scoping for escape platforms. These animals also exhibited impairments in spatial learning abilities, all evident by approximately 3 months of age (Chishti, Yang et al. 2001).

The TgCRND8 model of AD represents two very important attributes that mirror the human condition. By 6 months of age, these animals develop a total neuronal Aβ concentration anywhere between 3200-4600 pmol/g, fitting into the established range reported in humans of between 500-5000 pmol/g (Wang, Dickson et al. 1999). Secondly, the high Aβ1-42 to Aβ1-40 ratio as observed in human patients with AD (Wang, Dickson et al. 1999) is also exhibited in TgCRND8 mice (Chishti, Yang et al. 2001).

Decreased neonatal death often associated with overexpression of APP may have been accounted for in TgCRND8 animals through the use of permissive background strains (Chishti, Yang et al. 2001). Also, appearance of amyloid plaques were present in all animals positive for the APP mutation by 3 months of age in the TgCRND8, occuring quicker than other available models during that time (Games, Adams et al. 1995); (Hsiao, Chapman et al. 1996); (Chishti, Yang et al. 2001). Furthermore, the tandom activity of two mutations in TgCRND8, performing in concert with one another, works to accentuate the toxicity of amyloid beta in the brain (Chishti, Yang et al. 2001). Moreover, differences in promoter selection and APP isoform cassettes used may also add variation to the responses reported between different strains of transgenic models. For example,

the TgCRND8 mice were created using the Syrian hamster prion promoter expressing the APP695 isoform of APP, whereas some founders created using Thy-1 promoter expressed the APP isoform APP751 (Chishti, Yang et al. 2001).

In a recent publication by Supnet et al (2006), ryanodine-3 receptor mRNA was measured in 5-day old fetal primary cortical neuronal cells from TgCRND8 mice (Supnet, Grant et al. 2006). It was observed that endogenously generated Aβ1-42 in TgCRND8 mice contributed to increased ryanodine receptor-3 mRNA levels. Furthermore, when an Aβ1-42 antibody was applied to 5 day old primary cortical TgCRND8 neuronal cells the level of ryanodine receptor-3 mRNA levels decreased compared to non-transgenic neuronal cells (Supnet, Grant et al. 2006). In addition, these authors measured intracellular calcium levels in primary cortical neurons from 5 day old TgCRND8 mice after application of 250μM glutamate and 100nM ryanodine. It was demonstrated that after 5 days TgCRND8 primary neurons displayed increased intracellular calcium in response to treatment with glutamate when compared with non-transgenic littermates (Supnet, Grant et al. 2006). It has been well established that elevated intracellular calcium due to the effects of glutamate application is intimately related to increased neurotoxicity (Jarvis, Lilge et al. 1999); (Mattson, Gary et al. 2001); (Glazner, Chan et al. 2000); (Kim, Park et al. 2000); (Guo, Fu et al. 1999). Therefore Supnet et al's (2006) finding that there was increased intracellular calcium after 5 days in TgCRND8 primary cortical neurons due to a glutamate effect, may be a contributing factor in neurdegeneration in this model (Supnet, Grant et al. 2006). Following treatment with glutamate, agonistic concentrations of ryanodine (100nM) was applied to 5 day old primary cells and measured. Again, TgCRND8 primary cortical neurons exhibited

enhanced intracellular calcium levels after RyR stimulation, when compared to non-transgenic littermates (Supnet, Grant et al. 2006). Taken together, data from this recent set of experiments indicates that early in culture (day 5) TgCRND8 primary neurons possess elevated intracellular calcium levels as a response to glutamate or agonistic concentrations of ryanodine (Supnet, Grant et al. 2006). This information further suggests that endogenously generated Aβ1-42 in the TgCRND8 murine model of AD is intimately involved in disrupting intracellular calcium homeostasis (Supnet, Grant et al. 2006).

In vivo, the TgCRND8 model of AD has been widely characterized behaviourally and histopathologically (Janus, D'Amelio et al. 2000); (Chishti, Yang et al. 2001); (McCool, Varty et al. 2003); (Dudal, Krzywkowski et al. 2004); (Del Vecchio, Gold et al. 2004); (Lovasic, Bauschke et al. 2005); (Adlard, Perreau et al. 2005); (Ambree, Touma et al. 2006); (Sebastiani, Krzywkowski et al. 2006); (Bellucci, Luccarini et al. 2006); (Bellucci, Rosi et al. 2007), while very limited research exists regarding in vitro properties (Supnet, Grant et al. 2006).

In sum, the TgCRND8 transgenic mouse model of AD has lent, and continues to provide important opportunities for advancing the molecular and mechanistic knowledge of AD.

6.0 Therapeutic Interventions

Although there currently is no cure for AD, a number of therapeutic interventions have been developed in an attempt to eradicate the disease, slow the progression, or counteract symptoms in an effort to provide increased quality of life for the patient. A

number of pharmaceutical agents have been developed to slow the progression or address symptoms caused by AD, ranging from acetylesterase inhibitors to glutametergic receptor antagonists, and are designed to address symptoms at various stages in the disease. These medications may be used as stand-alone interventions or in conjunction with one another to maximize treatment.

6.1 Anticholinesterase Inhibitors

These agents decrease the degradation rate of acetylcholine thus allowing an increased concentration to remain in the brain. Acetylcholine is an excitatory neurotransmitter in the brain that is diminished in neurodegenerative diseases such as AD. Tacrine was one of the first anticholinesterase inhibitors developed to treat symptoms associated with AD but is no longer clinically used. Donepezil, also known as Aricept is a reversible anticholinesterase inhibitor that functions to increase the concentration of acetylcholine in the brain, and is used to treat mild to moderate AD (www.medicinenet.com). Side effects to usage of this drug include nausea, diarrhea, anorexic tendencies, and abdominal cramping (www.medicinenet.com). Galantamine, or Razadyne, is used to treat mild to moderate phases of AD and is a reversible, competitive anticholinesterase inhibitor, thereby also increasing the amount of acetylcholine available in the brain (Woodruff-Pak, Vogel et al. 2001). Additionally, this drug has been reported to stimulate the release of acetylcholine (Woodruff-Pak, Vogel et al. 2001). Rivastigmine, or Exelon, is another type of anticholinesterase inhibitor that is used to treat mild-moderate symptoms of AD as well, elimination of this drug does not include the hepatic system (Camps and Munoz-Torrero 2002), thereby decreasing propensity of possible liver toxicity.

6.2 Glutametergic Receptor (NMDA) Antagonists

Memantine is an N-methyl-D-aspartate (NMDA) receptor antagonist that exhibits uncompetitive binding, subject to voltage dependency (Robinson and Keating 2006). Alternatively, NMDA receptors may still be activated with high glutamate concentrations. Trade names for this drug include Akatinol, Axura, Ebixa, and Namenda (Cacabelos, Takeda et al. 1999). Memantine is used to treat mild to moderate symptoms of AD, using the liver as the major elimination route. Side effects may include dizziness, confusion, headache, agitation, drowsiness, and sleeplessness.

7.0 Hypotheses

This introductory chapter functions as a preamble providing necessary directive information pertinent to our investigative studies examining the core in vitro characteristics of the TgCRND8 mouse model of AD (transgenic vs. non-transgenic; which have not been previously established) along with probing measurable calcium differences and possible mechanistic pathways responsible for these variations.

Hypothesis 1:

The production of amyloid precursor protein and subsequent cleavage product, amyloid beta (1-42), and the generation of reactive oxygen species will be greater in TgCRND8 vs. non-transgenic littermate control primary neuronal cultures after 8 days in vitro. Neurite outgrowth and morphological cell survival will be reduced in TgCRND8 primary cells compared to non-transgenic littermates.

Hypothesis 2:

Resting calcium levels will be greater in TgCRND8 primary cortical neurons when compared to non-transgenic littermate controls, while treatment with 1mM glutamate will contribute to a further increase in calcium response in TgCRND8 vs. non-transgenic littermates.

Hypothesis 1 is addressed in chapter 2 and hypothesis 2 is addressed in chapter 3 of this document.

CHAPTER 2 - Characterization of TgCRND8

Abstract

Murine models imitate a variety of classic characteristics and biochemical markers of Alzheimer's disease (AD) including increased production of amyloid precursor protein (APP), augmented amyloid beta peptide (Aβ) generation, extracellular deposition of Aβ contributing to the formation of insoluble plaques, tau hyperphosphorylation, and occurrence of cognitive and behavioural decline. These models have and continue to provide critical tools for both in vivo and in vitro experimentation in the understanding of AD. No murine model is a perfect mimic to the human disease but each provide new insights due to differences in the genes inserted or mutated, the background strain, promoter, etc. It is known that overexpression of APP (and subsequent production of Aβ) in vitro is associated with toxicity and neurodeneneration, therefore the aim of this study was to characterize APP production, Aβ generation, cell survival, and neurite outgrowth (NOG), in cultured TgCRND8 and non-transgenic littermate neurons. Using Western blot and ELISA assays, we report that primary cortical neurons from TgCRND8 embryos produced significantly more APP ($p<0.01$) and secreted appreciably more Aβ1-42 ($p<0.001$) into media 8 days post-plating and onward than their non-transgenic littermates. Further, both TgCRND8 and non-transgenic cortical and hippocampal cells demonstrated similar initial survival, and neurite outgrowth in hippocampal cells were also comparable, thus eliminating possible initial growth discrepancies. However, differences were apparent later on in culture following increased Aβ1-42 production as there was a significant decrease in survival beginning 11 days post-plating in the TgCRND8 cells compared to non-transgenic littermate controls ($p<0.0001$).

Furthermore, the generation of reactive oxygen species (ROS) measured after 11 days in vitro, where significant cell death also began to occur, was significantly greater in TgCRND8 vs. non-transgenic littermate cortical cells ($p<0.0001$). Taken together, these results suggest early functional similarities between TgCRND8 and non-transgenic neurons in culture, while later differences in cell survival correspond with increased production of Aβ1-42.

Introduction

Alzheimer's disease (AD) is the most common cause of senile dementia (Jellinger 2001) manifested clinically by progressive, irreversible cognitive decline and disordered behaviours (Selkoe 2001); (Mattson 2004); (Tanzi and Bertram 2005). Currently, AD is only definitively diagnosed through post mortem examination and is characterized by accumulation of plaques made up of an insoluble, aggregated form of a protein called amyloid beta (Aβ) (Hardy and Selkoe 2002); (Selkoe 2001); (Tanzi and Bertram 2005); (Reddy and Beal 2005), intracellular mass of hyperphosphorylated tau protein, and cell death (Hyman, Van Hoesen et al. 1984); (Kamenetz, Tomita et al. 2003). Approximately 98% of all cases of AD are sporadic, occurring later in life (Hutton, Perez-Tur et al. 1998); (Behl 2000). The remaining 2% constitute early-onset familial autosomal dominant (FAD) cases, associated with mutations in the presenilin 1 or 2 (PS1, PS2) or the amyloid precursor protein (APP) genes resulting in elevated Aβ, which is widely thought to lead to events producing cognitive decline and neuronal death (Hutton, Perez-Tur et al. 1998); (Behl 2000).

Considerable time and effort has been spent genetically engineering mouse models of AD in an attempt to probe molecular pathways involved in the disease. The TgCRND8 mouse model was developed by Dr. Westaway and colleagues at the University of Toronto (Janus, Pearson et al. 2000); (Chishti, Yang et al. 2001). These animals house a double mutation (Swedish KM670/671NL and Indiana V717F) in the APP695 gene (Janus, Pearson et al. 2000); (Chishti, Yang et al. 2001), which leads to abnormal processing of APP (Citron, Oltersdorf et al. 1992), and increased production of Aβ1-42 (Citron, Oltersdorf et al. 1992); (Suzuki, Cheung et al. 1994). TgCRND8 generate Aβ1-42 three months postnatally, and display cognitive deficits as early as 11 weeks of age (Janus, Pearson et al. 2000); (Chishti, Yang et al. 2001). Other models share common expression of these mutations in one form or another under similar or variant promoters (Sankaranarayanan 2006). However, even though models may share common expression of genetic variants, it is the combinations of these modifications that determine APP production, deposition of Aβ and neonatal lethality (Chishti, Yang et al. 2001). For example, levels of Aβ1-42 in PDAPP (Indiana mutation driven by the PDGF-beta promoter) (Sankaranarayanan 2006) mice at 16 months of age (Johnson-Wood, Lee et al. 1997) are similar to that observed in TgCRND8 mice (driven by the prion promoter) found at 6 months of age (Chishti, Yang et al. 2001). In vitro study provides a focused system to investigate molecular pathways, providing a method to elucidate characteristics of both the present model and others, allowing a different platform to examine current or developing pharmacological therapies, as well as exploring signal transduction and/or biochemical pathways, involved in AD. The properties of TgCRND8 remain mostly unresolved in the TgCRDN8 mouse model of AD (Chishti, Yang et al. 2001), thus in the

current study we examined survival, reactive oxygen species, and other physiological parameters of embryonic cortical and hippocampal neurons from both TgCRND8 and non-transgenic littermate controls.

Methods

TgCRND8 Mice

The TgCRND8 mouse was developed by Dr. Westaway and colleagues at the University of Toronto (Chishti, Yang et al. 2001). These animals house a double mutated form of the major human brain amyloid precursor protein (APP) isoform, APP695. The APP transgene express both Swedish (KM670/671NL) and the Indiana (V717F) mutations driven by the Syrian hamster prion promoter (Chishti, Yang et al. 2001). The background strain of this model was on a C57BL/6-C3H hybrid cross. Wild-type (APP -/-) females were bred to transgenic (APP +/-) male. Newborn animals were weaned 3 weeks after birth, tattooed, and genotyped. Fetuses were genotyped for the mutant APP (mAPP) transgene before experimental use. All animals were maintained in the Animal Care Facility at the St. Boniface Hospital Research Centre. Approval for use of all animals was obtained through the University of Manitoba Care and Use of Laboratory Animals.

Genotyping

Colony Maintenance

DNA was extracted from weanlings via small tail snips (~5 mm) using a DNA extraction kit (REDExtract-N-Amp Tissue PCR kit, Sigma Aldrich, St. Louis, MO) and the DNA from mAPP transgene was detected using polymerase chain reaction (PCR).

The PCR primers used were: 5' (TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A) 3' and 5' (AGA AAT GAA GAA ACG CCA AGC GCC GTG ACT) 3' (Invitrogen, Frederick, MD). Amplification of PCR products was acquired through 30 cycles of 94/68/72° C. PCR products were separated on a 1% denaturing agarose gel (Fermentas, Hanover, MD) containing 3µl ethidium bromide (Fisher Biotech, Fair Lawn, New Jersey) at 100 V for 30 minutes using a Bio-Rad PowerPak 200 (Philadelphia, PA). PCR products were visualized with a Bio-Rad Gel Doc UV system (Philadelphia, PA).

Primary Cell Cultures

Brains from embryonic day 16 TgCRND8 and non-transgenic littermate control were aseptically removed. An appendage was removed from each embryo for genotyping. Brain tissue was grouped together as either transgenic or non-transgenic following genotyping, and cells were mechanically dissociated, counted and seeded on 6-12 well plates, coated with poly-d-lysine (Sigma Aldrich, St. Louis, MO) in borate, in Neurobasal media plus B27, gentamicin (Invitrogen, Frederick, MD), L-glutamine, HEPES (Fisher Scientific), and fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO). The media was removed and replaced with neurobasal without FBS the following day. Cells were kept in an incubator at 37° C in 5% CO2. All experiments using cortical cells were performed 8-15 days post plating. Neurite outgrowth (NOG) studies were performed using hippocampal cells beginning 6-12 hours after plating and ending after 4 days.

Neurite Outgrowth

Beginning 6 hours after culture, TgCRND8 mouse hippocampal neurons were photographed using phase contrast microscopy (Zeiss Axiovert 25) and a digital camera (Sony MPEG, DSC-S75). A grid was etched on the bottom of each well in a 6-well plate, to create a common reference point for each subsequent time point of photos. Photos were taken every 6-12 hours after plating for a period of 4 days, after which time neurites became too confluent to establish origin. A viability criterion was established a priori to ensure consistency. Using a grid overlay and Adobe Photoshop CS (version 8.0), relative neurite growth was measured over time based on the number of times each neurite crossed numbered lines.

Neuronal Survival

Beginning 4 days after plating, neuronal survival was determined using neuron morphology. Briefly, cells were considered viable if membranes were intact, no blebbing or vacuolization was present, and neurites were robust and not fragmented. Quadrant grids were etched on the bottom of each well to ensure subsequent consistency with baseline photos. Pictures were taken every 24 hours for an additional 7 days using a phase contrast microscope (Zeiss Axiovert 25) and digital camera (Sony MPEG, DSC-S75). Survival was measured based on total cells dead per well divided by number of total cells alive per well, multiplied by 100 and expressed as percent alive.

Trypan Blue Stain (Necrotic Cell Death Indicator)

Media was withdrawn from each well and washed twice with warmed phosphate buffered saline (PBS). Trypan blue stain (750μL; 0.4%, Invitrogen) was mixed with 750μL double-distilled water and 300μL of this diluted mixture was applied to each well and placed in an incubator at 37° C (5% CO2) for 5 minutes. Cells were washed once with 1ml of warmed PBS and analyzed immediately using a phase/contrast microscope (Zeiss Axiovert 25). Non-stained cells were contrasted against cells that were stained positive for Trypan blue (dead cells). Cell death was expressed as dead cell percentage.

Propidium Iodide Fluorescent Dye (Necrotic Cell Death Indicator)

Media was withdrawn from each well (200,000/well) and Propidium iodide labeling solution (20μL Propidium iodide plus 1mL Incubation buffer; 200-300μL of this mixture/well; Roche Applied Science) was added and incubated at room temperature for 10-15 minutes. Cells were imaged using scanning laser confocal microscopy with 20X objective, detector gain set at 948, pin hole set to 1, and amplifier gain at 1.1. Channel used was CY3 (543nm), excitation at 488nm. Propidium iodide evidence is presented as average cell number positive for Propidium iodide divided by number of live cells and reported as "% death" in TgCRND8 vs. Non-transgenic littermate controls.

ELISA for Extracellular Aβ

Media from primary cortical cells cultured for 3 and 8 days was collected. In addition, conditioned media from day 8 cortical neurons was removed and replaced with fresh media for 12 hours ("Fresh Media") and collected. All media samples were

prepared using manufacturer's guidelines and assayed using a human beta amyloid 1-42 colorimetric sandwich ELISA (BioSource International, Camarillo, CA).

TUNEL Apoptotic Marker

Cortical cells were cultured on glass coverslips as described previously (section 2.3) and allowed to mature at 37° C in 5% CO2 for 12 days. Cells were fixed using a Fixation Solution (Roche Applied Science) for 1 hr at room temperature. Cells were then rinsed with phosphate buffered saline (PBS) and then allowed to incubate on ice (2-8 degrees Celsius) in Permeabilisation Solution (Roche Applied Science) for 2 minutes. Cells were rinsed again with PBS and dried. TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) Reaction Mix (30-50μL; Roche Applied Science) was added to each well, and wells were then covered in parafilm. After incubation in a humidified environment for 1 hr at 37° C in the dark, cells were again rinsed twice with PBS. DAPI (4',6-diamidino-2-phenylindole) mix (1ml) was added to each well for 5 minutes. Slips were again rinsed in PBS and mounted to coverslides. Apoptosis was expressed as a percentage by dividing the number of TUNEL-stained cells (per well) by the total number of DAPI-stained cells.

MAP2, DAPI, and GFAP

Cortical cells were cultured on glass chamberslides and allowed to incubate at 37° C (5% CO2) for 8 days. Cells were washed with cold PBS and fixed using paraformaldehyde (4%) for 15 minutes. After washing with PBS, 1ml of 0.2% triton was added to each well for 15 minutes. Cells were washed again with PBS. 1 mL of 100%

methanol was then added to wells for 10 minutes. After washing, 1mL of 2% bovine serum albumin (BSA) blocking to each well and kept at room temperature for 45 minutes. Cells were washed, and primary antibody to microtubule associated protein 2 (MAP2) (Chemicon International) (rabbit polyclonal; 1:1000 dil.) was added to each well overnight at 4 degrees Celsius. Secondary antibody (anti-rabbit; FITC-tagged) was added the next day and allowed to incubate on cells for 1 hour at room temperature. Glial fibrillary acidic protein (GFAP) (Sigma-Aldrich, St. Louis, MO) mouse monoclonal (1:100 dil.) antibody was then added to cells overnight at 4° C. After washing, an additional secondary antibody (anti-mouse; Cy3 fluorscent-tagged) was added for 1 hour before washing. Coverslips were then mounted to glass slides using a mounting medium with DAPI (Vectashield; Vector, Burlingame, CA), and imaged using an upright Zeiss Axiovision fluorescent microscope.

Western Blotting

Cortical neurons were cultured as described above for 7-14 days then washed in ice-cold phosphate buffered saline (PBS) and lysed using 200μL lammeli buffer. Lysed cells were boiled for 15 minutes and centrifuged at 14,000 rpm for 10 minutes to remove membranes. Pellet was discarded and protein concentrations in the supernatant were determined using a detergent-based protein assay (DC protein assay; Bio-Rad, Hercules, CA). Proteins (20μg/well) were resolved on a 16% SDS tris-tricine gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose and incubated with primary antibody for either c-terminal fragment APP (Sigma-Aldrich, St. Louis, MO; or Zymed Laboratories Inc., San Francisco, CA) or Aβ 1-42 (Calbiochem, San Diego, CA) overnight. Blots

were incubated in secondary antibody for 2 hours, and imaged using Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology, Santa Cruz, CA) and Fluor-S MultiImager (Bio-Rad, Hercules, CA) or X-ray exposure. Blots were stripped and reprobed with actin to control for loading error (Santa Cruz Biotechnologies, Santa Cruz, CA).

Reactive Oxygen Species (ROS) Generation

Primary cortical cells were grown on glass coverslips or single glass-bottomed dishes (100,000 – 200,000 neurons/well) for 11 days. Media was changed to 1mL per well of either Hanks Balanced Salt Solution (HBSS) or neurobasal without serum. Cells were incubated with 5-10μL Dihydrorhodamine 123 (DHR123; Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37° C (5% CO2). Cells were washed twice with Locke's buffer or neurobasal without serum. Coverslips were removed from wells and fixed to single 60mm dishes with denture adhesive. This step was bypassed when using single glass-bottomed dishes. Cells were imaged using a scanning laser confocal microscope (Zeiss LSM 510) using a pinhole set to 1 and brightfield and FITC (488nm) channels, on a 40X oil objective. Data were analyzed by separating out brightfield from FITC and averaging the intensity of staining on the FITC channel (using Scion Imaging software) from each cell and comparing values obtained from TgCRND8 to those of non-transgenic littermate controls.

Statistical Analysis

Data were analyzed using GraphPad PRISM (version 4.02, San Diego, CA) or Excel. Differences between TgCRND8 vs. non-transgenic samples were analyzed using Student's t-test for differences between means in all experiments with the exception that 2-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to analyze morphological survival and neurite outgrowth studies. Differences in all cases were considered significant at $p < 0.05$; SEM's were reported in all experiments.

Results

1.0 In vitro, TgCRND8 and non-transgenic littermate controls possess similar neuron-to-glial ratio.

Immunostaining for neuronal and glial cells using microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) respectively, was employed to resolve the ratio of neuronal to non-neuronal cells, and to determine if there were any initial differences between transgenic and non-transgenic cultures. Staining revealed similar occurrence of MAP2 & GFAP in TgCRND8 (n=28 neurons, 5 positive for GFAP; 5.6% astrocyte content) and non-transgenic littermate controls (n=34 neurons, 6 positive for GFAP; 5.7% astrocyte content), thus indicating that a comparable culture environment existed between TgCRND8 and non-transgenic controls (Figure 1).

2.0 APP and Aβ1-42 expression are higher in TgCRND8 vs. non-transgenic primary cortical cells.

The expression of amyloid precursor protein (APP) from TgCRND8 and non-transgenic littermate primary cortical cells was measured using Western blot assay (against actin control). APP was found to be produced in both TgCRND8 and non-transgenic littermate controls, however APP levels were significantly higher in TgCRND8 eight days post-plating when compared to non-transgenic littermate controls ($p<0.01$) (Figure 2). APP generation was also found to be greater in TgCRND8 cortical cells compared to non-transgenic littermates during late-stage culture (day 14) (data not shown).

Aβ1-42 concentration in cell media was measured at 3 and 8 days post-plating using Aβ ELISA. Relatively small amounts of Aβ1-42 were produced 3 days after plating, but levels were not significantly different between TgCRND8 and non-transgenic littermates (Figure 3a). By day 8, there was a significantly greater concentration of Aβ1-42 in TgCRND8 cortical cells compared to non-transgenic littermate controls (2800 pg/ml vs. 1200 pg/ml; *$p<0.001 \pm$ SEM; Student's t-test) (Figure 3a).

To determine how quickly Aβ1-42 was secreted, fresh neurobasal media was added to 8 day-old TgCRND8 and non-transgenic control cortical cells for a period of 12 hours after which time media was collected and assayed. The concentration of Aβ1-42 in TgCRND8 was significantly greater when compared to non-transgenic littermate controls (1400 pg/ml vs. 62.5 pg/ml; *$p<0.001 \pm$ SEM; Student's t-test) (Figure 3b).

3.0 Cell survival, but not neurite outgrowth (NOG), is significantly different in TgCRND8 primary neurons vs. controls.

Cortical cells initially showed no difference in survival during the first week of culture between TgCRND8 and non-transgenic control neurons (Figure 4a). In agreement with our findings, White et al., (1998) observed that at 3 days in culture mouse APP -/- (knock-out) and APP +/+ (wild-type) neurons did not show significant differences in survival (White, Zheng et al. 1998). However, decreased survival in TgCRND8 cortical cells was observed beginning 11 days after culture and continuing through to day 16 ($p<0.0001$) when compared to non-transgenic littermates (Figure 4b). Additionally, Trypan blue staining (for necrosis) revealed significantly greater cell death in TgCRND8 (n=933 cells; 49.6% death) vs. non-transgenic control (n=474 cells; 38.6%

death) cortical neurons 12 days post-plating (Figure 5a), indicating a roughly 22.2% increase in cell death (two different experiments combined). Propidium iodide staining (also for necrosis) revealed a significant difference in necrotic cell death with TgCRND8 cells (n=388; 48% death) exhibiting more necrotic death than non-transgenic littermate controls (n=304; 33% death) 12 days post-plating (Figure 5b). This indicated a 20.2% increase in cell death in TgCRND8 vs. non-transgenic littermate controls. In the present study, evidence of apotosis (as indicated by cells immunolabeled with late-apoptotic indicator dye, TUNEL) in both TgCRND8 (n=246 cells; 31% death) and non-transgenic littermate (n=271 cells; 33% death) cortical cultures was identified, but no significant difference existed between the two groups 12 days post-plating (Figure 6). Taken together, these data indicate that increased cell death in TgCRND8 primary cortical cells, is not due to apoptosis but rather to necrosis.

To assess whether differences existed in neurite outgrowth between TgCRND8 and non-transgenic littermate cells, neurite outgrowth was observed over a period of 3 days. Hippocampal cells were chosen because of their ability to survive at very low plating densities and their robust growth of neurites. The length of this study was chosen to terminate by 4 days post-plating due to the inability of effectively determining neurite origin. It is acknowledged that the length of this study did not cover the latter time points when Aβ secretion became significantly greater in TgCRND8 vs. non-transgenic controls (i.e. 8 DIV). Notwithstanding the difficulty of morphologically assessing neurites in the longer term, examining initial biological culture activity between the two groups was the primary goal here. Using a phase contrast microscope, neurite outgrowth progression was documented every 6-12 hours and analyzed using a grid-overlay. There were no

significant differences in neurite outgrowth between TgCRND8 and non-transgenic littermate control hippocampal cells (Figure 7a, b). These data further substantiate observations in the current study that TgCRND8 and non-transgenic littermate cells are similar early on in culture.

4.0 Reactive oxygen species (ROS) generation was significantly higher in TgCRND8 cultures vs. non-transgenic littermate controls 11 days post-plating

To examine ROS generation in TgCRND8 primary cortical cultures, fluorescent intensity of dihydrorhodamine 123 (DHR123; a dye that detects the generation of hydrogen peroxide, a free radical that contributes to cellular stress) was measured using scanning laser confocal microscopy. After 11 days in culture, TgCRND8 cortical cells exhibited significantly greater generation of ROS (as indicated by greater intensity of DHR123 staining in mitochondria of each cell, averaged per well) compared to non-transgenic littermate controls ($*p<0.0001 \pm$ SEM; Student's t-test) (Figure 8).

Discussion

The distinct disadvantage of lack of naturally occurring rodent AD models has prompted the creation of several strains of transgenic mice to investigate the neurodegenerative mechanisms involved in the onset and progression of AD. One such transgenic mouse line is the TgCRND8. This model expresses a double mutant form of human APP695, leading to an accelerated production and accumulation of Aβ peptide. The aim of the present study was to characterize primary cortical and hippocampal neuronal function in TgCRND8 and non-transgenic littermate controls. This in vitro

system provides an advantageous means for studying molecular mechanisms involved in AD.

APP was normally expressed in non-transgenic primary cortical cells however APP expression was significantly greater in TgCRND8 cortical cells 8 days post-plating, as evidenced by Western blot assay. This result comparatively fits with what was observed in the in vivo TgCRND8 model whereby APP production in TgCRND8 brain tissue was much greater than in non-transgenic littermates (Chishti, Yang et al. 2001). APP maturation is extremely important in the pathogenesis of neurodegenerative diseases such as AD as it is after post-translation that APP is proteolytically cleaved at the plasma membrane to liberate toxic Aβ1-42 (Ling, Morgan et al. 2003).

The in vitro cleavage of APP by sequential secretase activity that liberates Aβ1-42 (Haass, Schlossmacher et al. 1992); (Shoji, Golde et al. 1992); (Seubert, Vigo-Pelfrey et al. 1992); (Busciglio, Gabuzda et al. 1993) made it imperative to ascertain whether TgCRND8 neuronal cells secreted Aβ. Using sandwich enzyme-linked immunosorbent assay (ELISA) to measure secreted Aβ in TgCRND8 cortical cells we demonstrated that Aβ1-42 was secreted into culture media early on in this system (by day 3) but that no differences in the amount of Aβ1-42 existed between TgCRND8 and non-transgenic littermate controls. However, a significantly greater concentration of Aβ1-42 was measured in media from TgCRND8 cortical cultures 8 days post-plating when compared to non-transgenics. Comparatively, other in vitro studies by Skovronsky et al (1998), Hartmann et al (1997), and Cook et al (1997), revealed similar results using different models of AD (Skovronsky, Doms et al. 1998); (Hartmann, Bieger et al. 1997); (Cook, Forman et al. 1997). Of interest in the current study, the rate of Aβ1-42 secretion was

very rapid reaching nearly half the amount assayed for TgCRND8 cells at 8 days post-plating, within 12 hours after the application of fresh media. This rapid secretion of Aβ1-42 into the media has also been reported in other models of Alzheimer's disease (Hartmann, Bieger et al. 1997); (Haass, Schlossmacher et al. 1992); (Haass, Hung et al. 1993); (Shoji, Golde et al. 1992).

Many functional roles for APP have been suggested. Among them is the proposed involvement in neurite outgrowth (NOG; (Araki, Kitaguchi et al. 1991); (Jin, Ninomiya et al. 1994). NOG was measured in the current study to indicate the general health and physiology of the cells. In the present study, no basal difference in neurite outgrowth was observed between TgCRND8 and non-transgenic littermates after 4 days post-plating. This was done to show that functionally there are no differences between the two cell groups until the production of Aβ. NOG is a straightforward indicator of neuronal health and function and was not used in the current study to measure the effects of overexpression of APP. Our results therefore support the idea that the later differences we observed in cellular health and function are due in some capacity to increased production and secretion of Aβ1-42.

Although controversial in vitro, in vivo age-related neuronal cell loss has been reported in various models of AD (Casas, Sergeant et al. 2004). Cell death appearance and mechanisms in vitro have been harder to delineate (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006). The current study observed no significant difference in early cell survival between TgCRND8 and non-transgenic cortical cells (4-10 days post-plating). Eleven days in culture the TgCRND8 cells began to die at a faster rate than non-transgenic littermate controls. This increase in cell death corresponds somewhat to

the sharp increase in Aβ1-42 secretion we observed in TgCRND8 cells, thus lending support to the hypothesis that increased cell death may be attributed to enhanced production of Aβ. Taken altogether, similar neurite outgrowth and initial survival trends between TgCRND8 and non-transgenic littermates indicates that fundamentally these two groups are similar early on in culture, thereby eliminating the existence of initial basal variance as a contributor to differences observed at later time points. These observations therefore provide strong supporting evidence that the later changes we reported in cell health and function can be attributed to augmented production of Aβ in this system.

To help explain the mechanism of cell death we observed in TgCRND8 primary neurons, both apoptosis and necrosis were measured. Some investigators have observed that Aβ can instigate an entire flow of apoptotic events in culture (Mattson 2000). However, in a review by Sankaranarayanan (2006), most murine models of AD do not reflect the full complement of features that exist in the human condition of AD, therefore very few studies report in vitro neuronal death at all (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006). There was evidence of apoptosis in our TgCRND8 and non-transgenic littermate cultures however there were no differences between the two groups. The presence of apoptotic cell death in our system does not fully explain the significant death in transgenic cells that we observed beginning 11 days in culture. Because of this, necrotic cell death was also measured. It was established that TgCRND8 primary cells displayed 22.2% greater increase in cell death, indicated by Trypan blue staining, than non-transgenic littermate cells. Moreover, TgCRND8 cells exhibited approximately 20.2% more necrotic cell death using fluorescent dye, propidium iodide, for necrosis, than what was observed in non-transgenic littermate controls. Therefore,

increased cell death in TgCRND8 primary neurons can to be attributed to a necrotic, rather than apoptotic, mechanism of cell death.

Importantly and perhaps of critical importance aimed at discerning possible mechanisms responsible for in vitro cell death in this model, damage from ROS generation and oxidative stress intermediates has been postulated to trigger swift necrotic neuronal demise (Behl 2000).

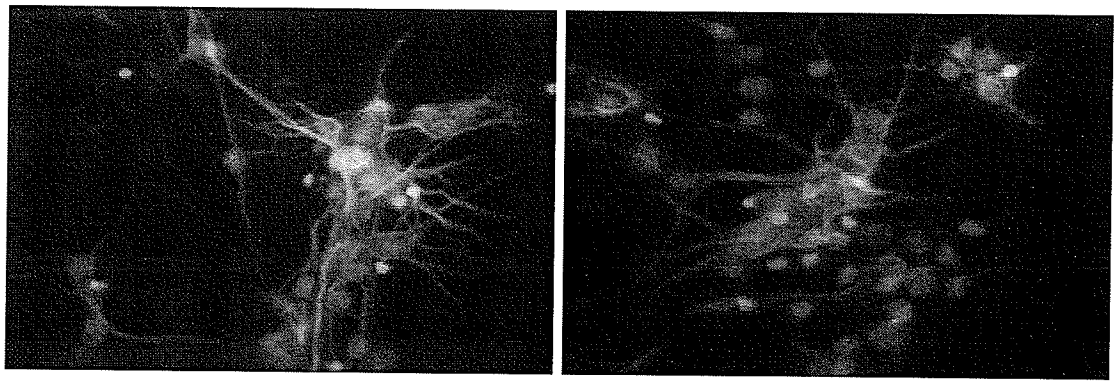
We measured reactive oxygen species (ROS) generation in our TgCRND8 and non-transgenic primary cortical cell cultures. In one neuronal in-vitro model example, Aβ peptide inserts into the lipid bilayer of neuronal cells and causes the generation of free radicals that can then incite lipid peroxidation and membranal damage (Varadarajan, Yatin et al. 2000). Of interest to note, the membrane damage caused by ROS can also lead to the dysfunction of cellular processes such as calcium homeostasis and the activation of cell death pathways (Varadarajan, Yatin et al. 2000). Keller et al (1997) and Mark et al, (1999) found that when Aβ was added to neurons in culture, ROS generation increased (Keller, Pang et al. 1997); (Mark, Fuson et al. 1999). Furthermore, hydrogen peroxide has been reported to be produced directly by Aβ peptide (Huang, Atwood et al. 1999). Similarly, in the current study ROS generation was present in our TgCRND8 in vitro system, and this was observed by at least 11 days post-plating. It is understood that this could simply be a product of cells dying in culture rather than an increase in reactive oxygen species precursors. Irrespective of cell death, the presence of ROS in our culture system coupled with the fact that there was a significant difference in ROS generation in TgCRND8 compared to non-transgenic cells is likely due to our finding that these cells produce and secrete more Aβ1-42 than their non-transgenic littermates. However,

because a control experiment using an Aβ antibody to control for possible effects of extracellular Aβ was not performed, the author cautions the reader that a definite conclusion can not be made at this time.

In general, this in vitro research intended to characterize the cellular activities of TgCRND8 has contributed valuable data to that which was previously unidentified. We report increased production of APP, increased generation and subsequent secretion of Aβ1-42, enhanced generation of ROS, and increased necrotic cell death in TgCRND8 primary cortical neurons compared to non-transgenic littermate controls. Given these data, TgCRND8 primary neurons provide a valid in vitro model with which to study molecular aspects of AD. However future studies will be necessary to help elucidate a number of biological functions, including measuring concentration of internal Aβ1-42 vs. secreted Aβ1-42, contribution of intracellular vs. secreted Aβ to cell damage and eventual death, determining whether calcium homeostasis is disrupted in culture in TgCRND8 primary neurons and the mechanism by which this may be occurring, presence of reactive oxygen species, the role(s) of various caspases in this system, and the calcium handling responses to various stimuli and blocking agents, to mention a few.

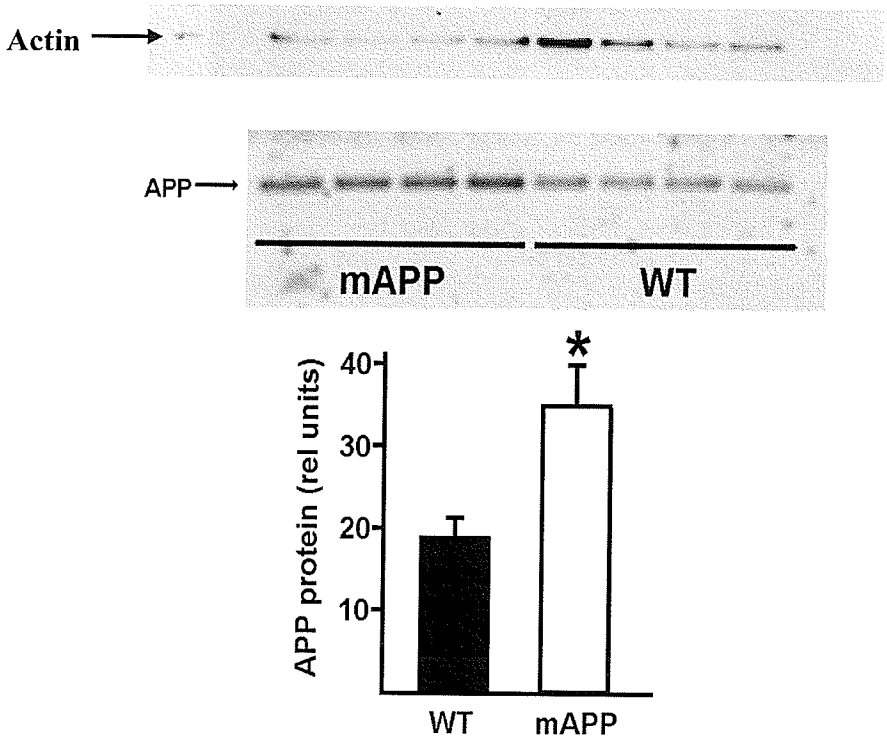
Chapter 2: Figures and Figure Legends

Figure 1: Immunocytochemistry of TgCRND8 and Non-Transgenic Cortical Neurons



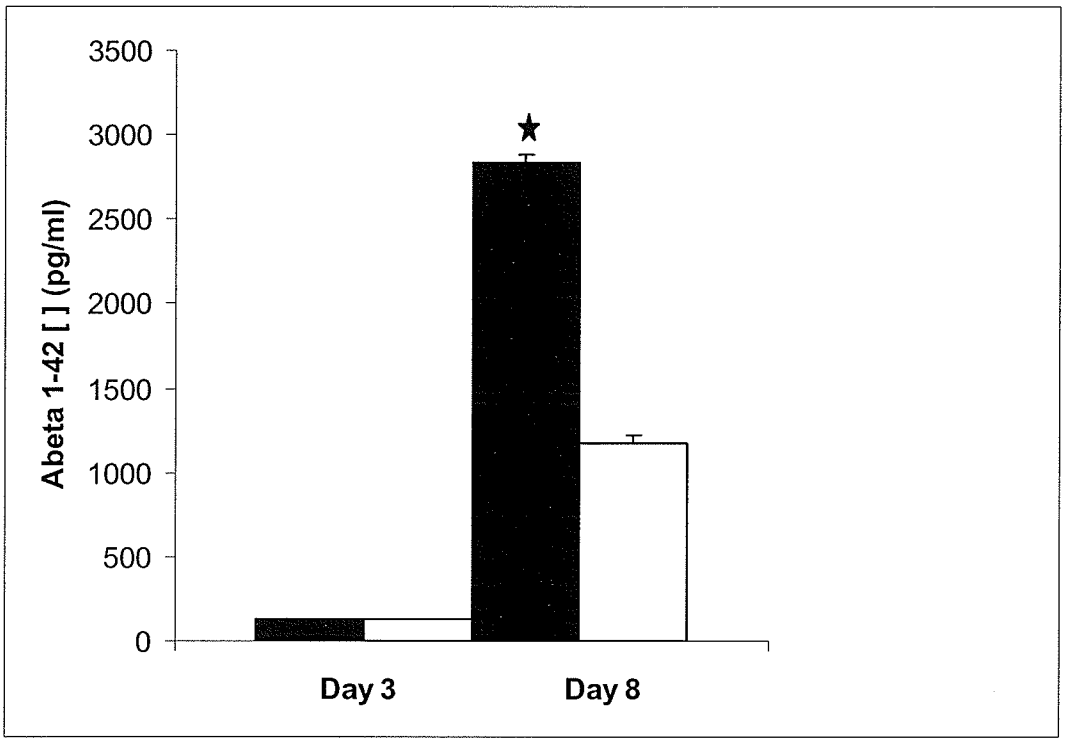
Panel on left is TgCRND8 cortical culture 8 days post-plating. Panel on right is non-transgenic littermate cortical controls from same culture. Blue stain (DAPI) is a nuclear stain, red (GFAP) indicates astrocytes, and green (MAP2) indicates neurons. Cells were imaged using an upright Zeiss Axiovision fluorescent microscope. TgCRND8 n=28 neurons, approximately 5.6% astrocytes; Non-transgenic n=34 neurons, approximately 5.7% astrocytes.

Figure 2: Expression of Mutant APP



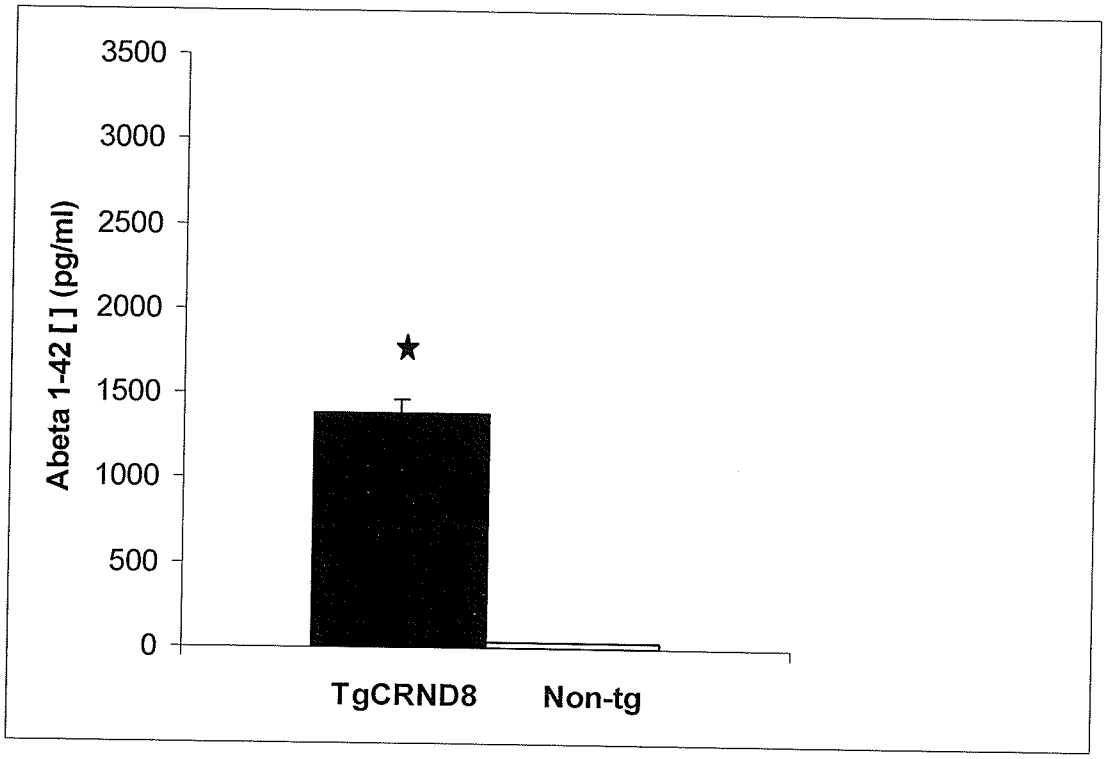
Western blot of mutant APP in TgCRND8 primary cortical cultures. Transgenic primary cortical neurons express significantly greater levels of APP 8 days in culture. Brains were removed aseptically from E16 transgenic TgCRND8 (mAPP) and Non-transgenic (WT) littermate embryos and separately dissociated. A sample of each was analyzed by polymerase chain reaction (PCR) to determine presence of mAPP transgene. Cells were grown for 8 days at which time total protein was isolated and analyzed by Western blot and standardized to actin control. Top panel is actin control. Middle panel are APP protein bands: first 4 wells are TgCRND8 (mAPP), latter 4 wells are Non-transgenic (WT). Histogram in lower panel shows quantitation of relative fluorescent intensity. APP expression was significantly higher in TgCRND8 (mAPP) compared to Non-transgenic (WT) littermates. Data are mean $\pm$ SEM; *$p < 0.01$ (n=4).

Figure 3a: Amyloid Beta 1-42 Concentration in Media



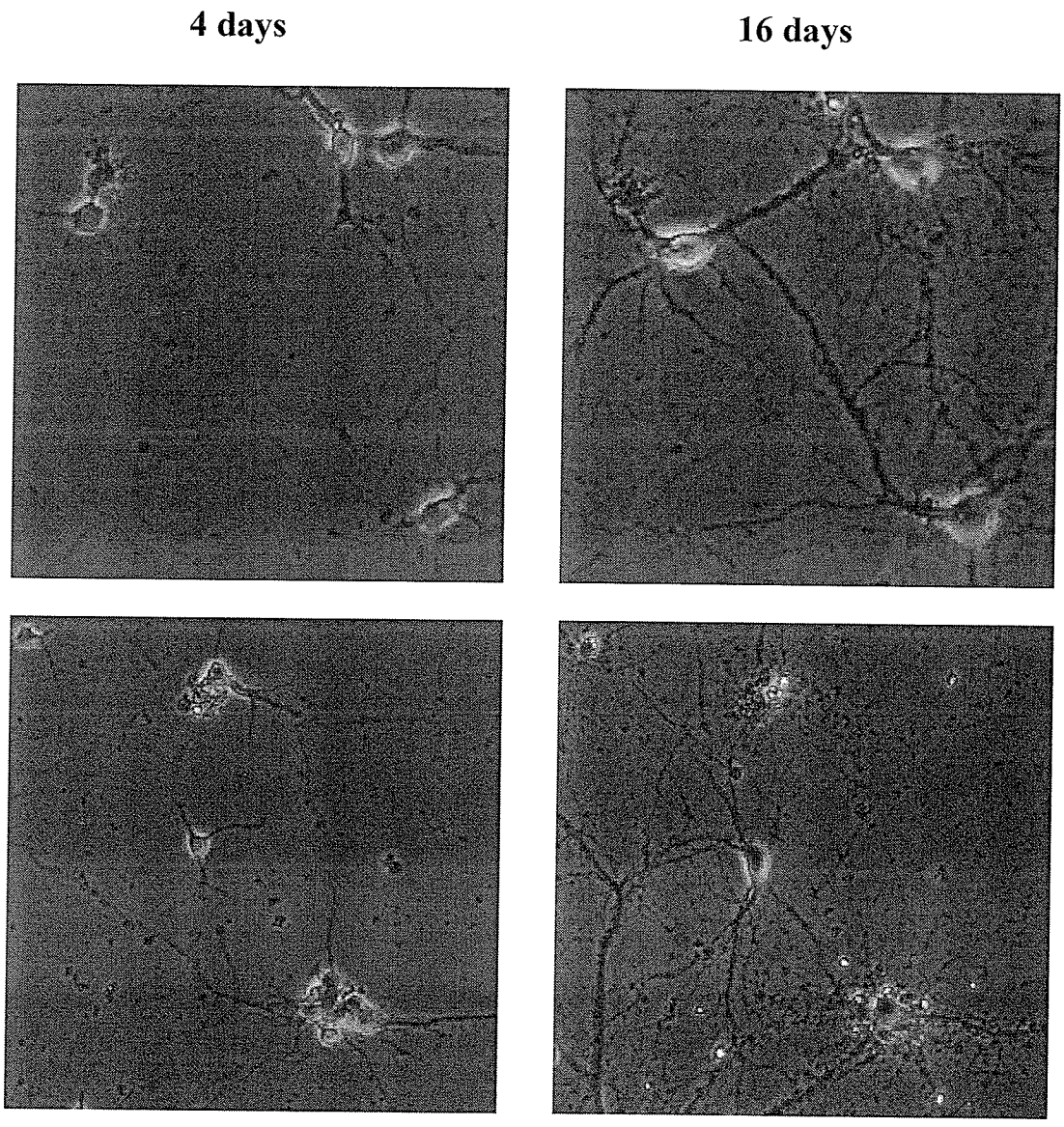
Amyloid beta (Aβ1-42) concentration in media from TgCRND8 and Non-transgenic littermates. Solid bars indicate TgCRND8, open bars indicate Non-transgenic Aβ1-42 concentration in media. Using sandwich enzyme-linked immunosorbent assay (ELISA) media from 3 and 8 day-old cortical cultures was collected and Aβ1-42 concentration analyzed. There was no difference in secreted Aβ1-42 concentration in media from 3 day-old cortical neuronal cultures. However, there was a significantly greater concentration of Aβ1-42 secreted in media from TgCRND8 cortical neurons (2840.775pg/ml) 8 days post-plating when compared to Non-transgenic littermates (1169.5pg/ml). Values are average means, $\pm$ SEM, n=4 wells; *$p < 0.001$, Student's t-test.

Figure 3b: Amyloid Beta 1-42 Concentration in Fresh Media



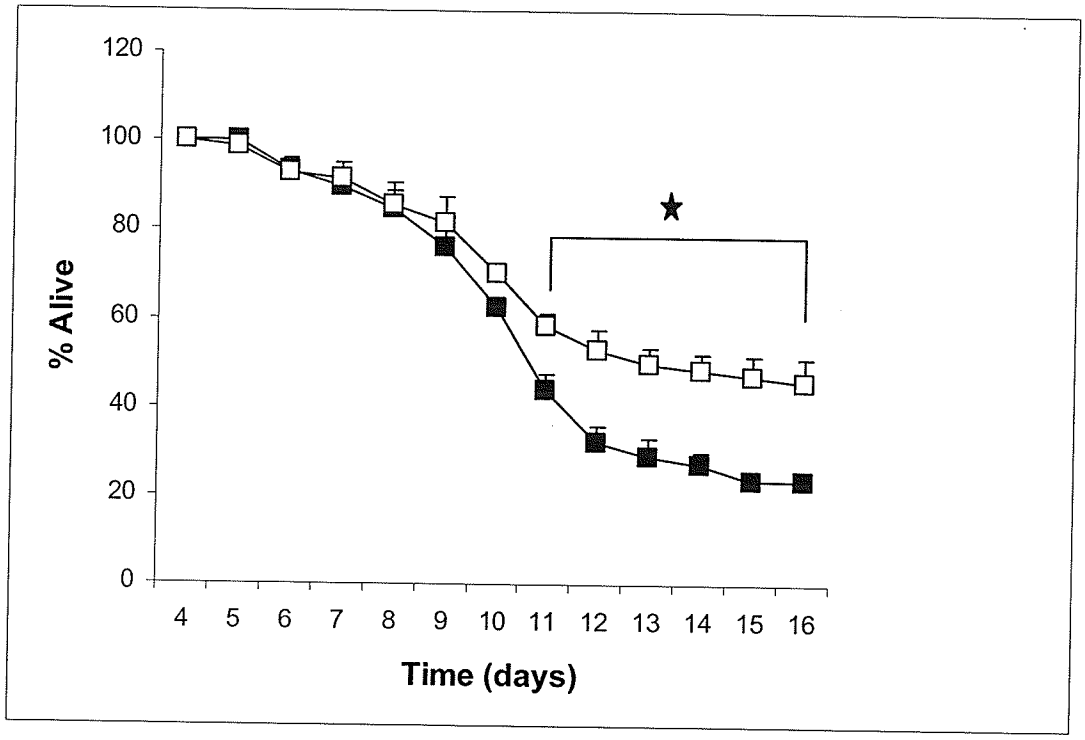
Amyloid beta (Aβ1-42) concentration in media from TgCRND8 and non-transgenic littermates. Culture media was removed from 8 day old cortical cultures and replaced with fresh neurobasal media. After 12 hours, media was collected and secreted Aβ1-42 was analyzed using sandwich enzyme-linked immunosorbent assay (ELISA). There was a significantly greater concentration of Aβ1-42 secreted into fresh media from TgCRND8 cortical neurons (1379.392pg/ml) compared to Non-transgenic littermates (31.7pg/ml). Values are average means ± SEM, n=4 wells; *$p < 0.001$; Student's t-test.

Figure 4a: Morphological Neuronal Cell Survival



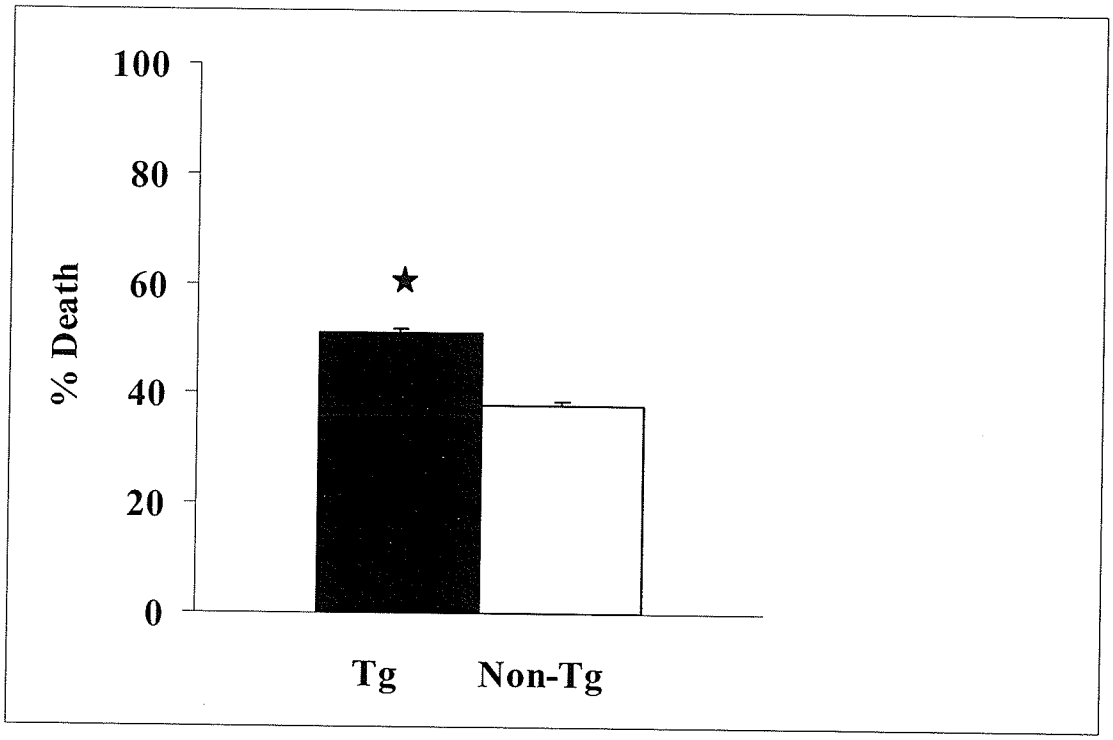
TgCRND8 and Non-transgenic littermate primary cortical neuron survival pictures using bright phase microscopy. Top two panels are non-transgenic cells; bottom two panels are TgCRND8 cells. Panel on the left is a photo of cells 4 days after plating. Panel on the right is a photo of cells 16 days after plating. Cells were in Neurobasal media (B27, L-glutamine, and antibiotic) without fetal bovine serum. Robust neurites and healthy cells are represented in the two left panels, while fragmented neurites, cellular debris, and membrane infirmity are evident after 16 days in culture (bottom right panel; TgCRND8).

Figure 4b: Neuronal Time Course Survival Trends



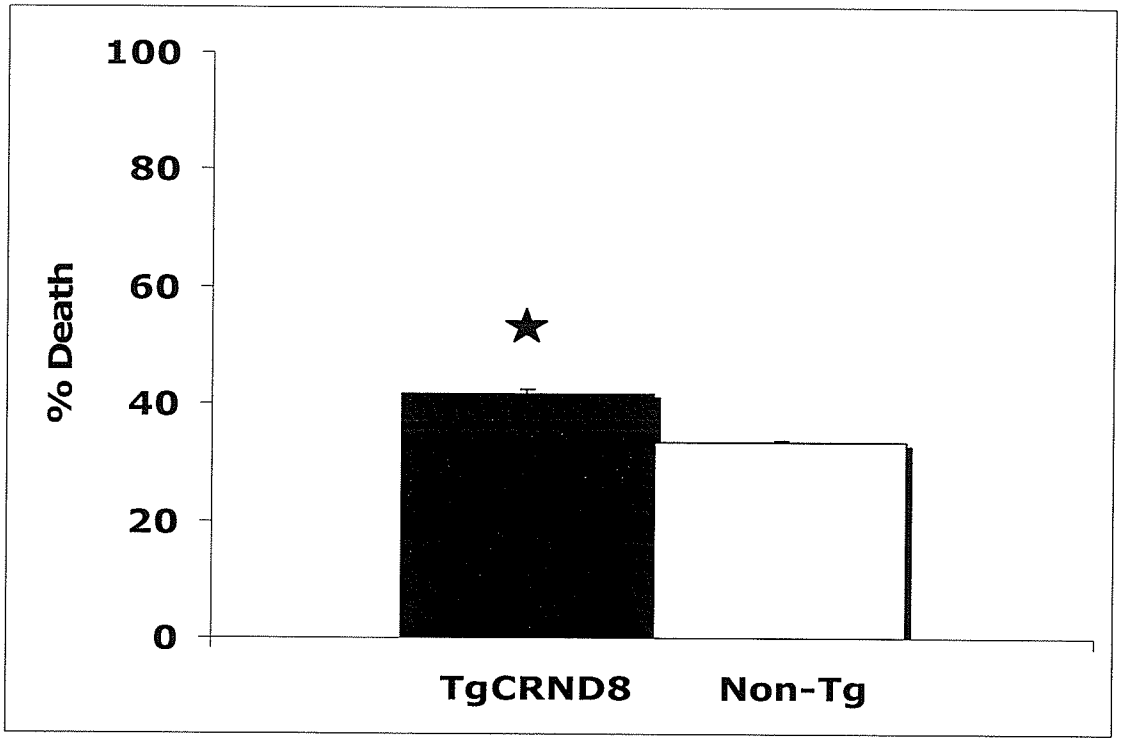
Line graph indicates survival trends for TgCRND8 and Non-transgenic littermate primary cortical cells. Solid squares are TgCRND8 cortical neurons, open squares are non-transgenic littermates. Initial survival is similar between TgCRND8 and Non-transgenic neurons for the first 9 days in culture. Survival decreases significantly in TgCRND8 cells beginning 11 days after culture compared to Non-transgenic littermate cells. Neurons were plated at a density of 100,000 cells per 35 mm diameter well. Starting 4 days after establishment of culture, photographs were taken of selected fields every 24 h, and survival determined by pre-established morphological criteria.
(* $p<0.05$, 2-way ANOVA, mean $\pm$ SEM, n=3).

Figure 5a: Trypan Blue Indicator Stain for Necrotic Cell Death



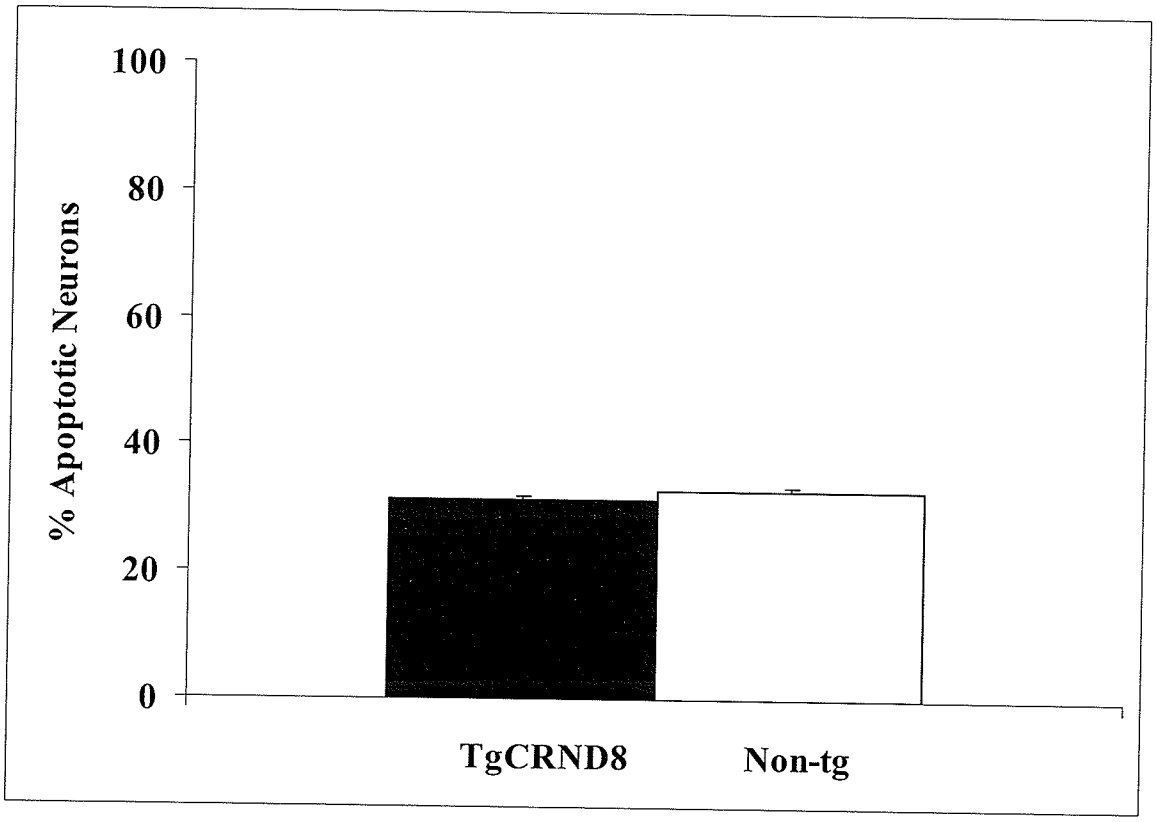
Trypan blue stain for necrotic cell death. Primary cortical cells were grown in culture for 12 days. Trypan blue was added to each well for 5 minutes. Cells were counted using a phase contrast microscope and data presented an average of dead cells divided by live cells per well and expressed as a percentage ("% Death"). There is a significant (22.2%) reduction in survival in TgCRND8 vs. Non-transgenic littermate controls (TgCRND8 n=933; 49.6% death, $\pm$ 0.735 SEM; Non-transgenic n=474; 36.6% death, $\pm$ 0.759 SEM; *$p<0.05$; Student's t-test).

Figure 5b: Propidium Iodide Stain for Necrotic Cell Death



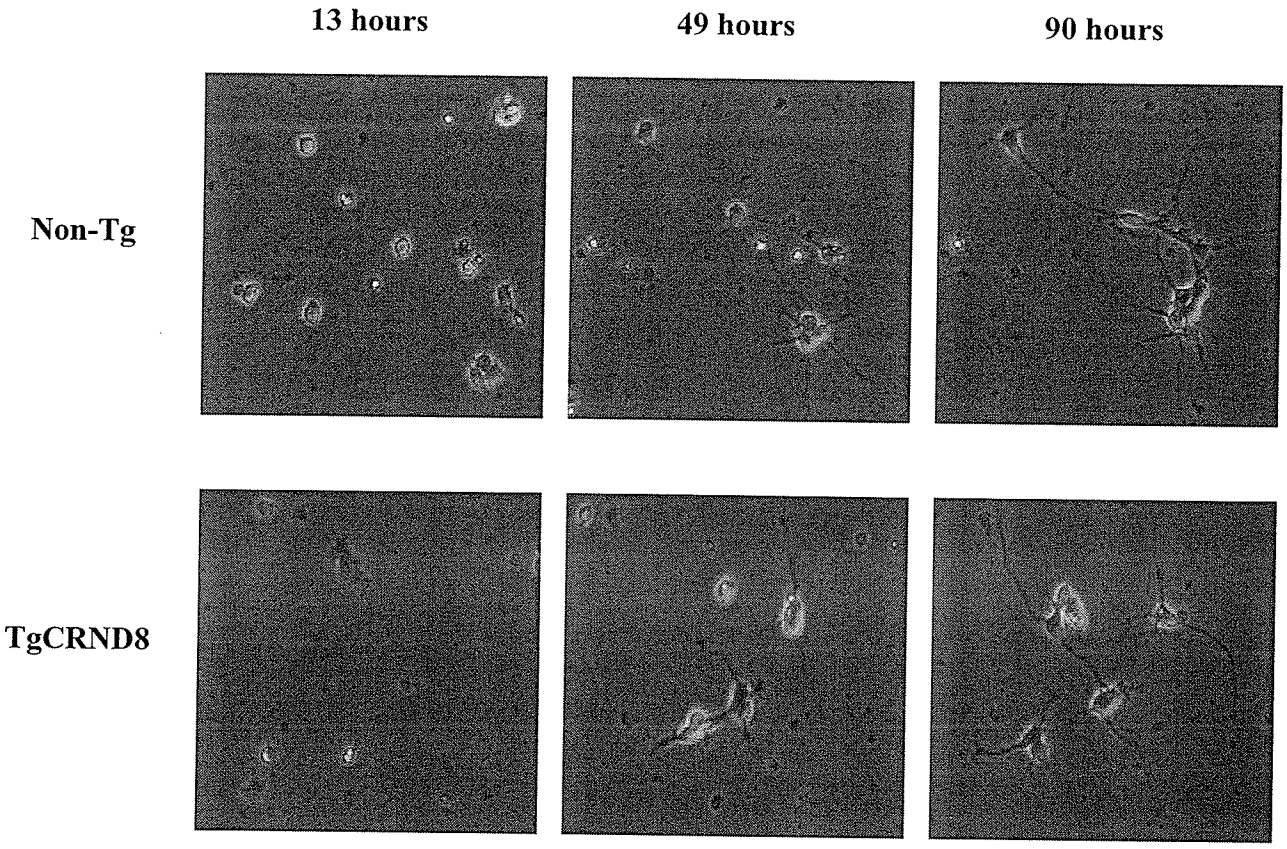
Propidium iodide stain for necrotic cell death. Primary cortical cells were grown in culture for 12 days. Propidium iodide was added to each well for 10-15 minutes and imaged using a scanning laser confocal microscope with 20X objective, detector gain set at 948, pin hole at 1, and amplifier gain at 1.1. Channel used was CY3 (543nm). Data presented as average of dead cells divided by live cells per well and expressed as a percentage ("% Death"). There is a significant (20.2%) reduction in survival in TgCRND8 vs. Non-transgenic littermate controls (TgCRND8 n=388; 42% death, $\pm$ 0.696 SEM; Non-transgenic n=304; 33.5% death, $\pm$ 0.481 SEM; *$p<0.05$; Student's t-test).

Figure 6: TUNEL Indicator Dye for Apoptotic Cell Death



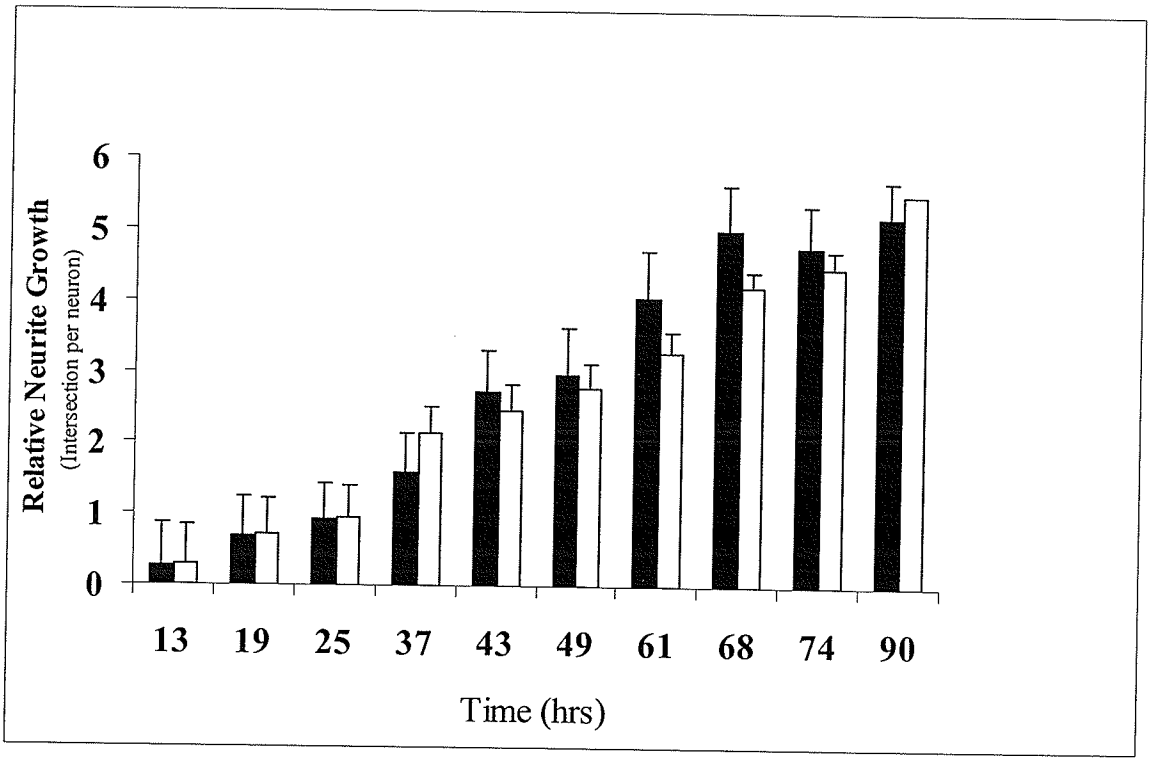
Primary cortical neurons stained with TUNEL, a late-apoptotic indicator. Primary cortical cells were grown in culture for 12 days, fixed using a Fixation buffer (Roche Applied Science), washed in phosphate buffered saline then bathed in Permeabilisation solution (Roche Applied Science) before being stained with TUNEL. Presence of apoptosis is presented here as number of TUNEL positive cells divided by the number of live cells and expressed as "% Apoptotic Neurons". Although apoptosis was evident in both TgCRND8 and Non-transgenic littermate primary cortical cells 12 days post-plating, there was no significant difference in death between the two groups. Values are means $\pm$ SEM, n=4 wells.

Figure 7a: Morphological Neurite Outgrowth Measurement



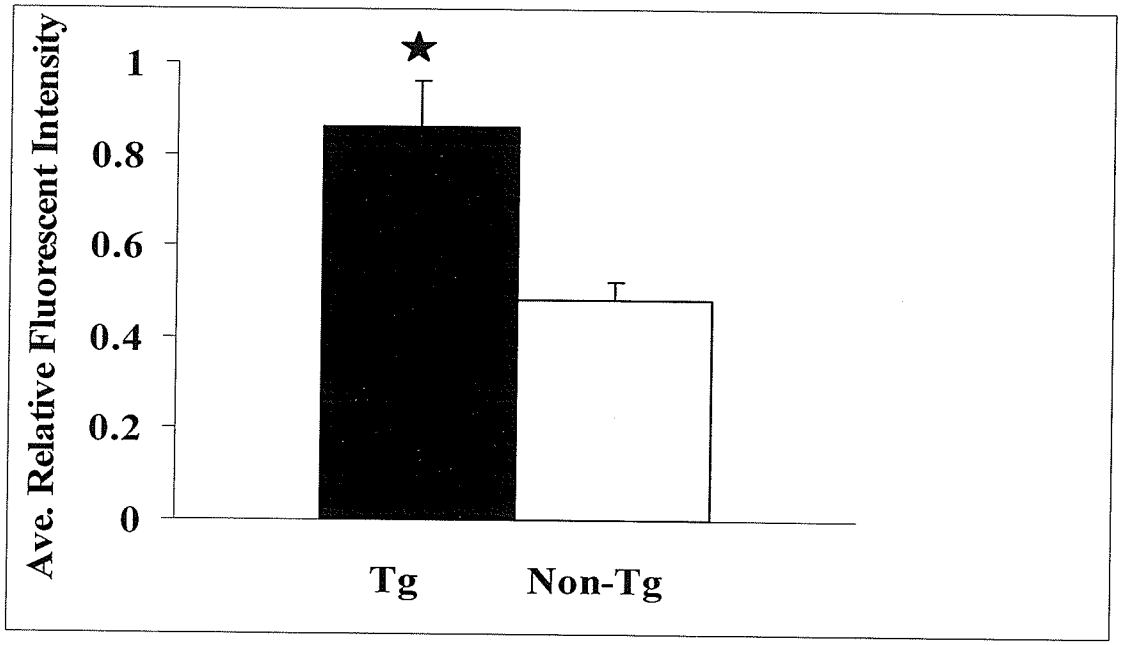
Neurite outgrowth photos from TgCRND8 and Non-transgenic primary hippocampal neurons. Top 3 panels are Non-transgenic, bottom 3 panels are TgCRND8. Panels on the left were taken 13 hours post-plating, middle panels were taken 49 hours post-plating, and last 2 panels were taken 90 hours post-plating. Pictures are reflective of beginning, mid, and end time points in neurite outgrowth study. No differences in neurite outgrowth between TgCRND8 and Non-transgenic littermate controls after 4 days in culture (Figure 7b depicts histogram of complete study time points).

Figure 7b: Histogram Depicting Neurite Outgrowth Over Time



Graph indicates neurite outgrowth in TgCRND8 and Non-transgenic littermate primary hippocampal cultures. Primary hippocampal neurons were plated at a density of 100,000 cells per 35 mm diameter well. Photos were taken starting 13 hours post-plating and every 6-12 hours thereafter until neurites became too confluent to establish origin. Using a grid overlay over each picture, numbers of neurites that crossed specific lines were counted and divided by total number of cells. Solid bars are TgCRND8 hippocampal neurons, open bars are Non-transgenic littermates. No significant differences were observed in neurite outgrowth between TgCRND8 and Non-transgenic hippocampal neurons after 4 days in culture (Data are presented as average means $\pm$ SEM; 2-way ANOVA with Bonferroni post-hoc).

Figure 8: Histogram Representing Reactive Oxygen Species Generation



Histogram showing relative fluorescent intensity of reactive oxygen species (ROS) using laser confocal microscopy in TgCRND8 (n=89 cells) and Non-transgenic (n=88 cells) primary cortical cells matured in culture for 11 days. Cells were incubated with Dihydrorhodamine 123 for 30 minutes. Cells on coverslips were imaged using scanning laser confocal microscopy using pinhole set to 1 and brightfield and FITC (488nm) channels, using a 40X oil objective. The Average Relative Intensity of fluorescence was calculated for each cell per well per group using Scion software. Significantly greater generation of ROS was evident in TgCRND8 primary cortical cells when compared to non-transgenic littermates (*$p<0.0001$, means $\pm$ SEM; Student's t-test)

CHAPTER 3 – Descriptive Calcium Handling in TgCRND8

Abstract

Loss of calcium homeostasis is a key pathological event in neurodegeneration, as observed in Alzheimer's disease (AD). Characterized by extracellular deposits of a toxic protein called amyloid beta (Aβ), intracellular hyperphosphorylated tau protein, and cell death, AD is the most frequent form of age-related dementia. It is recognized that genes involved in enhancing the preponderance of AD are in some way involved in regulating calcium signaling (LaFerla 2002). Data collected from human patients as well as from animal models compellingly suggest that calcium dyshomeostasis appears early in AD (Etcheberrigaray, Hirashima et al. 1998). Neurons normally maintain calcium homeostasis by regulating extracellular calcium influx and calcium release from intracellular organelles. One such organelle, the endoplasmic reticulum (ER) is an important calcium storehouse and key calcium source. Calcium uptake into the ER is maintained by SERCA pumps whereas calcium release is achieved primarily through inositol 1,4,5-triphosphate (IP3-R)- and Ryanodine (Ry-R) receptors. The chief plasma membrane receptors responsible for extracellular calcium entry include ionotropic and metabotropic receptors that are predominantly affected by the release (and/or binding) of a key neurotransmitter, glutamate. We used a transgenic mouse model of AD, the TgCRND8, that housed a double mutation (Swedish and Indiana) driven by the prion promoter to record resting basal calcium concentrations, descriptive calcium handling was examined. Here we report resting basal calcium levels, measured using a ratiometric calcium indicator dye, Fura 2, were higher both 8/9 DIV in TgCRND8 (0.132μM) vs.

non-transgenic littermate controls (0.095μM), and 12 DIV (TgCNRD8 = 0.123μM; non-transgenic = 0.078μM). N-methyl-D-aspartate (NMDA) receptor subunit NR1 protein expression was higher in TgCRND8 primary cortical neurons (2.1 ratio NR1/Actin) compared with non-transgenic littermate controls (0.75 ratio NR1/Actin). There was no difference in intracellular calcium influx in response to 1mM glutamate in TgCRND8 and non-transgenic littermates. However, after treatment with 100μM NMDA (mimicks the agonistic action of glutamate(Watkins and Jane 2006), intracellular calcium influx was significantly greater in TgCRND8 (peak ratio of 1.5) vs. non-transgenic controls (peak ratio of 1.29; $p<0.05$). Additionally, intracellular calcium influx was greater in TgCRND8 after pretreatment with 20μM 6-cyano-7-dinitroquinoxaline-2,3-dione (CNQX), a non-NMDA glutamate receptor AMPA competitive antagonist, followed by 1mM glutamate ($p<0.01$). Furthermore, intracellular calcium influx was significantly greater in TgCRND8 primary cortical cells (1.81 peak ratio vs. 1.57 peak ratio in non-transgenics) after pretreatment with MK801 (to distinguish between NMDA receptor activation) followed by 100μM AMPA. However, no differences existed between either group after pretreatment with 20μM [(+)-5-methyl-10,11-dihydro-5H-dibenzo [A,D] cyclohepten-5,10-imine maleate] (MK801; a non-competitive NMDA receptor antagonist) followed by 1mM glutamate. Moreover, after treatment with KCl (to stimulate metabotropic glutamate receptors) there was no difference in intracellular calcium influx between TgCRND8 and non-transgenic controls. After examination of intraorganelle contribution to intracellular calcium levels, TgCRND8 cells (2.18 peak ratio $\pm$ 0.09 SEM) exhibited greater intracellular calcium influx after application of 1μM thapsigargin, an ER SERCA pump antagonist, compared to non-transgenic controls (1.74

peak ratio ± 0.15 SEM), indicating that there may be more calcium stored in the ER in TgCRND8 cortical cells compared to non-transgenic littermates. The author acknowledges that plasma membrane calcium receptors were not inhibited during these experiments and therefore does not rule out the contribution of plasma membrane receptors to this increased intracellular calcium influx.

Collectively, the increased intracellular calcium influx generally observed in TgCRND8's vs. non-transgenic controls appears to depend heavily upon the activation of NMDA receptors when compared to all other tested glutamate receptors. Moreover, these data indicate that disruption in calcium homeostasis in TgCRND8 mice, likely due to increased Aβ secretion and subsequent ROS generation (as we've shown previously), may contribute to AD-like neuropathology and cell death in this model.

Introduction

Disturbances in the balance of intracellular calcium observed in Alzheimer's disease (AD), the most widespread, irreversible type of dementia correlated with age (Jellinger and Bancher 1998), has been implicated as an important contributor in the progression of this disease (Mattson, Cheng et al. 1992). One of the classic characteristics observed in AD is the production and deposition of amyloid beta (Aβ), a toxic protein liberated from a larger protein called the amyloid precursor protein (APP) (Smith, Green et al. 2005). Aberrant Aβ generation initiated by the proteolytic cleavage activities from enzymes collectively known as secretases (Smith, Green et al. 2005), has been implicated as being one of the major instigators of AD (Hardy and Selkoe 2002); (Smith, Green et al. 2005).

Studies have also suggested that increased intracellular calcium is pivotal to neuronal toxicity mediated by the accumulation of Aβ (Smith, Green et al. 2005). Conversely, calcium dyshomeostasis is affected by the amassing of Aβ peptide (Mattson, Cheng et al. 1992); (Smith, Green et al. 2005). Aβ has further been implicated in increasing ionotropic glutamate receptor activity, such as N-methyl-D-aspartate (NMDA) and kainate, leading to neurotoxicity in human primary cortical cells (Mattson, Cheng et al. 1992). The intracellular calcium milieu is highly regulated (Arispe, Rojas et al. 1993) but numerous studies have provided strong evidence that activity of calcium channels like NMDA receptors can be altered by Aβ (Wu, Anwyl et al. 1995), thus leading to increased neurotoxicity and cell death via increased intracellular calcium load. In vitro, it has been reported that glutamate-induced neurotoxicity can be heightened by the presence of Aβ (Brorson, Bindokas et al. 1995), and that the existence of extracellular calcium influences Aβ toxicity (Lin, Bhatia et al. 2001).

Inositol 1,4,5-triphosphate (IP3) is an endoplasmic reticulum (ER) membrane resident calcium release channel (Smith, Boyle et al. 2001). Further, ER calcium uptake is mediated by surface pumps called sarco/endoplasmic reticulum calcium ATPase or SERCA, whereas calcium release is accomplished primarily through IP3 and ryanodine receptors (LaFerla 2002). In a recent study by Supnet et al, 2006 it was observed that endogenously generated Aβ1-42 in TgCRND8 mice contributed to increased ryanodine receptor levels and activations, and that these neurons displayed increased intracellular calcium in response to treatment with glutamate (Supnet, Grant et al. 2006). It has been well established that elevated intracellular calcium as a result of glutamate application is intimately related to increased neurotoxicity (Jarvis, Lilge et al. 1999); (Mattson, Gary et

al. 2001); (Glazner, Chan et al. 2000); (Kim, Park et al. 2000); (Guo, Fu et al. 1999). Taken together, these data indicate that early in culture (day 5) TgCRND8 primary neurons possess elevated intracellular calcium levels in response to glutamate or agonistic concentrations of ryanodine (Supnet, Grant et al. 2006).

The aim of the present study was to characterize cellular functions including resting basal calcium levels, NMDA receptor subunit protein expression, and pharmacological manipulation of intracellular calcium handling in TgCRND8 and non-transgenic littermate control primary cortical neurons.

Methods

TgCRND8 Mice

The TgCRND8 mouse was developed by Dr. Westaway and colleagues at the University of Toronto (Chishti, Yang et al. 2001). These animals housed a double mutated form of the major human brain amyloid precursor protein (APP) isoform, APP695. The APP transgene expressed both Swedish (KM670/671NL) and the Indiana (V717F) mutations driven by the Syrian hamster prion promoter (Chishti, Yang et al. 2001). The background strain of this model was on a C57BL/6-C3H hybrid cross. Wild-type (APP -/-) females were bred to transgenic (APP +/-) males. Newborn animals were weaned 3 weeks after birth, tattooed, and genotyped. Fetuses were genotyped for the mutant APP (mAPP) transgene before experimental use. All animals were maintained in the Animal Care Facility at the St. Boniface Hospital Research Centre. Approval for use of all animals was obtained through the University of Manitoba Care and Use of Laboratory Animals.

Genotyping

Colony Maintenance

DNA was extracted from weanlings via small tail snips (~5 mm) using a DNA extraction kit (REDExtract-N-Amp Tissue PCR kit, Sigma Aldrich, St. Louis, MO), and the mAPP transgene was detected using polymerase chain reaction (PCR). The PCR primers used were: 5' (TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A) 3' and 5' (AGA AAT GAA GAA ACG CCA AGC GCC GTG ACT) 3' (Invitrogen, Frederick, MD). Amplification of PCR products was acquired through 30 cycles of 94/68/72° C. PCR products were separated on a 1% denaturing agarose gel (Fermentas, Hanover, MD) containing 3µl ethidium bromide (Fisher Biotech, Fair Lawn, New Jersey) at 100 V for 30 minutes using a Bio-Rad PowerPak 200 (Philadelphia, PA). PCR products were visualized with a Bio-Rad Gel Doc UV system (Philadelphia, PA).

Primary Cell Culture

Brains from embryonic day 16 TgCRND8 and WT littermate control were aseptically removed. An appendage was removed from each embryo for genotyping. Brain tissue was grouped together as either transgenic or non-transgenic and cells were mechanically dissociated. Cells were counted and seeded on 6-12 well plates, coated with poly-d-lysine (Sigma Aldrich, St. Louis, MO) plus borate, in neurobasal media plus B27, gentamicin (Invitrogen, Frederick, MD), L-glutamine, HEPES (Fisher Scientific), and fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO). The media was removed and replaced with neurobasal without FBS the following day. Cells were kept in an incubator

at 37° C in 5% CO2. All experiments using cortical cells were performed 8-15 days post plating. Neurite outgrowth (NOG) studies were performed using hippocampal cells beginning 6-12 hours after plating and ending after 4 days.

Calcium Imaging

Neurobasal media was withdrawn from wells and replaced with Locke's buffer plus bovine serum albumin (BSA) and 2μM Fura2-acetoxymethylester (AM) and incubated for 45 minutes. Cells were washed twice with Locke's buffer (without BSA) and allowed to equilibrate at 37 degrees Celsius (5% CO2) for 10 minutes. Coverslips were adhered to single well dishes and 1mL Locke's buffer was added. Calcium imaging was performed using a xenon arc lamp and 40x oil objective lens on an upright microscope (Zeiss). A baseline of approximately 20 seconds was established for all experiments before treatments were added. Fura2-AM bound to calcium excites at 340nm and Free Fura2-AM excites at 380nm. The resultant calcium data are presented as a ratio of bound to unbound calcium (340/380nm) with emission at 510nm. An average of 6-12 wells were imaged per cell type (transgenic vs. non-transgenic). Basal calcium concentrations were achieved by first calibrating the imager using a calcium standards kit (Molecular Probes, Invitrogen, Frederick, MD) and generating a standard curve. Cells were then loaded with Fura 2AM (as above) and imaging for an average of 20 seconds. Resultant numbers were pooled and averaged. The above standard curve was then used to convert experimental Fura2 fluorescence sample ratios into approximations of free calcium concentrations using the following equation:

$$[Ca2+] \text{ free} = Kd \times \left[\frac{R - R\,min}{Rmax - R}\right] \times \left[\frac{F380\,max}{F380\,min}\right]$$

Where, Kd is the dissociation constant, R is the ratio of 510nm emission intensity (340nm to 380nm excitation), R min is the ratio at zero calcium (0μM), R max is the ratio at saturating levels of calcium (39μM), F380 max is the intensity of fluorescence exciting at 380nm for no free calcium, F380 min is the intensity of fluorescence exciting at 380nm for saturating levels of free calcium. R-values obtained from each calibration was then be used to calculate free calcium in each experimental sample.

Western Blotting

Cortical neurons were cultured as described above for 7-14 days then washed in ice-cold phosphate buffered saline (PBS) and lysed using 200μL lammeli buffer. Lysed cells were boiled for 15 minutes and centrifuged at 14,000 rpm for 10 minutes to remove cell debris. Pellet was discarded and protein concentrations in the supernatant were determined using a detergent-based protein assay (DC protein assay; Bio-Rad, Hercules, CA). Proteins (20μg/well) were resolved on a 16% SDS tris-tricine gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose and incubated with primary antibody for either c-terminal fragment APP (Sigma-Aldrich, St. Louis, MO; or Zymed Laboratories Inc., San Francisco, CA) or Aβ 1-42 (Calbiochem, San Diego, CA) overnight. Blots were incubated in secondary antibody for 2 hours, and imaged using Western Blotting Luminol Reagent kit (Santa Cruz Biotechnology, Santa Cruz, CA) and Fluor-S MultiImager (Bio-Rad, Hercules, CA) or X-ray exposure. Blots were stripped and

reprobed with actin to control for loading error (Santa Cruz Biotechnologies, Santa Cruz, CA).

Statistical Analysis

Data were analyzed using GraphPad PRISM (version 4.02, San Diego, CA) or Excel. Differences between transgenic vs. non-transgenic samples were analyzed using two-way ANOVA with Bonferroni post-hoc tests (when appropriate) or Student's T tests. Differences were considered significant at $p < 0.05$; data presented as $\pm$ SEM.

Results

1.0 Resting basal calcium is elevated in TgCRND8 primary cortical neurons vs. non-transgenic littermates

As calcium dyshomeostasis is thought to be significant in AD pathology and is affected by every known clinical AD mutation (Smith, Green et al. 2005), it was therefore important to determine whether differences existed in resting calcium levels between TgCRND8 and non-transgenic littermate cortical cells. Calcium imaging was performed 8-9 and 12 days post-plating. Resting intracellular calcium levels were significantly higher in TgCRND8 cortical neurons (n=41 cells; 0.132μM) at 8/9 days post-plating when compared to non-transgenic littermates (n=53 cells; 0.095μM). *$p<0.05$; $\pm$ SEM; Student's t-test; data are pooled averages from 4 separate experiments. Resting calcium levels were also significantly higher in TgCRND8 at 12 days in vitro (n=26 cells; 0.123μM) compared to non-transgenic (n=17 cells; 0.078μM) littermate controls. *$p<0.05$; $\pm$ SEM: Student's t-test; data are pooled averages from 2 separate experiments (Figures 1a & 1b). Taken together these data further demonstrate that there are differences in late-stage cellular functions between TgCRND8 and non-transgenic littermate controls, and that these variations are associated at least in part, with increased secretion of Aβ1-42. These observations are supported by others who have reported that a disruption in calcium homeostasis may play a part in mediating the toxicity of Aβ and in turn, Aβ can affect calcium homeostasis (Mark, Blanc et al. 1996); (Supnet, Grant et al. 2006).

2.0 No difference in intracellular calcium levels in response to glutamate in TgCRND8 compared to non-transgenic littermate control primary cortical neurons.

To examine calcium response to glutamate (1mM), calcium imaging was performed using day 8 or 9 post-plating primary cortical cultures from TgCRND8 and non-transgenic littermate controls (Figure 2). Glutamate was not perfused out of this system. Data from 9 separate experiments were pooled and average mean fluorescence obtained using a ratio of bound vs. unbound calcium (340nm/380nm; Fura2) was analyzed. Cells responded quickly to glutamate, with enhanced intracellular calcium influx. However, there was no difference in intracellular calcium response to glutamate in TgCRND8 (n=131 cells) cells compared to non-transgenic littermate (n=105 cells) controls. Data were standardized to 1.0

3.0 Intracellular calcium is significantly increased in TgCRND8 primary cortical cells in response to NMDA

To determine if differences exist in intracellular calcium influx to glutamatergic agonistic stimulation by NMDA, Day 8 TgCRND8 and non-transgenic primary cortical cells were exposed to 100uM NMDA. There was an initial significant increase in peak intracellular calcium (as indicated by fluorescence ratio of bound to unbound calcium by Fura 2) in TgCRND8 (0.979 ± 0.07 SEM; n=33 cells) when compared to non-transgenic littermate controls (0.820 ± 0.07 SEM; n=23 cells) (Figure 3). Data are pooled means from 2 separate experiments, standardized to 1.0; *$p<0.01$

4.0 Intracellular calcium influx is not affected in TgCRND8 vs. Non-transgenic littermate controls after AMPA

To determine whether differences exist in intracellular calcium influx after application of AMPA (an AMPA receptor agonist), Day 8 TgCRND8 and non-transgenic primary cortical cells were pretreated with 20μM MK801 (a NMDA receptor antagonist used here to distinguish the activity of AMPA receptors without NMDA receptor activation), followed by 100μM AMPA. There was no difference in intracellular calcium influx in TgCRND8 when compared to non-transgenic littermate controls (Figure 4). Data were standardized to 1.0.

5.0 No difference in intracellular calcium levels in TgCRND8 vs. Non-transgenic controls after pretreatment with MK801

To examine intracellular calcium response to glutamate after NMDA receptor inhibition, primary cortical neurons from Day 8 TgCRND8 and non-transgenic littermate controls were pretreated with a non-competitive NMDA receptor antagonist, MK801 (20μM), for 5-10 minutes before the application of glutamate (1mM) (Figure 5) and imaged using calcium indicator dye Fura2 (ratiometric differences attained from bound vs. unbound calcium; 340nm/380nm). Glutamate was not perfused out in this system. Intracellular calcium did not reach the same level as was observed with glutamate alone, and there was no initial difference in calcium response to glutamate after inhibition of NMDA receptors by MK801 in TgCRND8 (n=63 cells) cells when compared to non-transgenic (n-57 cells) littermates. Data were standardized to 1.0

6.0 There is significantly more intracellular calcium influx in TgCRND8 vs. Non-transgenic littermate controls after pretreatment with CNQX.

To investigate the participation of AMPA receptors to intracellular calcium levels in TgCRND8 and non-transgenic littermate control neurons, AMPA receptor glutamatergic antagonist, CNQX (20μM), was applied to neurons for 5-10 minutes before the addition of glutamate (1mM) and imaged using fluorescence obtained from a ratio of bound vs. unbound calcium (340nm/380nm) using calcium indicator dye Fura 2 (Figure 6). Intracellular calcium response under these conditions was not as high as in the glutamate only experiments, however intracellular calcium levels in TgCRND8 (n=74 cells; 1.81 peak ratio) were significantly greater than in non-transgenic (n=62 cells; 1.57 peak ratio) littermates. *$p<0.0001$; Student's t-test; data are pooled averages standardized to 1.0

7.0 No difference in intracellular calcium influx between TgCRND8 and Non-transgenic littermate controls after metabotropic glutamate receptor stimulation with KCl

To determine whether metabotropic glutamate receptors contribute to increased intracellular calcium influx in TgCRND8 vs. non-transgenic littermate controls, primary cortical cells were treated with 20mM KCl (potassium chloride) 8 days post-plating. There were no differences in intracellular calcium influx between TgCRND8 and non-transgenic littermates after treatment with KCl (Figure 7). Data were standardized to 1.0.

8.0 Increased intracellular calcium response in TgCRND8 cortical cells in response to Thapsigargin

To investigate intracellular calcium levels in response to ER SERCA pump inhibition, TgCRND8 and non-transgenic littermate control primary neurons were treated with Thapsigargin (1uM) and imaged using Fura 2. TgCRND8 primary neurons exhibit a significantly elevated response in intracellular calcium when compared to non-transgenic controls. Peak response was significant at 2.25 ± 0.09 SEM for TgCRND8 cells (n=25 cells) compared to 1.74 ± 0.15 SEM in non-transgenic controls (n=19 cells; Student t-test at peak of 241 seconds; $*p<0.05$) (Figure 8). These results suggest that there may be more intracellular calcium stored in the ER in TgCRND8 primary cortical neurons compared to non-transgenic littermate controls, although there may be additional contributions from other methods of calcium influx (i.e. capacitative calcium influx).

9.0 There is increased NMDA NR1 subunit protein expression in TgCRND8 cortical cells compared to non-transgenic littermate controls

In order to elucidate in part a possible mechanism attributing to differences observed in calcium handling between TgCRND8 and non-transgenic littermate primary cortical cells, NR1 receptor subunit (a subunit of the NMDA receptor) protein expression was measured in both groups using Western blot assay. The NR1 subunit was chosen as it is of critical functional importance to NMDA receptor activation. It is the binding site for the NMDA receptor required co-agonist, glycine that, along with glutamate binding, activates the NMDA receptor. Protein levels of NR1 subunit were higher in the TgCRND8 cultures 8 days post-plating when compared to non-transgenic littermate

control cells (Figure 9). Cells were pooled from 2 separate wells using TgCRND8 and non-transgenic primary cortical neurons.

Discussion

In early indications of AD, well documented damaging alterations in intracellular calcium balance have been reported (LaFerla 2002). Studies have intimately linked ER-dependent calcium dyshomeostasis with AD-associated neurotoxicity (O'Neill, Cowburn et al. 2001). Normally, intracellular calcium homeostasis is regulated by plasma membrane calcium channels (i.e ionotropic glutamate receptors such as NMDA, and voltage-gated channels, such as L- and N-type), and ER membrane bound channels (i.e. inositol-1, 4, 5-triphosphate receptors (IP3 R) and ryanodine (Ry R) receptors) (Ghosh and Greenberg 1995). Recent in-vitro work in TgCRND8 primary neurons revealed that Aβ plays a part in activating calcium dyshomeostasis through ryanodine receptors, specific calcium release channels located on the membrane of the endoplasmic reticulum (Supnet, Grant et al. 2006). Comparatively, we observed a significant increase in resting basal calcium levels in TgCRND8 cells, which we previously established secreted greater levels of Aβ1-42, when compared to non-transgenic littermates (Olson, Schapansky et al. 2007). In human AD, it has been clearly established that dysregulation of calcium occurs early, before neuronal damage or loss (LaFerla 2002). Our results are in agreement with this as differences in resting calcium levels were apparent before cell death was detected. However, to further corroborate these data, earlier time points measuring resting calcium will need to be completed.

The neurotoxic effects of Aβ have been shown to augment intracellular calcium levels through the generation of reactive oxygen species, leading to lipid peroxidation resulting in subsequent damage and reduced activity of glutamate transporters on the plasma membrane (Mark, Hensley et al. 1995); (Mattson, Cheng et al. 1992). In the current study, the application of glutamate into the culture system was used to stimulate NMDA receptors on the plasma membrane thus allowing an influx of extracellular calcium into the cell. Although there are various means by which glutamate uses to move calcium into the cells such as through voltage-gated calcium channels after depolarization (Friel 2004), activation of other ionotropic glutamate receptors (i.e. AMPA), as well as metabotropic receptor activation (i.e. G-protein coupled receptors), we simply chose to stimulate NMDA receptors by adding glutamate to cultures. Different excitatory amino acids like glutamate are known to elicit unique patterns of degeneration (Coyle, Bird et al. 1981) that are mainly regulated by glutamate receptors (Olney 1994), therefore, we chose to examine NMDA receptors in a number of our experiments. Both TgCRND8 and non-transgenic intracellular calcium response to glutamate was high (i.e. greater calcium influx), but we report that there was no difference in calcium levels due to glutamate between TgCRND8 and non-transgenic littermate controls. We were puzzled by the lack of difference between TgCRND8 and non-transgenic controls. We had expected there to be a large increase in intracellular calcium influx in the TgCRND8 compared to non-transgenic littermates based on the fact that there was more Aβ being produced in the TgCND8 and studies have indicated that Aβ can cause significant elevations in intracellular calcium levels (Supnet, Grant et al. 2006); (Mattson, Cheng et al. 1992). Our experimental results differ from a recent paper stating that TgCRND8

primary cultures (5 days post-plating) exhibit increased intracellular calcium in response to 250uM of glutamate (Supnet, Grant et al. 2006). Differences between our results and those of this paper may be due to a number of variations that exist between experimental conditions. Age of cultured cells used was a prominent discrepancy as our primary cells were studied 8 days post-plating vs. 5 days in the other study. Also, we did not use a perfusion system when performing calcium imaging experiments, thereby preventing cells from recovering after treatment with glutamate, whereas a perfusion system was employed in the comparative study. Furthermore, glutamate dosages used were very different. In the current study we used 1mM glutamate, whereas a concentration of 250uM was utilized in the Supnet et al (2006) study (Supnet, Grant et al. 2006).

We speculate that the lack of differences in calcium levels after glutamate in our in vitro system may be due to TgCRND8 neurons possessing an increased capacity to take up extracellular glutamate over time compared to non-transgenic controls thereby resulting in equal calcium levels to applied glutamate in culture, as observed in other in vitro studies using different AD models (Mattson, Cheng et al. 1992). Why? Perhaps due in part to our finding that there is increased ROS generation in TgCRND8 primary cortical cells vs. non-transgenic littermate controls, and this increased ROS generation has been shown to disrupt glutamate transport (Mark, Hensley et al. 1995); (Mark, Pang et al. 1997). Furthermore, this lack of variation in intracellular calcium response to glutamate may be due to a different complement of NMDA receptor numbers in TgCRND8 vs. non-transgenic littermates, or that there may be more receptor recycling at the plasma membrane in TgCRND8 cells as opposed to non-transgenic controls, resulting in equal intracellular calcium between both groups, as observed in other studies using

various AD in vitro models (Snyder, Nong et al. 2005). Or, quite simply, the lack of difference in calcium between the two groups may be because no inherent difference exists between the two systems at that time in culture.

Additionally, we observed that TgCRND8 cortical neurons expressed greater levels of NMDA receptor subunit NR1 when compared to non-transgenic littermate controls (Figure 9). However, expression of other subunits was not analyzed in the current study therefore we cannot at this time exclude the possible contributions of other receptors.

Others have reported that accumulation of intracellular calcium is closely tied in with neurodegenerative pathways (Koh and Choi 1991). In vitro studies have reported increased intracellular calcium after treatment with glutamate that remains high for approximately 1 hour before descending to pre-glutamate concentrations (Dubinsky 1993). Cell death studies from these experiments have revealed that even though calcium returned to initial levels, over 2/3 of the neurons were dead before 24 hours (Dubinsky 1993). This is in agreement with preliminary work from our laboratory where we observed vast cell death in cortical neurons after the application of glutamate (data not shown). Previous studies have reported that neuronal primary cell death was arrested by either removal of calcium from the extracellular environment or by blockade of NMDA receptors thereby controlling intracellular calcium influx (Goldberg, Weiss et al. 1987); (Choi 1988). Furthermore, calcium influx from the extracellular environment and intracellular calcium release, have been hypothesized to increase glutamate release thus enhancing its neurotoxic effect (Choi 1990); (Strijbos, Leach et al. 1996). Additionally, Mattson et al (1993) and Koh et al (1990) suggested that cultured cortical cells

experienced increased vulnerability to glutamate toxicity due to the presence of Aβ (Mattson, Tomaselli et al. 1993); (Koh, Yang et al. 1990).

We further examined the difference in intracellular calcium levels between TgCRND8 and non-transgenic littermate controls after the application of NMDA. We observed a significant increase in intracellular calcium in TgCRND8 primary neurons compared to non-transgenic controls. Additional studies blocking and/or stimulating other voltage- or ligand-gated calcium membrane channels must be done in order to examine contributions from other membrane-based receptors. However, the current data suggests that enhanced intracellular calcium influx in more mature TgCRND8 primary neurons is influenced by NMDA receptor activation.

Other subtype of ionotropic plasma membrane receptors permeable to extracellular calcium are α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Collingridge and Lester 1989). To determine the contribution of AMPA receptors to increased intracellular calcium influx in TgCRND8 primary neurons, we blocked AMPA and measured resultant calcium. In primary cells pretreated with AMPA receptor antagonist CNQX, we did not find differences in intracellular calcium influx in response to glutamate in TgCRND8 and non-transgenic littermate primary cultures. Although there was a parallel rise in calcium in both TgCRND8 and non-transgenic control cells in response to glutamate it did not exhibit the same level of calcium as was observed in the glutamate alone experiments. Alternatively, when NMDA receptors were blocked with the antagonist MK-801, we observed an initial significant difference in intracellular calcium levels in TgCRND8 vs. non-transgenic littermate controls, suggesting that

intracellular calcium influx in TgCRND8 is more readily influenced by increased NMDA receptor activation when compared with non-transgenic littermate controls.

Primary cortical cells treated with an ER calcium uptake pump (SERCA) blocker, thapsigargin, displayed significantly greater intracellular calcium influx in TgCRND8 compared with non-transgenic littermate controls. This result falls in accordance with what Supnet et al (2006) observed when measuring ER calcium content in the presence of nominal extracellular calcium (Supnet, Grant et al. 2006). They report that the total amount of calcium released from the ER after glutamate was significantly higher in early cultures of TgCRND8 primary neurons vs. non-transgenic littermate controls (Supnet, Grant et al. 2006). Our results further demonstrate concordance with Supnet et al (2006) in that our mature primary TgCRND8 neurons display increased stored calcium in the ER when compared to non-transgenic control cells (Supnet, Grant et al. 2006). Smith IF et al (2005) suggested that increased calcium release from the ER may be a result of processes working in concert with one another, one being increased storage of intracellular calcium, initiated by the toxicity of Aβ (Smith, Green et al. 2005).

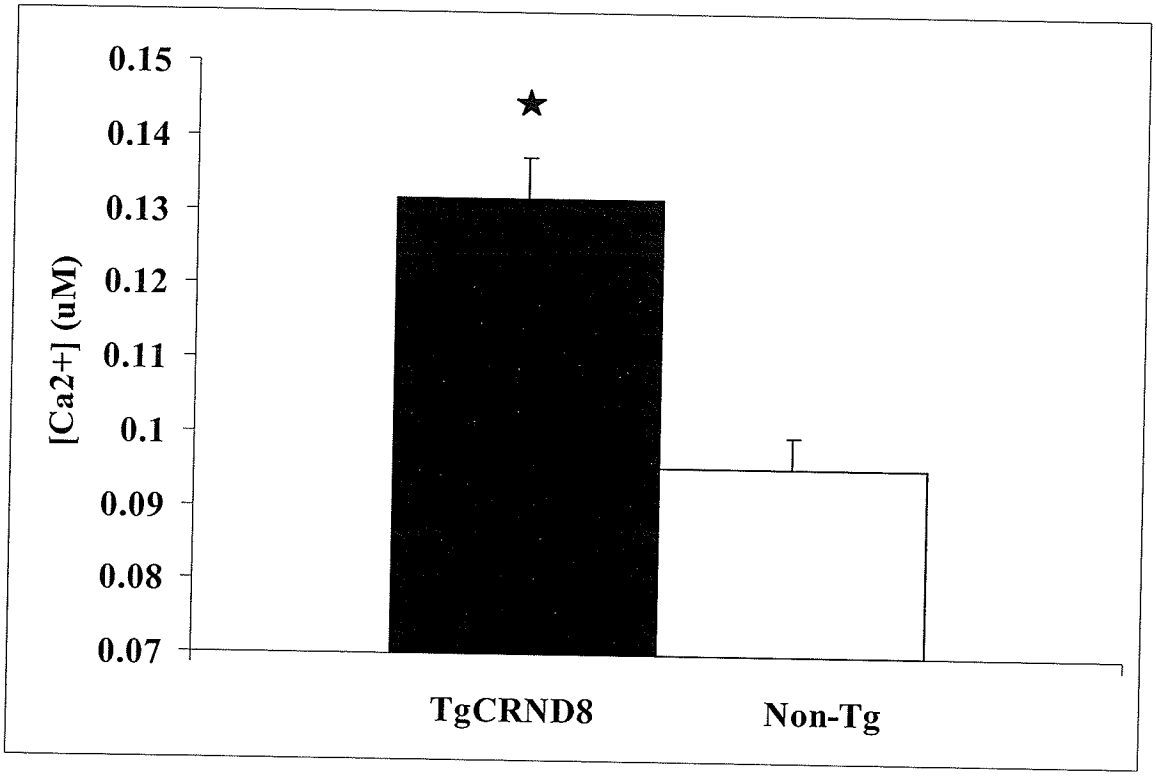
There are obvious limitations to this study. Globally, mechanisms describing calcium handling in the TgCRND8 mouse model were not expanded upon, rather, differences (or lack thereof) were reported here, but follow-up tracking the mechanistic properties for these differences were not performed. This will be imperative for future studies but satisfies the descriptive characteristic requirements set out by our initial hypotheses and curiousity. Another limitation comes from not properly dosing all the various pharmacological agents used in these studies. That said, we did dose glutamate (100μM, 500μM, and 1mM), MK801 and CNQX (15μM and 20μM each) in our calcium imaging

studies. The resultant doses from the above mentioned pharmacological agents, as well as all others used in these calcium handling studies, were taken from previous laboratory experience and from the literature.

In summary, there are differences in calcium concentration and handling in primary TgCRND8 vs. non-transgenic littermate controls and these aberrations in intracellular calcium homeostasis appear to correlate with increased Aβ1-42 production and subsequent ROS generation, but the mechanism(s) through which this is prompted and/or perpetuated remain undefined. Furthermore, the observed differences in intracellular calcium influx between TgCRND8 and non-transgenic littermates strongly appear to be a result of NMDA receptor activity diversity. Further study is required to establish molecular pathways and mechanisms involved in differential NMDA receptor activity and ER calcium load contribution to these observed differences.

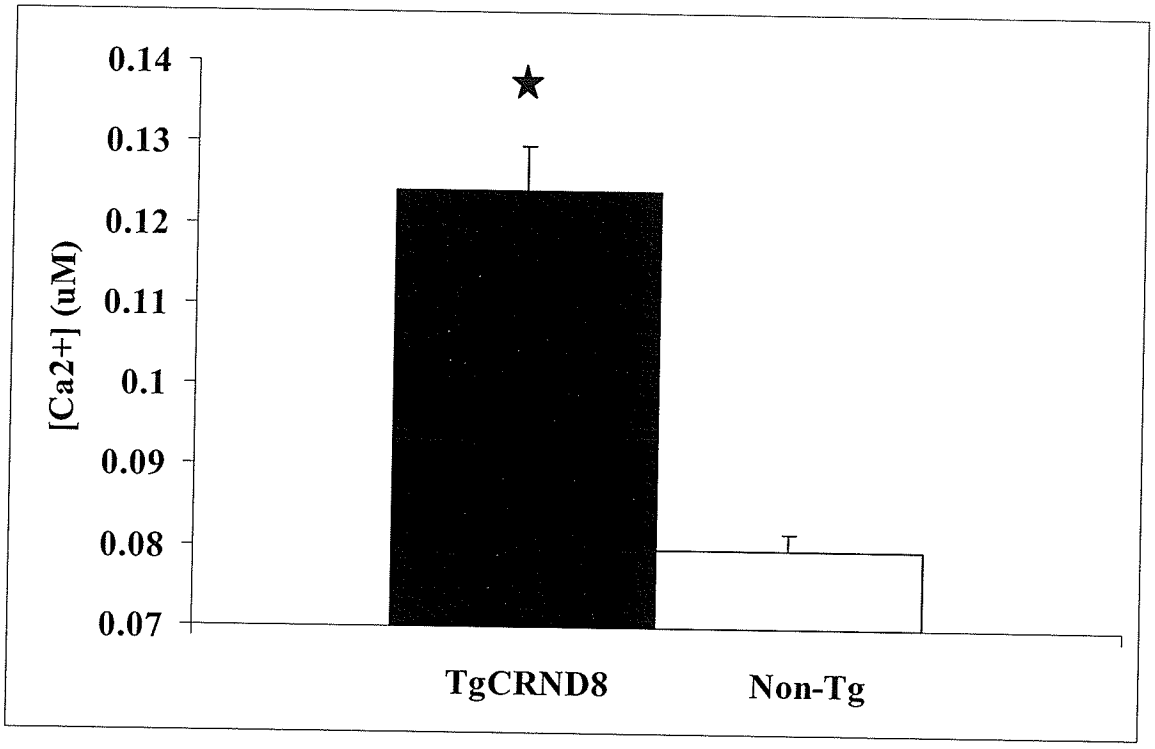
Chapter 3: Figures and Figure Legends

Figure 1a: In Vitro Day 8/9 Basal Calcium Concentration



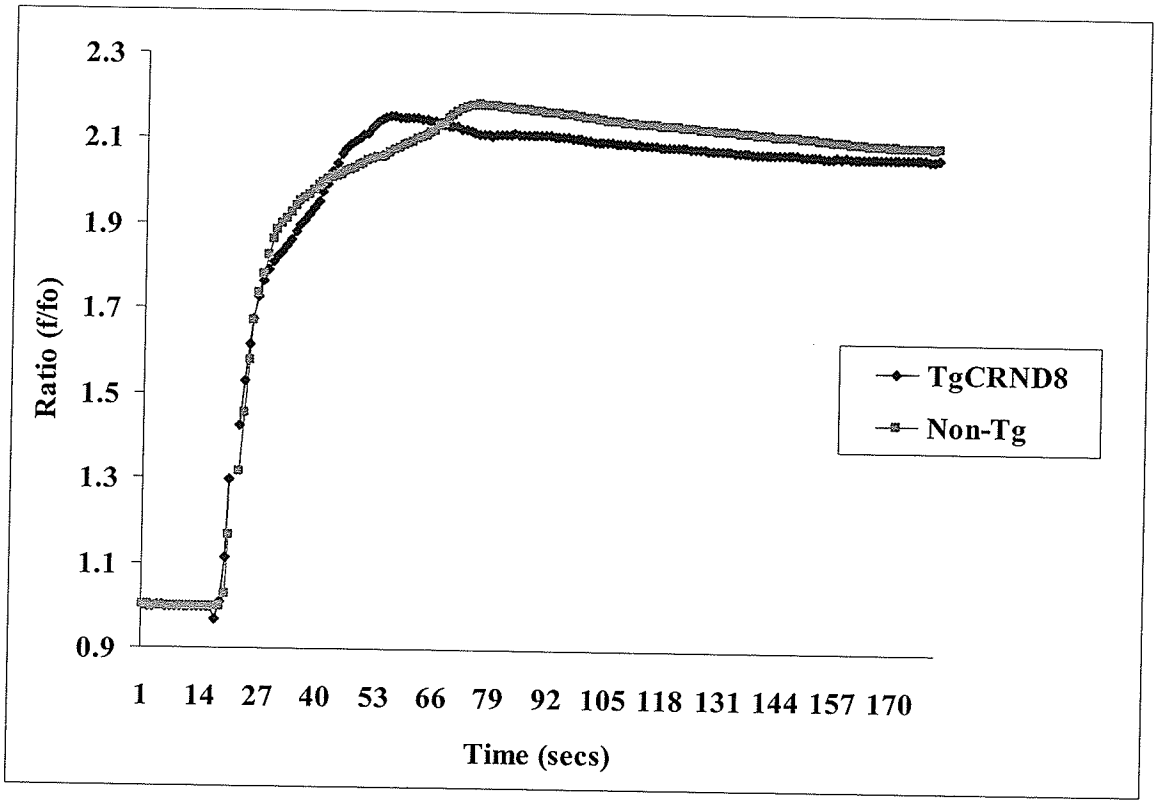
Histogram represents Day 8/9 resting calcium. TgCRND8 and Non-transgenic (Non-Tg) littermate controls were grown in culture for 9 days before being loaded with Fura 2 and imaged using a calcium imager. Calcium concentration was established using an established equation and accompanying calcium calibration kit from Invitrogen Molecular Probes with calcium standards. Data are presented as averaged pooled cell numbers from TgCRND8 and Non-transgenic cultures at one time point. There is significantly more basal calcium in TgCRND8 cortical cells (n=41 cells; 0.132μM calcium) than in Non-transgenic littermate controls (n=53 cells; 0.095μM calcium; *$p<0.05$; $\pm$ SEM; Student's t-test)

Figure 1b: In Vitro Day 12 Calcium Concentration



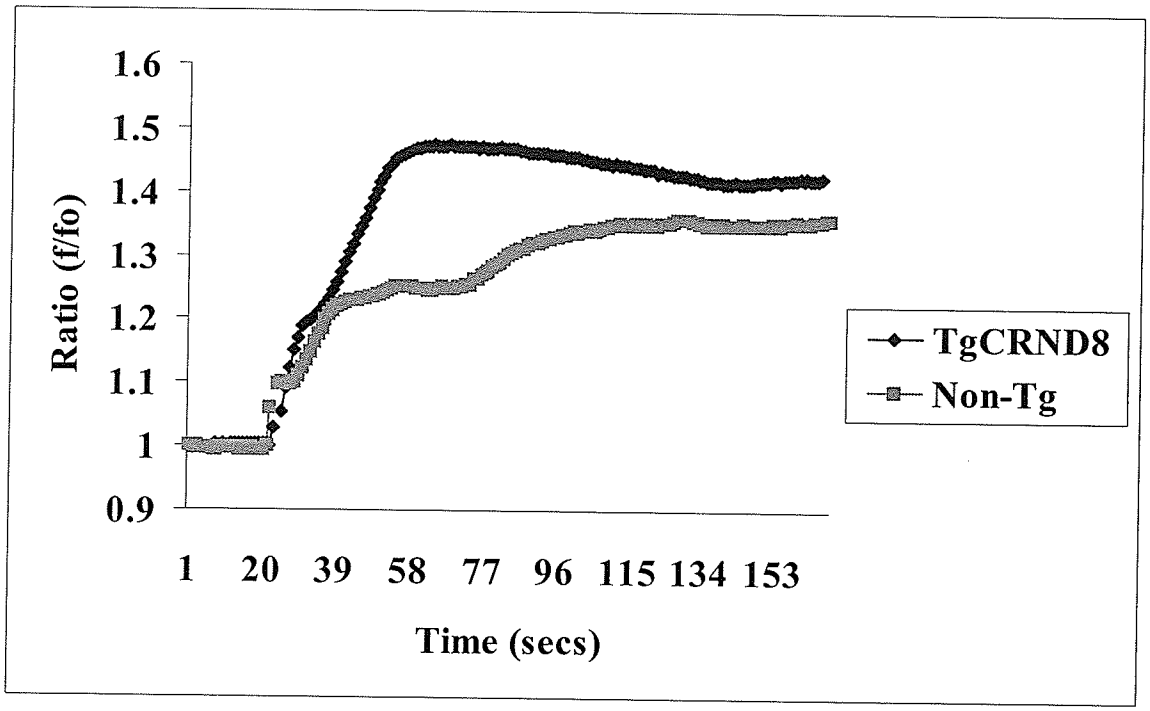
Histogram represents Day 12 resting calcium. TgCRND8 and Non-transgenic (Non-Tg) littermate controls were grown in culture for 9 days before being loaded with Fura 2 and imaged using a calcium imager. Calcium concentration was established using a kit from Invitrogen Molecular Probes with calcium standards. Data are presented as averaged pooled numbers from TgCRND8 and Non-transgenic cultures at one time point. There is significantly more basal calcium in TgCRND8 cortical cells (n=26 cells; 0.123μM calcium) than in Non-transgenic littermate controls (n=17 cells; 0.078μM calcium; *$p<0.05$; $\pm$ SEM; Student's t-test)

Figure 2: Intracellular Calcium Influx Following Glutamate



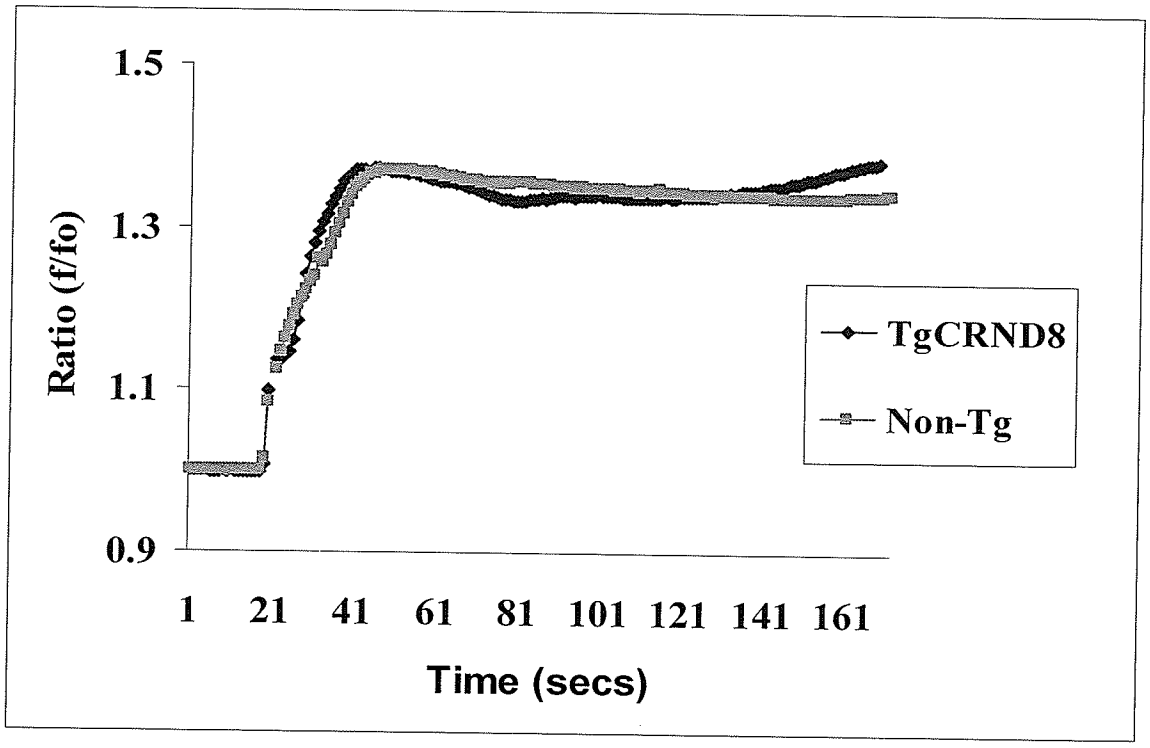
Calcium imaging analysis from primary cortical cells from TgCRND8 and non-transgenic littermates, 8-9 days post-plating. Data was gathered from 9 separate experiments, pooled and presented as a fluorescent ratio of bound to unbound calcium (340nm/380nm), using calcium indicator dye Fura2. Under these culture conditions and cell maturation there is no difference in calcium response to glutamate (1mM) in TgCRND8 (n=174 cells) vs. Non-transgenic (n=146 cells) littermate control cells. Non-responders were excluded. Glutamate was not perfused out. Data are pooled averages standardized to 1.0

Figure 3: Intracellular Calcium Influx in Response to NMDA



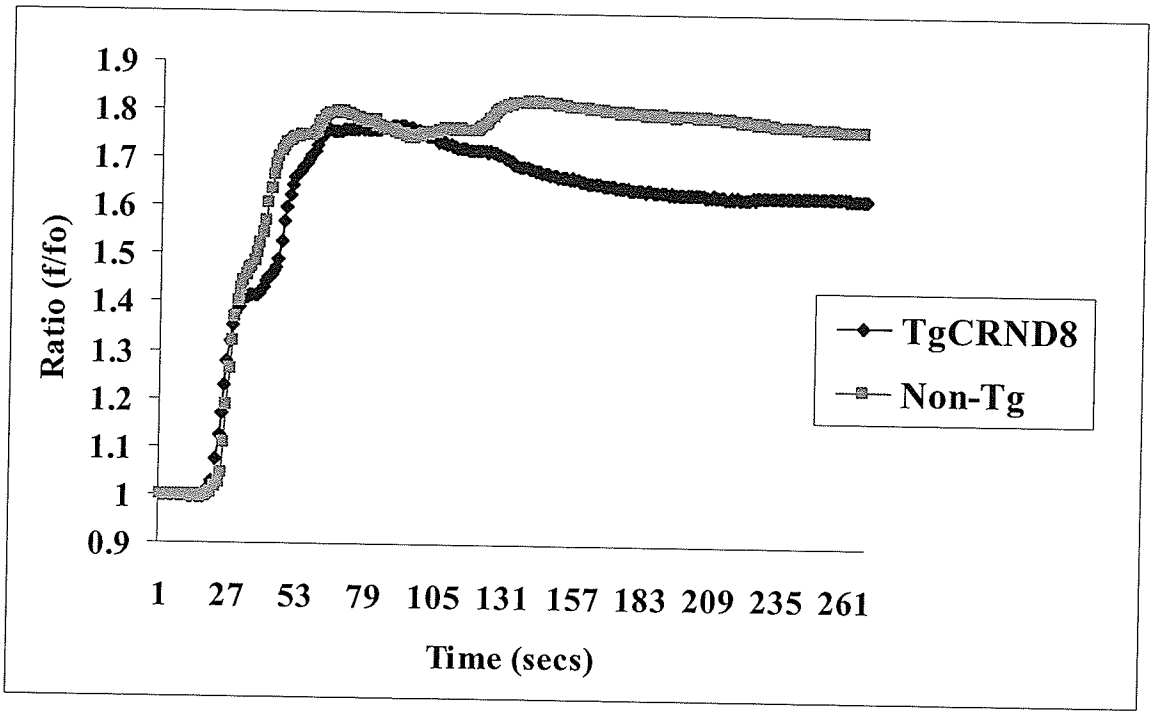
Intracellular calcium response in primary cortical cells (8 days post-plating) from TgCRND8 or Non-transgenic littermate controls. Calcium imaging trace exhibits fluorescence ratio of bound to unbound calcium to Fura 2 (340nm/380nm) after application of NMDA (100μM). There is significantly greater intracellular calcium in TgCRND8 (n= 33 cells) when compared to Non-transgenic littermate (n=23 cells) controls (*$p<0.01$; Student's t-test at 60 secs; 2 experiments; data are pooled averages standardized to 1.0).

Figure 4: Intracellular Calcium Influx in Response to AMPA



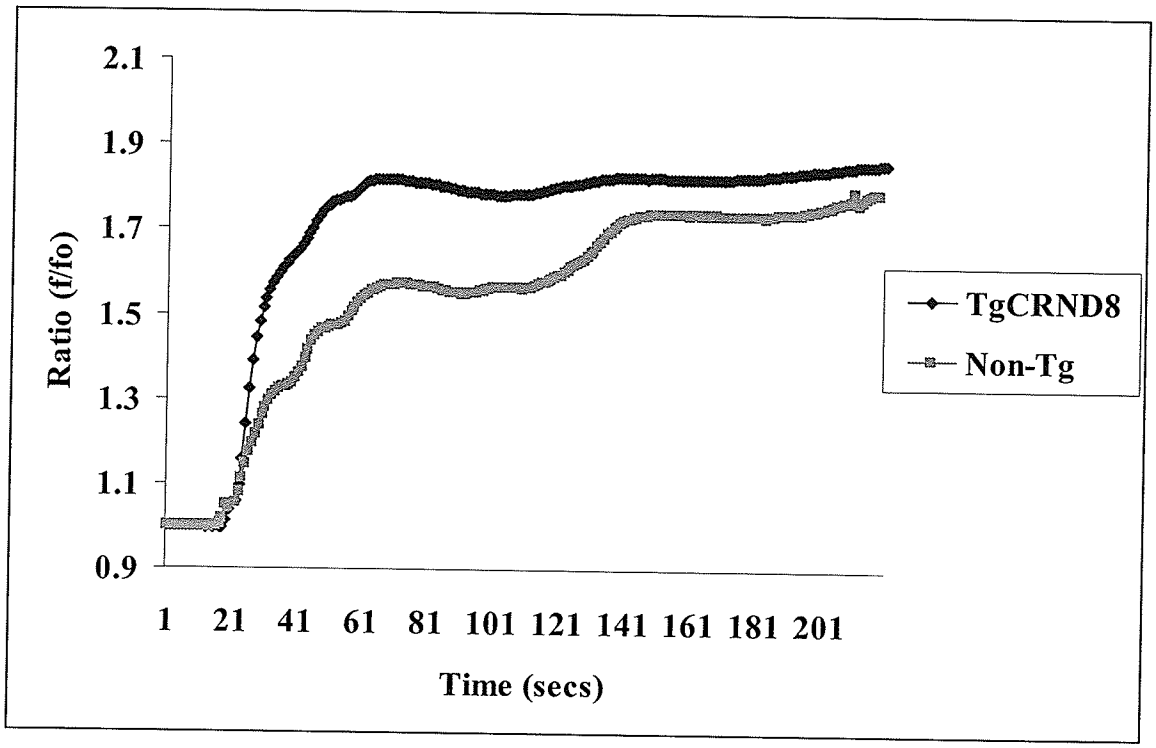
Intracellular calcium influx in primary cortical cells (8 days post-plating) from TgCRND8 or Non-transgenic littermate controls. Calcium imaging trace exhibits fluorescence ratio of bound to unbound calcium to Fura 2 (340nm/380nm) after pre-treatment with MK801 (20μM) followed by 100μM AMPA. There was no difference in intracellular calcium influx between TgCRND8 (n= 48 cells) compared to Non-transgenic littermate controls (n= 43 cells). Averages standardized to 1.0.

Figure 5: Intracellular Calcium Influx in Response to Glutamate Following Pretreatment with MK801



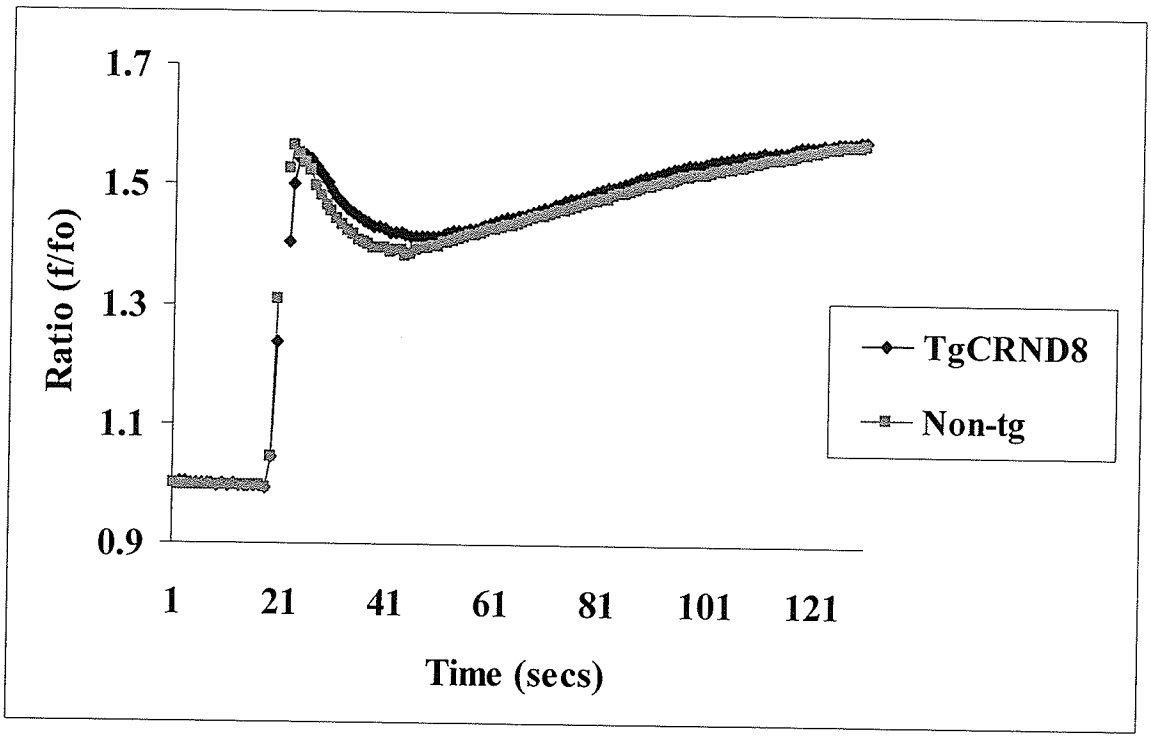
Calcium response to glutamate in 8-9 day old primary cortical cells from TgCRND8 and Non-transgenic littermate controls. Data are pooled averages standardized to 1.0 and presented as a fluorescent ratio of bound to unbound calcium using Fura-2. Cells were pretreatment with NMDA receptor antagonist, MK801 for 5-10 minutes, before the application of glutamate (1mM). Glutamate was not perfused out. Less calcium response was observed under these conditions when compared to glutamate alone (figure 1), however there was no difference between TgCRND8 (n=63 cells) cells and Non-transgenic (n=57 cells) littermate controls.

Figure 6: Intracellular Calcium Influx in Response to Glutamate Following Pretreatment with CNQX



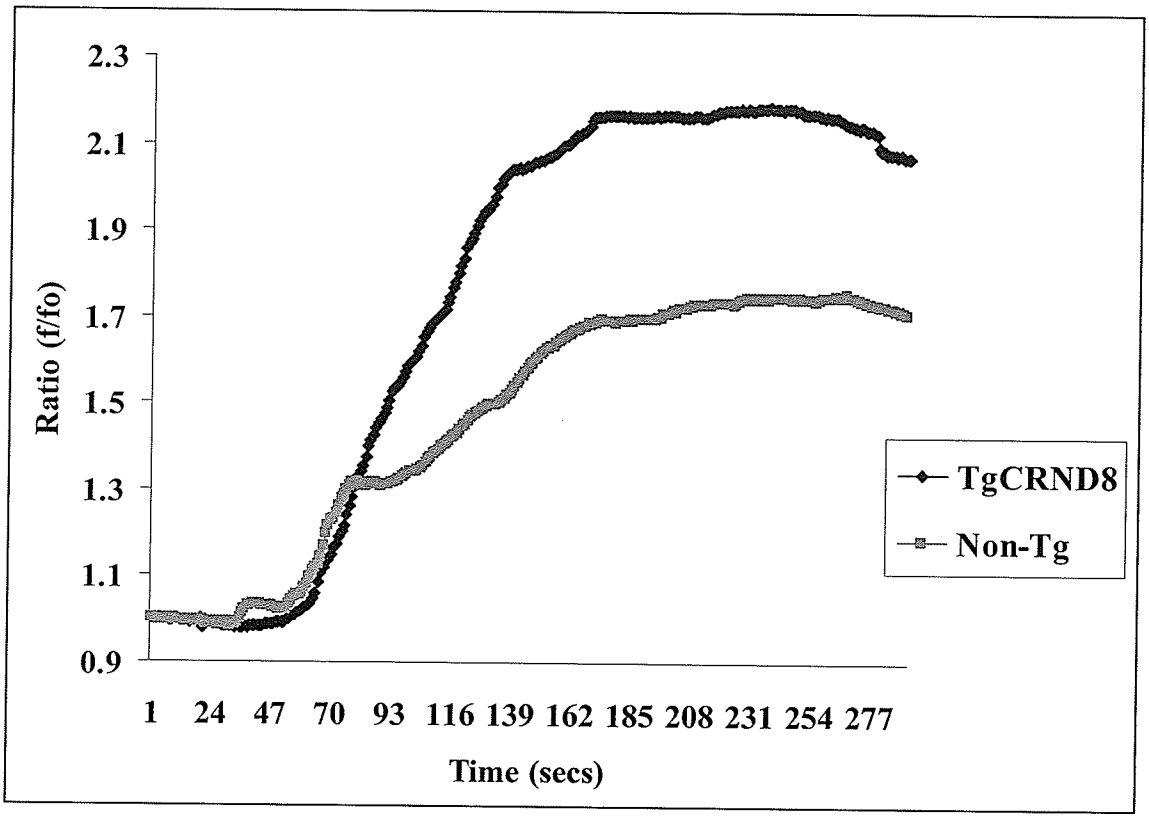
Calcium imaging from 8-9 day old TgCRND8 and Non-transgenic littermate control primary cortical neurons. Data was gathered from 3 separate experiments, means pooled, and presented as a fluorescent ratio of bound to unbound calcium using calcium dye indicator Fura-2. Cells were pretreated with AMPA receptor antagonist CNQX (20uM) for 5-10 minutes before application of glutamate (1mM). Glutamate was not perfused out. Calcium response in TgCRND8 cells (1.817 $\pm$ 0.057 SEM; n=74 cells) was significantly greater compared to Non-transgenic littermate controls (1.561 $\pm$ 0.06 SEM; n=54 cells; *$p<0.0001$; Student's t-test at peak of 67 secs). Averages standardized to 1.0.

Figure 7: Intracellular Calcium Influx Following KCl



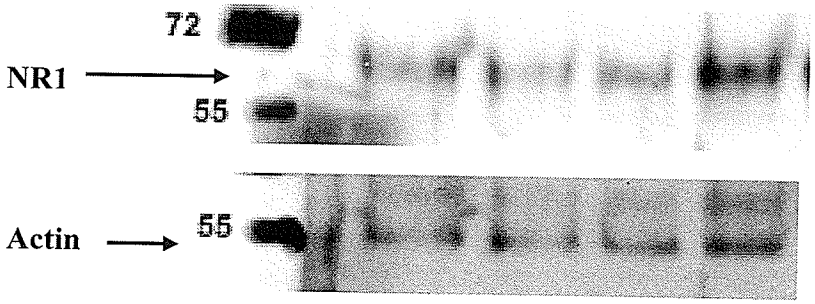
Calcium imaging from 8-9 day old TgCRND8 and Non-transgenic littermate control primary cortical neurons. Data are presented as a fluorescent ratio of bound to unbound calcium using calcium dye indicator Fura-2. Cells were treated with 20mM KCl that was not perfused out. There was no difference in intracellular calcium influx between TgCRND8 and Non-transgenic littermate control cortical neurons. Averages standardized to 1.0.

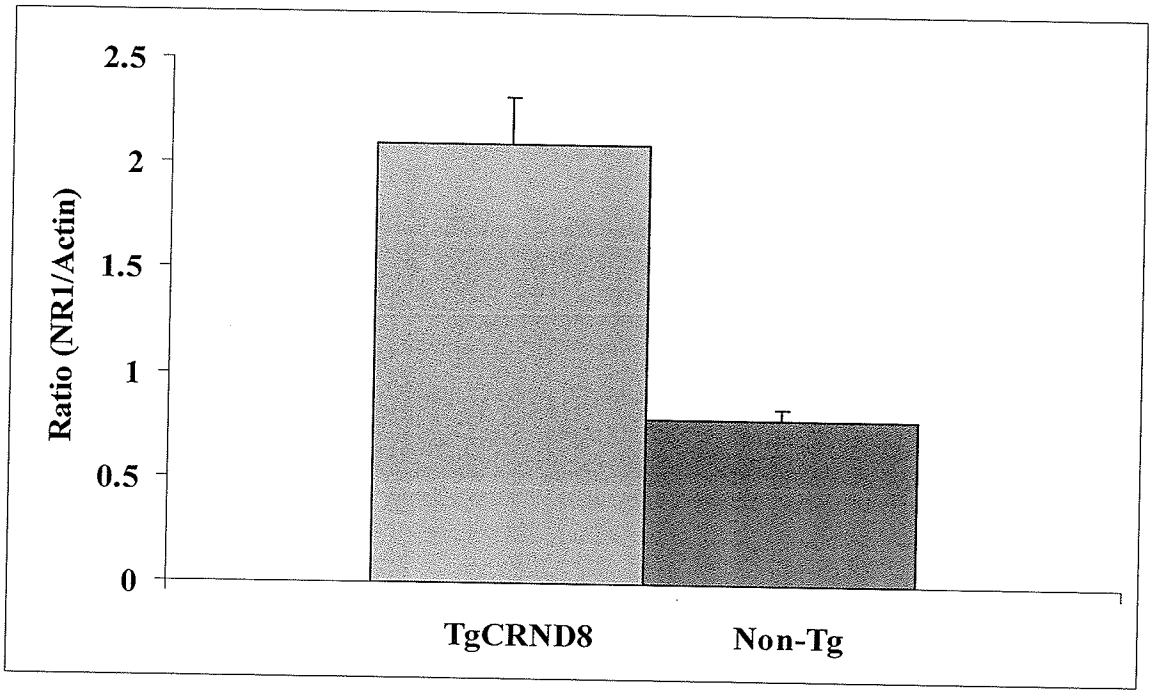
Figure 8: Intracellular Calcium Influx Following Thapsigargin



Intracellular calcium response in TgCRND8 and non-transgenic littermate control primary cortical neurons. Trace displays calcium response as fluorescent ratio of bound to unbound Fura-2 after application of endoplasmic reticulum membrane calcium pump, SERCA, inhibitor Thapsagargin (1uM). Data are pooled averages standardized to 1.0. TgCRND8 cells exhibit significantly greater intracellular calcium influx (2.18 $\pm$ 0.09 SEM; n=25 cells) compared to non-transgenic littermate controls (1.74 $\pm$ 0.15 SEM; n=19 cells). Student's t-test at peak of 241 seconds; *$p<0.05$.

Figure 9: NMDA Receptor Subunit NR1 Protein Expression





NMDA receptor subunit NR1 protein expression in TgCRND8 vs. Non-transgenic littermate controls, 8 days in vitro. Western blot analysis of protein derived from total cell lysates and expressed as total NR1 protein levels against actin control protein levels, and averaged per well. Two wells per group. There was greater NR1 protein expression in TgCRND8 vs. Non-transgenic littermate control primary cortical neurons.

CHAPTER 4 - General Discussion

Alzheimer's disease has been studied for over a century. Throughout its history, researchers have successfully uncovered numerous mysteries and developed a number of sophisticated diagnostic tools which are still used today in the pursuit of a cure and/or earlier, more accurate diagnosis. Despite the advances and pharmacological attempts, Alzheimer's disease remains incurable. Given that Alzheimer's can only be definitively diagnosed post-mortem, the difficulty in studying this disease has somewhat impaired investigative progress. The advent of transgenic models of Alzheimer's disease provides a foundational venue with which to manipulate and probe genetically altered cells with various pharmacological agents, thus enabling potential molecular pathway involvement to be studied from start to finish. Insight gleaned from transgenic models continues to help advance the study of this elusive disease.

In this thesis we used an early on-set familial mouse model of Alzheimer's disease called TgCRND8, developed in 2001 by Dr. David Westaway and colleagues at the University of Toronto (Chishti, Yang et al. 2001). This model was designed to overexpress mutant APP via APP restriction fragments introduced into oocytes using a cos.Tet vector system driven by the Syrian hamster prion promoter (Chishti, Yang et al. 2001). These animals house a double mutation (Swedish KM670/671NL and Indiana V717F) in the APP695 gene (Janus, D'Amelio et al. 2000); (Chishti, Yang et al. 2001), which leads to abnormal processing of APP (Qiu, Naten et al. 2001); (Citron, Oltersdorf et al. 1992), increased production of Aβ1-42 (Citron, Oltersdorf et al. 1992); (Suzuki, Cheung et al. 1994). TgCRND8 mice overexpress APP holoprotein to levels approximately 5 times higher than that observed in non-transgenic controls (Chishti,

Yang et al. 2001). Because of this overexpression of APP, TgCRND8 generate Aβ1-42 in concentrations that fall into the same range for human patients with AD (Chishti, Yang et al. 2001). These animals generate Aβ1-42 three months postnatally, and display cognitive deficits as early as 11 weeks of age (Janus, D'Amelio et al. 2000); (Chishti, Yang et al. 2001).

It has been well established that in vitro cleavage of APP by sequential secretase cleavage liberates toxic fragments of Aβ from the APP holoprotein (Haass, Schlossmacher et al. 1992); (Shoji, Golde et al. 1992); (Seubert, Oltersdorf et al. 1993); (Busciglio, Gabuzda et al. 1993). To begin our work using the TgCRND8 primary culture model of AD, it was critical that we first determine whether increased Aβ production and secretion was evident in these cultures. Although early on in culture there were no differences in Aβ1-42 concentration between TgCRND8 and non-transgenic littermate control cells, by 8 days post-plating there was a significantly greater concentration of Aβ1-42 in the media from TgCRND8 cortical cultures. Of interest in the current thesis, it was established that not only was Aβ1-42 produced and secreted in vitro, but the secretion occurred very rapidly (within 12 hours) reaching nearly half the amount assayed for TgCRND8 cells at 8 days post-plating. This swift secretion of Aβ1-42 into the media has also been reported in other models of Alzheimer's disease (Hartmann, Bieger et al. 1997); (Haass, Schlossmacher et al. 1992); (Haass, Hung et al. 1993); (Shoji, Golde et al. 1992). These experiments established a strong foundational baseline with which to build the in vitro TgCRND8 characterization story upon. The endogenous production of Aβ in vitro provides a cell culture model depicting the effects of Aβ which is more relevant then exogenously applied Aβ. To our knowledge ours is

the first to report TgCRND8 cultured neurons as generating Aβ in vitro and subsequently secreting the toxic fragments into the media.

In vivo age-related cell loss in neurons has been reported in various models of AD (Casas, Sergeant et al. 2004). Cell death appearance and mechanisms in vitro have been harder to delineate (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006). Experiments conducted for this thesis revealed no significant difference in early cell survival between TgCRND8 and non-transgenic cortical cells. However near the same time that Aβ1-42 levels increased in TgCRND8, morphological cell death differences between TgCRND8 and non-transgenic littermate controls became evident. One could therefore speculate that the increase in cell death could be attributed in some way with increased Aβ1-42 generation and secretion. Similar initial survival trends between TgCRND8 and non-transgenic littermates indicate that on a cellular level, these two groups are similar in early culture conditions, thus further strengthening our claim to being able to control for confounding factors

To help explain the greater incidence of cell death recorded in TgCRND8 primary neurons, both apoptotic and necrotic cell death mechanisms were measured and analzyed. Some researchers have observed that Aβ can instigate an entire flow of apoptotic events in culture (Mattson 2000), whereas others have reported that because most murine models of AD do not reflect the full complement of features as seen in the human condition of AD, there are few reports of transgenic in vitro neuronal death (Sankaranarayanan 2006); (McGowan, Eriksen et al. 2006). We found evidence of apoptosis in our TgCRND8 and non-transgenic littermate cultures however no differences existed between the two groups. The presence of apoptotic cell death in our

system does not explain the significant death in transgenic cells that we observed later in culture therefore necrotic cell death was also measured. We reported that TgCRND8 primary neurons displayed more necrotic cell death than what was observed in non-transgenic littermate controls. Thus increased cell death in TgCRND8 primary neurons can to be attributed more to a necrotic, rather than apoptotic, mechanism of cell death.

In in-vitro systems Aβ deposition triggers reactive oxygen species which can then generate lipid peroxidation (Hensley, Carney et al. 1994) leading to the malfunction of ATPases and other calcium delivery systems, thereby increasing cytoplasmic calcium (Mark, Hensley et al. 1995); (LaFerla 2002). Investigators have found that when Aβ was added to neurons in culture, reactive oxygen species generation increased (Keller, Pang et al. 1997). Similarly, in experiments conducted for the current thesis, the presence of reactive oxygen species in our TgCRND8 in vitro system was significantly higher when compared to non-transgenic controls. The significant difference in reactive oxygen species generation in TgCRND8 compared to non-transgenic cells is likely because these cells produce and secrete more Aβ1-42 than their non-transgenic littermates. Importantly, damage from reactive oxygen species generation and oxidative stress intermediates has been postulated to trigger swift necrotic neuronal demise (Behl 2000).

It has been shown that Aβ1-42 is produced by neurons (Skovronsky, Doms et al. 1998); (Cook, Forman et al. 1997); (Turner, Suzuki et al. 1996) and moreover that Aβ may be toxic to neurons before it is secreted (Hartmann, Bieger et al. 1997) thereby rendering neurons more susceptible to toxic insults as well as increased promotion of reactive oxygen species generation.

As a minor point in the characterization of the TgCRND8 in vitro model of AD, we chose to examine neurite outgrowth. Results from this thesis revealed that there was no basal difference in neurite outgrowth between TgCRND8 and non-transgenic littermates in early culture conditions. Because both TgCRND8 and non-transgenic littermate control cells express APP in culture (albeit significantly less in non-transgenic cells) the fact that we observed no difference in neurite outgrowth between TgCRND8 and non-transgenic littermates may possibly indicate that in our in vitro model, as well as in other models (Harper, Bilsland et al. 1998), APP may not be solely responsible for neurite outgrowth extension. This finding coupled with the observation that there were no culture differences in early survival rates, provided supporting evidence that TgCRND8 and non-transgenic littermate primary neurons were fundamentally similar early on in culture. Thus, these data help to eliminate the possibility of any existing basal differences serving as confounding factors. The data also further supports the premise that observed differences only occur after Aβ production.

Another important contributor to amyloidosis leading to neurodegeneration is increased levels of intracellular calcium concentration (Mark, Hensley et al. 1995); (Zhu, Lin et al. 2000). Recent in vitro work in TgCRND8 revealed that Aβ plays a role in activating calcium dyshomeostasis through a specific calcium release channel located on the membrane of the endoplasmic reticulum (ER) (Supnet, Grant et al. 2006). In agreement with this work, we observed significantly greater calcium concentration in TgCRND8 neurons which produced higher amount of Aβ, when compared with non-transgenic littermate controls. This difference was evident at both 8-9 and 12 days post-plating.

In the human condition of AD dysregulation of calcium occurs early in the pathological progression, before any neuronal damage or loss (LaFerla 2002). Our results are in direct agreement as differences in resting calcium levels were evident before cell death was detected. Earlier time points measuring resting calcium still need to be examined as the exact timing of this disparity in resting calcium concentrations will need to be elucidated.

Results in this thesis indicate that both TgCRND8 and non-transgenic cortical cells exhibit increased intracellular calcium as one would have expected after application of glutamate however there was no difference between TgCRND8 and non-transgenic littermates in this response to calcium. These experimental results differ from a recent paper stating that in TgCRND8 primary cultures (5 days post-plating) there was an increased amount of intracellular calcium in response to 250uM of glutamate (Supnet, Grant et al. 2006). However differing culture age, glutamate dosage, and calcium perfusion system may provide explanations for the recorded discrepancies. Based on our experimental conditions there was no difference between the two groups in response to glutamate.

Neurotoxicity is associated with increased intracellular calcium initiated by glutamate (Jarvis, Lilge et al. 1999); (Mattson, Gary et al. 2001); (Glazner, Chan et al. 2000); (Kim, Park et al. 2000); (Guo, Fu et al. 1999), and based on our previous observations whereby cell death was increased in primary hippocampal (and cortical) neurons 11 days after plating (Olson, Schapansky et al 2007 manuscript in progress), it could be proposed that this increase in cell death may be attributed, in part, to increased intracellular calcium via glutamate toxicity. It can further be hypothesized that increased intracellular calcium and

increased cell death could also be attributed to increased Aβ1-42 production and secretion that we observed previously after 8 days in culture. Others have reported that accumulation of intracellular calcium is closely tied in with neurodegenerative pathways (Koh and Choi 1991). Previous studies have revealed that neuronal primary cell death was arrested by either removing calcium from the extracellular environment or by blocking NMDA receptors (Goldberg, Weiss et al. 1987); (Choi 1988). Furthermore, calcium influx from the extracellular environment and intracellular calcium release, have been hypothesized to increase glutamate release thereby enhancing its neurotoxic effect (Choi 1990); (Strijbos, Leach et al. 1996).

Calcium response to glutamate in TgCRND8 vs. non-transgenic control neurons may be explained by another hypothesis stating that subpopulations of cells within the hippocampal and cortical regions vary in their response to Aβ-induced neurotoxicity (Mattson, Cheng et al. 1992). Yankner et al (1990) proposed that effects of Aβ toxicity were differentially tolerated by various subpopulations within the hippocampal and cortical brain regions (Yankner, Duffy et al. 1990), in other words neuronal neurotoxic susceptibility is not identical (Mattson, Cheng et al. 1992). Dubinsky (1993) added that there are neurons that lack glutamate receptors and that these cell subpopulations survive more readily than their glutamate-receptor possessing counterparts (Dubinsky 1993). This may indicate that the TgCRND8 cortical region may possess more cells without glutamate receptors or simply more "non-responders" than their non-transgenics controls. On the other hand, it may be that TgCRND8 primary cortical neurons have an increased capacity to take up extracellular glutamate over time compared to non-transgenic controls (for human cell work see Mattson, Cheng et al. 1992). It could also be postulated that

NMDA receptors on the plasma membrane of TgCRND8 cells are more transient in activity than those on non-transgenic membranes (MacDermott, Mayer et al. 1986); (Glaum, Scholz et al. 1990) and that inconsistency may be one of the reasons we do not see any difference in intracellular calcium influx after glutamate between TgCRND8 and non-transgenic littermates.

Based on data from Mattson et al (1992), it was hypothesized that processes which maintain calcium homeostasis may also be targeted by Aβ peptides (Mattson, Cheng et al. 1992). They provide an example whereby voltage- or ligand-gated membrane channel ability to allow calcium influx may be enhanced by Aβ (Mattson, Cheng et al. 1992). It is understood that self-aggregating Aβ1-42 causes neuronal impairment and demise by instigating apoptotic mechanisms (Mattson 1997); (Mattson 2000). During aggregation, Aβ1-42 can generate reactive oxygen species (Hensley, Carney et al. 1994); (Butterfield, Hensley et al. 1994); (Goodman and Mattson 1994); (Lynch, Cherny et al. 2000). Reactive oxygen species cause lipid peroxidation that can detrimentally alter membrane ion-motive ATPase function, as well as glucose and glutamate transport activities, thus culminating in lower ATP production, and membrane depolarization (Mark, Hensley et al. 1995); (Mark, Pang et al. 1997); (Blanc, Keller et al. 1998). Results from Aβ toxicity can lead to increased intracellular calcium concentration (Mark, Hensley et al. 1995). Chronic and pathological illnesses lead to constant cell membrane depolarizations in neurons (Hynd, Scott et al. 2004) leading to Mg2+ release from NMDA receptor channels allowing more calcium influx into the cell (Hynd, Scott et al. 2004). Increased NMDA receptor activation due to chronic depolarizations may eventually cause cortical "excitotoxic necrosis" or neuronal excitotoxicity (Dodd 2002). These data may help

explain why we see increased cell death, increased reactive oxygen species as well as enhanced resting calcium levels in TgCRND8, when compared to non-transgenic littermates.

We further examined the difference in intracellular calcium levels between TgCRND8 and non-transgenic littermate controls after the application of NMDA. We observed a significant increase in intracellular calcium in TgCRND8 primary neurons compared to non-transgenic controls. What remains to be understood is why we observed a significant increase in intracellular calcium in TgCRND8 after treatment with NMDA but we did not observe differences between TgCRND8 and non-transgenics after the addition of glutamate. It could be postulated that TgCRND8 cells possess a mechanism that dampens the influx of calcium into the intracellular milieu, or perhaps there was a type of counter-effect by metabotropic glutamate receptors, or conceivably the amount of glutamate added to cultures (both TgCRND8 and non-transgenic) caused a saturation of signal and thus overstimulated both cell types equally. However, this may not be plausible as preliminary studies using 100uM and 500uM glutamate instead of 1mM did not indicate higher calcium influx in the TgCRND8 when compared to non-transgenics. In fact, when we compared the resultant graphs from 100uM, 500uM, and 1mM dose-response glutamate treatments the tracings for each cell species was somewhat similar with non-transgenics displaying slightly greater calcium influx than TgCRND8's. What may be a more likely explanation for the lack of significant difference in intracellular calcium influx between TgCRND8 and non-transgenic littermates is that TgCRND8's have greater stored calcium in the endoplasmic reticulum (ER). When we blocked both NMDA and AMPA receptors and then added glutamate

there was more intracellular calcium in the TgCRND8 cells then when we added glutamate alone. Additional studies blocking and/or stimulating other voltage- or ligand-gated calcium membrane channels must be done in order to examine contributions from other membrane-based receptors. However, the current data suggests that enhanced intracellular calcium influx in more mature TgCRND8 primary neurons is influenced by NMDA receptor activation.

Other examples of ionotropic plasma membrane receptors permeable to extracellular calcium are α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Collingridge and Lester 1989). To determine the contribution AMPA receptors have on increased intracellular calcium influx in TgCRND8 primary neurons, we blocked AMPA with an antagonist called 6-cyan-7-dinitroquinoxaline-2,3-dione (CNQX) and measured resultant calcium. In primary cells pretreated with CNQX we found significantly greater intracellular calcium influx in response to glutamate in TgCRND8 vs. non-transgenic littermate primary cultures. Although there was a parallel rise in calcium in both TgCRND8 and non-transgenic control cells in response to glutamate, similar levels of calcium observed in the glutamate alone experiments were not achieved. Alternatively, when NMDA receptors were blocked with the antagonist MK-801, we observed no initial difference in intracellular calcium levels in TgCRND8 vs. non-transgenic littermate controls. Taken together this suggests that NMDA receptor activation in TgCRND8 cortical neurons appears to be the more important receptor type responsible for sanctioning increased intracellular calcium to flood the cell from the external environment.

Primary cortical cells treated with an ER membrane calcium uptake pump (SERCA) inhibitor, thapsigargin, displayed significantly greater intracellular calcium influx in TgCRND8 compared with non-transgenic littermate controls. This result falls in accordance with what Supnet et al (2006) observed after measurement of ER calcium content in the presence of nominal extracellular calcium (Supnet, Grant et al. 2006). They report that the total amount of calcium released from the ER after glutamate was significantly higher in early cultures of TgCRND8 primary neurons vs. non-transgenic littermate controls (Supnet, Grant et al. 2006). Our results further demonstrate concordance with Supnet et al (2006) in that our mature primary TgCRND8 neurons display increased stored calcium in the ER when compared to non-transgenic control cells (Supnet, Grant et al. 2006). Smith IF et al (2005) suggested that increased calcium release from the ER may be a result of processes working in concert with one another, one being increased storage of intracellular calcium, initiated by the toxicity of Aβ (Smith, Green et al. 2005). Furthermore, when ryanodine and IP3 receptors were blocked using pretreatment with dantrolene, there was increased intracellular calcium concentration in TgCRND8 when compared to non-transgenic littermates, however this increase was not as high as in the case of SERCA inhibition, thereby indicating that ER calcium concentration is greater in TgCRND8 cortical cells than in non-transgenic littermates. Also of interest, when IP3 and ryanodine receptors were blocked and treated with glutamate, there was a slow increase in intracellular calcium as opposed to a sharp increase after glutamate alone. This provides evidence that SERCA is attempting to maintain calcium uptake into the ER and thus calcium homeostasis, even under conditions of intracellular calcium flooding and blockade of ER calcium release channels.

It must not be dismissed, however, that there still remains some calcium release from the ER through slow leak channels.

Taken altogether, there are differences in calcium concentration and handling in primary TgCRND8 vs. non-transgenic littermate controls and aberrations in intracellular calcium homeostasis appear to be influenced by increased Aβ1-42 production and secretion, but the mechanism(s) through which this is prompted and/or perpetuated remain(s) undefined. Furthermore, the observed differences in intracellular calcium influx between TgCRND8 and non-transgenic littermates strongly appear to be a result of NMDA receptor activity diversity. The hypothetical model explaining our calcium differences is as follows:

TgCRND8 cortical cultures have increased NMDA receptor (NR1 subunit) expression on the plasma membrane and contribute to increased intracellular calcium concentration. TgCRND8 neurons may also have increased calcium binding capacity when compared to non-transgenic littermate controls and that is why we do not see more intracellular calcium in TgCRND8 vs. non-transgenic controls after application of glutamate. TgCRND8 cortical neurons also possess greater ER calcium concentration than non-transgenic littermates both at rest and after stimulation either by glutamate or by depolarization of the plasma membrane by potassium chloride (KCl; we observed a sharp increase in intracellular calcium concentration in both TgCRND8 and non-transgenic littermate controls, with an immediate tapering off of or loss of calcium---possibly by uptake into the ER). It may also be that non-transgenic littermates possess greater glutathione (antioxidant) stores than TgCRND8 and thus can combat reactive oxygen species generation more effectively and thereby promote increased cell survival.

In summary, the data from this thesis work will form a strong foundation upon which future studies will be able to build upon toward better understanding not only the TgCRND8 mouse model of AD, but also toward understanding how AD mutations affect cell death mechanisms and calcium handling. Ours, and other available in vitro models, provide solid, easily manipulatable mediums that closely mimic AD, providing a springboard to move pharmacological testing from rodent models to human models. Without the in vitro characterization of the TgCRND8 mouse model of AD, specifically whether this model produced and secreted Aβ1-42, further in vitro examination would be in jeopardy as baseline characteristics would have been virtually unknown. We now know that calcium handling is different between TgCRND8 and non-transgenic littermate controls due largely to the work that we were able to conduct. We are confident that this information will assist future studies in attempting to tease out mechanistic pathways that will build upon our observed differences (as well as lack of differences) in our experiments. Furthermore, it will be necessary to elucidate a number of biological functions in vitro in the TgCRND8 mouse model of AD and the data uncovered in our studies in the present thesis will most certainly assist in that search. These include measuring the concentration of internal Aβ1-42 vs. secreted Aβ1-42, examining the contribution intracellular vs. secreted Aβ has on cell damage and eventual death, determining the mechanism by which calcium dyshomeostasis occurs, precise time to onset of generation of reactive oxygen species, the role(s) of various cell death promoters and executioners in this system, and the calcium handling responses to various stimuli and blocking agents.

CHAPTER 5 - Future Directions

Future studies employing TgCRND8 mice will undoubtedly contribute to the progressive field of Alzheimer's disease research. As mentioned in this work, a number of studies have already been conducted using this model lending significant insight into the inner cellular and molecular workings, as well as whole animal exploration, of this mammalian facsimile of AD. Moreover, the insightful information investigators have obtained while testing the TgCRND8 model has led to the enhancement of scientific work previously laid out by existing models, thereby functioning to advance the work of AD and not to judge or deprecate other models. The beauty and benefit of possessing various murine models of AD to choose from is that each afford the ability to offer investigators a unique opportunity to study a particular pathway or mechanism using a full complement of testable foundations according to needs.

Results from our in vitro studies indicate that primary cortical TgCRND8 neurons generate more APP and secrete significantly more Aβ1-42 than non-transgenic littermate controls. This observation was of paramount importance as from it most other hypotheses depended upon it. One of the missing components to this discovery is that we are unsure as to the exact moment in time that the TgCRND8 mice begin to produce and secrete more APP and Aβ, respectively as APP expression and Aβ1-42 concentration was only taken 3 and 8 days after culture. At 3 days post-plating, there were no differences in Aβ1-42 concentration between TgCRND8 and non-transgenic littermate control cortical cells, with only a small amount of Aβ1-42 produced between the two groups. However at 8 days post-plating the TgCRND8 cortical cells exhibited much higher APP generation and significantly more Aβ1-42 production. What we are unclear about is when these

differences in expression and production began to occur. A time course study using Western blotting and ELISA assay will be required in the future in order to determine the exact time in culture that these differences between the two groups begins to emerge. With that information in hand, we would be able to establish a cultural baseline from which the earliest stages of amyloidogenesis would be occuring. This would ultimately provide us with a starting point to delve into the mechanistic changes that may be occuring within the cell before, during, and after the initiation of expression and concentration changes. Therefore, we propose that the next set of foundational experiments using TgCRND8 neurons would be to plan a time course whereby APP and Aβ1-42 levels could be tested between 3 and 8 days post-plating in order to secure moment of onset when these differences being to occur.

Additionally, one of the next steps would be to determine the concentration of Aβ1-42 generated in cell lysates in order to lay the foundation for future studies looking at intracellular vs extracellular Aβ pools and their toxicity to the cell and its environment. In a triple transgenic mouse model of AD, researchers have identified an inverse relationship involving Aβ found intracellularly and that found in the extracellular milieu (Oddo, Caccamo et al. 2006). Using immunotherapy, investigators found that clearance of Aβ from the extracellular environment took place before clearance of the peptide from the intracellular pool (Oddo, Caccamo et al. 2006). They concluded that intracellular levels of Aβ peptide may influence the concentration of the protein in the extracellular environment (Oddo, Caccamo et al. 2006). It was further conceded that we really do not know how extracellular Aβ assimilates into producing plaque formations in the brain (Oddo, Caccamo et al. 2006). Perhaps by understanding the relationship between

intracellular and extracellular Aβ, we may be able to shed light into how this process occurs. Oddo et al further lends credence to the applicability of transgenic mouse models of AD in the pursuit of understanding the birth of plaques in the brain, specifically through the use of immunotherapeutic interventions such as vaccines (Oddo, Caccamo et al. 2006). Interestingly, early information from these studies has proven that as intracellular Aβ levels decrease, the number of extracellular plaques increase (Oddo, Caccamo et al. 2006). It would behoove us to look at these effects in TgCRND8 mice as the answers may shed light as to the mechanism involved in this inverse relationship between intra- and extracellular pools of Aβ. Investigators have found that intracellular Aβ may be dangerous even before secretion therefore generating a plausible answer as to why neurons are more susceptible to neurodegeneration and perhaps why accumulations of Aβ are only observed in the brain (Hartmann, Bieger et al. 1997).

On the heels of the above suggested experiments another logical experiment would be to look at clearance mechanisms both in vitro and in the whole animal. Because the TgCRND8 genome does not include the "Kunitz protease inhibitor domain" (Chishti, Yang et al. 2001) there are no secreted forms of APP present (Mattson, Barger et al. 1993) in our system, therefore by controlling for this variable we are better equipped to study other forms of neurotrophism and clearance and degradative enzymes. It has been previously established that Aβ is swiftly cleared after production (Savage, Trusko et al. 1998); (Dovey, John et al. 2001; Farris, Mansourian et al. 2003), lending credence to the postulation that proteases responsible for degrading Aβ may play a significant part in maintaining the balance between production and degradation/clearance of this toxic peptide in the brain (Farris, Mansourian et al. 2003). In vitro, the degrading enzyme

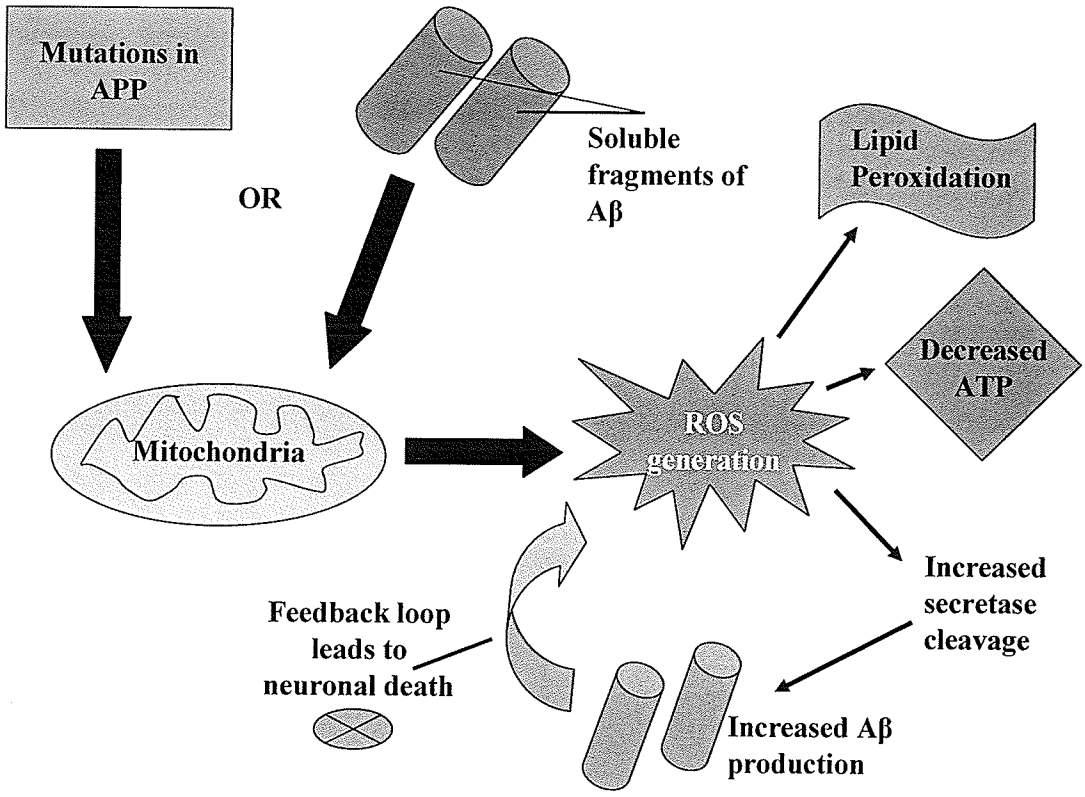
neprilysin has been studied using a number of cell systems including transfected cells. In a study by Hama et al, (2001) they found that in a dose-dependent fashion, wild-type neprilysin decreased Aβ1-40 and Aβ1-42 levels in transfected cells (Hama, Shirotani et al. 2001). They did not neglect to mention the possibility that some Aβ degradation may be occuring within the cell before it is secreted (Hama, Shirotani et al. 2001), as this will need to be controlled for. Neprilysin's active site is on the plasma membrane and therefore degrades Aβ at the surface of the cell (Fukami, Watanabe et al. 2002); (Iwata, Mizukami et al. 2004). Of importance for our future studies and comparisons with existing data, is that synthetic neprilysin is capable of degrading both man-made and endogenously secreted Aβ1-40 and Aβ1-42 (www.neuromics.com). In addition, investigating the activity and ability of insulin degrading enzyme (IDE) may also prove valuable when examining Aβ degradation and clearance as this enzyme has also been reported to reduce Aβ in vitro (Vekrellis, Ye et al. 2000); (Qiu, Ye et al. 1997); (Sudoh, Frosch et al. 2002). Furthermore, degradation of amyloid's neurotoxic effects has been linked to the activity of IDE (Mukherjee, Song et al. 2000).

Our data further suggested that survival in TgCRND8 and non-transgenic hippocampal and cortical cells were similar until 10 days post-plating. The next step would be to attempt to delineate the mechanism(s) responsible for the difference in survival observed after this time point. For example, it would be beneficial to look at gamma secretase by measuring its presence over time using Western blot analysis and immunostaining of cells and brain slices. As well, it would be useful to record gamma secretase activity in our in vitro system. This would provide valuable insight into the liberation activity of Aβ by gamma secretase, as well as possibly shedding light on why

we see more Aβ in TgCRND8 vs. non-transgenic littermate controls and when these differences begin to occur.

Our data revealed a difference in the generation of reactive oxygen species 12 days post-plating. It has been previously established that there is a relationship between oxidative stress mechanisms and AD (Lee, Zhu et al. 2006), therefore uncovering possible triggers responsible for differences in reactive oxygen species generation would undoubtedly prove very useful. Moreover, determining why antioxidant defenses are eventually bypassed in the TgCRND8 neurons in culture would also prove to be beneficial. In addition to investigating mechanisms associated with increased reactive oxygen species generation (for example, amyloid fragment involvement), it would further prove useful to record the activity of intracellular stress proteins, activated by insults such as upregulated reactive oxygen species generation.

Figure 1: Proposed Model of Reactive Oxygen Species' Contribution to AD



Mutations in APP or accumulated soluble Aβ promote reactive oxygen species (ROS) generation by mitochondria. ROS generation leads to impaired ATP production, lipid peroxidation, and initiation of aberrant secretase cleavage, thereby liberating increased Aβ1-42. Increased Aβ1-42 production feeds forward to produce more ROS creating a feedback loop that contributes to neuronal death.

Adapted from Crouch et al (2005), Takuma et al (2005), and Reddy (2006).

Detrimental cellular response to activation of the stress response and consequent upregulation of stress proteins such as GADD153 and caspase 12, is mediated through the endoplasmic reticulum (Glazner and Fernyhough 2002). Data we collected using another mouse model of AD, presenilin mutant mice, indicated that AD-linked presenilin mutations were associated with disrupting the balance normally held between death-inducing proteins such as GADD153 and pro-survival proteins such as nuclear factor kappa B (NFκB) (Schapansky, Morissette et al. 2007). Therefore given the association of disruption of this balance due to AD-linked mutations, it would be beneficial to measure the activity of NFκB and expression of GADD153 in the TgCRND8 in vitro system. Other associated 'stress proteins' important to measure include caspase 3 and 12, and members of the Bcl-2 family including both protective and destructive members. It would also be useful to uncover which genes NKκB and GADD153 affect when translocated to the nucleus or activated, respectively.

Results from our calcium experiments have led to answers to some questions but many still remain. One pressing question that remains not only in our studies but in the literature as well involves uncovering the mechanistic link between Aβ accumulation and calcium dyshomeostasis. Our results revealed that there is a basal resting difference in intracellular calcium concentration between TgCRND8 and non-transgenic littermates with the former having the highest concentration. Our data further suggested that there was more calcium in the endoplasmic reticulum in TgCRND8 cells when compared to non-transgenic littermates. Given that these differences in calcium concentration exist between TgCRND8 and non-transgenic littermates in our system, and that these differences may be attributable to an increase in Aβ production in the TgCRND8 primary

cells, it is important to determine the mechanism(s) involved in instigating these differences. Is the calcium dyshomeostasis an inbred part of the TgCRND8 makeup? Is the calcium concentration in the endoplasmic reticulum responsible for the increased death we see in the TgCRND8? Is the activation of NFκB affected by the level of intracellular calcium in TgCRND8? These are interesting questions awaiting equally interesting answers.

As follow-up calcium experiments are planned to move this project into the next phase, it is important to establish the contributions both plasma membrane and endoplasmic reticulum membrane calcium influx and efflux channels and exchangers have on influencing variant calcium levels between TgCRND8 and non-transgenic littermate controls. Equally as important, receptor densities will also need to be determined for receptors studied in this first phase as well as remaining receptors not observed. For example, in the current project we were able to manipulate many glutamatergic plasma membrane receptors such as N-methyl-D aspartate (NMDA) by either blocking extracellular calcium entry with antagonists, or by promoting calcium influx with NMDA or glutamate as the stimulus. We found that there was more intracellular calcium in TgCRND8 cells after the addition of NMDA (when compared to non-transgenic littermate controls). However we were somewhat surprised to see that there were no differences in intracellular calcium concentration between TgCRND8 and non-transgenic littermates after the addition of 1mM glutamate. It is known that glutamate is one of the most important excitatory neurotransmitters in the brain (Collingridge and Lester 1989) and that stimulation with this neurotransmitter will cause an influx of calcium into the cell (Choi 1992). However, it is also known that high

concentrations of glutamate may cause reduced binding affinity of a protein required for the tandem binding and subsequent activation of the NMDA receptors called glycine (Dingledine, Borges et al. 1999). Using a dose response method, the optimal concentration of glutamate needed for stimulation and not desensitization in vitro in TgCRND8 neurons will need to be elucidated.

Finally, because there were some differences in calcium handling in our system, it would provide another opportunity to further understand mechanisms involved in these differences if calcium binding proteins were measured. Calcium binding proteins (i.e. SERCA-2B, calbinin, calsenilin, and calreticulin) provide a buffering system which regulates calcium storage and intracellular calcium rises (Smith, Green et al. 2005). The expression of these proteins may be measured using Western blot analysis to determine specific levels of each in cell lysates.

There are many, many additional experiments that are required before the entire calcium story is evaluated, let alone a thorough understanding of Alzheimer's disease itself. Although the story has long been written, it has, and continues to demand, much more attention to elucidating the details. It would be ridiculous to assume that one dissertion such as this could hold the answers to the enigmatic nature of Alzheimer's disease but perhaps the results obtained throughout this document will help shed some light on previously unanswered questions such as the characterization of the TgCRND8 mouse model of Alzheimer's disease. The current experiments furthermore shed light on the in vitro system and the merits of studying such a system with regards to tracking and better understanding pathways and mechanisms involved in Alzheimer's disease.

ABBREVIATIONS

Aβ- amyloid beta

AMPA- alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

APP- amyloid precursor protein

CCE- capacitative calcium entry

CICR- calcium-induced calcium release

CNQX- 6-cyano-7-dinitroquinoxaline-2,3-dione

DAPI- 4',6-diamidino-2-phenylindole

DHR123- dihydrorohamine 123

ER- endoplasmic reticulum

GFAP- glial fibrillary acidic protein

GluR1- G-protein coupled metabotropic plasma membrane glutamate receptor

IICR- IP3-induced calcium release

IL-1A- interleukin-1 alpha

IL-1B- interleukin-1 beta

IP3- inositol 1,4,5- triphophate

KCl- potassium chloride

K/I- knock-in

K/O- knock-out

LRR- low-density lipoprotein receptor-related protein

MAP2- microtubule-associated protein 2

MK801- [(+)-5-methyl-10,11-dihydro-5H-dibenzo[A,D] cyclohepten-5,10-imine maleate]

mRNA- messenger RNA

NMDA- N-methyl-D-aspartate

NOG- neurite outgrowth

NR1- NMDA receptor glycine subunit

NSAIDS- non-steroidal anti-inflammatory drugs

PCR- polymerase chain reaction

PI- propidium iodide

PS1- presenilin 1

PS2- presenilin 2

PTP- permeability transition pore

ROS- reactive oxygen species

RyR- ryanodine receptor

SEM- standard error measurement

SERCA- sarco-endoplasmic reticulum calcium ATPase

TNF-α- tumor necrosis factor-alpha

TUNEL- terminal deoxynucleotidyl transferase dUTP nick-end labeling

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