# The Role of Metals and Aβ in Excitotoxicity and Alzheimer's Disease

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# Abstract


## Background

N-methyl-d-aspartate receptors (NMDARs) are ionotropic channels gated by the excitatory amino acid, glutamate. They play an essential role in synaptic plasticity, enhancing synaptic signal strength through long-term potentiation (LTP), a process thought to underlie learning and memory. At the synapse, NMDARs mediate neuroprotective signaling pathways including the regulation of calcineurin activity and inhibition of glycogen synthase kinase (GSK3). Under pathological conditions the prolonged and enhanced exposure of NMDARs to glutamate results in an excessive flux of calcium ($Ca^{2+}$) into the cell. This triggers a range of responses resulting in cell death, including increased oxidative stress, inappropriate activation of proteases such as calpain, dysregulation of $Ca^{2+}$-related pathways, mitochondrial damage and an apoptotic cascade. This process, termed excitotoxicity, contributes significantly to the acute neurodegeneration in ischemia and traumatic brain injury (TBI) and is believed to underlie the chronic neurodegeneration in Huntington's disease (HD) and more recently, Alzheimer's disease (AD).

Alzheimer's disease (AD) is characterised by progressive cognitive impairment resulting from synaptic degeneration and neuronal loss. A proposed key event in its aetiology is the formation of oligomeric species of the beta amyloid (Aβ) peptide. Recent work has demonstrated that the soluble Aβ oligomers induce excessive calcium influx across the cell membrane resulting in neuronal death by excitotoxicity. It is believed these toxic species of Aβ oligmomerise in the synaptic cleft between neurons in the hippocampus due to high levels of zinc and copper. These metals are released upon NMDAR activity from the pre- and post-synapse, respectively and can bind Aβ, increasing its rate of oligomerisation. Subsequent excitotoxic interactions between Aβ and NMDARs are copper ($Cu^{2+}$)-dependent. In contrast, $Cu^{2+}$ is also neuroprotective against excitotoxicity demonstrating the crucial role of metal homeostasis in specific regions of the brain affected by neurodegenerative diseases.

## Objectives

This PhD project has sought to determine the contribution of metals in excitotoxicity and whether modulating their levels could provide a mechanism to protect against this form of cell death. As excitotoxicity is strongly implicated in the aetiology of Alzheimer's disease subsequent research aimed to describe the involvement of excitotoxicity in Aβ-mediated cell death in cortical neural model and to establish whether metals played a necessary role in this process. The final goal of the research presented here was the development of a neural-based assay, which could be employed to screen various forms of Aβ to detect more toxic forms of the peptide.

## Results

In experiments with the metal chaperone PBT2, a therapeutic in clinical trials for chronic neurodegenerative diseases, neurons were protected against excitotoxic cell death by pretreatment with the drug. Subsequent experiments demonstrated that this was a metal-mediated effect that required zinc. Pretreatment with this drug induced preconditioning in neurons by moderate increases in intracellular levels of calcium that activated survival pathways and inhibited activation of calcineurin and GSK3 preventing cell death.

In further investigations the parameters for Aβ-induced excitotoxicity in cortical neurons were determined. In the presence of non-toxic levels of glutamate, Aβ induced significant toxicity that was dependent on the presence of metals, as demonstrated by metal chelation. These findings translated to the development of a calcium flux assay, which provided a functional readout of Aβ toxicity. Finally, this assay was validated by screening species of Aβ with varied degrees of toxicity to neurons.

## Conclusions

This work highlights the importance of metals in neurodegenerative disease and demonstrates modulation of both $Cu^{2+}$ and $Zn^{2+}$ levels in hippocampal synapses provide valid targets for future therapeutic approaches by preventing the formation of toxic oligomeric species. A concurrent finding has

been the identification of the parameters required for Aβ-induced excitotoxicity, which provides the tools to screen an array of both *in vivo* and *in vitro* Aβ species to determine their toxicity. This knowledge will enable targeted clearance of these forms of the Aβ peptide, which, along with therapies preventing oligomer formation, will show significant therapeutic affects in the treatment of Alzheimer's disease.

# Declaration

This is to certify that:

i. the thesis comprises only my original work towards the PhD except where indicated in the chapter prefaces,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

_____ _____

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# Abbreviations

| | |
|---|---|
| Aβ | Amyloid-beta |
| Aβ40 | Amyloid-beta Ending @ Valine40 |
| Aβ42 | Amyloid-beta Ending @ Alanine42 |
| AβOs | Amyloid-beta Oligomers |
| $A_{280}$ | Absorbance at 280 nm |
| ACh | Acetylcholine |
| AChEIs | Acetylcholinesterase Inhibitors |
| AD | Alzheimer's Disease |
| ADDLs | Aβ-Derived Diffusible Ligands |
| ANOVA | Analysis of Variance |
| APLP | Amyloid Precursor-Like Protein |
| ApoJ | Apolipoprotein J |
| APP | Amyloid Precursor Protein |
| APP695 | APP lacking exons 7 & 8 |
| APP751 | APP lacking exon 8 |
| APP770 | APP containing all exons (full-length APP) |
| ATP7a | Copper-transporting ATPase 1 |
| BACE | Beta-Site APP Cleaving Enzyme / Beta-Secretase |
| BCA | Bicincotinic Acid |
| β-CTF | Beta C-Terminal Fragment of APP |
| BL6 | Black-6 |
| BSA | Bovine Serum Albumin |
| CCK8 | Cell Counting Kit-8 |
| cDNA | Complementary DNA |
| CHCA | α-cyano-4 hydroxycinnamic acid |
| CNS | Central Nervous System |
| $CO_3$ | Carbonate |
| CQ | Clioquinol |
| CSF | Cerebrospinal Fluid |
| C-terminus | Carboxy-Terminus |

| | |
|---|---|
| $Cu^{2+}$ | Copper (II) |
| $CuCl_2$ | Copper Chloride |
| $ddH_2O$ | Double Deionised Water |
| DIV | Days In Vitro |
| DLB | Dementia with Lewy Bodies |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxyribonuclease |
| DO | Days Old |
| DS | Down's Syndrome |
| DTT | Dithiothreitol |
| ECL | Enhanced Chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-Linked Immunosorbant Assay |
| EPR | Electron Paramagnetic Resonance |
| EOAD | Early Onset Alzheimer's Disease |
| ER | Endoplasmic Reticulum |
| $F_0$ | Average background fluorescence |
| $F_1$ | Fluorescence immediately following treatment |
| FAD | Familial Alzheimer's Disease |
| FBS/FCS | Foetal Bovine Serum/Foetal Calf Serum |
| FDA | Food and Drug Administration |
| fEPSP | Field Excitatory Postsynaptic Potential |
| FTD | Fronto-Temporal Dementia |
| F/S | Filter/Sterilised |
| GDP | Gross Domestic Product |
| GFP | Green Fluorescent Protein |
| Glu | Glutamate |
| GnRH | Gonadotropin Releasing Hormone |
| $H_2O_2$ | Hydrogen Peroxide |
| HBSS | Hanks Buffered Saline Solution |

| | |
|---|---|
| HC | Healthy Control |
| HD | Huntington's Disease |
| HEK | Human Embryonic Kidney |
| HEPES | 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid |
| HFIP | 1,1,1,3,3,3-Hexafluoro-2-propanol |
| HPLC | High Performance Liquid Chromatography |
| HRP | Horseradish Peroxidase |
| ICP-MS | Inductively Coupled Plasma Mass Spectrometry |
| KCl | Potassium (Kalium) Chloride |
| kDa | Kilodalton |
| LOAD | Late onset Alzheimer's disease |
| LDH | Lactate Dehydrogenase |
| LTP | Long-Term Potentiation |
| MALDI-TOF | Matrix-Assisted Lazer Desorption Ionisation Time-Of-Flight |
| MCI | Mild Cognitive Impairment |
| MEA | Multielectrode Array |
| MEC | Molar Extinction Coefficient |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| Met35 | Methionine-35 |
| $MgCl_2$ | Magnesium Chloride |
| $MgSO_4$ | Magnesium Sulfateμ |
| mM | Millimolar |
| mRNA | Messenger Ribonucleic Acid |
| MS | Mass Spectrometry |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| mu | murine |
| MW | Molecular Weight |
| $N_2$ | Molecular Nitrogen |
| NaCl | Sodium Chlorde |
| $NaH_2PO_4$ | Sodium Phosphate |
| $Na_2HPO4$ | Sodium Phosphate dihydrate |
| NaOH | Sodium Hydroxide |

| | |
|---|---|
| NFT | Neurofibrillary Tangle |
| $NH_3$ | Ammonia |
| $NH_4OH$ | Ammonium Hydroxide |
| nm | Nanometre |
| NMDA | *N*-methyl-D-aspartate |
| NMR | Nuclear Magnetic Resonance |
| $NO_2$ | Nitrite |
| N-terminus | Amino-Terminus |
| $O_2$ | Molecular Oxygen |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PB | Phosphate Buffer |
| PBS | Phosphate-Buffered Saline |
| PBST | Phosphate-Buffered Saline, 0.1% Tween-20 |
| PBT2 | Prana Biotechnologies 2 |
| PCR | Polymerase Chain Reaction |
| PD | Parkinson's Disease |
| PDL | Poly-D-Lysine |
| pE | Pyroglutamate |
| pE-Aβ | Pyroglutamate-Aβ |
| PEI | Poly-Ethylene-Imine |
| PET | Positron Emission Tomography |
| PIB | Pittsburgh Compound B |
| $PrP^c$ | Prion Protein, Cellular form |
| PS1 | Presenilin-1 |
| PVDF | Polyvinylidene fluoride |
| RFU | Relative Fluorescence Units |
| ROS | Reactive Oxygen Species |
| RT | Room Temperature |
| sAPPα | Soluble Amyloid Precursor Protein (α-cleaved) |
| SDS | Sodium Dodecyl Sulfate |
| SELDI-TOF | Surface-Enhanced Lazer Desorption Ionisation Time-Of-Flight |
| SOD | Superoxide dismutase |

| | |
|---|---|
| TBS | Tris-Buffered Saline |
| TBST | Tris-Buffered Saline, 0.1% Tween20 |
| TFA | Trifluoroacetic acid |
| ThT | Thioflavin-T |
| Tris | Tris(hydroxymethyl)aminomethane |
| UV | Ultraviolet |
| v/v | Voloume/volume |
| w/v | Weight/volume |
| WT | Wild-Type |
| $Zn^{2+}$ | Zinc (II) |
| $ZnCl_2$ | Zinc Chloride |

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# 1 INTRODUCTION

## 1.1 Clinical Presentation of Alzheimer's Disease

### 1.1.1 Epidemiology of Alzheimer's disease

Alzheimer's disease (AD) is the leading cause of dementia with the number of people affected doubling every 5-year interval beyond 65 years of age (Ferri et al., 2006). It is estimated that as of 2013 there were 44.4 million cases worldwide in 2013 and this number increasing to 75.6 million in 2030, and 135.5 million in 2050 due to longer life expectancies and demographic changes. The dramatic increases in AD cases will have significant financial ramifications with the resulting costs associated with disease estimated at US$604 billion per year (World Alzheimer Report 2013). Recent data from the UK has emphasized the significant cost to society of AD, when considering the costs of healthcare, social care and the significant proportion of unpaid work by carers the average cost is £32,250 per person or £26.3 billion per year (Prince et al., 2014). To this end the research and development of greatly improved diagnostic tools and therapeutics for AD is of upmost importance.

### 1.1.2 Symptoms, stages and detection of Alzheimer's disease

Historically AD was defined by the three progressive stages; mild AD (lasts 2 - 4 years), moderate AD (lasts 2 - 10 years) and severe AD (1 - 3+ years). Since the early 1980s the progression of AD has also been described in seven stages by the Global Deterioration Scale (Reisberg et al., 1982). Both these classifications and the criteria for the clinical diagnosis of AD (McKhann et al., 1984) have remained until recently when the National Institute of Aging and Alzhiemer's Associations (U.S.) sought their revision to account for the advances in research over the preceding three decades (McKhann et al., 2011). Results from

biomarkers discovered in the interim as well as genetic mutations leading to early onset familial AD were now included in diagnosis. Today AD is classified by the categories of preclinical (Sperling et al., 2011), mild cognitive impairment (MCI) due to AD (Albert et al., 2011), mild, moderate and severe AD. In both preclinical and MCI stages of AD symptoms do not or only differ slightly from the slower mental processing and mild memory impairment accompanying normal aging. Biomarkers detected in the cerebrospinal fluid (CSF), or by Positron Emission Tomography (PET) scans and structural MRI are highly correlated with the neuropathological markers of AD (Clark et al., 2003; Strozyk et al., 2003; Tapiola et al., 2009) and allow the detection of these prodromal AD stages (MD et al., 2010) (Figure 1-1) In the most current research, data has been presented demonstrating the ability to predict phenoconversion to amnestic MCI or AD in normal older adults with greater than 90% accuracy using a panel of 10 lipid biomarkers in the peripheral blood, all involved in cell membrane integrity (Mapstone et al., 2014). Following this work a larger study has also identified a panel of 10 biomarkers that correlated strongly with disease severity present in the blood. This panel predicted conversion of MCI to AD with 87% accuracy (Hye et al., 2014). As the present accuracies of both screens is approximately 90% and the prevalence of AD in the population is considerably lower than this error, it could actually lead to more false positives than real ones. It may be that the significant impact these findings have is in the selection of candidates for clinical trials where the population already has presented with memory complaints and therefore prevalence of conversion from MCI to AD is significantly increased.

A common profile of people with mild AD is impaired episodic memory and the ability to retain new information (Welsh et al., 1991). As the disease progresses to moderate AD other cognitive, behavioural, and neuropsychiatric deficits develop and difficulties performing activities of daily living occur. Although moderate AD patients can be physically well their inability to perform complex activities such as cooking, coupled with their aggressiveness and agitation means they are reliant on caregivers during this stage. In the final

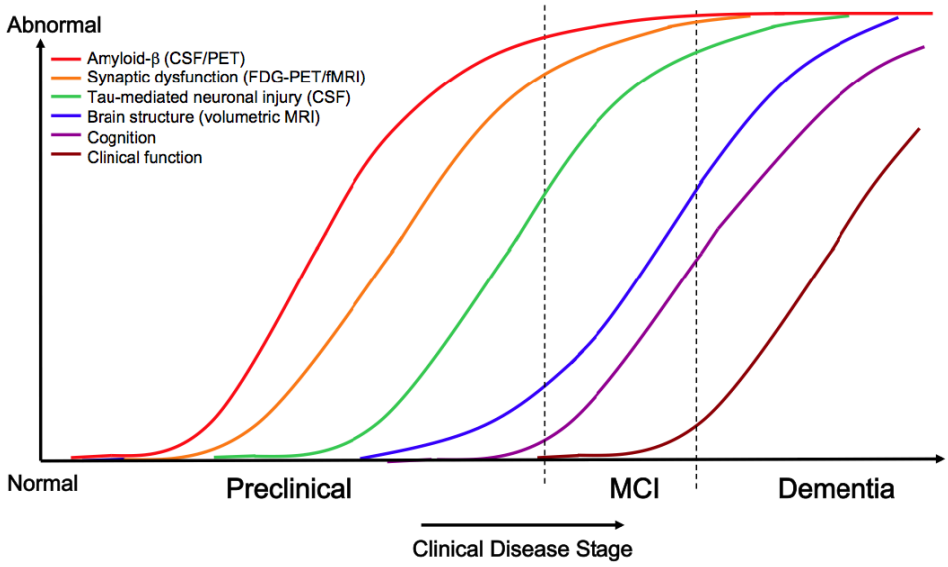



**Figure 1-1 A hypothetical model of biomarkers in preclinical stage of AD.** It parallels the hypothetical pathophysiological sequence of AD. Biomarkers change from maximally normal (y axis) to maximally abnormal as a function of disease stage (x axis). The two key clinical indicators of disease, cognition and clinical function, are also illustrated. Adapted originally from Cliff Jack (Sperling et al., 2011).

severe stage of AD nearly all cognitive functions are impaired, patients lose the ability to communicate, recognize family and basic motor skills. At the end stage impairment of swallowing can result in pneumonia due to food and beverage in the lungs and is the most common cause of death along with urinary tract infections and falls (Staff, 2013).

### 1.1.3 Risk factors for Alzheimer's disease

With the distinct lack of drugs that alter the progression of AD, modifying risk factors provides an effective strategy to minimize the effects of the disease. To delay the onset by one year could decrease AD prevalence by nearly 12 million in 2050 based on projections by Brookmeyer et al. and revised numbers of AD cases as of 2013 (Brookmeyer et al., 2007). At present a person's age is the strongest correlate with AD but there also exists an extensive field of research in to modifiable risk factors and their association with the disease. From metadata analysis of factors that increase the risk of AD it can be seen that those involved with cardiovascular disease including diabetes, midlife hypertension and midlife obesity all have significant effects (Whitmer et al., 2005; Beydoun et al., 2008; Lu

et al., 2009). Lifestyle factors including physical inactivity and smoking have also been found to increase the risk of AD (Peters et al., 2008). This is despite initial contrary findings for smoking and AD that were in line with the known effects of nicotine on enhancing learning and memory (Couey et al., 2007). The risk of AD can also be affected by cognitive factors with both depression and a low education significantly increasing the risk of AD. Meta analysis of traumatic brain injury (TBI) patients has established an increased risk of AD, which is higher among men and supported by increased levels in key phenotypes of AD in the post-mortem brain (Fleminger et al., 2003; Magnoni and Brody, 2010). Protective factors have been also observed with diet, physical activity and enhancing cognition through a Mediterranean diet, exercise and stimulating leisure activities, respectively, leading to a decreased risk of AD (reviewed by (Mayeux and Stern, 2012). If modifiable risk factors are to have an impact on the financial burden of disease it is essential that their individual prevalence in the population are accounted for to maximize effect (Barnes and Yaffe, 2011).

## 1.2 The Hallmarks of Alzheimer's Disease

To date, a definitive diagnosis of AD requires post-mortem histopathological confirmation of two cerebral markers; intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques (McKhann et al., 1984), although the recent discovery of biomarkers involving PET imaging and cognitive tests has allowed diagnosis of probable AD.

### 1.2.1 Neurofibrillary tangles of Tau

NFTs were first described as intraneuronal, lamentous aggregates in the perikarya and processes of neurons (Alzheimer et al., 1995). NFTs were later found to consist of Tau, a microtubule-associated protein, highly expressed in the nervous system, that is converted in to paired helical filaments that self assemble as a result of abnormal phosphorylation (Figure 1-2). This hyperphosphorylation of Tau, by kinases, such as GSK3, leads to destabilized

microtubules that impair axonal transport and neuronal function (Lee and Trojanowski, 1992). Unlike the widely varied Aβ plaque distribution in AD, NFT displays characteristic distribution patterns that correlate more closely with disease severity and are used in the diagnosis and staging of AD (Braak and Braak, 1991).

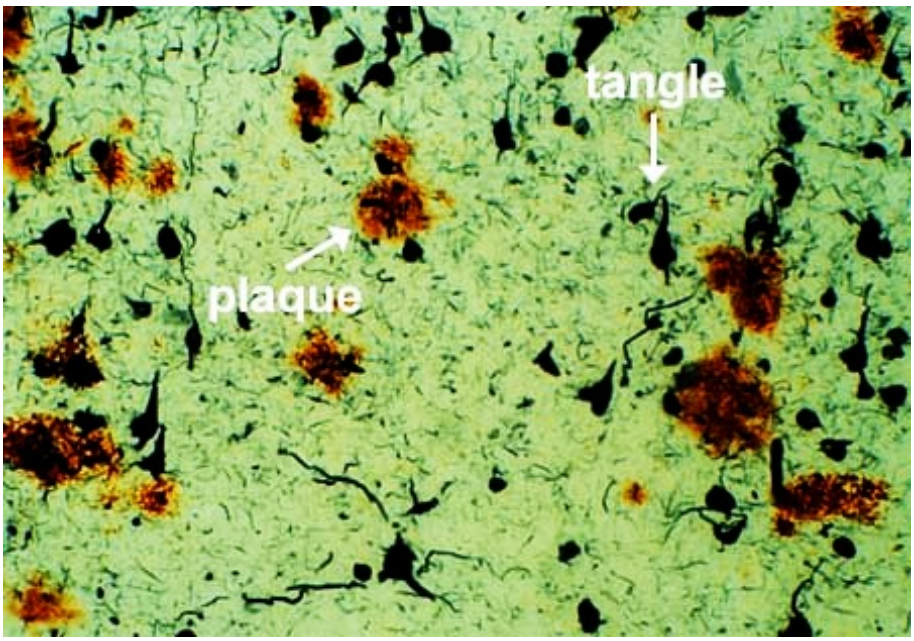



**Figure 1-2 Amyloid plaques and neurofibrillary tangles.** Extracellular plaques of amyloid, consisting mainly of the Aβ peptide and intracellular neurofibrillary tangles of Tau as visualised by immunohistochemistry and silver staining, respectively.

### 1.2.2 Beta amyloid

In Alois Alzheimer's seminal paper in 1906 he first described the "milary bodies" in the post mortem brain of the patient Auguste D. which is now considered one of the two key phenotypes of AD (Figure 1-2) Isolation and purification of the major subunit of amyloid plaque cores and blood vessel deposits followed by amino acid sequencing led to the discovery of a highly aggregating peptide of 4.5 kDa initially named Amyloid A4 protein (Glenner and Wong, 1984; Masters et al., 1985a; 1985b) and now referred to as beta amyloid (Aβ) due to its partial β-pleated sheet structure. Plaques in the AD brain are primarily composed of Aβ between 38 and 43 residues long. The two major

forms of Aβ present in the neuritic plaques are the longer Aβ1–42 (Aβ42) species that is the major and more toxic product and Aβ1–40 (Aβ40). The Aβ42 peptide has a higher propensity to aggregate which is attributed to the two additional hydrophobic residues an the C-terminal end (Li et al., 1999; Kim and Hecht, 2005). Currently PET imaging of these plaques is possible by the use of a radioactive analog of Thioflavin T, called the Pittsburgh B compound (Klunk et al., 2004), which distinguishes neuritic from diffuse plaques based on their forming beta-sheet structures (Figure 1-3).

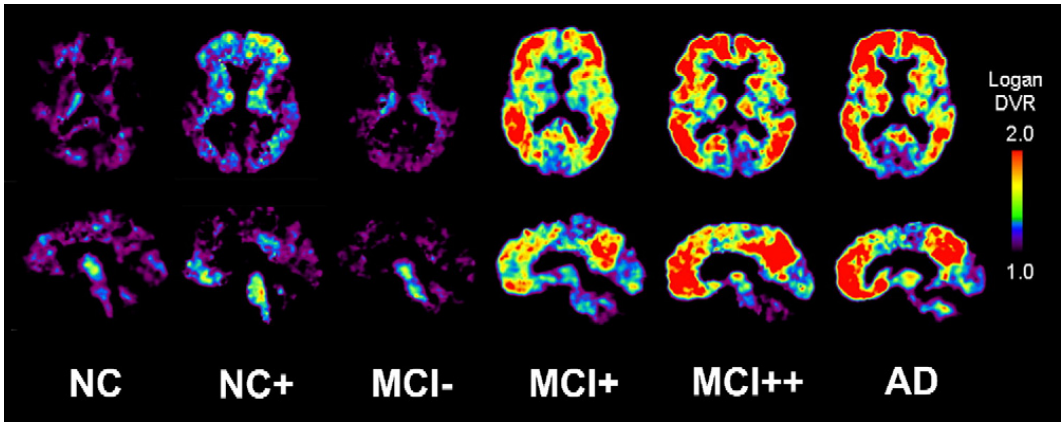



**Figure 1-3** PET images in the transaxial and sagittal planes of Logan distribution volumes (DVR) of [$^{11}$C]PiB in a normal control (NC), a [$^{11}$C]PiB-positive NC (NC+), a [$^{11}$C]PiB-negative MCI subject (MCI-), [$^{11}$C]PiB-positive (MCI+) and highly positive (MCI++) subjects and a [$^{11}$C]PiB-positive AD subject (AD). Adapted from (Mathis et al., 2007). Longitudinal studies with [$^{11}$C]PiB PET scans as well as MRI and neuropsychological examination have demonstrated a protracted preclinical phase of AD of 17 years and as AD progresses, Aβ deposition slowed towards a plateau with increasing Aβ burden (Villemagne et al., 2013).

This secondary structure of Aβ gives rise to the rapid aggregation of the peptide; inhibition of the beta sheet structures reduces preformed amyloid fibrils and their formation which prevents fibril-induced neuronal death (Soto et al., 1998).

### 1.2.3 The Amyloid precursor protein

In purification and identification of amyloid plaques it was noted that the same pathology was present in aged individuals with Downs syndrome (Masters et al., 1985b). Support for the argument that Aβ was a cleavage product of a larger precursor protein of neuronal origin came when the amyloid precursor protein (APP) gene was cloned from a cDNA library constructed from brain tissue

with Downs syndrome (Kang et al., 1987). Concurrent genetic mapping linked the Aβ peptide to chromosome 21 which explained the overexpression observed in the tissue which was caused by the increased gene dosage from the chromosome trisomy (Tanzi et al., 1987).

APP is an integral type I transmembrane protein with a single transmembrane domain, a large extracellular domain, and a short cytoplasmic tail (Figure 1-4). It has three isoforms from alternate splicing; APP695, APP751 and APP770 (Kang et al., 1987; Tanzi et al., 1988; Goate et al., 1991) that are ubiquitously expressed in embryonic development and adult tissue (Lorent et al., 1995). APP695 is major isoform in the brain with its expression mainly in neurons, the highest levels of these are seen in regions that correlate with those affected in AD, namely the hippocampus, cortices and cerebellum (Sola et al., 1993). APP is a member of an evolutionary conserved gene family in mammals that also includes the amyloid precursor-like proteins, APLP1 and APLP2 (Wasco et al., 1992; 1993). Within this family a functional redundancy has been observed with APLP2 and the remaining members. Studies of single knockout mice of each gene and double knockout mice of APP/APLP1 exhibit only subtle phenotypes compared with the lethality observed shortly after birth in the APLP2/APLP1 and APLP2/APP double knockout mice (Koch et al., 1997; Heber et al., 2000). A normal physiological role is yet to be fully elucidated for APP but it has been implicated in a variety of cellular processes including synaptic adhesion, trophic functions, axon remodeling, intracellular signaling and apoptosis (reviewed in Muller and Zheng, 2012). An essential role in synaptic function has been suggested by studies in APP knockout mice where impaired LTP and age-related cognition were observed (Dawson et al., 1999). Finally a neuroprotective function of APP was observed by its ability to protect against glutamate-induced excitotoxicity in neurons (Mattson et al., 1993b), recent work has proposed that this excitoprotective effect may result from the ability of APP to regulate intracellular iron homeostasis (Duce et al., 2010).

### 1.2.4 Amyloidogenic APP processing

In the amyloidogenic pathway cleavage of APP occurs at the amino terminus of Aβ by the β-site APP-cleaving Enzyme (BACE), a β-secretase identified independently by four groups in the late nineties (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). The site of cleavage appears to occur in specific cholesterol and sphingomyelin-enriched regions of the outer leaflet of the lipid bilayer, termed lipid rafts with a separate pool of APP outside of these rafts undergoing α-secretase cleavage (Ehehalt et al., 2003). Following the production of a soluble amino terminal ectodomain (sAPPβ) and a membrane bound c-terminal fragment (C99) by BACE, subsequent intramembrane processing by γ-secretase of C99 produces Aβ and the AICD (Haass, 2004) (Figure 1-4). Subsequently it has been shown that γ-secretase is a complex of four proteins; Presenilin (PS1 & PS2), presenilin enhancer-2 (PEN-2), anterior pharynx defective 1 (APH1) and Nicastrin (NCT) with all component proteins being required for proteolytic activity (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). In non neuronal cell lines the α-secretase pathway is dominant but due to high level expression of BACE, the products of the amyloidogenic pathway are favoured in neurons (Simons et al., 1996; Kuhn et al., 2010).

### 1.2.5 Non-amyloidogenic APP processing

The pathways believed be the non-toxic, physiological processing that prevents Aβ formation, involves the cell surface cleavage of APP by α-secretases, members of the ADAM family of zinc metalloproteases (Kuhn et al., 2010), within the Aβ region (Sisodia et al., 1990), leading to a large soluble ectodomain, termed soluble APP alpha (sAPPα) and a c-terminal fragment (C83) (Sisodia, 1992). Subsequent processing of the carboxy-terminal fragment by γ-secretase results in the production of p3, and the APP intracellular domain

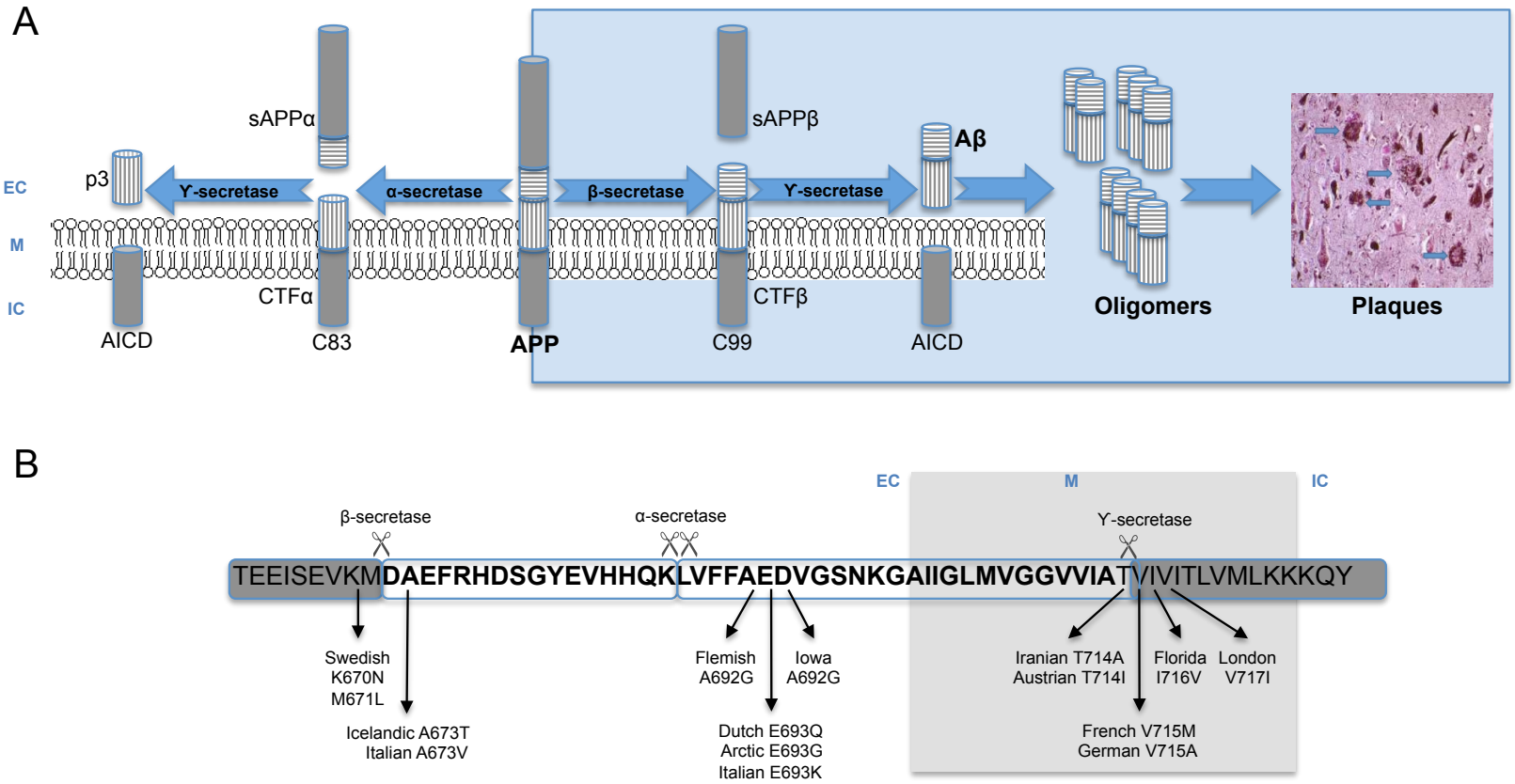



**Figure 1-4 The Amyloid Precursor Protein processing and mutations associated with AD** (**A**) APP amino acid sequence containing the Aβ peptide. The sequential proteolysis of amyloid precursor protein (APP) by β-secretase rather an α-secretase followed by γ-secretase generates Aβ, thought to be the underlying cause of toxicity in AD. (**B**) Genetic mutations associated with familial AD (FAD). Mutations near the β and γ-secretase cleavage sites increase Aβ production where those near the α-secretase site inhibit α-cleavage or increase Aβ aggregation (Van Dam and De Deyn, 2006) with the exception of a rare protective Icelandic mutation at 673 (Jonsson et al., 2012).

(AICD) (Figure 1-4). The AICD has been proposed to act on transcriptional regulation by analogy with the Notch protein and its intracellular domain, which is known to translocate to the nucleus and affect gene transcription (Selkoe and Kopan, 2003). Most recently the AICD has been linked to Aβ levels through epigenetic regulation of two Aβ degrading enzymes, neprilysin and transthyretin (Belyaev et al., 2010; Kerridge et al., 2014).

## 1.3 Genetics in AD

AD can be divided in to two major forms; familial (FAD) occurring prior to 65 years of age and sporadic for older patients. Whilst FAD can have a Mendelian pattern of inheritance non genetic factors play a significantly more important role in sporadic forms of AD.

### 1.3.1 Familial Alzheimer's disease

Early onset or FAD accounts for up to 6% of AD cases and may include non dominant forms of AD such as the apolipoprotein E4 allele or sporadic AD (see section 1.3.2) unlike autosomal dominant forms of AD (ADAD) that make up less than 1% of all AD cases (Bateman et al, 2011). In ADAD mutations occur in one of three genes; PSN1, PSN2 and APP on chromosomes 1, 14 and 21, respectively (Goate et al., 1991; Schellenberg et al., 1992; St George-Hyslop et al., 1992; Levy-Lahad et al., 1995; Sherrington et al., 1995; 1996). APP mutations account for 10 - 16% of the mutations described with the majority of these mutations located adjacent to the β and γ-secretase cleavage sites and resulting in increased production of Aβ. Internal mutations have also been described which result in decreased α-cleavage and can increase Aβ aggregation, a process thought to increase toxicity of the peptide (see Section 1.4.3) (Figure 1-4). Recently, a rare protective mutation was discovered in a genetic screen of nearly 2000 Icelanders. The mutation, adjacent to the β-secretase site, causes an approximate 40% reduction in Aβ levels (Jonsson et al., 2012)(Figure 1-4). PS1 mutations account for 30-70% of ADAD cases with PS2 mutations make up approximately 5% of cases (Schellenberg et al., 1992; Raux et al., 2005; Ferri et

al., 2006). Unlike APP these mutations are found throughout the presenilin genes and result in an increase in the ratio of Aβ42 to Aβ40 which increases the formation of toxic oligomeric species (Kuperstein et al., 2010) (see section 1.4.3). The prognosis of a child with an affected parent is poor with a 50% chance of inheriting the mutation and in doing so almost certainly developing the disease.

### 1.3.2 Sporadic Alzheimer's disease

Also referred to as late onset AD (LOAD), the majority of sporadic AD cases are patients over 65 years of age. Apolipoprotein E (ApoE) is by far the strongest genetic risk factor associated with sporadic AD and was implicated in AD through an association with Aβ and an overrepresentation of the ApoE4 allele, one of three ApoE alleles, when compared with control subjects (Corder et al., 1993; Strittmatter et al., 1993). The increase in risk of AD is three to eight times greater with one or two copies, respectively, when compared with a homozygous ApoE3 carrier (Farrer et al., 1997). Of the remaining two isoforms; ApoE2 and ApoE3, a genetic correlation has implicated ApoE2 as a protective isoform in AD (Corder et al., 1994; Royston et al., 1994). As well as the ApoE gene, large genome-wide association studies (GWAS) have identified nine other candidate genes that contribute to AD risk; *CR1, BIN1, CLU (previously APOJ), PICALM, MS4A4/MS4A6E, CD2AP, CD33, EPHA1, SorLA* and *ABCA7* (Harold et al., 2009; Lambert et al., 2009; Seshadri et al., 2010; Hollingworth et al., 2011; Naj et al., 2011). Studies of genetics and environmental effects on AD in monozygotic and dizygotic twins have predicted the genetic contribution to AD risk to be as high as 79% (Gatz et al., 2006). Given that the collective contribution to the genetic variance in AD of the genes described above is only 50% further research is still required to identify the additional genes involved (Kamboh et al., 2012).

## 1.4 The Role of Beta Amyloid in Alzheimer's Disease

### 1.4.1 A functional role of Aβ

Amyloid burden is only weakly related to cognitive decline (Villemagne et al., 2011) and although AD patients with low burden are rare, plaques are found in healthy controls without any overt cognitive impairments (Villemagne et al., 2013). These discoveries are central in alternate explanations for a physiological role of Aβ. One such example proposes that the formation of plaques is not the initiating pathogenic event but instead a secondary protective response to cell death (Lee et al., 2007). A less controversial hypothesis, with strong support in the literature, contends that toxicity is observed when Aβ is in excess but it demonstrates neurotrophic effects at low concentrations. Supporting this were findings that inhibition of endogenous Aβ by β and γ-inhibitors and immunodepletion resulted in toxicity. Moreover, this toxicity could be rescued by co-treatment with picomolar Aβ concentrations (Plant et al., 2003). Other physiological roles attributed to Aβ have included effects on cholesterol transport (Igbavboa et al., 2009), antimicrobial effects (Soscia et al., 2010) and modulating synaptic activity. In the latter it has been proposed that an activity-dependent Aβ production results in synaptic depression and provides a negative feedback on neuronal hyperactivity (Kamenetz et al., 2003). A causal link between synaptic activity and Aβ production is supported by brain imaging studies that demonstrated a correlation between the metabolic activity in young adults and amyloid deposition in elderly patients with AD (Buckner, 2005).

### 1.4.2 Aβ clearance

Along with overproduction of Aβ, an inability to clear the physiological levels of Aβ resulting from APP processing from the interstitial fluid surrounding neurons has also been strongly implicated in AD. In the first in vivo studies of Aβ production and clearance rates in the central nervous system (CNS) it was estimated the production rate of 7.6% per hour was exceeded by the clearance rate of 8.3% per hour (Bateman et al., 2006). Subsequent research from the same group found the clearance rate to be impaired for both Aβ42 and Aβ40 in

CSF in Alzheimer's disease compared to controls with no observable difference in production between the two groups (Mawuenyega et al., 2010).

Evidence that the inhibition of Aβ clearance may be receptor-mediated across the blood brain barrier (BBB) was provided by research implicating the low-density lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE). These receptors are the major transporters from brain to blood and blood to brain, respectively (reviewed in Deane et al., 2009; 2009). LRP binds Aβ directly and preferentially effluxes Aβ42 over Aβ40 due to high beta-sheet composition in its secondary structure. Aβ42 also promotes LRP degradation at pathological levels (>1 μM) (Deane et al., 2004). As described previously, risk factor genes associated with AD risk are not all linked to Aβ but those that have the highest associated risk and have been shown to affect clearance. As is the case for the ApoE and ABCA7 genes with ApoE also being linked to inhibition of LRP1 and subsequent decrease of receptor-driven clearance of Aβ (Jiang et al., 2008; Kline, 2012).

Proteolytic degradation provides another mechanism in which Aβ is removed from the brain. Key amyloid clearing proteases involved in this process include insulin degrading enzyme, neprysilin and transthyretin and all been implicated in AD (reviewed in Nalivaeva et al., 2014; 2014).

A third mechanism, recently described by Nedergaard and colleagues, could have a substantial impact on the progression of disease. It has been understood for some time that the brain clears Aβ to the CSF from the parenchyma by bulk flow along the perivascular interstitial (ISF) drainage pathway. A failure of this pathway has been implicated in AD as well as Cerebral Amyloid angiopathy (Weller et al., 2007). This recent pivotal research has utilised two-photon microscopy of fluorescent tracers allowing real-time three-dimensional imaging of Aβ clearance through a paravascular pathway (Iliff et al., 2012). In this and subsequent work, a system has been described in which interstitial solutes are cleared by para-arterial influx of CSF in to the parenchymal space resulting in efflux of ISF along paravenous drainage s. This system has been termed the 'Glymphatic system', due to the requirement for astrocyte-mediated water movement between the influx to efflux pathways. The activity of the

system is far greater during sleep where the brain's interstitial space is increased 60% allowing a substantial increase in the CSF-ISF exchange. This exchange has been shown to result in two-fold increase in the ability of the brain to clear Aβ. This is not due to circadian rhythm as anesthesia increases glymphatic activity to the same extent as sleep but involves adrenergic signaling as in peripheral tissues (Xie et al., 2013).

Given that age remains the strongest correlate with disease and the ability to fall asleep and remain so decreases significantly after the age of 60 (Ohayon et al., 2004) suggests a potential role for sleep in AD and is supported by the diurnal characteristics of the glymphatic system. A review of multiple cross sectional studies indicates that sleep disruption can result in impaired cognition providing support of a link between the activity of the glymphatic system and AD (Lucey and Bateman, 2014) but sleep disturbances have also been implicated as a symptom of AD as opposed to initiating the disease. Levels of ISF Aβ are regulated by neuronal activity and sleep/wake cycle. Studies in Aβ over-expressing mice showed marked deterioration of their normal sleep cycle following Abeta plaque formation and loss of diurnal fluctuations of Aβ levels in the ISF that was rescued by active immunisation. This effect on the sleep-wake cycle was also seen in the CSF of young adults with presenilin mutations and together suggests that in the prodromal stage of AD, Aβ aggregation affects the sleep pattern which in turn results in impaired clearance of Aβ accelerating the disease. (Roh et al., 2012).

### 1.4.3 Soluble Aβ Oligomers

As discussed previously Aβ is generated from normal physiological processing of APP. In familial AD the mutations described all have the effect of increased production of toxic Aβ, described above and also highlighted by research in ADAD cases with PSN mutations. Measurement of CSF showed increases in Aβ42 to Aβ40 production and turnover correlates with plaque formation and reduced Aβ in the CSF compared with healthy controls (Bateman et al., 2012; Potter et al., 2013). Whether it is evidence of overproduction of Aβ

or diminished ability to clear the peptide that underlies the aetiology of AD is still not fully understood but together they both strongly support the role of Aβ in the toxicity of AD.

In the Alzheimer's research field today a wide array of Aβ species have been studied in both in vitro and in vivo models of AD and in the last fifteen years it has become evident that it is the soluble oligomeric species and not the fibrillar form found in plaques is most likely responsible for its toxicity. This is supported not only by the fact that soluble oligomers better correlate with disease than insoluble fibrils and plaques (Lue et al., 1999; McLean et al., 1999) but also that they're more toxic (Deshpande, 2006; Lesné et al., 2008). Although the exact mechanism of how Aβ causes toxicity is not entirely understood it has been shown that Aβ's toxicity is dependent upon its aggregation state. Soluble oligomers are defined as Aβ assemblies that are not pelleted from physiological fluids by high speed centrifugation (Haass and Selkoe, 2007). Early work on Aβ analogues determined that only peptides that include part of the transmembrane sequence form stable aggregates at pH 7.4 that are SDS-resistant (Burdick et al., 1992). Structural-activity studies refined the region required for stable aggregates to the highly hydrophobic 29 – 35 amino acid region of Aβ and demonstrated that toxicity in neuronal cultures was dependent on the formation of aggregates (Pike et al., 1993). Subsequent experiments with synthetic Aβ peptides demonstrated that neurotoxicity is due to soluble, prefibrillar forms of Aβ (Roher et al., 1996; Lambert et al., 1998; Hartley et al., 1999). Further agreement that oligomeric species of Aβ are the toxic form in AD comes from research showing levels of soluble Aβ and not plaques, consisting of fibrillar Aβ in the human AD brain, strongly correlate with the severity of dementia (McLean et al., 1999; Wang et al., 1999). Of the synthetic peptide preparations Aβ-derived diffusible ligands (ADDLs) have been the most extensively studied (Lambert et al., 1998). The molecular weights of these preparations are consistent with oligomers ranging from trimers through to 24mers (Lambert et al., 1998; Gong et al., 2003). Memory loss is thought to be a failure of the synapse caused by soluble Aβ (Lacor, 2004), by modelling synaptic plasticity in vitro with hippocampal cells from rodents, it has been shown that

ADDLs can inhibit long term potentiation (LTP), a model system for synaptic strengthening and memory (Lambert et al., 1998; Walsh et al., 2002). Several other soluble Aβ preparations have been described, these include prefibrillar oligomers of approximately 80 kDa and generally termed AβOs (Kayed et al., 2007). Aβ*56 (Aβ star 56) oligomers, named after their apparent size, were purified from brains of transgenic mice and were shown to induce memory impairment in young rodents (Lesné et al., 2006). Annular Protofibrils

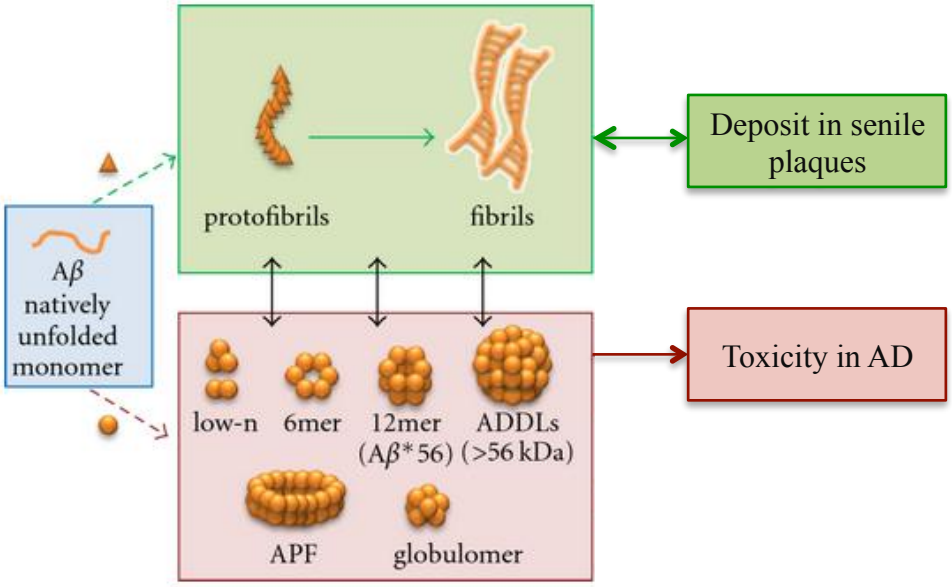



**Figure 1-5 Aβ Aggregation Pathways**. Monomeric forms of Aβ, themselves non toxic or neuroprotective (Giuffrida et al., 2009), undergo conformational changes leading to either protofibrils then insoluble fibrils which deposit in plaques or to toxic soluble oligomers. Theses oligomeric species include; low-n oligomers such as dimers, trimers and tetramers, hexameric and dodecameric (Aβ*56) forms, Aβ-derived diffusible ligands (ADDLs) and annular protofibrils (APFs). In the brain there exists a dynamic equilibrium between insoluble and soluble pools of Aβ with plaques contributing to toxic soluble forms of Aβ in the ISF (Hong et al., 2011). Adapted from (Rushworth and Hooper, 2011).

(APFs) are circularized non-fibrillar Aβ species over 90 kDa that resemble a class of pore-forming bacterial toxins (Lashuel et al., 2002; Kayed et al., 2009). Their morphology is consistent with a membrane-permeabilising activity previously proposed for Aβ (Glabe and Kayed, 2006). Finally Globulomers, a 60 kDa Aβ species, can be prepared synthetically and antibodies raised against them were shown to stain Aβ deposits in the brains of AD patients transgenic and AD mice

(Barghorn et al., 2005). These globulomers were shown to bind neuronal processes specifically and block LTP in rat hippocampal slices (Figure 1-5).

### 1.4.4 Aβ and Oxidative Stress

Oxidative stress has been widely described in the literature as a major feature of AD. Resulting from an imbalance in pro-oxidant and anti-oxidant homeostasis, oxidative stress leads to the upregulation of reactive oxygen species (ROS) such as hydrogen peroxide, nitric oxide, superoxide and highly reactive hydroxyl radicals. The oxidative stress observed in the AD brain results from increased levels of protein carbonyls, nitration of tyrosine residues, lipid peroxidation and nucleic acid oxidation (Butterfield et al., 2006). As the brain is the most aerobically active organ, utilising 20% of total oxygen in an individual, perturbations to the tightly regulated oxidative metabolism as seen in the AD brain has serious implications. The formation of ROS comes from the reaction of molecular oxygen with the redox metals, Copper ($Cu^{2+}$) and Iron ($Fe^{3+}$) (Halliwell and Gutteridge, 2007). Aβ through its redox activity can reduce $Cu^{2+}$or $Fe^{3+}$ directly producing hydrogen peroxide ($H_2O_2$) setting up conditions for Fenton-type chemistry. A biological electron donor is required in $H_2O_2$ production and its production can be attenuated by metal chelation (Opazo, 2002; Barnham et al., 2003; Ciccotosto et al., 2004; Puglielli et al., 2005). In vitro studies have confirmed the ability of Aβ to generate ROS with Aβ $1\text{-}42_{human}$ generating more than Aβ $1\text{-}40_{human}$ which in turn generates more than Aβ $1\text{-}40_{rat}$, this correlates with the involvement of these peptides in amyloid toxicity (Huang et al., 1999). Oxidation of Aβ can also result from its coordination and reduction of $Cu^{2+}$. One site of this oxidation is the sulfur atom of Methionine-35 (Met-35) as determined by mass spectrometry (Nishino and Nishida, 2001), and initial cell based assays with synthetic Aβ mutants of Met-35, that increased cell binding, showed increased toxicity (Barnham et al., 2003; Ciccotosto et al., 2004). Interestingly, recent in vivo studies with transgenic mouse models have shown the requirement for Met-35 of Aβ for oxidative stress but that this is neither required nor sufficient for memory abnormalities (Butterfield et al., 2010). Of the

other adducts that can be formed tyrosine at position 10 on the Aβ is of particular interest as its conjugated aromatic ring makes it susceptible to free radical attack. Reacting Aβ42 in the presence of $Cu^{2+}$ and $H_2O_2$ forms dityrosine cross-linked oligomers (Atwood et al., 2004). These oligomers are resistant to proteolytic degradation and the dityrosine linkage facilitates further aggregation resulting in higher order oligomers (Barnham et al., 2004a) (Barnham et al., 2004b; Jomova et al., 2010).

### 1.4.5 Aβ Toxicity and Metals

The toxicity and aggregation state of Aβ is modulated to varying degrees by the metal ions Zinc ($Zn^{2+}$), $Cu^{2+}$ and $Fe^{3+}$. These transition metals are highly concentrated in the AD brain and concentrated further in amyloid plaques (Lovell et al., 1998). Studies by Bush et al. in the nineties demonstrated that these metals rapidly precipitate Aβ in a pH-dependent manner. Initially $Zn^{2+}$ was shown to induce formation of protease-resistant aggregates at low micromolar concentrations (Bush et al., 1994a; 1994b). This effect was not observed with peptides with the rodent's sequence and was found to be due to changes in the peptide's physiochemical properties bought on by substitutions at positions 5 (Arg to Gly), 10 (Tyr to Phe) and 13 (His to Arg) of the sequence (Bush et al., 1994a). Zinc's binding to Aβ in humans is mediated by histidine at position 13 and this substitution most likely confers resistance to aggregation seen in rodents (Bush et al., 1993; Liu et al., 1999). Cu and Fe, normally exist in biology bound to proteins such as ceruloplasmin and ferritin but under mildly acidic conditions, as is observed in AD or head injury they are released from metalloproteins and cause aggregation of Aβ (Atwood et al., 2000). Metal chelation reverses this aggregation in vitro (Huang et al., 1997; Atwood et al., 1998) and in vivo (Cherny et al., 2001) and increases solubilization of the aggregates in human AD brain tissue compared with age-matched controls (Cherny et al., 1999).

With the clear role that metals have in modulating Aβ aggregation defining the metal binding site on Aβ was of particular interest. It was initially

shown that there are 2.5 metal binding sites on Aβ (Atwood et al., 2000) and nitrogen ligands from the three histidines at position 6, 13 and 14 and an oxygen ligand mediate $Cu^{2+}$ and $Zn^{2+}$ binding (Curtain, 2001; Curtain et al., 2003). Subsequent spectroscopic studies proposed conflicting structures for the metal binding site(s) which electron paramagnetic resonance (EPR) experiments have now resolved. These indicated that there is not a single structure of coordination to Aβ but instead a interconverting ensemble of structures (Drew et al., 2009a; 2009b).

### 1.4.6 Receptor-mediated Aβ Toxicity

It is unclear how Aβ induces neuronal degeneration at present. In support of a specific membrane interaction as opposed to indiscriminate pore-formation in the membrane (Glabe and Kayed, 2006), is recent research that has identified several receptors as binding partners implicating receptor-mediated toxicity in AD.

One such receptor that has been shown to bind Aβ is Alpha 7 nicotinic Acetylcholine receptor (α7nAchR). Pyramidal cells in the cerebrocortex have an abundant expression of α7nAChR, which is reduced in the AD-affected brain (Burghaus et al., 2000) and bind with exceptionally high affinity to Aβ42 (Wang et al., 2000). In cell culture this binding has been shown to facilitate internalization and accumulation of exogenous Aβ1-42 but not Aβ1-40 (Nagele et al., 2002). In a transgenic model of AD (3xTg-AD mice), that exhibits robust plaques and tangles, treatment with an α7nAChR agonist had no effect on the pathology in the mice but completely restored cognition to the same level of their age-matched controls (Medeiros et al., 2014).

The actions of advanced glycation endproducts (AGEs) through their receptor, RAGE, can explain many of the pathological features of AD such as protein cross-linking and oxidative stress. Accumulation of these AGEs is accelerated in AD compared with normal aging (Luth, 2004) and experiments with increased expression of RAGE suggested its involvement in mediating Aβ

toxicity on neurons and microglia (Yan et al., 1996) (Takeuchi and Yamagishi, 2008; reviewed in Srikanth et al., 2009; 2009).

The pan neurotrophin receptor, p75NTR has been shown to be another candidate as it has been reported to directly bind Aβ and mediates its toxicity by inducing apoptosis in cell lines (Yaar et al., 1997) (Coulson, 2006; Coulson et al., 2009). Along with p75NTR the TrkA receptor is concomitantly activated with increased protein expression and phosphorylation in the early stages of Aβ42 treatment (Bulbarelli et al., 2009). These primary rat hippocampal cells underwent apoptosis and resulted in activation of the Akt/ GSK-3β pathway.

#### *1.4.6.1 PrP and Aβ*

Over the last two decades research has strongly implicated the role of Prion protein (PrP) in synaptic toxicity. PrP knockout mice showed impaired spatial learning (Criado et al., 2005), increased LTP and increased excitability (Collinge et al., 1994). They also displayed an elevated susceptibility to neuronal damage that was reduced by the NMDA antagonist, MK801 (Rangel et al., 2007) suggesting a role in excitotoxicity (refer to 1.6). More recently and extending on this research, experiments with PrP-ablated mice exhibited enhanced and prolonged NMDA-evoked currents due to upregulation of NMDA receptors. This resulted in an increase in neuronal excitability and enhanced excitotoxicity in vitro and in vivo (Khosravani et al., 2008).

Evidence of direct interaction between PrP and Aβ was provided the following year by library screens against Aβ42 in which the two positive clones isolated were both full-length PrP clones (Laurén et al., 2009). Interestingly it was demonstrated in this research that other cell surface molecules bind Aβ42 as immunofluorescence measurements showed only a 50% reduction to punctate binding of Aβ42 in PrP-ablated mice. By , antibodies directed against PrP to block Aβ42 binding and rescue synaptic plasticity further evidence was provided to support the role of PrP. This has been demonstrated with both ADDL preparations of Aβ42 (Laurén et al., 2009; Freir et al., 2011) and with SDS-stable Aβ42 dimers isolated from water-soluble extracts from human AD brains (Freir et

al., 2011). These dimers from AD brains were also injected in to rat brains demonstrating an in vivo rather than in vitro effect on LTP that could be prevented by immunodepletion of PrP (Barry et al., 2011).

Two recent publications propose mechanisms in which Aβ42, PrP and the NMDA receptor (NMDAR) are required for toxicity. Resenberger and colleagues show cell surface localized $PrP^{C}$ can mediate toxic signaling of β-sheet-rich conformers of completely different origin in a structural rather than sequence specific manner. The toxic signaling is dependent on the GPI-anchor of PrP and can be inhibited by the NMDAR antagonist, Memantine (Resenberger et al., 2011). Another mechanism by which NMDAR is linked to both PrP and Aβ42 is implied by experiments using neurons with an inactivated form of PrP, mice with high levels of natural Aβ42 due to over-expressing five familial mutations in APP (5XFAD), synthetic Aβ42 or $Cu^{2+}$ chelation. In each of these cases an increase in glycine affinity for the NR1 subunit of NMDARs was observed. This in turn resulted in excitotoxicity by the slowing of NMDA desensitization. In their proposed model, under normal conditions, $Cu^{2+}$-bound PrP reduces glycine affinity to the NMDA receptor complex, enhancing desensitization and reducing toxic influx of calcium (You et al., 2012).

Lastly, research to further elucidate the involvement of PrP and Aβ has demonstrated a requirement for the low-density lipoprotein receptor-related protein 1 (LRP1), an important transmembrane protein required for $Cu^{2+}$-mediated PrP internalization, in the toxicity of Aβ oligomers. This work also demonstrated the necessity for lipid rafts in the binding of Aβ oligomers to PrP (Rushworth et al., 2013).

All of the preceding Aβ binding partners have strong evidence suggesting a potential role in the toxicity observed in AD but for over a decade now there has been extensive evidence accumulating supporting the role of the glutamatergic system and more specifically, the N-methyl-D-aspartic acid (NMDA) receptor, as the principle target for toxic forms of Aβ. Supporting this role are findings that Aβ inhibits LTP (Lambert et al., 1998; Walsh et al., 2002)

and that this inhibition is NMDA-receptor (NMD-R) dependent (Cullen et al., 1997; Hsia et al., 1999; Raymond et al., 2003).

## 1.5 The Glutamatergic System

Glutamate is the primary excitatory neurotransmitter and is present at around 0.6 μM in the synaptic cleft at resting conditions (Bouvier et al., 1992). Synaptic transmission occurs when localised concentrations of L-Glu are increased to greater than 10 μM for 1 – 2 ms (Clements et al., 1992). Glutamate uptake by synaptic and post-synaptic cells terminates this excitatory action (Danbolt, 2001). Glutamate acts on two types of receptors in the synaptic cleft; metabotropic glutamate receptors (mGluRs), a G-protein coupled receptor type and ionotropic receptors. The latter are ligand-gated ion channels located at the post synapse. Named after their agonists that bind them preferentially, they are kainate, α-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) (Alzheimer, 2003).

### 1.5.1 Metabotropic Glutamate Receptors

mGluRs are dimeric complexes and members of the G-protein-coupled receptor (GPCR) family that mediate intracellular signaling, upon interactions with glutamate, by activation of heterotrimeric G-proteins. They are divided in to three groups based on sequence homology and intracellular signaling mechanisms. Group 1 comprise of mGluR1 and 5 that are positively coupled to phospholipase C (PLC) and primarily located post synaptically in the hippocampus. Group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8) receptors are negatively coupled to andenylate cyclase, inhibit cyclic AMP (cAMP) with Group II receptors localised mainly to the presynapse (Manahan-Vaughan, 1997). Targeting group II mGluRs has been suggested as a potential therapeutic approach based on an observed increase in production of the more amyloidogenic form of Aβ, Aβ42 following activation (Thathiah and De Strooper, 2011). mGluR1 and 5 appear to play a more significant role in AD and this is believed to be due to the $Ca^{2+}$-mediated intracellular signaling cascades that are

utilized upon activation of both receptor subtypes. Selective antagonism has implicated both mGluR1 and 5 receptors in LTP and spatial memory (Naie and Manahan-Vaughan, 2004; 2005). Furthermore recent research has indicated a co-receptor role of the mGluR5 receptor providing a link to Aβ and downstream toxic events at the postsynaptic density (Um et al., 2012).

### 1.5.2 Kainate receptors

Often grouped together with AMPA as non-NMDA inotropic receptor family members, kainate receptors (KARs) were initially implicated in epileptogenesis (Meldrum and Garthwaite, 1990). The subsequent development of AMPAR-selective antagonists and knockout mice has provided a better understanding of their functional role. As with its fellow inotropic family members, KARs exists as homo- or hetero-tetrameric complexes comprised of the subunits; GluK1 to 3 and 4 or 5. They're expressed ubiquitously throughout the brain and are located at both the pre and postsynapse. Currently functional roles that are attributed to KARs involve mediating synaptic transmission at the postsynapse and presynaptically, the modulation of neurotransmitter release, both of which contribute to a role for the receptors in established forms of plasticity such as non-NMDA LTP in the hippocampus (reviewed in Sihra et al., 2014). Research in to the involvement of KARs, specifically in AD, is relatively sparse. A review of the literature indicates that in AD patients there is reduced expression of KARs in the hippocampus and this is increased in the cerebral cortex, suggesting their possible involvement (Matute, 2010).

### 1.5.3 LTP

The importance of the glutamatergic system in AD is highlighted by the critical role it has in modulating the induction of long-term potentiation of synaptic transmission in neurons (Figure 1-6). In 1949 Hebb postulated that "When an axon of cell A is near enough to excite cell B or repeatedly or persistently takes part in firing it, some growth process or metabolic change

takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Hebb, 1949). Experimental proof of this was published in 1973 (Bliss and Lomo, 1973). Today this process of long-lasting enhancement in signal

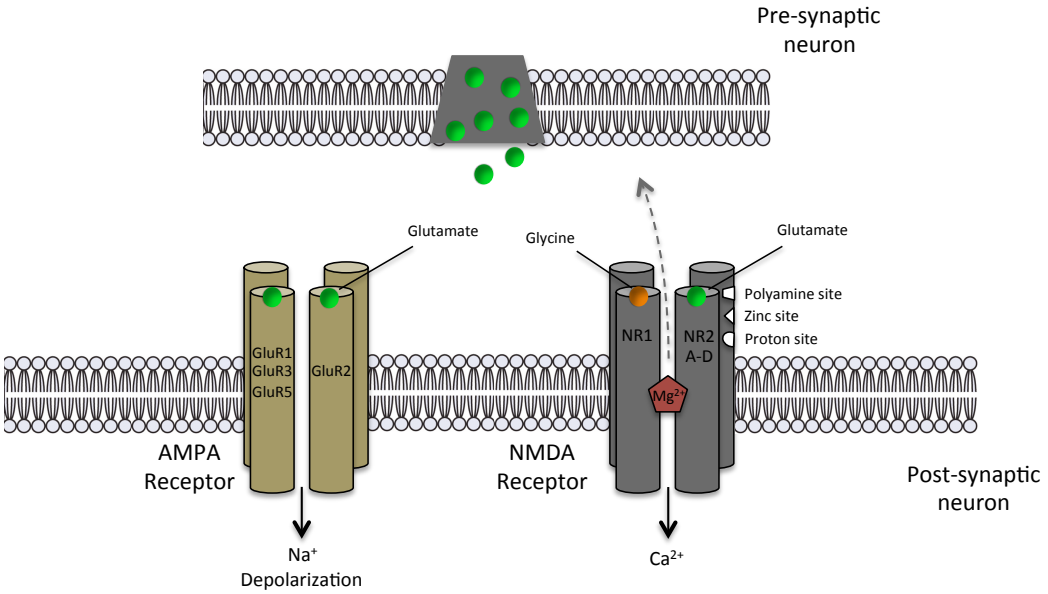



**Figure 1-6 Long Term Potentiation – Strengthening of Synaptic Transmission.** Glutamate is released in to the synaptic cleft where it binds the post-synaptic receptor, AMPA and along with the co-agonist, glycine, it binds the NMDA receptor. Given a pre-synaptic electrical signal of sufficient strength and frequency the corresponding prolonged activation of the AMPA receptor by glutamate results in depolarization of the post-synaptic neuron. The magnesium then withdraws from the NMDA receptors and allows large numbers of calcium ions ($Ca^{2+}$) to enter the cell that activate downstream proteins. This leads to enhanced transmission by the promotion of AMPA receptors in to the post-synaptic membrane.

transmission between two neurons that results from stimulating them synchronously is known as Long Term Potentiation (LTP) (Cooke, 2006) and has been strongly implicated in the neuronal functions of memory and learning (Riedel, 2003).

### 1.5.4 AMPA receptors

The ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptors (AMPARs) are mainly found as tetrameric complexes containing a dimer of dimers of GluA2 and GluA1, GluA3 or GluA4 (also termed GluR1-4) (Derkach et al., 2007). These receptors are involved in the majority of

fast excitatory synaptic transmission in the CNS and their modulation, along with NMDARs, is believed to be the crucial in neuronal plasticity. This can be seen in the pivotal role they play in the enhancement of signal transmission observed in LTP. An action potential travels along the axon and causes the release of glutamate from the presynapse. Upon glutamate activation at the postsynapse AMPA receptors open to allow intracellular flux of sodium ($Na^+$). LTP will result when there is sufficient depolarization due to glutamate concentration and $Na^+$ influx to depolarize the cell and remove the magnesium block of the NMDA receptor by electrostatic repulsion allowing a large influx of $Ca^{2+}$ (Figure 1-6). The connection between the neurons is further strengthened by a $Ca^{2+}$-mediated increase in trafficking of AMPARs from an adjacent non-synaptic pool to the synaptic membrane in early phase LTP. In late phase LTP there is a $Ca^{2+}$-mediated increase in gene transcription and protein expression of AMPARs in the postsynapse (Frey and Morris, 1997; Malenka and Bear, 2004). It has also been demonstrated that the increase in synaptic transmission in LTP results from increasing the single channel conductance of AMPARs (Benke et al., 1998). Subsequent research has demonstrated that both forms of modification to AMPAR function is achieved by the insertion of $Ca^{2+}$-permeable AMPARs resulting with increased insertion leading to an increase in overall conductance (Plant et al., 2006).

The subunit composition of AMPARs has been implicated in AD through early experiments that sought to detect differences in AMPAR subunit expression in the hippocampus and entorhinal cortex of normal controls and AD brains. Collectively they showed a marked reduction in GluR1 and GluR2/3 (Armstrong et al., 1993; García-Ladona et al., 1994). Regions of the hippocampus considered “vulnerable” to AD pathology (CA1 and subiculum) were found to have lower levels of GluR1 and GluR2/3 subunits in AD patients which was not observed in regions that are “resistant” (CA2/3 and dentate gyrus) (Ikonomovic et al., 1995). In these experiments total levels of AMPARs were analysed which do not account for cellular localisation and therefore receptor functionality as seen in LTP. This is a possible explanation for earlier experiments which found no significant differences in mRNA expression in the hippocampus of normal and AD

brains (Pellegrini-Giampietro et al., 1993). The importance of the AMPAR subunit composition was highlighted in preceding experiments that demonstrated expression of an edited form of the GluR2 subunit confers $Ca^{2+}$-impermeability. (Hollmann et al., 1991; Hume et al., 1991). The edited form of GluR2 is the only form present in the mature brain and therefore its expression could play a significant role in the cells ability to gate calcium and maintain intracellular $Ca^{2+}$ homeostasis and also confer protection against glutamate-induced excitotoxicity (Burnashev et al., 1992). Later research has provided evidence for a role for AMPARs in AD through its down regulation through endocytosis. AMPARs have been shown to be down regulated by endocytosis resulting from Aβ-induced long-term depression (LTD) (Hsieh et al., 2006). The early-expression activity-regulated cytoskeletal (Arc) gene has also been shown to reduce the activity of neuronal networks by mediating AMPAR endocytosis (Wu et al., 2011).

### 1.5.5 The NMDA receptor

The NMDARs are a subclass of ionotropic receptors gated by glutamate with high calcium ($Ca^{2+}$) permeability. Along with AMPARs they are the major receptors required for LTP induction, and therefore play an essential role in memory, learning and synaptic transmission (Bear and Kirkwood, 1993; Bliss and Collingridge, 1993). NMDARs consist of obligatory NR1 subunits and combinations of two to three NR2A-D or NR3A-B subunits (reviewed in Paoletti et al., 2013; 2013). In the hippocampus and cortex, complexes composed of NR1/NR2A or NR1/NR2B subunits are the predominant form and indicate the importance of these subunits in synaptic plasticity (Monyer et al., 1994). The function of the NMDARs is tightly regulated with evidence of several mechanisms contributing to the levels of calcium entering the cell. Phosphorylation/ dephosphorylation of serine/ threonine and tyrosine residues of NMDAR subunits is well documented and effects the gating, permeability to $Ca^{2+}$ and cell surface expression of NMDARs reviewed in (Van Dongen et al., 2009). The expression, trafficking and subunit composition of NMDARs provides a central mechanism in the control of NMDAR function. With neuronal

development NMDARs are increasingly localised synaptically to postsynaptic densities (PSDs) and consist predominantly of NR2A subunits but a significant

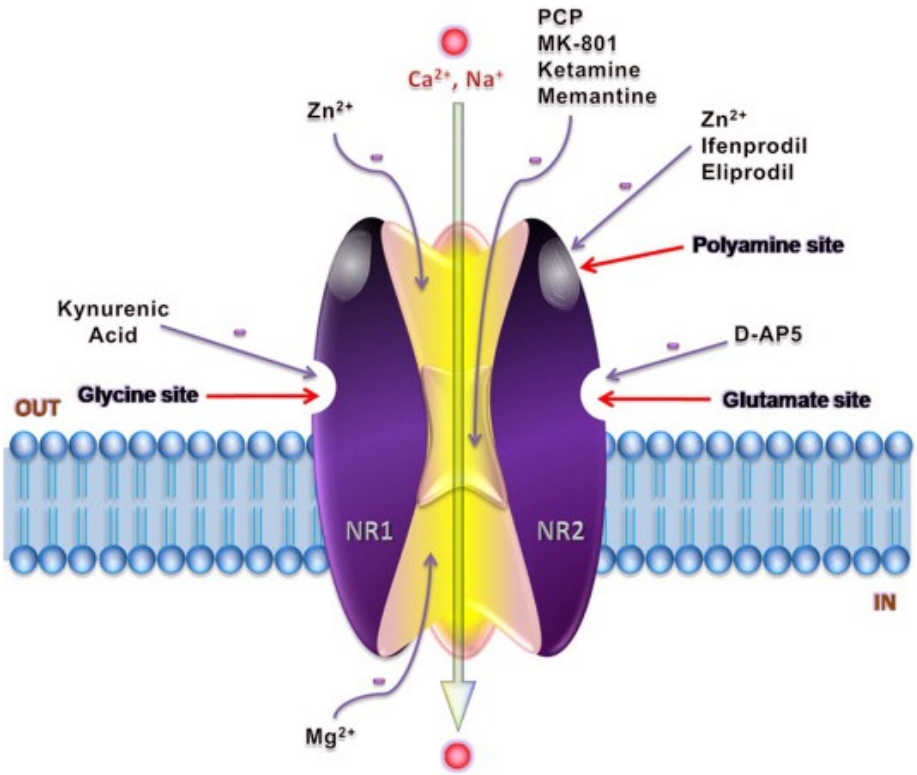



**Figure 1-7 The NMDA receptor complex. A tetrameric complex consisting of obligatory NR1 subunits and varying expression of NR2A-D subunits or, to a lesser extent, NR3A-B subunits. Multiple binding sites on the complex include glutamate, glycine, polyamine, $Mg^{2+}$ and $Zn^{2+}$. The antagonists, Dizocilpine (MK-801) and Memantine have a non-competitive action with binding sites within the ion channel where as Ifenprodil has NR2B-selectiove action. At higher concentrations $Zn^{2+}$ no longer acts as an agonist through its n-terminal binding site on NR2A subunits but can inhibit NMDAR activity (see section 1.6.2). Adapted from** (Ghasemi and Schachter, 2011).

population remain located extrasynaptically (Tovar and Westbrook, 1999) and are NR2B-containing NMDAR complexes (Wittmann et al., 2004). Up to 65% of these complexes located in the synapse are not anchored but can move laterally in to the extrasynaptic pools (Tovar and Westbrook, 2002). Subsequent research showed that these dynamic receptor complexes were largely composed of NR2B subunits which are highly mobile and move in and out of extrasynaptic pools unlike NR2A subunits, which are stable in the synapse (Groc et al., 2006). The movement of the NR2B receptor complexes from extrasynaptic pools specifies one way in which NMDAR levels are regulated in the post synaptic membrane. Another form of NMDA regulation was demonstrated by research utilising

NMDAR antagonists, which showed NMDAR activation to highly regulate NMDAR levels in the membrane and this to be NR2A subunit-specific effect with NR2B complexes unaffected. Taken together these results describe a cellular environment in which NR2B subunits are the main components found extrasynaptically and the NR2A subunits remain located at the synapse and are regulated by NMDAR activity.

## 1.6 Excitotoxicity

NMDARs are dichotomous in nature and preceding their involvement in cell survival was research in to the toxicity resulting from their excessive activation. It was first identified in the inner layers of retinal neurons in 1957 (Lucas and Newhouse, 1957), this receptor-mediated form of cell death was termed excitoxicity and found to occur throughout the brain and was not just limited to the retina as initially described (Olney, 1969). By definition, excitotoxicity is

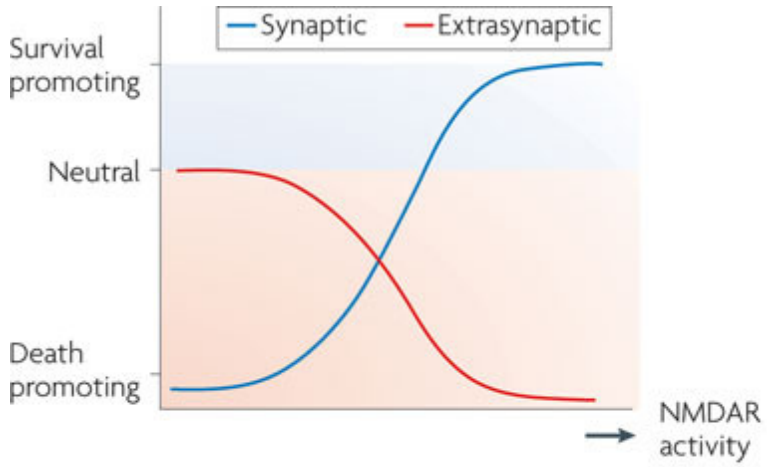



Figure 1-8 Model of opposing effects on neuronal survival mediated by synaptic and extrasynaptic NMDAR activity. Increased synaptic NMDAR activity leads to activation of neuroprotective pathways and cell survival, the opposite is observed due to increased activation of extrasynaptic receptors. Reduced activity of synaptic receptors is neurotoxic but survival is not effected by low level activation of extrasynaptic NMDARs. Adapted from (Kleckner and Dingledine, 1988; Hardingham and Bading, 2010).

the prolonged and enhanced activation of NMDARs by an excitatory amino acid EAA), that results in an excessive influx of $Ca^{2+}$ and neuronal injury or death. In neuronal excitotoxicity this EAA is glutamate, as it is the major neurotransmitter of the central nervous system (Choi et al., 1988; Dong et al., 2009). It was the formative work by Choi et al. that established the intracellular flux of $Ca^{2+}$ to be the key mediator of the glutamate-induced neurotoxicity in the late 1980s (Choi, 1985; 1987; Choi et al., 1987; Johnson and Ascher, 1987). Subsequent experiments demonstrated $Ca^{2+}$ flux was mediated by NMDARs by the use of specific ionotropic antagonists (Choi et al., 1988; Hirai et al., 1996). The excessive influx of $Ca^{2+}$ triggers a range of intracellular responses resulting in cell death, including increased oxidative stress, inappropriate activation of proteases, dysregulation of $Ca^{2+}$-related pathways, mitochondrial damage and an apoptotic cascade.

### 1.6.1 Excitotoxicity and Glycine

Using primary mouse cortical neurons it was shown that glycine potentiates the NMDAR response (Johnson and Ascher, 1987; Mayer et al., 1989) and further studies using Xenopus oocytes expressing NMDARs observed that it is a requirement for NMDAR activation (Kleckner and Dingledine, 1988; Mayer et al., 1989). Subsequent site-directed mutagenesis studies by Hirai and colleagues identified the glycine recognition site located on NR1 subunit with the glutamate recognition site on subunit NR2 (Hirai et al., 1996; You et al., 2012). It acts as a co-agonist with glutamate and regulates the NMDA receptor in a concentration-dependent manner by increasing peak current amplitude and slowing the uncoupling or desensitization (Mayer et al., 1989; Frederickson et al., 2006). This is an intrinsic mechanism to protect neurons against prolonged agonist activation and subsequent toxic influx of $Ca^{2+}$. Of important note for experimental design is that this effect can be masked in vitro by $Ca^{2+}$ influx which can trigger secondary mechanisms leading to glycine-resistant desensitization (Pérez-Clausell and Danscher, 1985; Mayer et al., 1989).

More recently the ability of glycine to regulate NMDAR activity by its desensitization has been shown to be involved in Aβ toxicity. In this work You et al. demonstrated the ability of soluble Aβ, decreased $Cu^{2+}$ and the inactivation of $PrP^{c}$ to induce non-desensitising NMDAR currents. By using low and normal physiological glycine concentrations (0.1 & 0.3 μM), as opposed to the higher concentrations (10 – 50 μM) used in most investigations of NMDAR activity, they have observed a potential physiological role for $Cu^{2+}$ and $PrP^{c}$ in neuronal protection under excitotoxic conditions (Peters et al., 1987; Westbrook and Mayer, 1987; You et al., 2012).

### 1.6.2 Excitotoxicity and Zinc

Presently $Zn^{2+}$ is known to be abundant in synaptic boutons and its levels rise to high micromolar concentrations in the synaptic cleft during activity (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Paoletti et al., 2000; Frederickson et al., 2006) indicating a strong link to synaptic transmission. Initially this link was determined through ultrastructural studies localising $Zn^{2+}$ to synaptic vesicles (Pérez-Clausell and Danscher, 1985; Frederickson et al., 2005). Experiments that followed in cortical and hippocampal neurons found low micromolar concentrations of $Zn^{2+}$ act as a voltage-independent, non-competitive agonist of NMDA responses unlike $Mg^{2+}$, which acts a channel blocker. These results provided the first evidence of a direct receptor-mediated action (Peters et al., 1987; Westbrook and Mayer, 1987; Lee et al., 2000). Following cloning of the NMDAR and its subunits a high affinity $Zn^{2+}$ binding domain was mapped the to the N-terminal domain (NTD) of NR2A preceding the glutamate binding domain. $Zn^{2+}$ inhibition of the binding site is only partial at saturating concentrations in a potential mechanism that would allow excessive NMDA activity in a high $Zn^{2+}$ environment. Another paradoxical effect of $Zn^{2+}$ is a delayed increase in agonist activation of NMDARs through tyrosine phosphorylation of NR2A and NR2B. The NTD of NR2B but not NR2C and NR2D, also forms a $Zn^{2+}$ binding site, albeit of much lower affinity (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Paoletti et al., 2000; Cote et al., 2005). $Zn^{2+}$ has been implicated in excitotoxicity but the

role it plays is not clearly understood. It has been proposed that $Zn^{2+}$ released from presynaptic vesicles to post synaptic neurons contributes to zinc-induced cell injury in excitotoxicity in several models of brain injury (Frederickson et al., 2005; Deshpande et al., 2009). Studies in $ZnT_3$ knockout mice suggested this not to be the case as these mice completely lacked $Zn^{2+}$ in synaptic vesicles indicating that it had originated from other sources (Trombley and Shepherd, 1996; Lee et al., 2000). A more likely explanation is that it is $Zn^{2+}$ levels that determines whether its effect is neurotoxic or neuroprotective. Research by Cote et al. demonstrated that the action of intracellular $Zn^{2+}$ is cell-type specific and concentration-dependent with low levels providing protection and high levels causing cell death (Vlachová et al., 1996; Cote et al., 2005). Following this work, research by Desphande et al. proposed an alternative mechanism that links $Zn^{2+}$ with the NMDAR through Aβ42 oligomers to the toxicity in AD. Immunofluorescence experiments in rat and mouse hippocampal slices and primary human cortical neurons found that synaptic activity enhanced oligomer formation and localization at synaptic sites. This targeting was reduced upon treatment with Clioquinol, a member of the quinoline class of drugs with $Zn^{2+}$ chelating properties, and in mice deficient in the zinc vesicular transporter ZnT3. In their work Desphande et al. (2009) propose that increased levels of $Zn^{2+}$ as a result of synaptic activity increased Aβ42 oligomerization due to the peptide's metal binding properties, which results in increased toxicity mediated by the NMDAR by multiple mechanisms. These included direct interaction between Aβ oligomers and the receptor, altering permeability of neuronal membranes or the activation of the mitochondrial death pathway (Schlief, 2005; Deshpande et al., 2009).

### 1.6.3 Excitotoxicity and copper

$Cu^{2+}$ plays a vital role in the central nervous system as illustrated in Menkes disease, a fatal disease characterised by a loss of hippocampal neurons resulting from the loss of function of mutations in a Cu-transporting ATPase required for cellular $Cu^{2+}$ homeostasis. Several groups have published evidence

of $Cu^{2+}$ involvement in synaptic signaling regulated by the NMDAR through varying mechanisms of interaction. Whole cell recordings of rat olfactory bulb neurons, a region with high concentrations of $Cu^{2+}$, demonstrated for the first time that $Cu^{2+}$ has an inhibitory effect on NMDAR and importantly that this was masked by desensitising concentrations of glycine (>30 μM) (Trombley and Shepherd, 1996; Hardingham et al., 2002). Published the following month was work with free $Cu^{2+}$ that showed it to act as a non-competitive antagonist that preferentially binds glutamate/glycine-bound NMDARs. Using whole cell recordings of hippocampal neurons they demonstrated that $Cu^{2+}$ has no effect when complexed to either glutamate or glycine (Vlachová et al., 1996; Schlief et al., 2006). Schlief, Gittlin and colleagues in 2005-6 provided a better understanding of the cellular mechanisms involved in $Cu^{2+}$ homeostasis in two publications focusing on Menkes ATPase (Atp7a). In their first publication they provided evidence of a mechanism for control of Atp7a trafficking by the activation of NMDAR and subsequent exocytic efflux of $Cu^{2+}$. They suggest that Atp7a gathers $Cu^{2+}$ in readily available intracellular pools for exocytosis upon NMDAR activation and the resulting $Ca^{2+}$ influx (Gibson and Peterson, 1987; Schlief, 2005). Atp7a trafficking was induced only by synaptic or total cellular and not extrasynaptic NMDARs which suggested the possibility that it was acting through a pro-survival pathway, referred to earlier, involving synaptic NMDARs and CREB (Koh et al., 1990; Hardingham et al., 2002). In their subsequent publication Schlief et al. went on to demonstrate the neuroprotective capability of $Cu^{2+}$ (Schlief et al., 2006). In hippocampal neurons under glutamate/glycine-induced excitotoxic conditions, co-treatment with $Cu^{2+}$ showed total rescue of cell death. Experiments with the addition of zinc chloride ($ZnCl_2$), nickel chloride ($NiCl_2$) and the depletion of $Fe^{3+}$ by a metal chelator showed the protection to be $Cu^{2+}$-specific and inhibition by l-nitroarginine demonstrated the necessity for nitric oxide production for the $Cu^{2+}$-mediated protection. In order to observe the protective effects of $Cu^{2+}$ in vivo, functionally null Atp7a mice were used. These mice showed impaired trafficking of $Cu^{2+}$ upon NMDAR activation and an increased sensitivity to excitotoxic conditions that could be ablated by treatment with $Cu^{2+}$ prior to, or during the insult. In these experiments intracellular, rather

than extracellular, $Cu^{2+}$ is neuroprotective and the findings provide a direct link between NMDAR-mediated excitotoxicity and impaired $Cu^{2+}$ homeostasis (Mattson et al., 1992; Schlief et al., 2006).

### 1.6.4 Aβ-mediated Excitotoxicity in Alzheimer's disease

The involvement of calcium homeostasis and NMDA signaling in AD was first recognized in the late 1980s (Gibson and Peterson, 1987; Walsh et al., 2002) and was followed by work in mouse cortical neurons which demonstrated the enhancing effects of pretreatment with Aβ on both glutamate and NMDA-induced excitotoxicity. This effect of Aβ was dependent on both concentration and the duration of exposure (Koh et al., 1990; Snyder et al., 2005). To overcome the limitation of the rodent model that does not develop symptoms of AD, Mattson and colleagues performed extensive studies with human cerebral cortical neurons at 14 – 16 week gestation. Their work using the 1-38 and 25-35 synthetic Aβ peptides confirmed the effect of Aβ on glutamate-induced excitotoxicity. Experiments monitoring intracellular $Ca^{2+}$ levels with the fluorescent dye, Fura-2 and depleting media of $Ca^{2+}$ demonstrated that calcium flux was required for the neuronal damage elicited by the co-treatment with glutamate and Aβ peptides (Mattson et al., 1992; De Felice et al., 2007).

It remains unclear as to whether NMDARs are activated by the direct or indirect action of Aβ. Contrasting research has demonstrated that Aβ binds to or in close proximity to NMDARs (De Felice et al., 2007), requires NMDARs for binding to neurons with no direct interaction (Decker et al., 2010) and can have a secondary effect on NMDAR activity through altered synaptic composition (Roselli, 2005). Research to date has proposed various mechanisms in which Aβ exerts its effect on the NMDA receptor. Supporting an indirect effect is research showing Aβ promoted endocytosis of NMDA receptors causing toxicity by inhibiting normal signaling through the receptor. Exposing primary neurons to cultured media from an over-expressing N2A cell line similar to previous work by Walsh and Selkoe (Walsh et al., 2002; Snyder et al., 2005), demonstrated a time-dependent decrease in surface receptor expression of NR1. Treatment with a γ-

secretase inhibitor to decrease Aβ levels was able to restore surface expression (Roche et al., 2001; Snyder et al., 2005). In contrast, research using ADDLs to treat hippocampal neurons induced excessive formation of reactive oxygen species (ROS). This effect was could be blocked by anti-ADDLs and anti-NR1 antibodies and the NMDAR antagonist, Memantine, but did not impact on NMDAR-induced ROS generation. Results from co-immunoprecipitations suggest that the induction of ROS by ADDLs is due to an increase in intracellular calcium (De Felice et al., 2007; Ittner et al., 2010).

Phosphorylation of the NR2B subunit is another mechanism implicated in mediating Aβ excitotoxicity. STEP, a tyrosine phosphatase that has been shown to regulate NMDAR, is dephosphorylated and activated by Aβ treatment which subsequently increases dephosphorylation of tyrosine 1472 (Y1472) on the NR2B receptor (Snyder et al., 2005; Li et al., 2011). This is of particular importance as it resides in a region involved in NMDAR endocytosis and binding to the synaptic scaffolding protein (PSD95) (Roche et al., 2001). Further evidence for the role of NR2B in Aβ-mediated excitotoxicity is described in research involving the Src kinase, Fyn. In this work a mechanistic link between Tau and Aβ toxicity was established by demonstrating an uncoupling of the NMDAR/PSD95 complex with normal phosphorylation due to a disruption to targeting of Fyn in Tau mutant and knockout mice (Ittner et al., 2010). The uncoupling of the NMDAR/PSD95 complex by both the Tau mice and peptide-targeted perturbation was shown to reduce excitotoxic seizures and Aβ toxicity, respectively. In subsequent work this pathway was further elucidated by research that detailed Aβ activation of Fyn mediated through PrP binding. Oligomeric Aβ isolated from AD brains resulted in neurotoxic signaling through the phosphorylation of the NR2B subunit. This activation led to a transient excitotoxic increase in cell surface NR2B followed by dendritic loss, decreased surface receptors and increased cell death (Um et al., 2012). The role of extrasynaptic NR2B-containing NMDARs is given more support by research showing that oligomers of Aβ inhibit LTP. In this work toxicity occurs due to a glutamate “spill-over” from the synapse to the extrasynaptic receptors that results from Aβ oligomers inhibiting the astrocytic reuptake of glutamate (Li et al., 2011). Supporting this work was the finding that Aβ acts on astrocytic

α7nAchRs to stimulate excessive glutamate release resulting in increased intracellular calcium and nitric oxide levels. This in turn led to dendritic spine loss which could be prevented by the NMDAR-antagonist, Memantine (Talantova et al., 2013).

## 1.7 Preventing Excitotoxicity in AD

Currently only two types of drugs have been approved for the treatment of AD. Acetylcholinesterase inhibitors were the first of these to be prescribed and treat mild to moderate AD with varying degrees of efficacy. Clinical trials with the current acetylcholinesterase inhibitors; Aricept (donepezil), Razadyne (galantamine) and Exelon (rivastigmine) have demonstrated significant but marginal improvement in cognition (Birks, 2005; Raina et al., 2008). One meta data analysis has questioned the scientific basis for their use in treating AD at all, arguing the methodology used in available trials was poor and the level of improvement in patients, although significant, fell well below the minimum level proposed by the FDA as a clinically important effect (Kaduszkiewicz et al., 2005).

NMDA antagonists are the second class of drugs for the treatment of AD of which Namenda (memantine) is the only member with current approval. Memantine is indicated for the treatment of moderate to severe AD but often incorrectly prescribed for patients with mild AD (Schneider et al., 2011). NMDARs have become an appealing therapeutic target over the last two decades as the body of evidence supporting their role in neurodegeneration has increased. Prior to testing in AD, clinical trials of NMDAR antagonists had been performed in the acute neurodegenerative diseases of stroke and traumatic brain injury (TBI), with antagonists to the glutamate site (selfotel), the glycine site (gavestinel), the ion channel site (aptiganel) and the NR2B subunit (traxoprodil). Each of these trials failed to demonstrate a therapeutic effect with the trials of selfotel and aptigangel being ended prematurely due to trends toward high mortality (Muir, 2006; Kalia et al., 2008 for review; 2008). Memantine was originally designed as an antidiabetic by Eli Lily in 1963 but was ineffective at lowering blood sugar. Merck later showed efficacy in Parkinson's disease (PD) but it was in 1989 that it

was shown to act as NMDA antagonist (Kornhuber et al., 1989). It is believed that memantine avoids the significant side effects of other NMDA antagonists due to being a low-affinity, uncompetitive antagonist that avoids blocking normal physiological activity of NMDARs necessary for normal function. In the proposed mechanism of action for memantine, the drug is described as open-channel blocker with a fast off rate and this property enables the drug to be cleared from the receptor ion channel following excessive glutamate activation so as to not block critical normal function of the receptor (Lipton, 2005; 2006). In the years to date further research has provided better understanding of how the drug can be well tolerated yet still act to block NMDA-mediated excitotoxicity. It has now been demonstrated that memantine acts specifically on extrasynaptic NMDARs, the NR2B-containing receptors, preventing excitotoxic cell death (Hardingham et al., 2002; Xia et al., 2010; Talantova et al., 2013).

## 1.8 Hypothesis, Aims and Research Questions

### 1.8.1 Hypothesis

That beta amyloid peptide underlies the aetiology in Alzheimer's disease and causes gradual cell death through excitotoxicity, which results from increased concentrations of the peptide and metals in the synapse and subsequent dysregulation of the neurotransmitter, glutamate. The regulation of metal levels in the synapse could therefore provide a novel therapeutic target in the treatment of Alzheimer's disease.

### 1.8.2 Aim 1: Describe the role of metals in protection against excitotoxicity

- Does PBT2 treatment affect glutamate-induced excitotoxicity?
- Is this a metal-mediated event?
- How can metals affect excitotoxicity?

To answer these questions mouse primary cortical neurons will be employed to establish an in vitro model of excitotoxicity. Subsequent questions will investigated by using metal chelators and the reintroduction of specific divalent cations known to be at high concentrations in the synapse.

### 1.8.3 Aim 2: Characterise the Parameters Required for Aβ Toxicity in Cortical Neurons

- Does Aβ Aggregation time or protein expression resulting from the number of days in vitro (DIV) of the neurons contribute more to toxicity?
- What type of toxicity results from Aβ treatment?
- Are metals involved in this toxicity?

Mouse primary cortical neurons will be employed and the effects of DIV and Aβ Aggregation on toxicity will be observed by cell viability assays and compared with the expression of key proteins implicated in Aβ toxicity. Subsequent questions will investigated by the use of antagonists to determine receptor involvement and metal chelators against specific divalent cations known to be at high concentrations in the synapse.

### 1.8.4 Aim 3: Develop a Neural Assay to Screen Varying Aβ Peptides

- Robust and reproducible.
- Sensitive – can detect varying levels of toxicity.
- Short lag time – rapid Aβ42 oligomerization.

This assay will be developed in mouse primary cortical neurons taking advantage of their functioning synapses in order to better replicate neurodegeneration in the human brain. It will hope to be a medium to high throughput assay with an immediate readout of Aβ toxicity, which once validated on wild-type Aβ42 can be used to screen different forms of the peptide associated with AD.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals

PBT2 was a gift provided by Prana Biotechnology Limited. 6-cyano-7-nitroquinozaline-2,3-dione (CNQX), Dizocilpine (MK-801) and Memantine Hydrochloride were purchased from Sigma Aldrich (Australia) and dissolved in $H_2O$. Zinc Chloride ($ZnCl_2$) (Sigma Aldrich, Australia) was used dissolved in $H_2O$. Diamsar, (1,8-diamino-3,6,10,13,16,19-hexaaza-bicyclo(6,6,6)eicosane) was prepared as described previously (Bottomley et al., 1994).

### 2.1.2 Primary culture

Poly-D-lysine stock (100X) (Sigma P-0899):

Dissolve 25 mg poly-D-lysine in 50 mL dd$H_2O$. Filter sterilised (F/S) through 0.22 µm filter and stored in 5 mL aliquots at -20°C. A working stock of 0.5 mg/mL (in dd$H_2O$) final concentration was then made.

AraC (Cytosine β-D-arabinofuranoside, C1768 Sigma):

0.5 mg/mL stock. F/S through 0.22 µm filter and stored in 50 µl aliquots at -20°C.

Trypsin (20x) (Sigma T-4665) (~7500 units/mg):

25 mg in 10 mL 1x Krebs, F/S through 0.22 µm filter and stored in 0.75 aliquots at -20°C.

DNaseI/SBTI (10x) (Sigma D-5025; Sigma T-9003):

8 mg DNaseI, 26 mg SBTI, dissolved in 10 mL 1x Krebs, F/S through 0.22 µm filter and stored in 0.5 mL aliquots at -20°C.

10 x Kreb's stock (500 mL):

36.25 g NaCl 2.0 g KCl; 0.7 g $NaH_2PO4.H_2O$; 13.0 g D-glucose; 0.05 g phenol red; 29.7 g HEPES acid (Sigma-Aldrich). Dissolved in 450 mL $ddH_2O$. Adjusted pH to 7.4, made up to 500 mL, F/S through 0.22 µm filter, stored at 4°C.

1X Kreb's stock (500 mL):

50 mL 10x Krebs stock; 446 mL dH2O; 1.5 g BSA; 4 mL 3.85% $MgSO_4$ (Sigma-Aldrich). Adjusted pH to 7.4; F/S through 0.22 µm, stored at 4°C.

Hanks Balanced salt solution (HBSS) (500 mL):

10 mL 10x HBSS; 88 mL ddH20; 1 ml 0.3 M Hepes. 100 µL gentamycin (10 mg/mL). pH to 7.4; F/S 0.22 µm, at 4°C.

Plating Media (500 mL)

345 mL $ddH_2O$; 50 mL 10 x MEM; 5 mL 200 mM glutamax; 14.7 mL 7.5% $NaHCO_3$; 0.5 mL gentamycin (10 mg/mL); F/S (0.22 µm); Add filtered and heat inactivated 50 mL FCS, 25 mL Horse Serum, stored at 4°C.

NB Culture Media (#1 and #2) (50 mL):

NB#1: 50 mL Neurobasal media; 1 mL B27 supplement; 50 µl Gentamicin (10 mg/mL); 125 µL 200mM glutamax; Stored unused media at 4°C. Discarded if not used within 14 days.

NB#2: (Following at least 4 DIV): 50 mL Neurobasal media; 1 mL B27 supplement (+/- Antioxidants); 50 µL Gentamicin (10 mg/mL); 50 µL 2mM AraC; Stored unused media at 4°C. Discarded if not used within 14 days.

All cell culture reagents from Life Technologies unless otherwise stated

## 2.2 Methods

### 2.2.1 Preparation of Aβ

Human Aβ peptides were synthesised by the W. M. Keck Laboratory (Yale University, New Haven, CT) unless otherwise stated. Aliquots of lyophilised Aβ peptides were weighed in $N_2$-purged microfuge tubes under zero-static conditions by use of an anti-static gun (Zerostat 3, Milty). Aβ stock solutions were prepared by dissolving lyophilised peptide to 1 mM in HFIP (10 mg/ 2.217 mL) and incubating at room temperature for 30 min in 1.5 mL microfuge tubes with the lids closed. At 30 min the peptide solutions had become clarified and the lids were then opened to air-dry overnight in a fume-hood; a cover was placed above the tubes to prevent aerosolised particulates from entering the tubes. The following day the peptide films were further dried by vacuum centrifugation on a low heat for 20 min and stored at -80°C until use.

For Aβ treated with $NH_4OH$, peptides were dissolved directly in to 10% w/v $NH_4OH$ at 0.5 mg/mL and lyophilised following 10 min at RT, 5 min sonication in 1. 5 mL microfuge tubes and as with HFIP-treated Aβ, stored at -80°C until used

For fresh, HFIP or $NH_4OH$ Aβ the lyophilised peptides were dissolved to 5 mg/mL in NaOH (20 mM) and incubated at RT for 5 min to break down aggregated material. The solution was then diluted to 1 mg/mL in $ddH_2O$ and 10 x PBS (PBS is defined as 50 mM sodium phosphates, 2.7 mM KCl, 137 mM NaCl) at a v/v/v ratio of 2:7:1. The preparation was sonicated for 10 min in a ice water bath and then centrifuged at 16,500 x g for 10 min at 4 °C. Supernatants (upper 90 % of solution) were taken and kept on ice for immediate use. Peptide concentrations were determined by measuring the UV spectrometry absorbance values (at 214 nm) and calculated with molar extinction coefficients predetermined by amino acid analysis (Aβ40 91,462; Aβ42 94,526; Aβ3-42 91462 and Aβ3pE42 90925) (McColl et al., 2009) and using the Beer-Lambert Law ($A = \varepsilon.b.c$, where A = absorbance, ε = molar extinction coefficient, b = path-length of light and c = concentration).

### 2.2.2 Primary neuronal culture

Mouse primary cortical neuronal cultures were prepared under sterile conditions. On the day prior to isolations (or 2 h at 37°C) plates were coated with poly-D-lysine to enable adherence of neurons. 15 min before plating neurons, poly-D-lysine was removed and plates air-dried with lids partially removed.

#### *2.2.2.1 Hippocampal isolation*

On the day of isolation embryonic day 17 C57BL/6J mouse cortices were removed, dissected free of meninges in Krebs buffer and dissociated in 0.025% trypsin (w/v) (Sigma) in HBSS. The dissociated cells were triturated using a pipette tip, allowed to settle at the bottom of the tube, aspirated and resuspended in HBSS containing trypsin inhibitor and DNase I. The cells were then allowed to again settle to the bottom before aspirating off buffer and resuspending in plating media resuspended in plating medium (minimum Eagle's medium, 10% fetal calf serum, 5% horse serum), and counted. Hippocampal neuronal cells were plated into poly-D-lysine coated 48-well at a density of 150,000 cells/well in 200 μL plating medium/well in the inner 24 wells of the plate and dd$H_2O$ in the outer wells to avoid evaporation.

#### *2.2.2.2 Cortical isolation*

Embryonic day 14 C57BL/6J mouse cortices were removed, dissected free of meninges, and dissociated in 0.025% trypsin (w/v) (Sigma) in Krebs buffer (124 mM NaCl, 5.1 mM KCl, 1.0 mM $NaH_2PO_4.H_2O$, 14.4 mM D-glucose, 0.001% phenol red (w/v), 25 mM HEPES (4-(2 Hydroxyethyl)piperazine-1-ethanesulfonic acid), 0.3% BSA (w/v), 2.6 mM $MgSO_4$, pH 7.4). The dissociated cells were triturated using a pipette tip, pelleted, resuspended in plating medium (minimum Eagle's medium, 10% fetal calf serum, 5% horse serum), and counted. Cortical neuronal cells were plated into either poly-D-lysine coated 48-well or 96-well plates. In 48-well plates cells were at a density of 150,000 cells/well in 200 μL plating medium/well in the inner 24 wells of the plate and dd$H_2O$ in the outer wells to avoid evaporation. In 96-well plates cells were at a density of 225,000 cells/ well

in 100 μL plating medium/well in wells in rows B – G of the plate and $ddH_2O$ in the outer wells of rows A and H. All cultures were maintained in an incubator set at 37°C with 5% $CO_2$. After 2 hours, the plating medium was replaced with fresh Neurobasal medium containing B-27 supplements, gentamicin, and 0.125 mM glutamax (NB#1) (all tissue culture reagents were purchased from Life Technologies unless otherwise stated). Following 6 days *in vitro* (DIV) media was replaced with medium containing B-27 supplements minus antioxidants, gentamicin and cytosine-β-D-arabinofuranoside (to halt glial growth) (NB#2). This method resulted in cultures highly enriched for neurons (>95% purity) with minimal astrocyte and microglial contamination (Ciccotosto et al., 2004).

### 2.2.3 Cell Viability Assays

Cell survival was monitored by phase contrast microscopy, and cell viability was quantitated using the Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies Inc.). The CCK8 is a colourimetric assay that uses the water soluble tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) which has a higher detection sensitivity than competing tetrazolium salts; MTT, XTT, MTS or WST-1. WSTs form an orange formazon end product following extracellular reduction directly dependent on NAD(P)H-dependent oxidoreductase enzymes in the cytosol and therefore is a measure of mitochondrial activity. It is advantageous to MTT as the formazon product is formed extracellularly and therefore less toxic to cells (Berridge et al., 2005).

In performing the assay, the medium on neurons was replaced with fresh Neurobasal medium (NB#2) supplemented with B27 lacking antioxidants, and 10% v/v CCK-8 was added to each well and incubated for 3 hours at 37 °C in a 5% $CO_2$ incubator. Plates were gently shaken, and a 150-μl aliquot from each well was transferred to separate wells of a 96-well plate. The colour change of each well was determined by measuring the absorbance at 450 nm using a FLUOstar OPTIMA plate reader (BMG Laboratories), and background readings of CCK8 incubated in cell-free medium were subtracted from each value before

calculations. The data were normalized and calculated as a percentage of untreated vehicle control values. Data are represented as mean ± standard error means.

### 2.2.4 Toxicity assays

Neurons were cultured between 6 to 15 days in vitro (DIV) in NB#1 (Life Technologies) with media changes at 6 days. To observe Aβ toxicity, freshly prepared synthetic Aβ peptide stock solutions (200 μM, see 2.2.1) and were diluted to the final concentration in the neurobasal medium (Life Technologies). Cell viability was measured using Cell Counting Kit 8 (Dojindo, Japan).

### 2.2.5 Caspase Assays

Neurons were cultured for 9 DIV as described above (see 2.2.2). In order to determine caspase activity as a readout of apoptosis the Caspase-Glo® 3/7 Assay was employed (Promega). This is a luminescent reagent, which lyses cells releasing intracellular caspases. The reagent contains a substrate that upon caspase cleavage releases aminoluciferin, a substrate for luciferase, which results in the production of light through the two-step luciferase reaction.

*(i)* *Luciferin + ATP → luciferyl andenylate + $PP_i$*

*(ii)* *luciferyl andenylate + $O_2$ → oxyluciferin + AMP + light*

Light is emitted because the reaction forms oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state (Gould and Subramani, 1988).

In the experiments performed here, cell lysates were harvested as described below (see 2.2.9) from samples treated for 48 h, unless otherwise stated, with vehicle, Aβ40 or Aβ42 (10μM). Staurosporine treatment for 4h prior to harvesting was used as a positive caspase 3 activity control (2 μM). Then 50 μL of Caspase-Glo 3, 7 reagent, made fresh or at 4°C, as per manufacturer's instructions, added to 50 μl of lysate per well of a 96-well, white-walled, clear

bottom T/C plate with or without 5 μM caspase 3 inhibitor, Z-DEVD-FMK (Calcbiochem). White-walled plates were necessary to avoid cross-talk between wells and maximize luminescence. The lysate/ reagent solution is then agitated for 5 min at RT to ensure thorough mixing and then incubated at RT for a further 30 - 40min to allow luminescence to reach a maximum. A FLUOstar OPTIMA plate reader (BMG Laboratories) was used to measure then luminescence with no filter set chosen.

### 2.2.6 Excitotoxicity assays

Neurons were cultured for 9 DIV as described above (see 2.2.2). All drugs and glutamate were diluted in water. Neuronal cultures were pretreated with PBT2 (3 μM) or Memantine (10 μM) for 6 h and (MK-801) (10 μM) for 20 min prior to 1 h excitotoxic exposure to glutamate (50 μM). Cell viability was measured using Cell Counting Kit 8 (Dojindo, Japan) following 18 h in fresh neurobasal media. Metals were depleted by addition of Diamsar (10 μM), a cell impermeable metal chelator, to neurobasal media ≥ 1 h prior to treatments. $ZnCl_2$ and $CuCl_2$ were dissolved in water and added to neurons with PBT2.

### 2.2.7 Calcium flux assays

Neurons were cultured as described above (see 2.2.2) and then loaded with Fluo4-NW dye (Life Technologies) according to the manufacture's recommendations. Briefly, cells were incubated for 30 min in complete Fluo-4 loading solution (containing HBSS loading buffer, Fluo-4 dye reagent and probenecid) at 37°C followed by a 30 min incubation at RT. To measure fluorescence the inner 72 wells of 96-well plates were measured taking 19s for each cycle. To establish background ($F_0$) measurements were averaged for the 10 cycles preceding injection of glutamate. The $11^{th}$ cycle, the first post injection, was used for fluorescence ($F_1$), which was measured by a FLUOstar OPTIMA plate reader (excitation at 490 nm, emission at 520 nm) (BMG Laboratories) with values expressed as the difference in fluorescence over initial fluorescence

($\Delta F_1/F_0$). In PBT2 assays neurons were pretreated with the drug at 3μM for 6h prior to injection of 100 μM Glu/10 μM Glycine. Treatment with Diamsar at 10 μM to remove metals was performed for 1 h prior to PBT2 treatment

### 2.2.8 Fluorometric analysis of *in vitro* Aβ aggregation

Analysis of self-aggregation of the Aβ peptide a modified protocol was employed (Hortschansky et al., 2005). Solutions of 5 μM Aβ peptide in PBS, pH 7.4, were incubated at 37 °C in a 96-well black-walled microtitre plate (Greiner) with 20 μM thioflavin-T (ThT, Sigma) at a volume of 150 μL for 40 h. Measurements of ThT binding to aggregated Aβ were obtained using a FLUOstar OPTIMA plate reader (BMG Laboratories) and measuring fluorescence at 482 nm (excitation = 450 nm) with a 475 nm emission cut-off filter. Data points were collected in 5-min intervals via bottom reading, with each cycle consisting of 5 s of orbital shaking immediately followed by the fluorescence measurement. Plates were sealed with acetate adhesive seals (MP Biomedicals) to minimize evaporative loss. ThT binding was represented as the mean relative fluorescent units from n = 6 replicate wells following subtraction of the vehicle background fluorescence.

### 2.2.9 Protein harvesting and quantitation

Following treatments, cultures in 6 or 48-well plates (Nunc) at 150,000 neurons/mL were washed three times with ice cold 1 x PBS and then lysed by adding 120 μL (48-well) or 200 μL (6-well) lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 0.1% Triton-X) and 1% protease inhibitor mix set 1 (Calbiochem) to each well. After 20 min ice shaking protein was harvested by scraping with either a pipette tip (48-wells) or cell scraper/rubber policeman (6-wells).

Protein concentrations of proteins were performed in duplicate or triplicate by using a BCA protein assay kit (Pierce, Thermo Scientific). This colourimetric assay employs the biuret reaction where $Cu^{2+}$ forms a complex

with proteins in an alkaline environment and is reduced to $Cu^{+}$. The reagent, bicinchoninic acid (BCA) reacts with $Cu^{+}$ to form a purple-colored end product that absorbs strongly at 562 nm in a linear fashion with increasing protein. Standard curve were generated for each assay with 2 mg/mL bovine serum albumin (BSA) with concentrations of 0 (blank), 31.75, 62.5, 125, 250, 500 and 1000 μg/mL. Samples and protein, in 25 μL cell lysis buffer, were made up to 200 μL with BCA assay reagent made up at 1:49 as per manufacturer's instructions. Absorbances of samples were read at 562 nm following 1 h at RT. Concentration was determined from the slope of the graph from the standard absorbances after taking in to account dilution factors.

### 2.2.10 Biotinylation assay of cell surface receptor expression

Cortical mouse neurons were cultured as described above (see 2.2.2) for 9 DIV in 6-well plates at 2.25 x $10^6$ cells/ well. Treatments with PBT2 (3 μM), PBT2 (3 μM) + Diamsar (10 μM) and PBT2 (3 μM) + Diamsar (10 μM) + $Zn^{2+}$ (7μM) were performed for 0, 1, 3 and 6 h in fresh NB#2 media. Diamsar was added 1 h prior to and during PBT2 and/ or $Zn^{2+}$ treatments where indicated. Following treatments, cells were washed twice with ice-cold PBS (+ $Ca^{2+}$, $Mg^{2+}$) and incubated with 1 mL sulfo-NHS-SS-biotin at 1 mg/mL (Pierce, Thermo Scientific). This performed at 4°C to prevent internalization and trafficking of receptors at the cell surface. Unreacted biotinylation reagent was quenched and removed with two washes with ice-cold TBS followed by two ice-cold PBS washes and then harvested as described above (see 2.2.9)

Protein concentrations for each samples were determined by BSA assays and 50 μg of protein (from soluble lysates), made up to same volume as most dilute sample with 1 x PBS, were mixed with 50 μL of streptavidin beads (BcMag) at 4°C rocking for O/N. Samples were placed into magnetic separator for 2-3 mins to separate supernatants containing the nonbiotinylated proteins. The remaining pellets containing streptavidin conjugated beads were washed five times with ice-cold 1 x PBS (+ $Ca^{2+}$, $Mg^{2+}$). Biotinylated (surface) proteins were eluted from the beads with 50 μL 4 x Laemmli buffer containing 50 mM

DTT (i.e. 25 μL 1M DTT + 125 μL 4 x Laemmli buffer, 350 μL 1 x PBS) and heated at 56°C for 30 min to reduce disulphide bonds. Surface (Surf) and total (Tot) proteins were analysed using anti-AMPA (GluR2/3/4) and NMDA (NR1, NR2A and NR2B) antibodies (adapted from De Felice et al., 2009; 2009).

### 2.2.11 Poly-acrylamide gel electrophoresis (PAGE) and western blotting

Cell lysates were separated by SDS-PAGE using 4-12% Bis-Tris gels (Criterion, Bio-rad and NuPage, Life Technologies) according to the manufacturer's instructions. Western blots were performed on total cell extracts (20 μg/lane) harvested from mouse cortical neuronal cultures described above (see 2.2.2). Briefly, samples were heated to 90 °C for 5 min in NuPage LDS sample buffer (Life Technologies) and 2% beta-mercaptoethanol (v/v), is added to reduce disulfide bonds, then electrophoresed at 150 V for 60 min. The gels were transferred to pre-assembled nitrocellulose membrane stacks using a Trans-Blot® semi-dry transfer apparatus (Bio-rad). Blots were blocked in TBST (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% Tween-20) containing 5% skim milk. Primary antibodies were incubated on blots overnight at 4 °C. HRP-conjugated rabbit anti-mouse (Dako) was diluted 1:10,000 in TBST and incubated for 1 -2 h at room temperature. All antibodies were diluted in TBST containing 0.05% sodium azide as a preservative. Blots were washed 3 x 5 min in TBST after each primary and secondary antibody-binding step, and ECL (Immobilon, Millipore) was used to detect the chemiluminescence signal. The images were captured and analysed by densitometry using a ChemiDoc MP imagining system (Bio-Rad). Stain free gels (Bio-Rad) were used and in-gel activation allowed for protein normalization to control for loading. Comparisons to housekeeping proteins commonly used as loading controls; beta actin and GAPDH, produced unchanged results and the stain-free technology is thought to be a more robust and reliable control method (Gürtler et al., 2013).

### 2.2.12 Antibodies

Primary antibodies were as follows: polyclonal rabbit anti-GluR2/3/4 (2460), polyclonal anti-GSK3α (9338), monoclonal mouse anti-phospho (Ser21) - GSK-3α (9337), monoclonal rabbit anti-GSK3β (9315), polyclonal rabbit anti-phospho(Ser21/Ser9) GSK3α/β (9331) (all at 1:5000 dilution, Cell Signaling Technology); polyclonal rabbit anti-Calcineurin A (ab3673) (1:1000 dilution, Abcam); rabbit polyclonal anti-NR1 (G8913) and anti-NR2A (G9038) (1:2000 dilution, Sigma); rabbit polyclonal anti-NR2B (06-600) (1:2000 dilution, Millipore). Secondary antibodies were from mouse or rabbit and conjugated to Horseradish Peroxidase (GE Healthcare, UK). Proteins were visualized using Immobilon chemiluminescent HRP substrate (Millipore, Australia) on Amersham Hyperfilm XP (GE Healthcare UK). Western blot data were quantified by densitometric analysis of at least three different blots per experiment.

## 2.3 Multielectrode Array Experiments

Neocortex was dissected from C57BL/6 pups (postnatal days 1-3) under sterile conditions. Cortices were cut into pieces of about 1 $mm^3$, prior to dissociation using papain and trituration. Cells were plated at 5000 cells/$mm^2$ on standard 8 x 8 titanium arrayed MEAs (Multi Channel Systems, Reutlingen, Germany) coated with poly-ethylene-imine (PEI) and laminin. Cultures were maintained for 2–3 weeks prior to recording, in the following medium: high glucose DMEM with 10% Horse Serum, 0.5 mM GlutaMax, 1 mM sodium pyruvate, and 2.5 μg/ml insulin (All from Life Technologies, Australia). Cultures were kept for 21 days in an incubator at 37 °C, 65% relative humidity, 5% $CO_2$, and 9% $O_2$.

The MC_RACK software and the MEA-2100-60-System (Multi Channel Systems, Reutlingen, Germany) were used to record and analyse data. The MC_RACK software was used to detect and record unit activity. Data was gathered from neuronal cultures pre-treated with or without PBT2 (3 μM) for 5 min pre and post exposure to glutamate (100 μM) and glycine (10 μM). A -20 μV threshold was set for the detection of spike and an inter spike interval

detection threshold set to greater than 10 ms to avoid contamination with burst firing. Channels that had a baseline greater than -10 μV were denoted as noisy channels, and were excluded from the data set.

## 2.4 Data Analysis

Densitometric analysis was performed using ImageJ software (Rasband, 1997). All data was normalised to an internal control (vehicle or glutamate only) set at 100%. Differences between groups were evaluated using either one-way, two-way or repeated measures analysis of variance (ANOVA). These analyses were followed by Dunnett's multiple comparisons posthoc tests, paired t-tests or unpaired t-tests, as indicated against data not used for normalising to ensure error is not lost. All statistical calculations were performed using GraphPad Prism (v5.0; GraphPad Software, Inc.) Data are presented as mean ± standard error of the mean (SEM).

# 3 The Role of Zinc in a Glutamate-induced Model of Excitotoxicity

## Preface

The majority of this chapter has been published in the journal, Neurobiology of Disease (see Appendix, section 7.2)

**Figure 3-6** was produced from collaborative research by Timothy Johanssen and Xiang Liu.

**Figure 3-7** was produced from collaborative research by Timothy Johanssen and Nuttawat Suphantarida.

All remaining figures in this chapter have been produced solely from the research conducted by Timothy Johanssen.

## 3.1 Introduction

In the search for an AD therapeutic, the majority of research since the 1990s has focused on decreasing the amyloid load in the brain by targeting plaques that are comprised of aggregated beta-amyloid (Aβ) peptides found extracellularly in the post mortem brain of patients. The plaques are initially deposited in the neocortex and subsequently in the entorhinal cortex and hippocampus (Braak and Braak, 1991). It is believed that the progressive memory deficits associated with AD result from pathological changes in the entorhinal-hippocampal system, a region of the brain crucial for memory formation and the most severely affected in the disease (Hyman et al., 1984). $Cu^{2+}$ and $Zn^{2+}$ levels are increased in this region in AD and found at exceptionally high concentrations in amyloid plaques (Lovell et al., 1998; Dong et al., 2003). Both $Cu^{2+}$ and $Zn^{2+}$ induce the rapid aggregation of Aβ (Bush et al., 1994a) causing fibrillisation and precipitation of the peptide. By exploiting this property Cherny et al. were able to solubilize Aβ extracted from AD brains using metal chelators (Cherny et al., 1999). This led to the screening and selection of the quinoline and quinolone class of drugs from the US Pharmacopoeia that, although not termed metal chelators, displayed chelating properties. As proof of concept an example of these drugs, clioquinol (CQ), was used in transgenic mice experiments resulted in a significant and rapid decrease in Aβ deposition of nearly 50% (Cherny et al., 2001). A subsequent phase II clinical trial with clioquinol exhibited a significant decrease in plasma Aβ (Ritchie et al., 2003). These findings validated the therapeutic targeting of $Cu^{2+}$ and $Zn^{2+}$ in AD and from this the second-generation quinoline compound, PBT2 was developed.

As the mechanism of action of PBT2 was further refined it was hypothesised that the drug prevented the oligomerisation and precipitation of Aβ and therefore the formation of toxic soluble oligomers that had just been described by Walsh and Selkoe et al. (Walsh et al., 2002). It was proposed that PBT2 prevented Aβ aggregation by reducing the high concentrations of $Zn^{2+}$ and $Cu^{2+}$ previously reported to be released in to the synaptic cleft from the pre and postsynapse, respectively (Schlief, 2005; Frederickson et al., 2006) through its

moderate affinity for both the metals. This return to metal equilibrium in the synapse was possible due to the ability of PBT2 to increase intracellular levels of $Zn^{2+}$ and $Cu^{2+}$ through its ionophoric capabilities (Adlard et al., 2008). The effect of the drug in the glutamatergic system was first implicated in this research by Adlard et al. (2008), which demonstrated the ability of PBT2 to rescue Aβ-induced inhibition of long-term potentiation (LTP), a cellular model of memory and learning (Riedel, 2003). The inhibition of LTP, by so-called soluble Aβ oligomers (Lambert et al., 1998; Walsh et al., 2002; Wang et al., 2002; Shankar et al., 2008) provides significant evidence that Aβ toxicity in AD is mediated through the NMDA receptor (NMDAR). The receptor-specificity of the inhibition of LTP by Aβ was further supported by the peptides failure to also inhibit NMDAR-independent LTP (Raymond et al., 2003).

The normal activation of NMDARs, through regulated moderate influx of calcium, is essential for synaptic transmission and initiating an array of cell signaling pathways. Evidence of this has been the failure of NMDAR antagonists in clinical trials of stroke and traumatic brain injury, particularly those of selfotel and aptiganel which were ceased due to safety concerns as both trials trended towards higher mortality rates (Muir, 2006; Kalia et al., 2008). These severe clinical side effects are most likely due to the drugs' near complete block of the NMDAR and subsequent inhibition of the prosurvival pathways reliant on NMDAR activity (Lipton, 2004). The phosphoinositide-3-kinase (PI3) - Akt kinase cascade is one such pathway by which NMDARs are reported to mediate neuroprotective activity. $Ca^{2+}$-dependent activation of PI3 leads to activation by phosphorylation of Akt which in turn inhibits the activity of GSK3β as well as the pro-apoptotic protein BAD and p53 (Hardingham, 2009). Synaptic NMDAR activity has also been shown to induce activity-dependent gene expression and cell survival through activation of the transcription factor, CREB and increased BDNF gene expression in a key finding by Hardingham et al. (2002). In this work $Ca^{2+}$ influx through synaptic NMDARs, led to activation of neuroprotective pathways, whereas $Ca^{2+}$ entry through extrasynaptic NMDARs acted to inhibit the same gene expression involved in cell survival (Hardingham et al., 2002).

An alternative mechanism by which NMDAR signaling through calcium mediates neuroprotection is through induced tolerance. Also referred to as preconditioning, it can be obtained by oxygen-glucose deprivation, chemical induction or electrical stimulus and is a mechanism in which a sublethal concentration or mild stimulation provides a window of protection against a subsequent severe insult. This tolerance is dependent on both concentration and time with the latter indicating that secondary or indirect responses such as gene expression may be required to precondition the cell and provide neuroprotection. Preconditioning was first observed in the canine heart where a series of brief ischaemic episodes protected against a subsequent extended exposure (Murry et al., 1986). The role of calcium in preconditioning was first alluded to in experiments with verapamil, a non-specific $Ca^{2+}$ channel antagonist (Nishi and Berg, 1981) blocked enhanced survival. Importantly the removal of extracellular $Ca^{2+}$ did not alter the effect of the agonist whereas it was negated following chelation of $[Ca^{2+}]_i$ indicating the requirement of internal $Ca^{2+}$ stores (Koike et al., 1989). The involvement of moderate increases in $[Ca^{2+}]_i$ mediated by NMDARs was later described in models of ischaemic and NMDA-stimulated preconditioning in cultured primary neurons (Chuang et al., 1992; Grabb and Choi, 1999). The moderate increase in $[Ca^{2+}]_i$ by synaptic NMDAR activity (Soriano et al., 2006) or by a $Ca^{2+}$ -selective ionophore (Bickler and Fahlman, 2004) resulted in the activation of shared neuroprotective signaling pathway. In both instances this was through activation of the PI3K-Akt pathway, previously shown to promote neuroprotection against apoptotic insults via activation of the (Papadia, 2005). These significant findings present the modulation of $[Ca^{2+}]_i$ as a novel mechanism in which to target in therapeutic design.

The key intracellular calcium sensors; Calpain, a $Ca^{2+}$-dependent cysteine protease, and calcineurin, a $Ca^{2+}$/calmodulin-dependent protein phosphatase play vital roles in normal cellular signaling but also are strongly implicated in $Ca^{2+}$-mediated neuronal death (reviewed in the introduction and (Wu et al., 2007)). Under excitotoxic conditions calpain cleavage generates a constitutively active form of calcineurin which acts through the PI3-Akt kinase pathway and GSK3 to induce caspase activity and neuronal death (Wu et al., 2003; Shioda et

al., 2006; Park et al., 2008). Deregulation leading to increased activity of GSK3, a serine/threonine kinase, plays an important role in facilitating this excitotoxic cell death and is strongly implicated in both sporadic and familial forms of AD (reviewed in (Hooper et al., 2007)). Calcineurin has been shown to activate GSK3 by dephosphorylation at the inhibitory site, Serine 9, and therefore has the potential to disrupt the cellular responses of GSK3 which include targeted protein degradation by phosphorylation of proteins including the key AD protein, Tau (Kim et al., 2009). Calcineurin has also been reported to inhibit the cell survival pathway mediated by the transcription factor, CREB (Lin et al., 2003).

The deregulation of synaptic zinc ($Zn^{2+}$), as with $Ca^{2+}$, has been observed in acute and chronic neurodegeneration (reviewed in (Corona et al., 2011; Vogler and Busciglio, 2014)) and its modulation has been investigated as a therapeutic target for the treatment of both AD and HD. To this end PBT2 has recently been shown to induce $Ca^{2+}$-mediated neuroprotective signaling cascades involving calcineurin, CREB and GSK3 inhibition by chaperoning zinc from extracellular pools into cells (Crouch et al., 2011a; Adlard et al., 2013). PBT2 has been shown to rescue disease phenotypes in transgenic mouse models of both AD (Adlard et al., 2008) and HD, and is currently undergoing clinical evaluation for HD. This finding suggests an alternate underlying mechanism of the drug other than directly reducing Aβ load. To this end excitotoxicity has been implicated in the pathogenesis of both diseases raising the question as to whether protection against excitotoxicity is a common mechanism of action resulting in these therapeutic benefits.

The work presented in this chapter investigates the ability of PBT2 to ameliorate glutamate-induced excitotoxicity in cortical neuronal cultures and delineate a mechanism by which PBT2 elicits its neuroprotective effects. To that end I have focused on the ability of the drug to affect NMDAR-mediated changes in the cell. $Ca^{2+}$ flux-based assays and multielectrode array experiments have been employed as a functional readout of PBT2 treatment on $Ca^{2+}$ signaling. In conjunction with this the activation of key proteins in the neuroprotective pathways have been analysed and together they have demonstrated a novel mechanism by which PBT2 can protect neurons from cell death.

## 3.2 Results

### 3.2.1 The metal chaperone activity of PBT2 is required for its protection against excitotoxicity

Previous research has demonstrated that metals can protect against glutamate-induced excitotoxicity (Schlief et al., 2006) and have either neurotoxic or neuroprotective actions depending on concentration and cell type (Cote et al., 2005). As PBT2 acts as a metal chaperone to significantly increase intracellular levels $Zn^{2+}$ (Crouch et al., 2011a) we sought to further elucidate the drug's mechanism of action investigating whether PBT2 protected primary mouse cortical neurons from glutamate-induced excitotoxicity. Experimental conditions were optimized to induce approximately 50% cell death upon 1-hour treatment with glutamate (40 μM), as neurons at this cell viability showed maximal protection. Following 6 hours pretreatment of neurons with increasing concentrations of PBT2 (1, 3 and 7.5 μM), its maximum protection against excitotoxicity was conferred where no effect on excitotoxicity by PBT2 was observed at 1 h. This was in contrast to MK-801 and Memantine, both direct NMDAR channel blockers, that demonstrated full protection in 1 hour (Fig. 1, A and B). Pre-treatment with PBT2 protected against excitotoxic insults restoring cell viability of glutamate-treated neurons (56 ± 6%) in a dose-dependent manner (glutamate + PBT2 at 0, 3 and 7.5 μM = 59 ± 6%, 87 ± 5% and 88 ± 2%, respectively, $n = 6$, $p < 0.001$) (Fig. 1B). These levels were comparable to the non-competitive NMDA antagonists, MK-801 and Memantine (89 ± 4% and 90 ± 2%, $n = 6$, $p < 0.001$) (Fig. 1A). To test whether the protective effect of PBT2 against excitotoxicity was dependent on its metal chaperone activity, metals were depleted from the cell culture media by 1-hour treatment with $(NH_2)_2$sar (Diamsar) (10 μM) prior to incubation with PBT2 and then glutamate (Fig. 1C). Diamsar is a high affinity metal chelator, unable to permeate the cell membrane (Crouch et al., 2011a). Upon sequestration of metals by Diamsar, glutamate-treated cells (44 ± 6%)were no longer protected by PBT2 (1 μM, 49 ± 9%, 3 μM,

49 ± 9% and 7.5 µM, 53 ± 8%) demonstrating the requirement of metals as co-factors for PBT2 to mediate protection. As expected the NMDA antagonists have

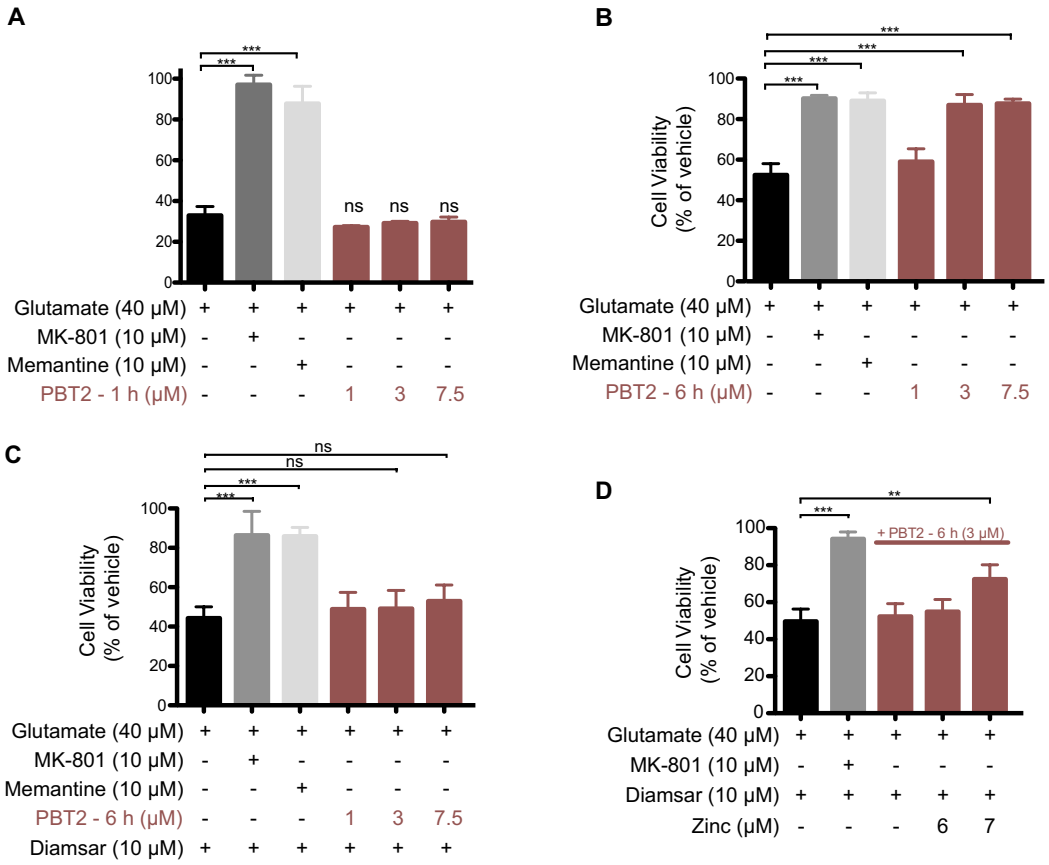



**Figure 3-1. PBT2 protects against glutamate-induced excitotoxicity by its metal chaperone activity.** Mouse primary cortical neurons were treated with the NMDA-specific antagonists, MK-801 (dark grey) and Memantine (light grey) for 1 h and PBT2 at increasing concentrations for 6 h prior to exposure to glutamate (40 µM). Cell viability was measured and results were normalized to the untreated vehicle. ***A,*** The non-competitive antagonists, MK-801 and Memantine prevent excitotoxicity where as 1 h pre-treatment with PBT2 is not sufficient to effect cell viability. ***B,*** 6 h pre-treatment with PBT2 prevents excitotoxicity in a concentration-dependent manner equivalent to the levels of the non-competitive antagonists, MK-801 and Memantine. ***C,*** Removal of metals from neuronal media by pre-treatment with the chelator, Diamsar abolishes PBT2 protection against excitotoxicity. ***D,*** Titrating zinc in to metal-depleted media restores PBT2 protection against excitotoxicity. Data represent mean ± SE; n = 3 - 6; **p < 0.01, ***p < 0.001; ns, not significant compared to glutamate treated values by one-way ANOVA using Dunnett's post hoc test.

no requirement of metals to mediate their effect (MK-801, 86 ± 12% and Memantine, 86 ± 5%, n = 4, $p < 0.01$, $p < 0.001$, respectively) (Fig 1C). To further illustrate the role of metals in PBT2 protection $Zn^{2+}$ was titrated back in to the metal-depleted media and restoration of protection by PBT2 against excitotoxicity was observed ($Zn^{2+}$ at 7 μM, 50 ± 7% to 72 ± 8%, n = 5, $p < 0.05$) (Fig 1D). Given that previous ICP-MS analysis had detected 3 – 5 μM $Zn^{2+}$ in neurobasal media, the 7 μM $Zn^{2+}$ supplemented back in to the metal-depleted media was sufficient to overcome chelation by 10μM Diamsar.

### 3.2.2 PBT2 significantly reduces NMDAR-mediated $Ca^{2+}$ flux.

MK-801 protects against excitotoxicity by directly acting on NMDA receptors preventing the excessive influx of $Ca^{2+}$ required for NMDA-mediated cell death (Stout et al., 1998). To investigate whether PBT2 protects by this mechanism we measured intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) flux in cultured mouse cortical neurons using the Fluo-4 dye. Neurons were pre-treated with vehicle, MK-801 (10 μM), PBT2 (10 μM) for 1 hour (Figure 3-2A) and 6 hours (Figure 3-2B) prior to dye loading and treatment of cells with a concentration of glutamate and the co-agonist, glycine (100 μM/ 10 μM) to ensure maximum $Ca^{2+}$ flux (light grey line). Background levels of fluorescence were established prior to the injection of glutamate/glycine treatment. By treating for 1 hour we sought to determine whether PBT2 immediately antagonized the NMDA receptor as with MK-801. Upon 1-hour treatment with MK-801, glutamate-induced $Ca^{2+}$ flux was significantly attenuate by 48.5% (51.5 ± 7.5%, n =4, $p < 0.001$). The remaining $Ca^{2+}$ flux can be attributed to recovery from MK-801 blockade during extended agonist exposure (McKay et al., 2013) and to a lesser extent, extrasynaptic NMDRs, non-NMDA ionotropic receptors and metabotropic receptors. Treatment for the same duration with PBT2 however resulted in a reduction in $Ca^{2+}$ flux of only 16% (84 ± 4.5%, n = 4, $p < 0.001$) (Figure 3-2A). In previous excitotoxicity experiments it was observed that PBT2 required 6 hours for

maximal protection with no effect seen at 1 hour, indicating a second messenger mediated event. When neurons were pre-treated for 6 hours with MK-801,

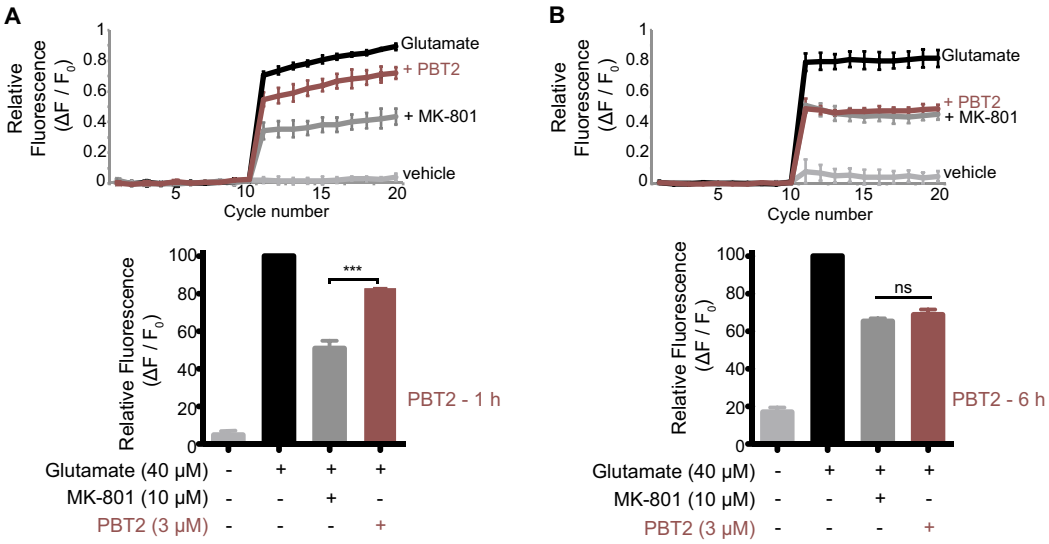



**Figure 3-2. The time-dependent effect of PBT2 on $Ca^{2+}$ flux through the NMDA receptor.** Intracellular $Ca^{2+}$ levels in cortical neurons preloaded with the $Ca^{2+}$ indicator Fluo4, were measured upon stimulation by glutamate/glycine (100 µM/10 µM) and reading fluorescence at 490 nm excitation and 520 nm emission. The top panels of ***A*** and ***B*** show representative traces with measurements taken at t = 209 s ($F_1$) and the average background fluorescence measured from t = 0 - 190 s ($F_0$), data is expressed in bottom panels as bar graphs where $\Delta F = F_1 - F_0$. ***A,*** Prior to Fluo4 dye loading neurons were treated for 1 h with PBT2 or a non-competitive NMDA antagonist, MK-801. ***B,*** Neurons were also pre-treated with PBT2 for 6 h, the time required for PBT2 to protect against glutamate induced excitotoxicity, and MK-801 for 1 h. Data represent mean ± SE; n = 4; ***p < 0.001; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

glutamate-induced $Ca^{2+}$ flux remained significantly attenuated (65.3 ± 3.3%, n = 4, $p < 0.001$) however the longer treatment with PBT2 resulted in a significant reduction in $Ca^{2+}$ flux of 31.2% (68.8 ± 5.6%, n = 5, $p < 0.001$) compared with the 1-hour treatment (Figure 3-2B).

### 3.2.3 PBT2 requires zinc to reduce NMDAR-mediated $Ca^{2+}$ flux.

The results obtained from the excitotoxicity experiments clearly demonstrated a requirement of metal for the protective effect of PBT2 on cell viability of neurons. To determine whether metals were also a requirement for the observed effect of PBT2 on $Ca^{2+}$ flux, experiments were performed in the presence and absence of metals by using the metal chelator, Diamsar. The reduction in $Ca^{2+}$ flux previously seen following a 6-hour treatment with PBT2 (Figure 3-2B) is ablated by the removal of metals (87.3 ± 9.3%, n = 4, $p < 0.001$) (Figure 3-3A). Although experiments performed in the 1990s with ionotropic receptor agonists support the role of NMDAR mediating glutamate-induced excitotoxic cell death (Choi, 1992), glutamate activation of AMPA and Kainate receptors could still contribute to $Ca^{2+}$ flux. To ensure the effects of PBT2 were mediated solely by NMDARs neurons were concomitantly treated with the AMPA/Kainate receptor antagonist, CNQX (Figure 3-3, B and C). We then tested whether the reintroduction of zinc to metal-depleted neurons would restore the ability of PBT2 to block $Ca^{2+}$ flux. A concentration of $Zn^{2+}$ shown to rescue glutamate excitotoxicity (7 μM) (Figure 3-1C) in metal-depleted media was used during PBT2 pretreatment of cells (Figure 3-3C). The presence of $Zn^{2+}$ fully restored the ability of PBT2 to block $Ca^{2+}$ flux and this was shown to be an NMDAR-mediated event by concomitant treatment with CNQX.

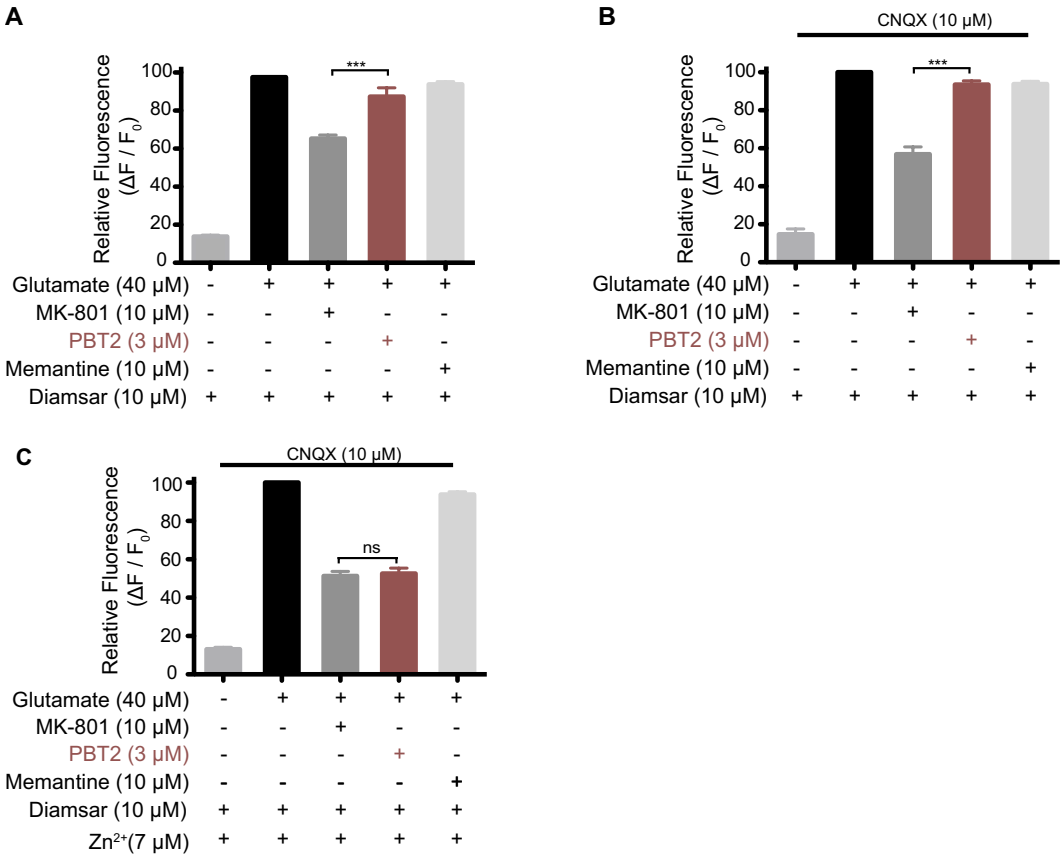



**Figure 3-3. Zinc is required for the inhibition of $Ca^{2+}$ flux by PBT2.** Intracellular $Ca^{2+}$ levels in cortical neurons preloaded with the $Ca^{2+}$ indicator Fluo4, were measured upon stimulation by glutamate (100 μM)/ glycine (10 μM) and reading fluorescence at 490 nm excitation and 520 nm emission. Measurements were taken at t = 209 s ($F_1$) and background fluorescence measured from t = 0 - 190 s ($F_0$), data is expressed as bar graphs where $\Delta F = F_1 - F_0$. ***A,*** Prior to Fluo4 loading neurons were treated with PBT2 (3 μM) or the non-competitive NMDA antagonist, MK-801 (10 μM) for 6 h in media depleted of metals by Diamsar (10 μM). ***B,*** To demonstrate NMDA-specificity neurons were co-treated with the competitive AMPA/Kainate antagonist, CNQX (10 μM). ***C,*** Neurons were treated with CNQX (10 μM) and PBT2 (3 μM) or MK-801 (10 μM) for 6 h in media depleted of metals by Diamsar (10 μM) then exogenous $Zn^{2+}$ (7μM) added. Data represent mean ± SE; n = 4 - 6; ***p < 0.001; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

### 3.2.4 The reduction of calcium flux by PBT2 is not mediated by glutamatergic cell surface expression.

Excitatory glutamatergic synapses are involved in most neurotransmission in the central nervous system and as such the activity through the glutamatergic receptors is tightly regulated. As well as altering function of the receptor complexes by changes in subunit composition, activity through the glutamatergic receptors, namely; AMPA, NMDA-NR1, NMDA-NR2A and NMDA-NR2B can be upregulated by increased expression at the cell surface. Conversely, a reduction of these receptors at the postsynaptic membrane by cycling to intracellular or extrasynaptic pools as with AMPAR and NMDA-NR2B, respectively, or by endocytosis as with synaptic NMDARs can significantly attenuate activity (Groc et al., 2006; Wang et al., 2010b). In order to determine whether PBT2 attenuated $Ca^{2+}$ flux by affecting the cell surface expression of these receptors, surface biotinylation experiments were performed on cortical neurons. Cells were treated with PBT2 (3 μM), then biotin and streptavidin magnetic beads were used to separate cell surface expressed proteins from total protein. As PBT2's ability to reduce $Ca^{2+}$ flux was shown to be time-dependent (Fig 3.2) these conditions were tested by treating neurons for 0, 1, 3 & 6 h with PBT2. In order to observe the zinc-dependent nature of PBT2 on $Ca^{2+}$ flux, neurons were treated with PBT2 in the presence of metals (Figure 3-3A), absence of metals (Figure 3-3B) or with metals removed and $Zn^{2+}$ (7 μM, from Figure 3-1C) reintroduced back in to the media (Figure 3-3C). Protein levels were probed with antibodies for the key ionotropic glutamate receptors, AMPAR and NMDAR and the NMDAR subunits; NR1, NR2A and NR2B. In addition, biotinylation was controlled for by probing for beta actin (β-actin). In each case there was no consistent observable change in cell surface expression or total expression of the proteins during the 6 h treatment whether metal were present or not (Figure 3-3). This finding provides evidence that PBT2 must reduce $Ca^{2+}$ flux and protect against excitotoxicity by a mechanism other than modulating glutamatergic receptor expression.

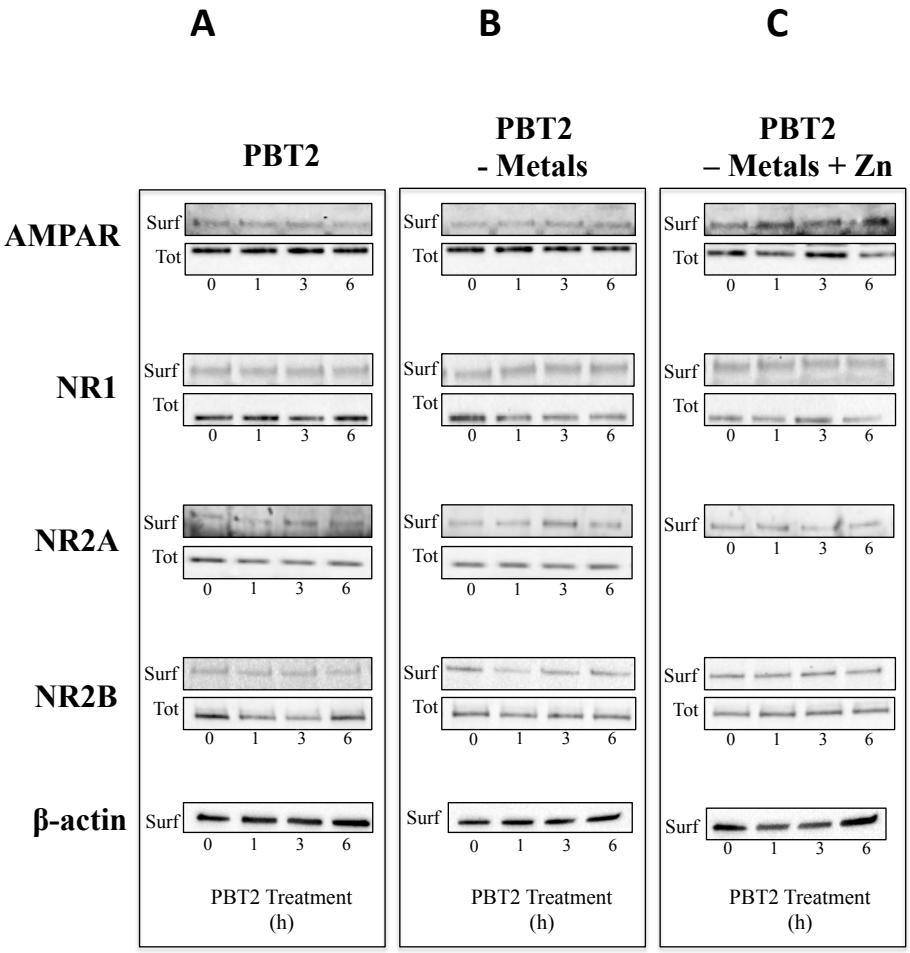



**Figure 3-4. PBT2 treatment does not effect cell surface glutamatergic receptor expression.** Cortical neurons (DIV 9) were treated with PBT2 (3 μM) for 0, 1, 3 and 6 h in media containing metals (***A***) media pretreated with 10 μM Diamsar to deplete metals (***B***) or metal-depleted media with $Zn^{2+}$ (7 μM) reintroduced (***C***). Proteins were separated in to surface (Surf) and total (Tot) protein (see materials methods, Chp. 2) and cell surface expression of the key glutamatergic receptors; AMPAR, NMDA-NR1 (NR1), NMDA-NR2A (NR2A) and NMDA-NR2B (NR2B) and the biotinylation control protein, beta actin (β-actin) observed by immunoblot analysis. PBT2 treatment at no time point showed any effect on the expression of the receptors tested at the cell surface or on their total protein levels. Results in ***A***, ***B*** and ***C*** show representative blots from 3 to 6 independent experiments.

### 3.2.5 Preconditioning by PBT2 treatment is neuroprotective against excitotoxicity.

The phenomenon of neuroprotection by preconditioning has been well documented in ischemic models (Murry et al., 1986; Kirino et al., 1991; Katsura et al., 2001) in which glutamate-induced excitotoxicity underlies neuronal death. Additionally, sublethal levels of intracellular zinc have been shown to be neuroprotective in rats (Lee et al., 2008) and to increase $[Ca^{2+}]_i$ by release from the endoplasmic reticulum (Qin et al., 2011). Given that moderate increases in $[Ca^{2+}]_i$ can precondition neurons (Bickler and Fahlman, 2004) and the strong ionophore action of PBT2 on $Zn^{2+}$ we sought to determine whether PBT2 treatment would have a metal-dependent effect on $[Ca^{2+}]_i$ levels in our neuronal model. These data were obtained from observing the $[Ca^{2+}]_i$ levels in the $Ca^{2+}$ flux experiments (Figure 3-2 and 3) prior to treatment with glutamate/glycine. A significant increase (113.0 ± 3.9%, n = 5, $p < 0.001$) in $[Ca^{2+}]_i$ was observed in neurons pretreated with PBT2 but not in vehicle-treated (99.05 ± 1.5%, n = 5) or neurons treated with the NMDAR antagonists; MK-801 (97.8 ± 2.2%, n = 5) and Memantine (97.7 ± 3.2%, n = 5) (Figure 3-5A). The metal-dependent effect of PBT2 was again demonstrated by depletion of metals by supplementing media with Diamsar prior to and during drug treatment which ablated the increase in $[Ca^{2+}]_i$ levels due to PBT2 (Figure3-5B). $Zn^{2+}$ was supplemented back in to metal-depleted media at 7 μM, the concentration shown to protect against excitotoxicity in Figure 3-1C. Following treatment with PBT2 under these conditions $[Ca^{2+}]_i$ levels were significantly increased (120.6 ± 4.6%, n = 4, $p < 0.001$) compared with neurons treated with; vehicle (99.13 ± 1.4%, n = 4), MK-801 (99.42 ± 1.8%, n = 4) and Memantine (100.4 ± 3.7%, n = 5) (Figure 3-5C).

### 3.2.6 Effect of PBT2-induced Preconditioning on Network Activity

To further investigate PBT2 treatment 64-channel MEAs were employed to observe network-wide extracellular activity. Results were obtained by measuring spike activity of three separate cortical neuron preparations cultured

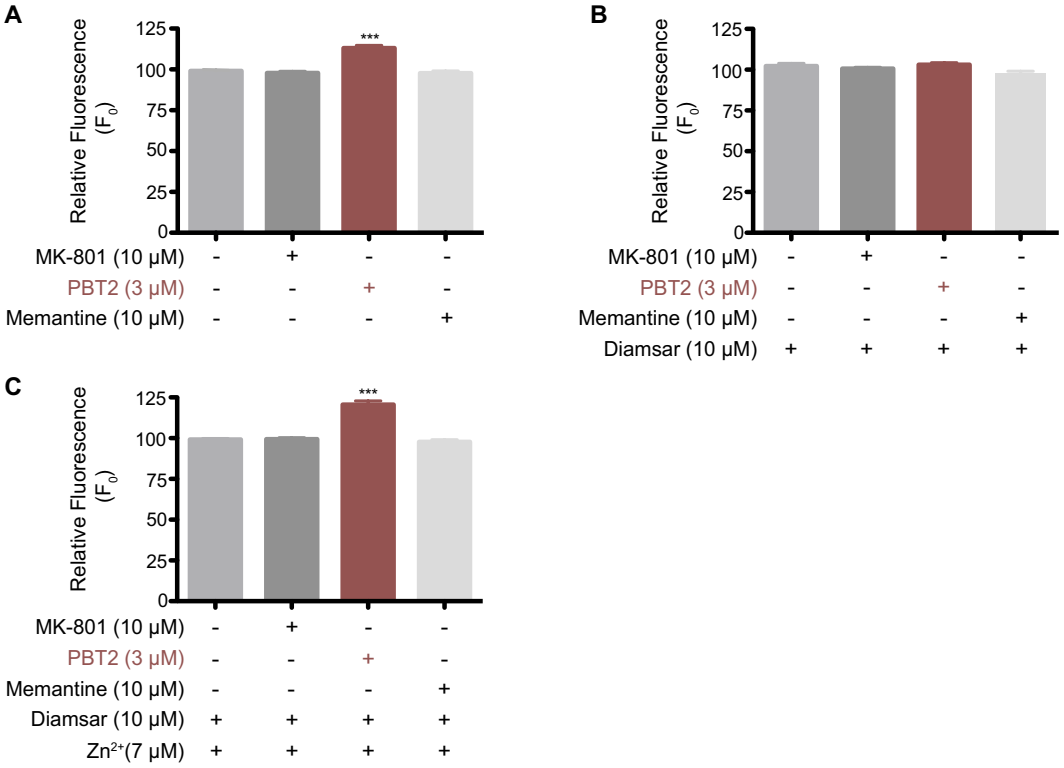



**Figure 3-5. PBT2 preconditioning protects against excitotoxicity by increasing intracellular $Ca^{2+}$ levels.** ***A,*** Following pre-treatment with PBT2 (3 µM), the non-competitive NMDA antagonists; MK-801 (10 µM) or Memantine (10 µM for 6 h) cortical neurons were loaded with the $Ca^{2+}$ indicator Fluo4 for 1 h and fluorescence measured at 490 nm excitation and 520 nm emission. Mean fluorescence was expressed as a bar graph with values normalised to untreated vehicle. ***B,*** Metals were depleted from neuronal media by pre and co-treatment with Diamsar (10 µM). ***C,*** Exogenous $Zn^{2+}$ (7 µM) was supplemented in to Diamsar-treated neurons during treatment. Data represent mean ± SE; n = 4 - 6; ***p < 0.001; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

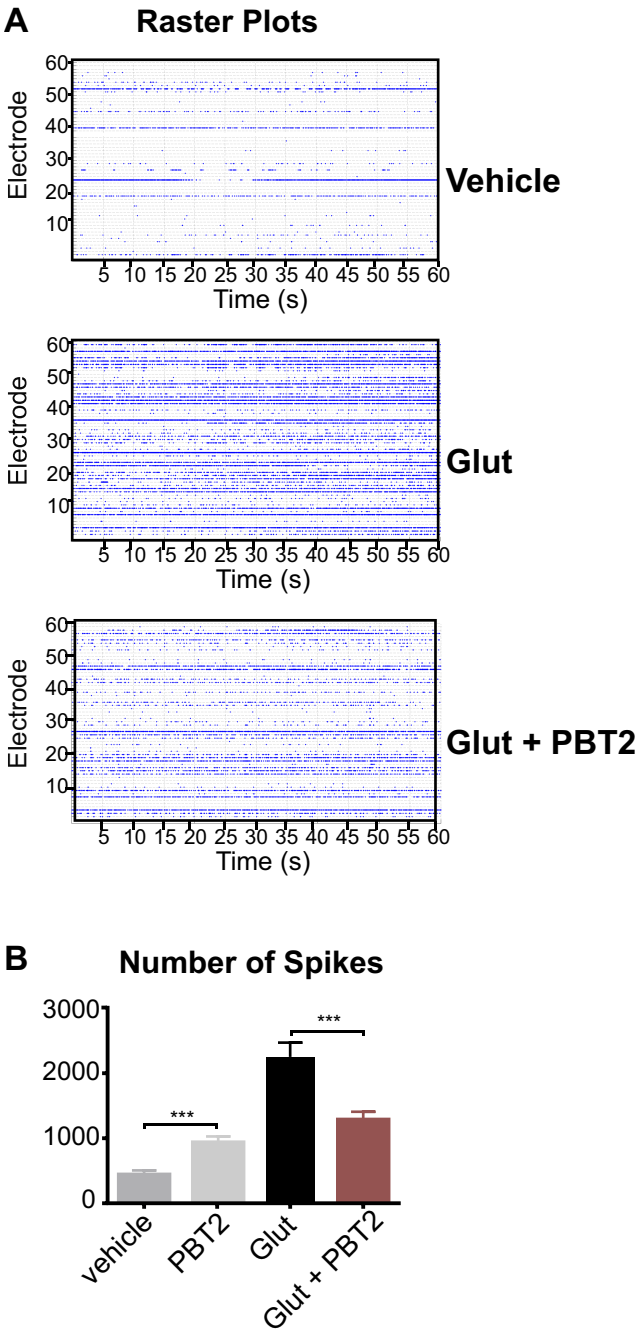



**Figure 3-6. PBT2 induces moderate synaptic activity and reduces excitotoxic network excitation following glutamate exposure.** ***A,*** Example of Raster plots of a single MEA experiment with cortical neurons treated with vehicle, excitotoxic levels of glutamate (100 μM)/ glycine (10 μM) (glut) or pre-treated for 6 h with PBT2 (3 μM) prior to treatment with glutamate/glycine (glut + PBT2). The raster plots show neural activity by recording spikes at each of the 60 electrodes (blue dots) as a function of time with each dot representing a single action potential. ***B,*** Graphical representation of data obtained from the above MEA experiments. The number of spikes was recorded over the period of 5 mins pre and post application of glutamate/ glycine for each treatment. Data represent mean ± SE; n = 3; ***p < 0.001 compared to vehicle and glutamate treated values, respectively, by one-way ANOVA using Dunnett's post hoc test.

on MEAs and correlate directly with data from the $Ca^{2+}$ flux assays (Figure 3-2 and 3-3).The data shows a significant decrease in excitotoxic network activity induced by glutamate/ glycine following pretreatment with PBT2 for 6 h (Figure 3-6, A and B). In further support of PBT2 preconditioning neurons, a moderate but significant increase in synaptic activity was observed in neurons treated with PBT2 alone compared with vehicle, as measured by the number of spikes (Figure 3-6B). This increase in spontaneous synaptic activity corroborates the increases in $[Ca^{2+}]_i$ levels that were observed previously (Figure 3-5, A and C) and also past findings demonstrating the requirement of prolonged and marked elevation of electrical activity for preconditioning in an ischemic model (Tauskela et al., 2008).

### 3.2.7 PBT2 restores normal levels of protein expression and phosphorylation under excitotoxic conditions

Calcineurin, a $Ca^{2+}$-dependent protein phosphatase, and glycogen synthase kinase 3 (GSK3) both mediate survival in primary neurons from rodents (Endo et al., 2006; Wu et al., 2007). Previous work by colleagues in cultured SH-SY5Y cells has demonstrated that an increase of GSK3 phosphorylation by PBT2 is a result of inhibition of calcineurin (Crouch et al., 2011b). To determine whether PBT2 activates cell survival pathways by preventing the cleavage of calcineurin and inhibiting of GSK3α and GSK3β activity by phosphorylation, we analysed their respective protein expression following pre-treatment with drugs and exposure to excitotoxic glutamate in mouse cortical neurons. PBT2 pretreatment inhibited activation/cleavage of calcineurin significantly increasing protein levels in glutamate treated neurons when normalized to vehicle (from 21 ± 7% to 81 ± 9%, $n = 3$, $p < 0.05$, $p < 0.01$ for PBT2) (Figure 3-6A). Further evidence of neuroprotection by PBT2 mediated by GSK3 was seen by its ability to increase phosphorylation of both GSK3α (from 8 ± 4% to 85 ± 19%, $n = 3$, $p < 0.01$ for PBT2) and GSK3β (from 55 ± 9% to 92 ± 8%, $n = 3$, $p < 0.001$ for PBT2, respectively) (Figure 3-6, B and C).

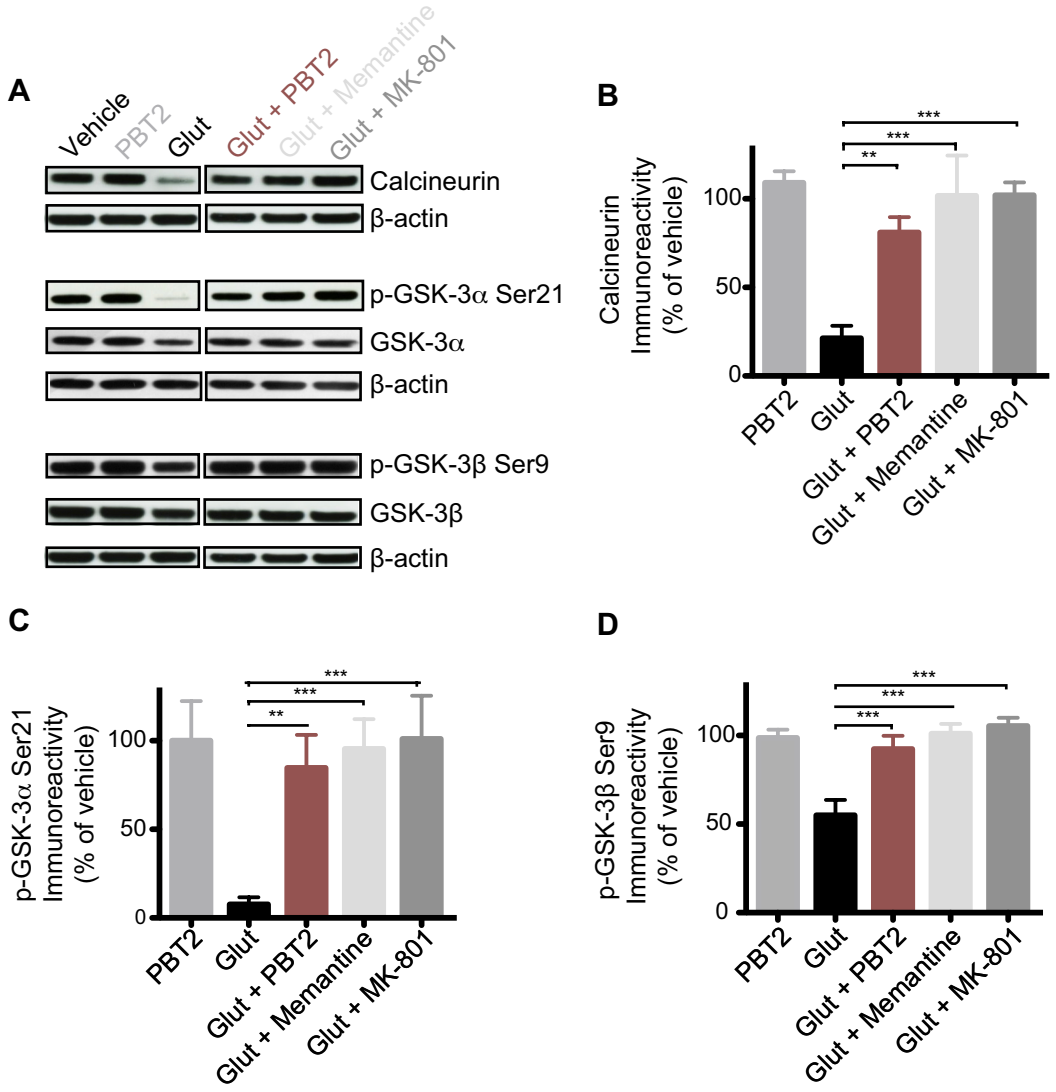



**Figure 3-7. PBT2 inhibits calcineurin and GSK3 activation.** ***A***, Western blot analysis of calcineurin, p-GSK3α/β, total GSK3α/β, and the control protein, β-actin in cortical mouse neurons (D.I.V. 9). Neurons were pre-treated with PBT2 (3 μM) and with the NMDA-specific antagonists; Memantine (10 μM) for 6 h and MK-801 (3μM) for 1 h prior to treatment with glutamate (glut, 40 μM)) for 1 h. Protein was harvested after an 18 h incubation in fresh media. ***B-D***, Densitometry analysis of western blot data shown in panel ***A***, PBT2 treatment inhibited calcineurin activity by preventing its calpain-mediated cleavage demonstrated by restored protein levels (***B***). PBT2-treated neurons showed significantly increased levels of inactive phosphorylated GSK-3α (***C***), to a lesser extent GSK-3β (***D***) compared with glutamate-treated samples. All samples were normalized to the β-actin to control for loading. Western blots are representative of 3 or more separate experiments. Data represent mean ± SE; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to glutamate treated values.

## 3.3 Discussion

Currently the underlying aetiologies of the neurodegenerative diseases, AD and HD are not yet fully understood. Although protein aggregation and deposition are a common feature in the diseases emerging evidence has implicated the dysregulation of $Ca^{2+}$ flux through the NMDA receptors and the resulting excitotoxic cell death as a shared mechanism and more probable cause of the neurodegeneration underlying both indications (Milnerwood and Raymond, 2010; Um et al., 2012).

The involvement of the glutamatergic system in the neurodegeneration underlying AD is well documented. Toxic soluble forms of the peptide responsible for plaques in the AD brain, Aβ that have been isolated from cell lines, rodents or human diseased brains have been shown to inhibit LTP mediated through NMDARs. (Lambert et al., 1998; Walsh et al., 2002; Shankar et al., 2007; 2008). PBT2 acts on this key phenotype of AD, reducing levels of Aβ oligomers and also decreasing phosphorylated Tau (Crouch et al., 2011a). A role for the drug's action in glutamatergic signaling was indicated by its ability to inhibit the Aβ-induced inhibition of LTP in rodents (Adlard et al., 2008). PBT2 was originally identified by compound screens for its ability to inhibit toxic extracellular Aβ-metal interactions, a mechanism thought to involve the sequestration $Zn^{2+}$ from the extracellular Aβ aggregates and drug-mediated intracellular metal delivery (Adlard et al., 2008). However in the work presented here we provide evidence of an alternative mechanism action of the drug, one that supports the findings in which PBT2 has shown efficacy in both AD and HD.

The initial experiments in this the ability of PBT2 to protect against glutamate-induced excitotoxicity and that this protection is metal dependent. As evidence of this, the protective effect of PBT2 was ablated when the metal chelator, Diamsar, removed metals in the cell culture media. The removal of metals had no effect on the actions of NMDA-specific antagonists, MK-801 and Memantine (Figure 3-1).

NMDA receptors play a vital role in the normal function of the nervous system including their role in cognition, learning and memory through LTP.

Clinical trials of NMDA antagonists such as MK-801 in stroke, Huntington's disease and traumatic brain injury failed to show safety resulting in psychotomimetic effects including hallucinations, agitation, sensory disturbance and catatonia (Lees et al., 2000; Muir, 2006). These severe side effects have been attributed to the slow "off-rates" of the drugs leading to prolonged blockage of the normal signaling by NMDA receptors. Memantine is an NMDA antagonist that has EU and FDA approval for moderate to severe AD. The drug is clinically tolerated reportedly due to a faster "off rate' (Lipton, 2006) Unlike MK-801, Memantine has been shown to act selectively through extrasynaptic NMDARs (Talantova et al., 2013). This mechanism is supported by our findings in which glutamate-induced $Ca^{2+}$ flux, mediated through NMDARs at the post synapse, is not altered in neurons pretreated with Memantine (Figure 3-2 and 3-3).

To further understand the phenomenon of excitotoxic protection by PBT2 an intracellular $Ca^{2+}$ flux assay was employed and demonstrated that an extended 6 h, as opposed to 1 h, pre-treatment of neurons with PBT2 is required to block NMDA receptor-mediated flux to the extent seen in neurons pretreated with MK-801 (Figure 3-2, A and B). This was also the case in the initial optimisation of the excitoxicity assays where 6 h pretreatment with PBT2 was required for protection. Such a requirement of PBT2 suggested it was acting primarily on downstream components in the $Ca^{2+}$-signaling pathway to protect against excitotoxicity, discussed below, rather than directly antagonising the NMDAR as with 1 h MK-801 treatment.

The subsequent $Ca^{2+}$ flux assays demonstrated a significant metal-dependent reduction in $Ca^{2+}$ flux by PBT2 to levels seen in neurons treated with NMDAR antagonist, MK-801 (Figure 3-3A). In these experiments metals were chelated using Diamsar, which has a high affinity for $Zn^{2+}$ and showed negligible effects on $Ca^{2+}$ levels. Analysis of raw fluorescence data established that co-treatment with Diamsar had no effect on $Ca^{2+}$ levels and therefore had no effect on the observed changes in $Ca^{2+}$ flux between samples (Figure 3-2B and 3-3A). Non-specific effects of PBT2 on $Ca^{2+}$ in this experiment were also negated by the observation that when metals were removed $Ca^{2+}$ flux was not significantly different to vehicle (Figure 3-3, A and B). It was also important to determine

whether the intracellular flux of $Ca^{2+}$ was mediated solely by NMDARs or also by the two remaining classes of glutamatergic receptors; AMPA and Kainate receptors. Co-administration with the competitive AMPA/ Kainate receptor antagonist, CNQX, demonstrated no effect on $Ca^{2+}$ flux in untreated and treated neurons indicating the $Ca^{2+}$ flux to be NMDAR-mediated (Figure 3-3, A and B). In the initial drug screens PBT2 was selected based on its action as an ionophore to restore levels of intracellular $Zn^{2+}$ and $Cu^{2+}$ in a neuronal cell line (Adlard et al., 2008). PBT2 is believed to work in AD through its ability to remove zinc bound to Aβ in doing so prevent a rapid induction of Aβ fibril formation (Adlard et al., 2008) and transport the metal into cells and activate neuroprotective signaling cascades (Crouch et al., 2011a). In the initial excitoxicity experiments (Figure 3-1C) reintroduction of 7 μM $Zn^{2+}$ was sufficient to overcome the chelation of exchangeable metals in the cell culture media by 10 μM Diamsar and significantly restore protection by PBT2 against glutamate. The same concentration of $Zn^{2+}$ completely restored the reduction in $Ca^{2+}$ flux by PBT2 in metal-depleted neuronal culture. Given PBT2 has also been shown to markedly promote the uptake of $Cu^{2+}$ by cells we also tested whether it was required for reduction of $Ca^{2+}$ flux by PBT2, however cortical neurons were not viable due $Cu^{2+}$ toxicity. .

It is possible that PBT2 is mediating its effect on $Ca^{2+}$ flux by various mechanisms; including by altering either the activity or expression of NMDARs. The NMDARs mediate the majority of intracellular $Ca^{2+}$ flux across the cell membrane upon glutamate exposure as well as during synaptic activation (Rogers and Dani, 1995). The modulation of this receptor through one of its subunits would therefore account for the substantial reduction in $Ca^{2+}$ flux observed following PBT2 treatment (Figure 3-2, B and 3-3, B). Zinc is known to antagonize NMDARs by two independent mechanisms; a voltage-dependent mechanism and a voltage-independent mechanism with the latter being dependent on NMDAR subunit composition with $Zn^{2+}$ acting on the amino-terminal domain of NR2A with far greater affinity than the NR2B subunit (Choi and Lipton, 1999; Paoletti et al., 2000). Given PBT2's action on $Zn^{2+}$ as an ionophore it would be expected treatment with the drug would reduce extracellular $Zn^{2+}$ in the synaptic cleft and cause reduction of any voltage

dependent-inhibition of NMDAR activity which in turn would lead to an increase in $[Ca^{2+}]_i$. In the work presented here a converse effect was observed upon PBT2 treatment with $[Ca^{2+}]_i$ significantly decreased (Figure 3-2, B and 3-3, B). Further investigation of PBT2's action on $Ca^{2+}$ flux was undertaken with biotinylation experiments which observed the changes in cell surface expression of the key receptors mediating intracellular flux, namely; AMPAR and NMDAR. Given that the subunit composition can affect NMDA receptor function, the expression of NR1, NR2A and NR2B were also studied (Paoletti et al., 2013). These experiments clearly demonstrated that PBT2 was not mediating its effect at the cell surface or by altering total expression of the receptors (Figure 3-4) and that PBT2 was not acting via an NMDA-mediated mechanism.

Preconditioning by pre-exposure to subtoxic levels of glutamate and NMDA (Chuang et al., 1992; Rocha et al., 1999) or brief ischemic insults (Murry et al., 1986; Kirino et al., 1991) is neuroprotective against excitotoxic trauma. Further work describing this phenomenon has demonstrated the involvement of cellular pathways mediated by NMDARs. Preconditioning with low doses of NMDA induces key proteins in neuroprotective pathways including the phosphatidylinositol 3 kinase-protein kinase B (Akt) signaling to GSK3β inhibition (Soriano et al., 2006). These survival signals can also be activated by using $Ca^{2+}$ ionophores to moderately increase $[Ca^{2+}]_i$ conferring long-term tolerance of ischemia or other stresses and this occurs independently of NMDAR-mediated $Ca^{2+}$ flux (Bickler and Fahlman, 2004). A central finding of this work is that PBT2 causes a moderate but significant increase in $[Ca^{2+}]_i$ levels following treatment (Figure 3-5). In this data PBT2 treatment did not protect against excitotoxicity by attenuating total $[Ca^{2+}]_i$ mediated by NMDARs as with MK-801 (Figure 3-2 and 3-3), rather it reduced flux by increasing initial levels of $[Ca^{2+}]_i$ and in turn preconditioned neurons against excitotoxic exposure to glutamate (discussed below). This is because it is the total net flux of $Ca^{2+}$ in to the cell rather than total levels of ($[Ca^{2+}]_i$) that correlates with the extent of excitotoxic cell death (Abdel-Hamid and Baimbridge, 1997; Dugan and Choi, 1999; Soriano et al., 2006).

There are several potential mechanisms by which PBT2 could result in the zinc-dependent increase in $[Ca^{2+}]_i$. It can rise by crossing the cell membrane through NMDARs, AMPAR, store-operated $Ca^{2+}$ channels (SOCCs), voltage-gated $Ca^{2+}$ channels (VGCCs), ionotropic glutamate receptors (NMDARs and AMPARs) or it can be released from intracellular stores in the endoplasmic reticulum (Corona et al., 2011). Another obvious candidate recently described involves the post synaptic $Zn^{2+}$-sensing receptor ($Zn^{2+}$R)/G-protein coupled receptor (GPR39) and as with many metabotropic receptors, the resulting increase $[Ca^{2+}]_i$ is mediated through phospholipase C and subsequent generation of inositol 1,4,5 triphosphate (IP3) (Hershfinkel et al., 2001; Chorin et al., 2011). In our work we did not see an increase in $[Ca^{2+}]_i$ in neurons other than those treated with PBT2 in normal, metal–depleted and metal-depleted/$Zn^{2+}$ conditions (Figure 3-5). This observation in conjunction with the metal-dependent nature of the drug's action makes it is less likely that PBT2 acts directly on an extracellular receptor to increase $[Ca^{2+}]_i$ but instead acting through its ionophoric ability to increase intracellular zinc triggering the release of $Ca^{2+}$ from the ER via the inositol 1,4,5-trisphosphate (IP3R) and the ryanodine (RyR) receptors. In support of this mechanism cytosolic levels of $Zn^{2+}$ have been shown to modulate ER $Ca^{2+}$ stores (Qin et al., 2011).

In work delineating the effects mediated through synaptic and extrasynaptic NMDARs by Hardingham and colleagues, elevated levels of electrical activity were shown to precondition neurons against apoptosis from serum deprivation, excitoxicity or oxidative stress (Hardingham et al., 2002; Papadia et al., 2008; Tauskela et al., 2008). My results demonstrating an increase in $[Ca^{2+}]_i$ indicate that PBT2 is effecting synaptic activity to a moderate extent (Fig 3-5). In subsequent experiments studying the effect of PBT2 on network activity with MEAs it was confirmed that PBT2 indeed can reduce excitotoxic electrical signaling due to glutamate/ glycine as was originally hypothesised. An important finding that provided further evidence of preconditioning as a mechanism of action of PBT2 was a moderate but significant increase in electrical activity across the MEAs due to PBT2 treatment (Figure 3-6).

As discussed prior moderate increases in $[Ca^{2+}]_i$ (Bickler and Fahlman, 2004) result in preconditioning of neurons by activation of the neuroprotective pathways involving Akt, CMK, & GSK3 and previous research has demonstrated the capability of structurally diverse metal chaperone compounds to activate neuroprotective signaling pathways (White et al., 2006; Adlard et al., 2008; Donnelly et al., 2008; Crouch et al., 2009; 2011b). The restoration of normal calcineurin levels indicates that PBT2 acts on a key pathway associated with neurodegeneration involving the $Ca^{2+}$-activated cysteine protease, calpain ((Bradley et al., 2012)). The cleavage and subsequent constitutive unregulated activation of one of its downstream targets, calcineurin, has been shown to induce dephosphorylation and activation of GSK3α and β. GSK3 dysregulation has been implicated in AD (Avila et al., 2004) and other relevant neurodegenerative diseases including HD (Carmichael et al., 2002) and frontotemporal dementia with parkinsonism (Engel et al., 2006). In this study we have shown that PBT2, in protecting against glutamate-induced excitotoxicity by increasing $[Ca^{2+}]_i$, inhibited cleavage and therefore activation of calcineurin and restored its normal protein levels (Figure. 3-7, B). Further evidence of the drug acting to inhibit neurotoxic signaling was observed by the increase of phosphorylation of both GSK3 isomers (Figure 3-7, C and D).

In summary the metal chaperone PBT2 can protect against excitotoxic insults by inducing preconditioning in neurons. PBT2 acts by transporting $Zn^{2+}$ into cells, increasing $[Ca^{2+}]_i$ and activating neuroprotective pathways involving Calcineurin and GSK-3 and this process does not block the channels of the glutamatergic system that are required for normal synaptic function. Given that excitotoxicity is implicated in a number of neurodegenerative diseases this work suggests that metal chaperones have the potential to be effective therapeutic agents across the spectrum of these disease.

# 4 Describing the Necessary Conditions of Beta Amyloid Toxicity in Neurons.

## 4.1 Introduction

In Alzheimer's disease of the varied hypotheses for its cause, a large body of work has centered on the involvement of Aβ in attempts to determine whether it is its overproduction or failure to clear Aβ from the brain that underlies neuronal loss initially in the hippocampus and cerebral neocortex. This is highlighted in a recent review collating the number of publications each year that focused on Aβ as opposed to the remaining significant markers of Alzheimer's, Tau and apoE4. Between the years 2010 - 2013 there was an average of 1750 Aβ publications (Michaelson, 2014). An array of transient and intermediate Aβ species, which exist in a dynamic equilibrium on the pathway to fibril and extracellular amyloid plaques formation, have previously been described and demonstrated to cause synaptic dysfunction and neurotoxicity (Lambert et al., 1998; Lashuel et al., 2002; Walsh et al., 2002; Barghorn et al., 2005; Lesné et al., 2006; Shankar et al., 2008; Kayed et al., 2009). Evidence indicates that the neurotoxicity from Aβ can result from many varied mechanisms and through either a direct or indirect action results in the neuronal loss observed in AD. The main neurotoxic effects that have been described for Aβ include; neuroinflammation, oxidative and nitrosative stress, synaptic dysfunction, mitochondrial damage, impaired autophagy and disturbances to glutamatergic neurotransmission, with the latter leading to excitotoxicity (Walsh et al., 2002; Nixon and Yang, 2011; García-Escudero et al., 2013; Carrillo-Mora et al., 2014; Macchi et al., 2014). Subsequently cell death then occurs by either of two distinct processes, apoptosis or necrosis. Characteristic of classical apoptosis is the activation of cysteine-dependent, aspartate-directed proteases called caspases, which lead to phagocytosis of the cell following DNA condensation and

fragmentation. Necrosis is an accidental or pathological form of cell death that results from significant ion influx, mitochondrial swelling and plasma membrane rupture (Majno and Joris, 1995; Martin, 2001). One specific pathway is still to be proven solely responsible for the toxicity observed in AD, it is more likely that cell death is multifactorial involving several different mechanisms. In support of such mechanistic complexity are findings that different conformations of oligomeric Aβ namely, ADDLs (Lambert et al., 1998), AβOs and fibrils (Demuro et al., 2005) act via distinct pathways and different time-courses to affect toxicity in human cortical neurons (Deshpande, 2006).

The current assays that are routinely employed globally to screen Aβ-induced cytotoxicity are unable to distinguish specific signaling pathways or conformational changes to Aβ that play a role in toxicity. Instead they indirectly measure total cell viability or death through the reduction of colorimetric dyes or by way of loss of membrane integrity, as is the case for MTT/MTS/CCK-8 and LDH/Calcein assays, respectively. The former assays are based on the reduction of the tetrazolium salts; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the subsequent, more sensitive analog, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8 or CCK-8). They act as indicators of intracellular redox activity and therefore are used as a measure of cell viability.

Assaying Aβ cytotoxicity by measuring cell viability of cultured neurons has been one of the most commonly used assays in many laboratories and as been used in AD research internationally for more than two decades (Yankner et al., 1990; Walsh et al., 1999; Chromy et al., 2003; Wogulis, 2005; Sakono et al., 2008; Noh et al., 2009; Zhang et al., 2010). Primary cultured neurons provide a relatively simple and fast option to investigate the role of Aβ aggregation state and the mechanism by which Aβ mediates its toxicity as well as providing valuable tools for therapeutic inhibitors of Aβ toxicity. Despite these positive attributes, the time frame necessary to observe statistically significant decreases in cell viability assays is substantial limitation to the assays. Publications have described Aβ toxicity in neurons in as short as 5 h (Walsh et al., 1999; Song et al.,

2008) but treatment times ranging from 24 h to 96 h are more representative of the norm (Ferreira et al., 2012; Mozes et al., 2012; Ryan et al., 2013; Giordano et al., 2014). In the standard toxicity assays performed in our laboratory primary mouse cortical neurons are treated with 10 - 15 μM synthetic Aβ for 96 h (Smith et al., 2007; Barnham et al., 2008; Hung et al., 2008; Ciccotosto et al., 2011). Such a protracted treatment time in this standard Aβ assay makes it near impossible to correlate aggregation state with its cytotoxicity. After 96 h the majority of Aβ would have formed fibrils, whereas to study the toxic oligomeric species will require an instant toxicity readout instead.

Measurement of apoptotic cell death provides a possible alternative to overcome the shortfalls of cell viability assays in response to Aβ treatment. Apoptosis, rather than necrotic cell death has been strongly implicated in Aβ toxicity (Mattson, 2000; Yuan and Yankner, 2000). In support of this were experiments in which the knockout of either caspases 2 or 12 in mice afforded resistance to Aβ toxicity (Nakagawa et al., 2000; Troy et al., 2000) These are two key caspases that regulate the extrinsic (receptor-mediated) and intrinsic (mitochondrial) pathways, respectively. Both pathways converge at the proteolytic activation of the executor protease, caspase 3 from the inactive procaspase 3. The outcome of this is regulation of caspase 3 activation and therefore apoptotic cell death (Porter, 2006). The measurement of this activation provides an indirect readout of Aβ toxicity as has been demonstrated in experiments in human cortical neurons (Deshpande, 2006).

In order to understand the necessary parameters for Aβ toxicity it was necessary to further characterise the standard cell viability assay employed in our laboratory. The requirement of 96 h treatment with Aβ to observe toxicity hinders the ability to determine whether the aggregation state of the peptide or the maturation stage of the primary cortical cultures, or both, are necessary for toxicity. Together with the investigation of alternative assays for toxicity the aim of the subsequent research presented in this chapter has been to test these parameters by comparing the days in vitro (DIV) of the primary neuronal cultures with the duration of treatment. Additionally, to investigate whether excitotoxicity underlies this cell death as has been proposed by several

significant publications recently (Ittner and Götz, 2010; Um et al., 2012; You et al., 2012; Tackenberg et al., 2013) (For review see Introduction, 1.6), the expression profiles of the key glutamatergic proteins were studied.

## 4.2 Results

### 4.2.1 The standard Aβ42 Toxicity Assay

In the standard cell culture method employed by our laboratory, primary mouse cortical neurons cultured for 6 days *in vitro* (DIV) and exposed to synthetic Aβ peptide at 10 and 15 µM that has previously been treated with hexafluoro-2-propanol (HFIP) to monomerise the peptide. This preparation is added immediately and mainly monomeric (De Felice et al., 2007) (see chapter 2 for preparation). This results in a significant decrease in cell viability by 72 h and a more substantial decrease of approximately 35 – 40% at 96 h (Figure 4-1A). It has been necessary to use both 10 and 15 µM concentrations of Aβ peptide to ensure toxicity due to frequently observed interbatch variations in peptide quality from our provider, Keck (Yale University, New Haven). The considerable lag time of this assay of 72 – 96 h means it is not possible to correlate toxicity to a specific oligomeric form of Aβ due to its propensity to rapidly aggregate. The aggregation profile of synthetic Aβ was observed by the binding of Thioflavin T (ThT) to β-sheet rich structures formed during Aβ aggregation, enhancing fluorescence. It can be seen that the ThT fluorescence, indicating peptide aggregation, reaches a plateau by 24 h with freshly prepared Aβ and by 30 h with HFIP-treated Aβ, as measured by the binding of ThT fluorescence (Figure 4-1B). In the ThT assays Aβ was prepared in a buffered saline solution, with phosphate to 3 mM unlike in Neurobasal media. The presence of salts increase Aβ aggregation therefore it would be predicted that the aggregation of Aβ on cells would be reduced but not sufficient to halt the peptides aggregation so that a specific oligomeric species of Aβ associated with toxicity can be detected.

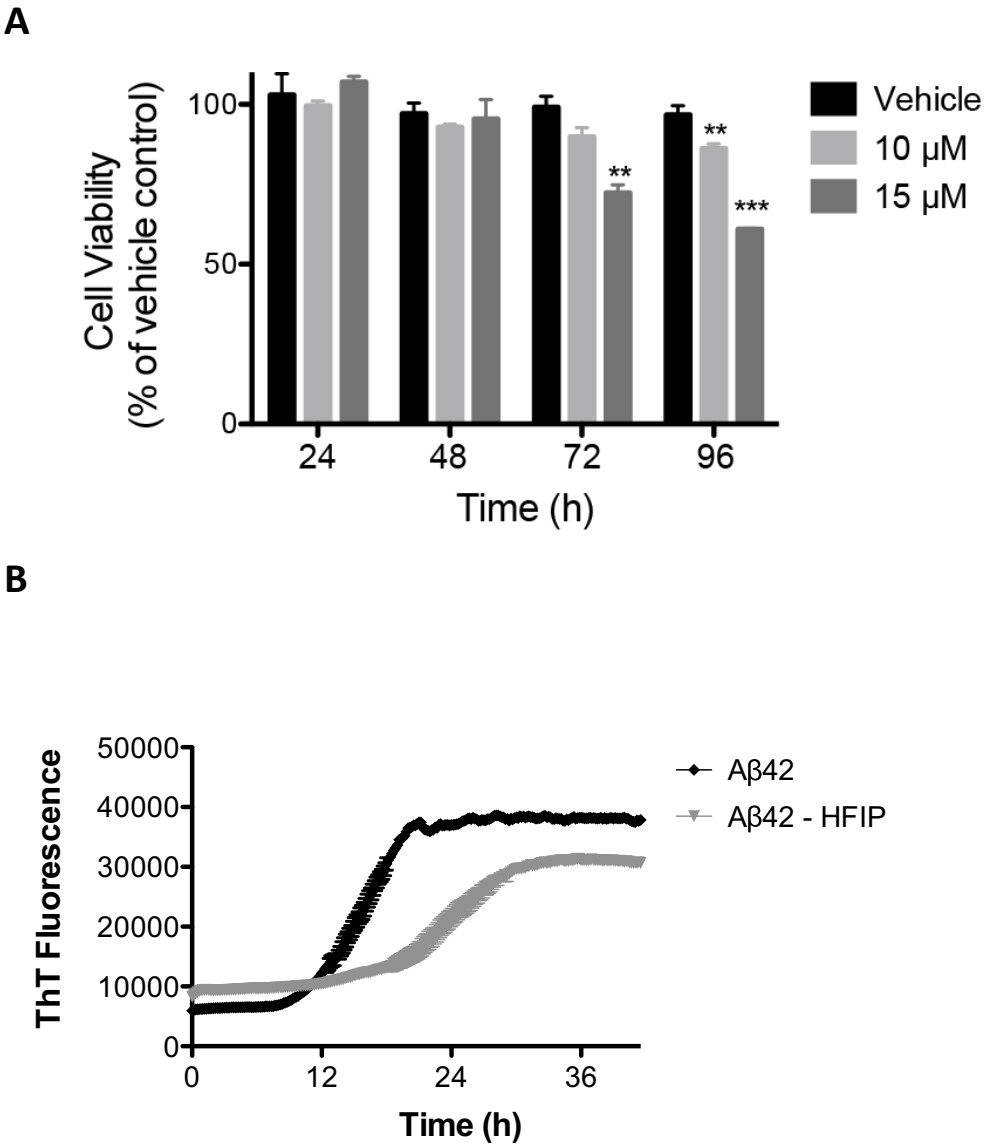



**Figure 4-1. Our standard Aβ42 cell viability assay cannot detect specific oligomeric species associated with toxicity.** ***A,*** In this Aβ42 toxicity assay cortical neurons are cultured for 6 DIV and then treated with 10 μM or 15 μM of synthetic Aβ42 with the latter often required due to lower purity in the preparation of peptide by the provider. Cell viability is assessed by CCK8 assay to determine toxicity and a significant decrease relative to time-matched vehicles is not observed until at least 72h (9 DIV) and consistently by 96h (10 DIV). ***B***, Rapid aggregation of the Aβ42 peptide was measured by Thioflavin T (ThT) fluorescence, which increases proportionally with aggregation. The aggregation rate of Aβ42 was reduced in the presence of HFIP. ThT binding was represented as the mean relative fluorescent units from *n = 6* replicate wells, ± SE following subtraction of the vehicle background fluorescence. Cell viability is expressed as the mean of ± SE; n = 3 - 6; **p < 0.01, ***p < 0.001; ns, not significant compared to glutamate treated values by one-way ANOVA using Dunnett's post hoc test.

### 4.2.2 Aβ-induced caspase 3 activity

Apoptosis has been widely implicated as a process that underlies neuronal cell death in neurodegenerative disease (Mattson, 2000; Deshpande, 2006; Eimer and Vassar, 2013). To determine whether caspase 3 activity could be used to assay Aβ toxicity, primary hippocampal neurons were exposed to the Aβ40 and Aβ42 peptides as well as vehicle and the positive apoptotic control, Staurosporine (STS), for 48h. Samples were also treated with a caspase 3 inhibitor to demonstrate specificity. Treatment with vehicle and Aβ40 showed no statistically significant increase of caspase 3 activity whereas Aβ42 induced a substantially significant increase in caspase 3 activity to the levels comparable to the STS positive control (Figure 4-1A). To make this assay more amenable to screening larger numbers of samples the Aβ42-induced activity was measured in primary mouse cortical, rather than hippocampal, neurons that provided a ten-fold increase in the number of cells from each embryonic isolation. Subsequent time-course experiments exhibited a significant Aβ42 increase in caspase 3 activity at 4 and 6 h post treatment compared with vehicle treated controls (Figure 4-2B). Despite the considerable reduction in time for an observable Aβ42-induced effect from 72-96 h to 4-6 h, this duration still affords enough time for considerable Aβ42 aggregation that prevents the identification of specific oligomeric forms. More importantly the toxicity induced by treatment with Aβ42 did not correlate with the levels of caspase 3 activity. Treatment of hippocampal and cortical neurons with 10 μM Aβ42 results in between 20 - 40% decrease in cell viability whereas treatment with STS kills all neurons, yet both activate caspase 3 to similar levels. This indicates the caspase activity assay is not suitable for the purposes of screening Aβ42 toxicity. It also suggests that the cell death is not due to apoptosis.

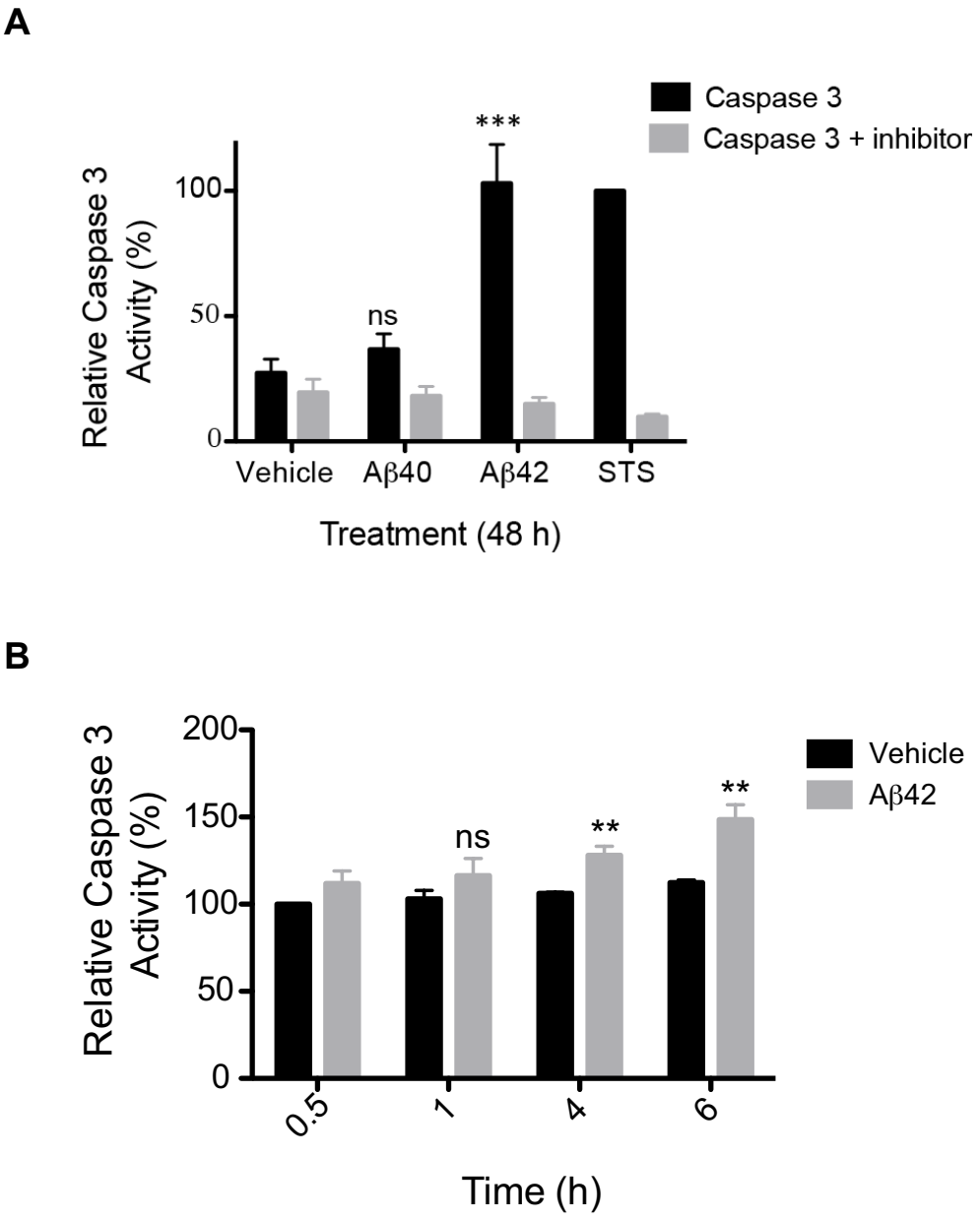



**Figure 4-2. Effect of Aβ on caspase 3 activity in primary mouse neurons.** ***A***, A significant increase in caspase 3 activation was observed following treatment with Aβ42 (10 μM) or the positive caspase control, Staurosporine (STS) (2 μM) for 48 h. Treatment with the non-toxic Aβ40 peptide (10 μM) showed no significant increase in caspase activation relative to vehicle. The caspase 3 inhibitor, Z-DEVD-FMK (5 μM), was used to demonstrate caspase-specific activation. ***B***, It was also possible to observe a significant increase in caspase 3 activity in cortical neurons. Cells treated for 4 h and 6 h with Aβ42 followed by an 18 h incubation in fresh media showed significant increases in activity relative to time matched vehicle. Data represent mean ± SE; n = 3 - 4; **p < 0.01, ***p < 0.001; ns, not significant compared to vehicle- treated values by one-way ANOVA using Dunnett's post hoc test.

### 4.2.3 Aβ-induced $Ca^{2+}$ flux through the NMDAR

To identify an instant readout of functional change resulting from Aβ treatment that paralleled its toxicity, a $Ca^{2+}$ flux assay was employed as previously described (Section 3.2.2). In attempts to induce a specific $Ca^{2+}$ flux, varied preparations of synthetic Aβ that have previously been described as the major components of the peptide's toxicity were screened. The oligomeric state was first compared by preparing the peptide either by dissolving directly in to NaOH or by pretreatment with hexafluoro-2-propanol (HFIP) to monomerise the Aβ (Chromy et al., 2003; De Felice et al., 2007). A time-course over 4 h was performed with both preparations of Aβ42 without any of the treatments affecting a significant change to the calcium flux when the data of 3 independent experiments were combined with the results normalised to glutamate-treated neurons and compared to vehicle treated neurons (Figure 4-3A). Along with these several preparations of Aβ that have been implicated as the toxic species underlying the aetiology of AD were tested for their ability to affect $Ca^{2+}$ flux. Firstly, the Aβ dimer, thought to be a potent toxic species (Walsh et al., 2002),(Shankar et al., 2008), was tested by using dityrosine linked Aβ40 dimers synthesized in our laboratory (Smith et al., 2007; Kok et al., 2013). Following this Aβ-derived diffuse ligand (ADDLs) preparations, a stable preparation of soluble oligomeric Aβ42 (Lambert et al., 1998) was tested. Also screened was a stable oligomeric form of Aβ42 of approximately 60 kDa, which was detected in the brain and mice and termed globulomers (Barghorn et al., 2005). The Aβ42Y10A mutant, which prevents the dityrosine cross linkage and therefore dimer formation (Barnham et al., 2004a) was tested as a negative control. The Aβ42M35V mutant was also tested, again acting as a negative control by changing the methionine to a valine at position 35, which has been implicated in oxidative stress, one of the mechanisms believed to underlie Aβ42 toxicity (Ciccotosto et al., 2003). As was observed in Figure 4-3A, no $Ca^{2+}$ flux was observed, however, to control for these experiments, ADDLs prepared in Ham's F12 (Lambert et al., 1998) media were compared to ADDLs in Neurobasal (NB media). These experiments revealed the increased $Ca^{2+}$ flux not to be specific

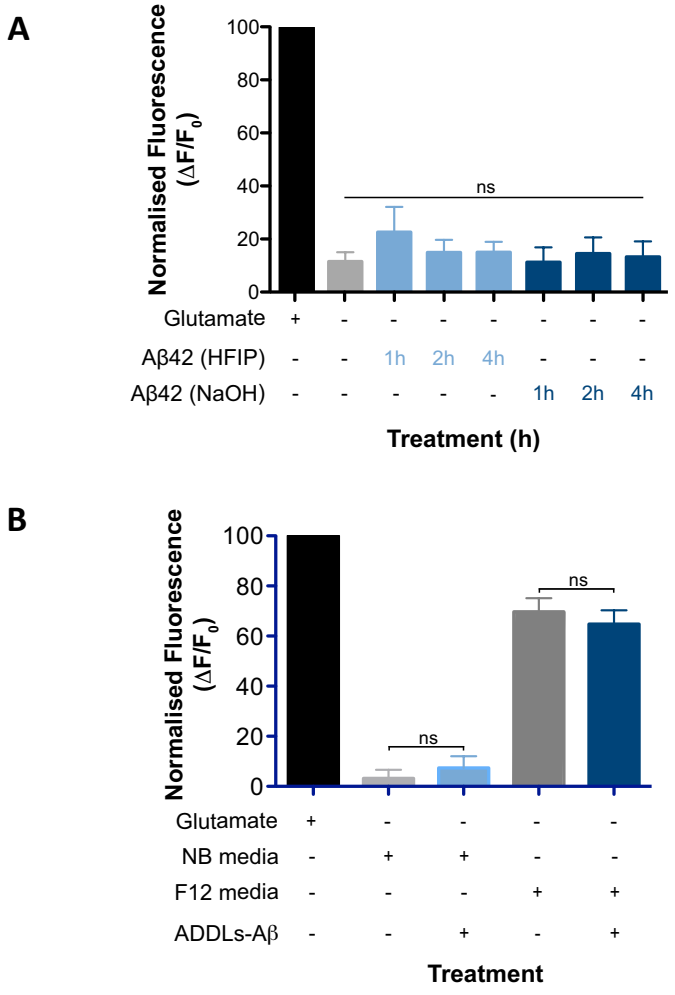



**Figure 4-3. Aβ42 preparations, in isolation, are not sufficient to induce $Ca^{2+}$ flux in neurons.** ***A,*** Aβ42 peptides prepared with HFIP, to increase proportion of monomers, or fresh by dissolving directly in to NaOH were tested over 4 h for their ability to induce $Ca^{2+}$ flux. Results were expressed normalised to neurons treated with 100 μM glutamate. No statistically significant changes were observed with either of the Aβ42 preparations at any of the time points (light and dark blue bars) compared to vehicle (grey). ***B,*** A significant increase in $Ca^{2+}$ flux due to treatment with ADDLs (10 μM) was not specific and due to the F12 media used in their preparation. This is indicated by the substantial increase in $Ca^{2+}$ flux in neurons treated with F12 media only (dark grey bar) which is not significantly different to the ADDLs treated neurons (dark blue bar. ADDLs prepared in NB media have no effect on $Ca^{2+}$ flux compared with their vehicle control (light blue and grey bars, respectively). Data is expressed normalised to glutamate treated neurons (black bar) as the mean of ± SE; n = 3; ns, not significant compared to glutamate treated values by one-way ANOVA using Dunnett's post hoc test.

Aβ42 effect but an artefact resulting from the media used (Figure 4-3C).

### 4.2.4 Aβ-induced cell death through activation of glutamatergic receptors.

To investigate whether Aβ42 toxicity were mediated through the glutamatergic system, the effects on cell survival were studied following Aβ42 treatment. Several key publications had recently implicated the NMDAR in the cell death associated with Aβ42 (Ittner and Götz, 2010; Um et al., 2012; You et al., 2012). This had also been clearly demonstrated in the recent publication by Alberdi et al., (Alberdi et al., 2010) who had employed a cortical neuron model and the stable preparation of Aβ42, ADDLs. In their manuscript, together with describing an effect on $Ca^{2+}$ flux (section 4.2.3), they demonstrated NMDAR-mediated toxicity in cortical neurons induced by ADDLs. In my attempts to induce a specific toxicity due to Aβ42 conditions identical to those used in their experiments my experimental conditions were identical to those employed in their manuscript. In agreement with the findings in their work, the ADDLs preparations of Aβ induced cell death following 24 h treatment in cortical neurons as measured by cell viability (Figure 4-4A) However when the vehicle was treated identically according to the preparation of ADDLs (described in Lambert et al., 1998) it too decreased cell viability to the same extent as the Aβ42 preparations. Moreover this toxicity was completely prevented by pretreatment with the NMDAR-antagonist, MK-801. This NMDAR-mediated toxicity was also observed in neurons exposed to the ADDLs preparations with specificity demonstrated by pretreatment with MK-801 (Figure 4-4B). These findings demonstrate that the ADDLs-Aβ42 preparations did not cause NMDAR-mediated cell death, instead this effect was due to the vehicle rather than the ADDLs in contrast to what was concluded in the research by Alberdi and colleagues.

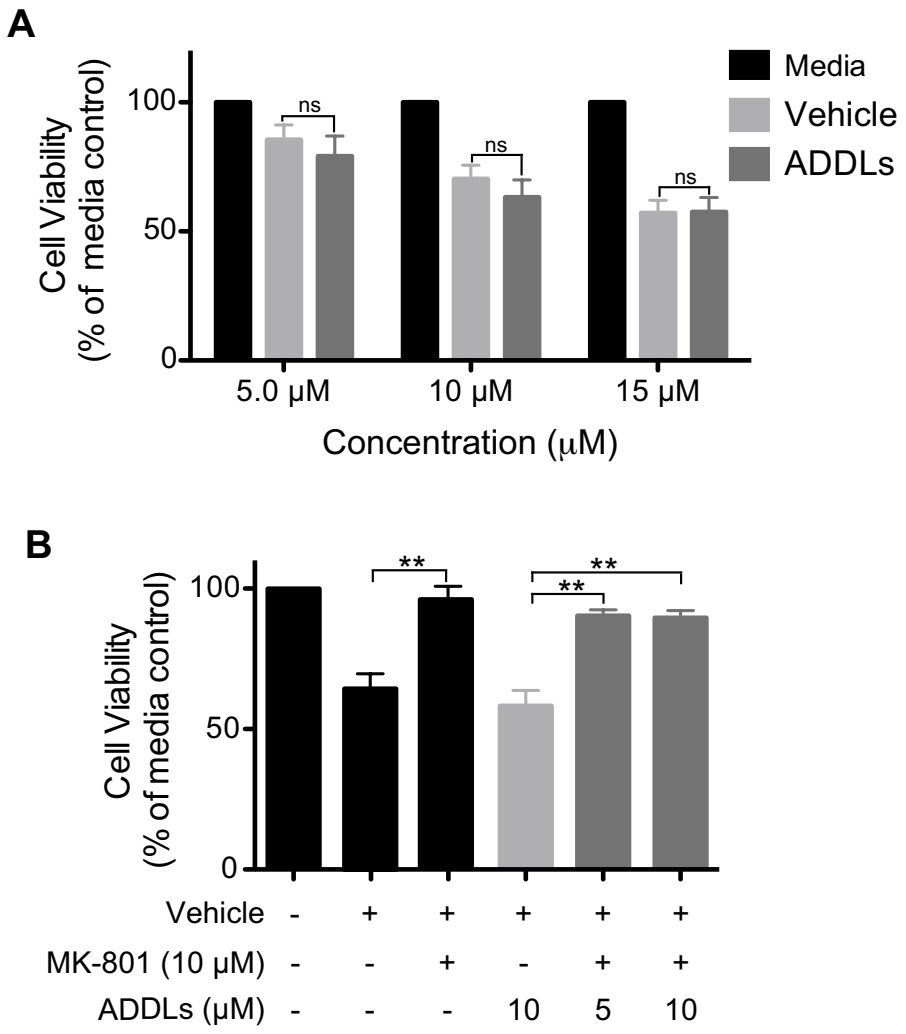
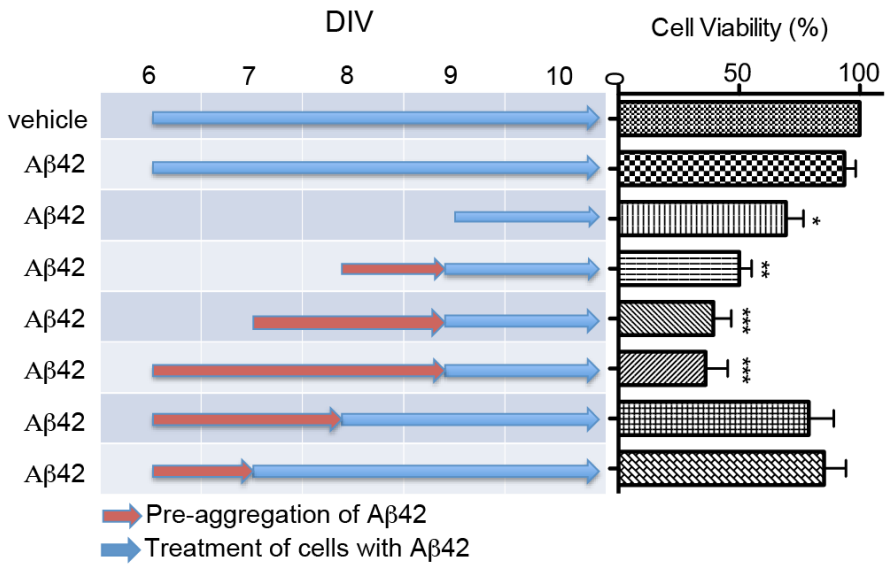



**Figure 4-4. The toxicity of ADDLs Aβ in primary cortical neurons is attributed to it's vehicle.** ***A***, Treatment with increasing concentrations (5 - 15 μM) of ADDLs or the corresponding volumes of vehicle results in a significant reduction in cell viability. As there was no significant difference between ADDLs-treated neurons and its F12 media vehicle cell death cannot be attributed to the ADDLs Aβ42. ***B***, Further experiments in neurons demonstrate that the observed toxicity of ADDLs was mediated by the NMDAR as cell viability was significantly prevented by pre-treatment with the uncompetitive NMDAR antagonist, MK-801 (10 μM) for 1 h. The toxicity observed in F12 media vehicle-treated neurons was also significantly inhibited by pre-treatment with MK-801. Data represent mean ± SE; n = 3 - 6; **p < 0.01; ns, not significant by unpaired, two-tailed Students t-test (A) or one-way ANOVA using Dunnett's post hoc test (B).

### 4.2.5 The effect of the number of days in vitro (DIV) on toxicity in cortical neurons.

In concurrent experiments to those with the ADDLs-Aβ42 preparations, the parameters for Aβ42 toxicity in the standard toxicity assay (section 4.2.1) were investigated to determine whether the duration of Aβ42 treatment or the number of days cortical neurons were cultured (DIV) contributed more significantly to Aβ42 toxicity. The aim of these experiments was to resolve which parameter/s of the standard toxicity assay, following optimisation, could significantly reduce the Aβ42 treatment time. Three parameters were examined in these experiments; Aβ42 treatment time (blue arrows, Figure 4-5A), the effect of aggregation by incubating Aβ42 in media prior to treatment of neurons (red arrows, Figure 4-5A) and the DIV of cortical neurons in the standard assay (6 -10 DIV) (Figure 4-5A). Treatment of cortical neurons in cultures fewer than DIV 9 with Aβ42 (10 μM) had no significant effect on cell viability. In cells treated at DIV 9, significant toxicity was observed which increased with the number of days the Aβ preparation had been incubated for prior. In these experiments the vehicle control was added at DIV 6 and treatment was for 4 days, which had no observable effect on cell viability. Importantly, subsequent attempts with these newly optimised conditions, that is Aβ42 added to neurons at DIV 9 without pre-aggregation, failed to induce specific Aβ42 toxicity above that of the vehicle (Figure 4-5B). The vehicle control was in fact responsible for the toxicity as was observed by its significant decrease in cell viability compared with neurons treated only with Neurobasal media (Figure 4-5B). Interestingly, DIV influenced the extent of this toxicity indicating that necessary components of the toxic pathway may not be fully expressed in immature cultures such as those ≤ DIV8.

**A**




**B**

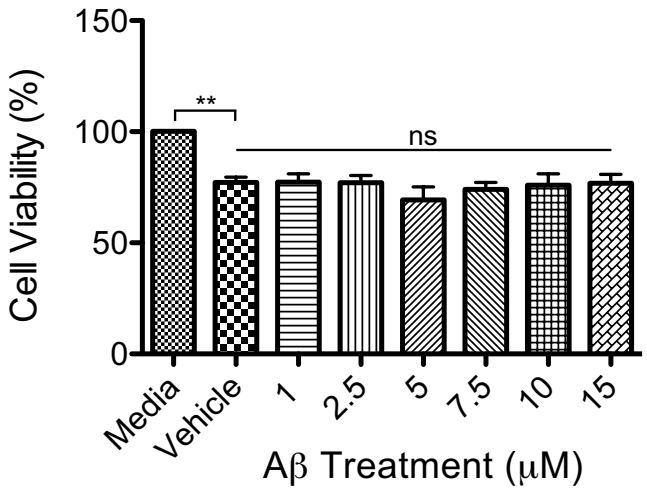



**Figure 4-5. Toxicity observed in cortical neurons is dependent on DIV.** ***A***, Cortical neurons were treated at DIV6 with vehicle and at DIV 6 and 9 with fresh Aβ42 (10 μM) (blue arrows). To test the effect of aggregation Aβ42 was pre-incubated for 24, 48 and 72 h (red arrows) and then added to cells from DIV 7 – 10 (blue arrows). The cell viability in neurons treated at DIV 6, 7 and 8 with Aβ42 aggregated for 0, 24 and 48 h, respectively were not significantly different to vehicle demonstrating a greater requirement of DIV compared with aggregation in Aβ42 toxicity. The effect of DIV on toxicity was confirmed in neurons treated with fresh Aβ42 or Aβ42 aggregated for 24, 48 or 72 h at DIV 9. ***B***, Subsequent experiments with Aβ42 & vehicle added to neurons at DIV 9 without pre-aggregation (same as in ***A***, blue arrow, third from top) demonstrated the toxicity was not specific to Aβ42 treatment when compared with neurons treated with media only. Data represent mean ± SE; n = 3; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant; compared by one-way ANOVA using Dunnett's post hoc test.

### 4.2.6 The effects of the developmental age of cultures and the duration of Aβ42 treatment on cell viability in the standard toxicity assay.

The maturity of cortical cultures has been shown to significantly affect Aβ toxicity (Figure 4-5) and in the standard toxicity assay 72 h to 96 h treatment was necessary for an Aβ42-induced effect. The next set of experiments aimed to determine whether the DIV or treatment time affected Aβ42 toxicity more significantly as was previously attempted (section 4.2.5). Cortical neurons were cultured for 6, 8, 10 and 12 DIV and then for each of the 4 differently aged cultures, treated with Aβ42 (10 μM) for 24, 48, 72 and 96 h (Figure 4-6, A-D). The Aβ42 batch (Keck, Harvard, USA) used in these experiments demonstrated lower toxicity than expected as seen in neurons at 6 DIV treated for 96 h (Figure 4-6D), which were not affected by treatment, unlike the previously described decrease in cell viability to 86% with the same concentration of Aβ42 (Figure 4-1A). The effect of DIV on Aβ42 toxicity was clearly demonstrated in neurons DIV ≥ 8 and treated for just 24h (Figure 4-6A) With exception to DIV 10 neurons, which required 48 h Aβ42 treatment to show significant toxicity (Figure 4-6B), the 8 and 12 DIV cultures had a significant reduction in cell viability compared with the neurons at 6 DIV (Figure 4-6A). Interestingly, no significant increase was observed due to treatment time in neurons at DIV12. Statistical comparisons show no difference between cultures that were the equivalent to 12 DIV or greater (for example; DIV 10 neurons treated for 96 h = DIV 14) when the cell viability was measured, with the exception of neurons treated for 96 at DIV 10. In all comparisons cell viabilities were expressed normalised to the vehicle control and statistical analysis compared treatments with neurons at 6 DIV for each treatment time (Figure 4-6, A-D).

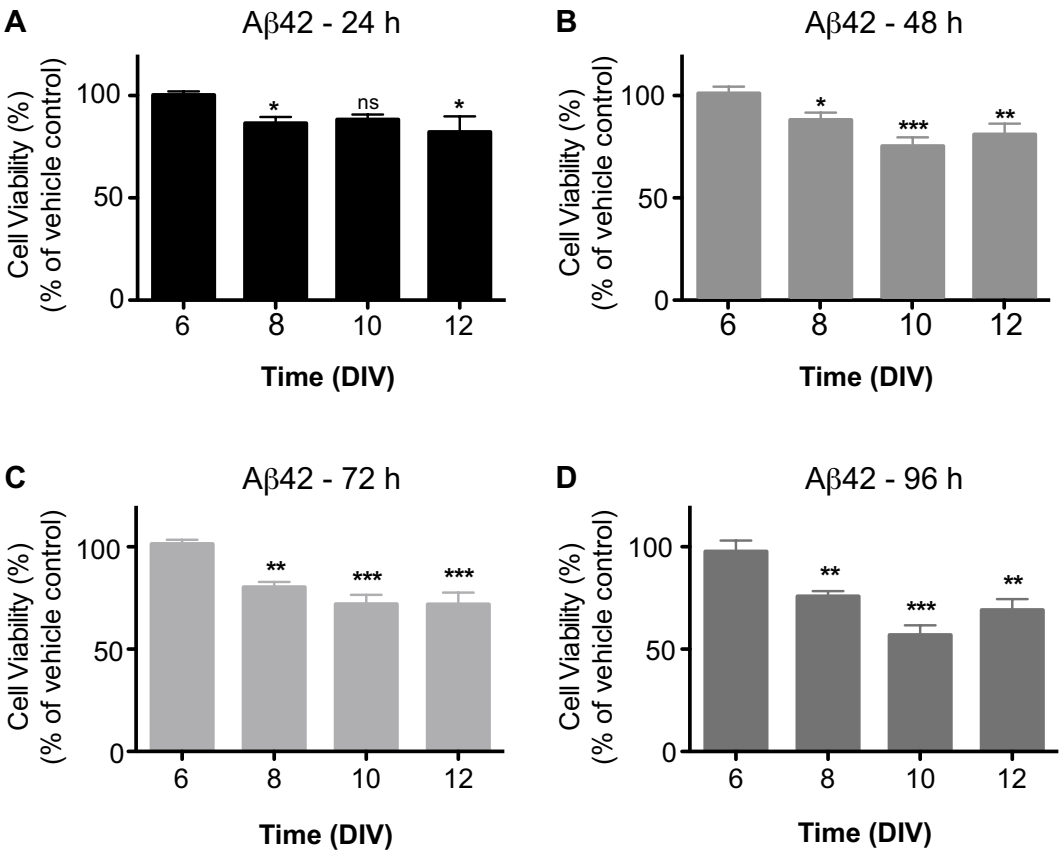



**Figure 4-6. Aβ42 Aggregation versus DIV of cortical neuron**s. ***A – D***, Aggregation-dependent Aβ42 toxicity was observed by treating cortical neurons for 24, 48, 72 and 96 h with 10 μM Aβ42 as in the standard Aβ42 toxicity assay. To observe the effect of DIV on toxicity, the time-course experiments were performed on neurons previously cultured for 6 to 12 DIV. The toxicity of the synthetic Aβ42 batch used in these experiments was significantly lower than normally observed in our standard toxicity assays as seen in ***D*** where neurons initially at 6 DIV were treated with Aβ42 for 96 h but showed not significant decrease in cell viability. Despite the decreased toxicity of the Aβ42, significant toxicity occurred in all neurons 8 DIV or more (equivalent to 9 DIV or more in total when taking in to account duration of Aβ42 treatment) with the exception of neurons at 10 DIV treated for 24h. There was no significant difference between neurons at DIV12 treated for 24 - 96 h (***A-D***) Cell viability is expressed normalised to time-matched vehicles (not shown on graphs). . Data represent mean ± SE; n = 4-5; *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant; compared by one-way ANOVA using Dunnett's post hoc test.

### 4.2.7 The effect of DIV on the expression of key glutamatergic receptors.

Following the finding that toxicity was affected by the age of the mouse cortical cultures and given the involvement of excitotoxicity in Aβ-mediated cell death, expression levels of receptors changed with DIV were investigated. In doing so the protein expression profiles of the glutamatergic receptors; AMPAR and the NMDAR subunits; NR1, NR2A and NR2B from DIV6 – DIV14 were determined. Recent research lends significant support to the involvement of excitotoxicity in Aβ42-induced cell death. This pathway is mediated through NMDARs and importantly, through NR1/NR2B receptor complexes with NR1/NR2A complexes believed to mediate cell survival signaling (Hardingham, 2009). Expression levels of the obligatory NR1 subunit and the NR2A and B subunits in these experiments were consistent with those previously described in rat cortical neurons (Ferreira et al., 2012). Apart from an anomaly observed in DIV12 neurons, NR1 total protein expression remained consistent with maturation time points (Figure 4-7A) In keeping with observations in rat cortical neurons, the expression of NR2A is relatively low at DIV6 – 10 before levels are significantly increased from DIV12 (Li et al., 1998). This was also the case for AMPAR expression with mature levels not being reached until DIV 12. The relatively low levels of both NR2A and AMPAR at DIV 9 supports the proposition that the previously observed toxicity is not mediated through these receptors. The expression profile of NR2B subunit, in contrast to NR2A, indicates total protein levels begin to increase at DIV 9 and are significantly increased by DIV11. This profile associated closely with Aβ toxicity indicating that it may be NR2B-mediated.

### 4.2.8 An equivalent developmental age of cultures is required for excitotoxicity and Aβ42-induced toxicity.

To determine whether development of cortical neuronal cultures affected glutamate-induced excitotoxicity to the extent it affected Aβ toxicity,

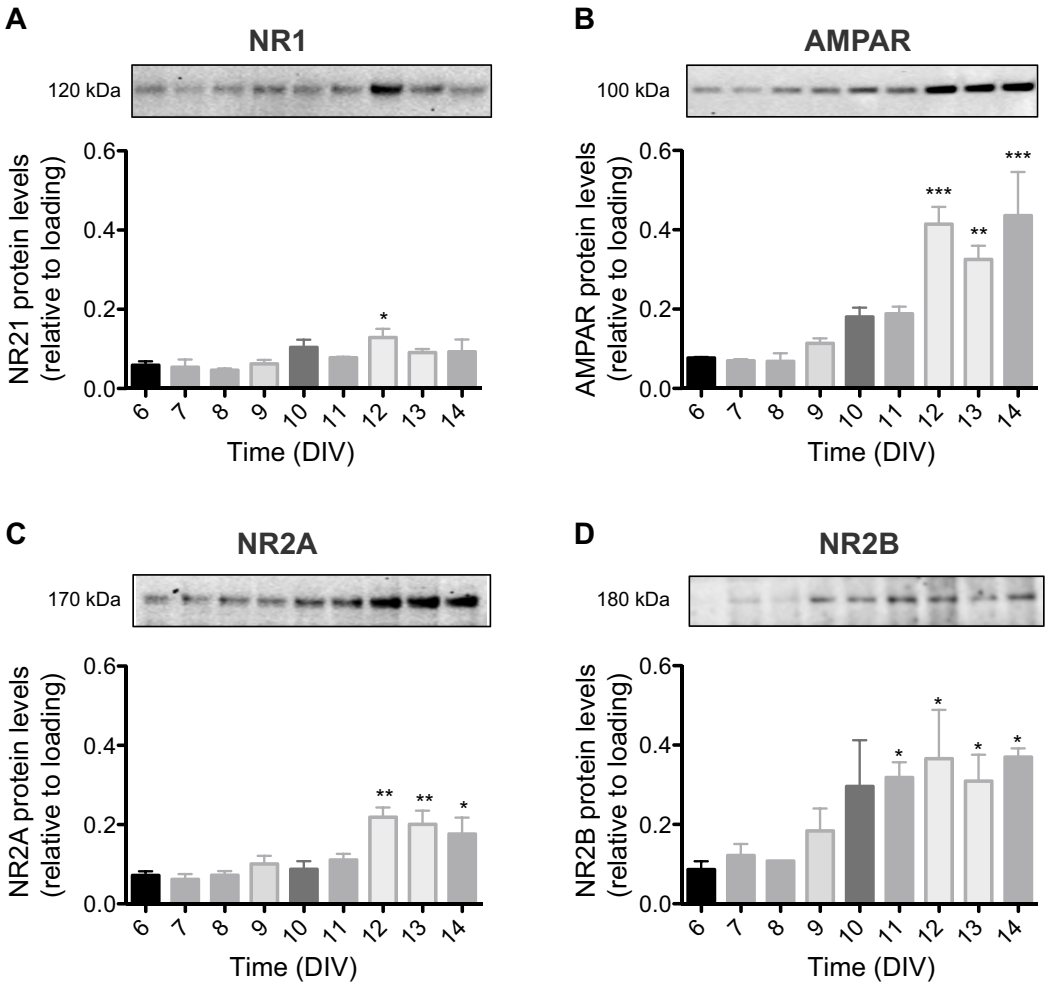



**Figure 4-7. Protein expression levels of key glutamatergic receptors in cortical neurons. *A – D*, Time**-dependent total expression levels of the obligatory NMDAR subunit NR1 (120 kDa), AMPAR (100 kDa) and the NMDAR subunits NR2A (170 kDa) and NR2B (180 kDa) in cultured cortical cells for 6 to 13 days. Proteins were analysed by western blot with values obtained for DIV 6, 7, 9, 11 and 13 expressed as arbitrary units normalised to total protein transferred to membrane used for antibody detection (see methods, chapter 2). Representative blots of protein levels at DIV 6 to 14 are shown in panels above graphs. Data represent mean ± SE; n = 3; *p < 0.05, **p < 0.01, ***p < 0.001; compared to DIV 6 neurons values by one-way ANOVA using Dunnett's post hoc test.

cell death that was mediated by the NMDAR in the cultures, was examined. Neurons were grown in culture for 6, 7, 8, and 9 DIV and then exposed to

excitotoxic levels of glutamate (50 μM). NMDA-specific cell death was determined by the use of the NMDAR-specific antagonist, MK-801 (Figure 4-8). The decrease in cell viability observed in vehicle-treated neurons compared with MK-801-treated neurons (black and grey bars, respectively, Figure 4-8) indicated cell death mediated through pathways other than the NMDARs. A small but significant decrease in cell viability is observed at DIV6 and 7 but as was observed in the protein expression profiles, it is not until DIV9 that substantial excitotoxicity is induced by glutamate exposure. This observation parallels the effect of DIV on Aβ42-induced toxicity (Figure 4-6) and strongly suggests that excitoxicity underlies the cell death observed in this model.

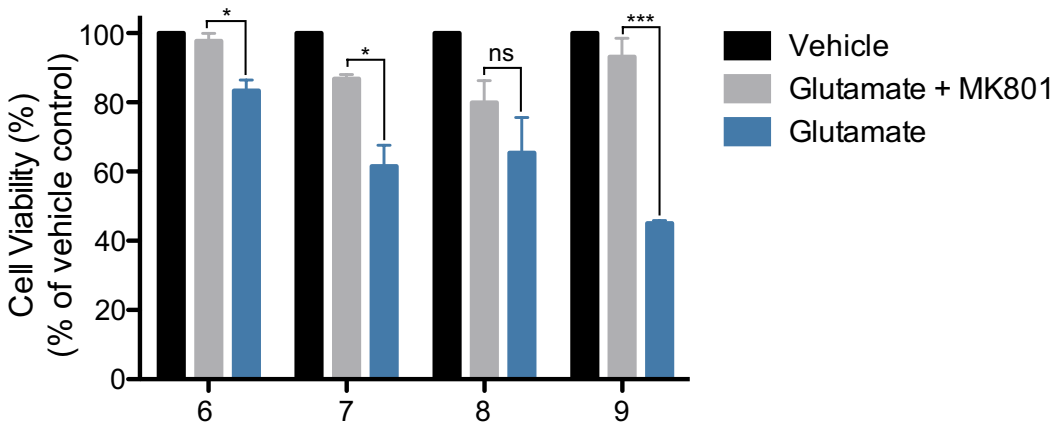



**Figure 4-8. The effect of Days in vitro (DIV) on excitotoxic cell death in primary cortical neurons.** Glutamate (50 μM, 1 h) (blue bars) was used to induce excitotoxicity and the effect of DIV was observed. Cortical neurons were pre-treated with the NMDAR specific antagonist, MK-801 (10 μM, 1 h) (grey bars) to demonstrate glutamate-induced cell death was entirely NMDAR-mediated and therefore excitotoxic. Significant decreases in cell viability are observed at DIV 6 and 7 but it is only at DIV 9 that a marked toxicity that is fully prevented by MK-801. Data represent mean ± SE; n = 3; *$p < 0.05$, ***$p < 0.001$; ns, not significant by unpaired, two-tailed Students t-test.

## 4.3 Discussion

To date cell culture-based screens have been employed in parallel with biophysical assays to detect Aβ aggregation in efforts to characterise the oligomeric species of Aβ underlying the toxicity observed in AD. Such assays have been performed routinely in our laboratory using primary mouse cortical neurons and measuring cell viability with the tetrazolium salt, WST-8. In conjunction, aggregation of Aβ, prepared fresh or treated with HFIP to monomerise the peptides, has been determined by fluorometric analysis of ThT binding (Figure 4-1, A and B). It has been reported that coupling these cell biology and biophysical techniques with Transmission electron microscopy (TEM) and cytotoxicity assays measuring LDH release provides necessary secondary controls but also allows investigators to correlate Aβ cytotoxicity with its aggregation (Jan and Lashuel, 2012). Another analytical technique, which has been used to observe molecular species of Aβ is surface-enhanced laser desorption/ ionization – time of flight (SELDI-TOF) mass spectrometry. This technique overcame possible artefacts that may have arisen from gel based anaylsis such SDS-induced dimers as reported recently (Watt et al., 2013). Despite this the propensity of the Aβ42 peptide to aggregate has meant that each one of these techniques, either independently or in conjunction with others, were unable to immediately assign a particular assembly form of Aβ to the corresponding neurophysiological effects.

The development of an assay to screen caspase activity as a readout of Aβ toxicity initially appeared to be a superior alternative to our current method. Caspase 3 activation specifically by the Aβ42 form, as opposed the less toxic Aβ40, in hippocampal neurons was potent and observed in the much shorter time frame of 48 h (Figure 4-2A). This finding could be reproduced in cortical cultures affording a 10-fold higher throughput due to increased cell numbers. In these neurons a significant effect on caspase 3 activity could be observed in as little as 4 hours post Aβ42 treatment (Figure 4-2B). Despite the apparent increased sensitivity and reduced lag time, the assay was unable to demonstrate correlation between caspase activation and Aβ42 toxicity. Staurosporine (STS)

was used in these experiments to positively control for apoptotic cell death mediated by caspase 3 (Chae, 2000; Belmokhtar et al., 2001). In my previous work and in published research STS (≥ 0.5 μM) treatment of neurons, as described in figure 4-2A, has been shown to induce as near to total cell death as possible to measure by cell viability assays (Prehn et al., 2002). Aβ42 (10 μM), despite activating caspase 3 to comparable levels (Figure 4-2A), decreased cell viability by no more than 20% under the similar conditions (Figure 4-1A). In support of non toxic activation of caspase 3 by Aβ is the discovery of a role of caspase 3 in a non-apoptotic pathway involving the BAD-BAX cascade that mediates long-term depression (LTD) by the NMDAR (Li et al., 2010; Jiao and Li, 2011). This alternate function, although quite interesting as it raises novel mechanistic pathways for Aβ, excluded the detection of caspase 3 activity as an assay for Aβ toxicity.

In search of an assay with very little or no lag time between the preparation of fresh Aβ42 until detecting its toxic effect research concentrated on the glutamatergic system and its role in AD. The body of evidence asserting the involvement of NMDARs in mediating Aβ42 toxicity had been increasing significantly with an array of publications describing an effect of Aβ42 on the intracellular flux of $Ca^{2+}$ (reviewed in Introduction, 1.6). A substantial majority of the research has reported increases in intracellular $Ca^{2+}$ flux following treatment with Aβ42 (De Felice et al., 2007; Alberdi et al., 2010; Texidó et al., 2011; Ferreira et al., 2012). One publication contradicts these findings, in this work the authors propose that dendritic spine loss follows partial NMDAR inhibition by Aβ42 treatment (Shankar et al., 2007). Inhibition of the NMDAR was subsequently shown to occur and the resulting reduction in $Ca^{2+}$ flux was specific to Aβ42 oligomers with monomeric Aβ42 and controls remaining unchanged (Shankar et al., 2007). A potential explanation for these contrary findings may arise from a recent and publication from the same group, which describes the major constituent of their naturally secreted oligomeric preparations were, in fact, extended Aβ42-containing APP fragments rather than dimeric and trimeric forms of Aβ42 (Welzel et al., 2014). These fragments were also found to be toxic, significantly inhibiting LTP, as with Aβ42. Despite their apparent toxicity these

APP-containing fragments may have protective affects on intracellular calcium as was observed in previous experiments with soluble APP alpha that would explain their opposing results (Mattson et al., 1993a; Duce and Bush, 2010)

Attempts to replicate the findings that Aβ specifically induces $Ca^{2+}$ flux with the Dimeric, ADDLs and globulomer synthetic preparations of Aβ42, each reported to have different effects on toxicity, (Figure 4-3, A - C) and show effects on intracellular calcium levels, were unsuccessful. Time-course experiments, designed to test the effect of increased Aβ42 aggregation, were also unable to affect changes in $Ca^{2+}$ flux in this model (Figure 4-3A). Research that has reported effects of Aβ on $Ca^{2+}$ flux revealed in each case have used ADDLs preparations in Ham's F12 media as originally described by Lambert et al. (1998). The significance of the media the ADDLs was prepared in was seen in $Ca^{2+}$ flux (Figure 4-3C) and cell viability experiments (Figure 4-4). In both sets of experiments the F12 media vehicle demonstrated a significant effect on the readout of the assay and these effects were not significantly different to the ADDLs preparations. When the formulation of the Ham's F12 media was compared to the normal NB media used in all my experiments the important difference between the two culture media was observed. The Ham's F12 media contains 1 mM glutamine and 100 µM glutamate (see Supplementary chapter, 7.1). Two of the four publications (De Felice et al., 2007; Ferreira et al., 2012) have indicated that Ham's F12 without glutamine was used but according to the suppliers they have cited, the formulations show the media still contains 100 µM glutamate. In these experiments they have used between 300 nM (De Felice et al., 2007) and 5 µM ADDLs (TexidÃ³ et al., 2011). As the concentration of glutamate in Ham' F12 media, with or without glutamine, is the same as the initial concentration the ADDLs added to cells in each of the publications, they are adding the same concentration of glutamate as they are ADDLs. Given the sensitivity of the assay systems they have described glutamate at 300 nM would most likely affect their results. Taking into account the presence of glutamate in the media does not explain the discrepancy between my results (Figure 4-3C and Figure 4-4, A and B) and those publications where ADDLs treatment was adequately controlled in $Ca^{2+}$ flux assays (De Felice et al., 2007) and neural toxicity assays (Alberdi et al.,

2010). To speculate, one explanation may be that the F12 vehicle in both instances has not been treated identically to the ADDLs and not incubated for 24 h at 4°C then centrifuged to remove insoluble aggregates. Instead it may have been added the same day as the experiment is performed, particularly as ADDLs preparations are often stored for some time at 4°C prior to their use.

Such an example of non-specific toxicity due to Aβ pre-incubation was observed in subsequent cell viability experiments. These experiments aimed to determine the contribution of the aggregation state of Aβ and the DIV of the cortical cultures to Aβ toxicity (Figure 4-5A). Initially it appeared that the DIV of the cultures had a potent effect on Aβ toxicity and the number of days the Aβ was aggregated also having a significant effect. This toxicity was subsequently determined to be an artefact of the vehicle (Figure 4-5B) but the cell death increased with the number of days the samples were incubated, emphasizing the effect of time on the toxicity resulting from the media. Along with these observations the maturation of the cultures demonstrated a potent impact on cell death from the media and indicated a requirement of receptor expression to mediate this toxicity (Figure 4-5A).

Further indications that receptor expression may be involved in mediating Aβ toxicity was seen in the subsequent, appropriately controlled experiments investigating the effects of DIV and Aβ incubation time in cortical neurons (Figure 4-6). Despite Aβ42 toxicity being less than normal, significant toxicity is still observed in neurons that have been cultured for 8 DIV or more when compared to those at 6DIV, strongly implicating the maturation of the culture in mediating toxicity (Figure 4-6). One of the major biophysical properties of Aβ42 is attributed to the two hydrophobic amino acids at its c-terminus. These additional amino acids dramatically increase the aggregation rate of Aβ42 compared to Aβ40 as well as its toxicity to neurons (Yan and Wang, 2007). To test the effects of Aβ42 aggregation on toxicity, the neurons were also treated for 24, 48 and 96 h and normalised to vehicle-treated neurons (Figure 4-6, A-D). Assessment of the data shows significant time-dependent increases in toxicity for DIV 8 and 10 when compared to 24 h. The significant finding from these

experiments however can be seen in neurons at DIV 12 where there is no significant increase in Aβ42 toxicity due to the duration of treatment. This result provides evidence that the toxicity of Aβ42 is due to the DIV of the culture and not the treatment time. The significant increases observed in the less mature neurons (DIV 8 and 10) treated for 24 – 96 h are therefore attributed to the increasing age of culture during the treatment, in this comparison DIV 8 cultures treated for 96 are equivalent to DIV 10 treated for 48h etc. Given neurons treated for the same time at DIV 12 it is also concluded that the necessary time for aggregation of Aβ42 is less than 24 h given no further increase to toxicity at cultures at DIV12 or greater.

The strong correlation between Aβ toxicity and DIV of culture led to an investigation of the glutamatergic receptor expression profiles in cortical neurons at the same stages of maturation in the previous Aβ toxicity experiments (Figure 4-7, A-D). The expression profile of NR2B subunit of NMDAR not only directly parallels Aβ toxicity but also the non-specific toxicity observed in F12 vehicle-treated neurons (Figure 4-5) implicating the involvement of excitotoxicity mediated through this receptor. Of the remaining glutamatergic receptors studied all maintained were expressed at normal basal levels during this timeframe. Both the AMPA and NR2A receptor profiles required extended culturing of ≥ DIV12 before protein levels rose above basal expression. Given significant toxicity was observed following 24 h treatment of neurons at DIV 8 (DIV 9 equivalent) it is improbable they mediate toxicity. The same conclusion can be drawn for the NMDAR-NR1 (Figure 4-7A) which shows no change in expression as would be expected given its obligatory role in NMDA complex. In further support of the ionotropic NMDARs' involvement in Aβ-induced cell death in this cortical neuronal model is seen in published expression profiles, in neurons, of two other key receptors implicated in Aβ toxicity; α7nAchR (Oz et al., 2013), mGLuR1 and 5 (Renner et al., 2010). Protein levels of both of these receptors demonstrated significant increases although they occurred at a considerably earlier time (DIV 5-7) than what corresponds with Aβ toxicity (Voulalas et al., 2005; Mielke and Mealing, 2009). It is important to note that due

to differences between cell type, age and species of animal it is not possible to draw any conclusions with regard to their involvement in Aβ toxicity.

In experiments to optimise the conditions for glutamate-induced excitotoxicity presented here it can be clearly seen that neuronal vulnerability is significantly enhanced during development (Figure 4-8). This is in agreement with comparable research in the last 25 years, both in vivo and in vitro (Peterson et al., 1989; Liu et al., 1996; Mizuta et al., 1998). In these data a non-NMDAR mediated component of cell death was observed at earlier time points which could not be prevented by the use of the NMDAR antagonist, MK-801. However by DIV9 the significant excitotoxic cell death resulting from glutamate treatment was entirely NMDAR-mediated and, more importantly, this corresponded with both the expression profile for the NMDAR-NR1 subunit expression and Aβ toxicity.

The work presented in this chapter has sought to elucidate the crucial components in a cortical neuronal model for mediating Aβ toxicity. As a result a developmental relationship between the NMDARs and neuronal cell death in cortical cell cultures treated with Aβ has been demonstrated. The significant impact of these findings will be realized in subsequent research, which can now focus on the glutamatergic system and the role of Aβ in excitotoxic cell death.

# 5 A Glutamate-induced Calcium Flux Assay to Screen Toxicity of Aβ Preparations

## 5.1 Introduction

Dysregulation of $Ca^{2+}$ has been implicated in aging and AD for several decades. The $Ca^{2+}$ hypothesis of AD and aging was first postulated by Khachaturian in the early eighties and further revised a decade later (Khachaturian, 1994). The main principle of the hypothesis is that sustained alterations to intracellular $Ca^{2+}$ $[Ca^{2+}]_i$ homeostasis accounts for both the cognitive impairment and increase in neuronal cell death in AD. Supporting evidence was initially provided in previous work by Landfield and colleagues during the late 1980s and early 1990s, which proposed that calcium flux through voltage sensitive channels could be increased due to aging or AD (Landfield et al., 1989; 1990; 1992). During this period Mattson et al. published an extensive body of work that strengthened the $Ca^{2+}$ hypothesis and underlies many research perspectives in the field of AD today. In their initial investigations they described the involvement of $Ca^{2+}$ in the molecular mechanisms that regulate the neuronal architecture and proposed the loss of this function resulted in impaired dendritic arborization, pruning or complete elimination and neuronal loss in AD (reviewed in Kater et al., 1989; Mattson and Rychlik, 1989a). In their early research to explain how these findings pertained to AD they demonstrated that $Ca^{2+}$ influx, induced by the excitatory amino acid glutamate, led to sustained elevations of intracellular $Ca^{2+}$. This caused similar cytoskeletal changes as NFTs in AD (Mattson and Rychlik, 1989b; Mattson et al., 1991). It was the discovery from their subsequent research into the effects of Aβ on glutamate-induced cell death in human neurons that has been of considerable importance to the research presented in this chapter. Building upon the recent discovery that Aβ was neurotoxic to hippocampal neurons (Yankner et al., 1990) Mattson et al., used

primary human cortical neurons to demonstrate the ability of Aβ to render cells more susceptible to calcium influx and glutamate toxicity (Mattson et al., 1992). This Aβ-specific effect on excitotoxicity occurred in neurons co-treated with non-toxic levels of glutamate and resulted in a significant increase in $[Ca^{2+}]_i$ compared to neurons treated only with glutamate.

When considering the effects of Aβ on glutamate induced signaling, the microenvironment of the synaptic cleft, where glutamate diffuses from pre to post synapse, must be considered. Membrane-bound Aβ oligomers accumulate in the cleft and have been reported to exert their effects through a variety of mechanisms including the inhibition of glutamate reuptake (Li et al., 2011) and altering $[Ca^{2+}]_i$ by affecting glutamergic receptors at the post synapse (Ittner et al., 2010; Renner et al., 2010; Um et al., 2012). The targeting to the synapse and rapid aggregation of Aβ has shown to be dramatically enhanced by the presence of the transition metals, $Cu^{2+}$ and $Zn^{2+}$ (Bush et al., 1994a; Atwood et al., 1998; Deshpande et al., 2009). $Zn^{2+}$ is released in to the presynapse during neuronal excitation at concentrations between 10 and 100 μM (Watt et al., 2010). It has also be established that upon a neuron firing the resulting NMDAR activation causes a rapid efflux of $Cu^{2+}$ in to the synapse (Schlief, 2005). The concentration of $Cu^{2+}$ in the cleft has been estimated at 15 μM (Hartter and Barnea, 1988) and also at much higher levels of 100 - 250 μM (Kardos et al., 1988). Both $Zn^{2+}$ and $Cu^{2+}$ bind Aβ at the same histidine residues located within the first 16 residues of the N-terminus (reviewed in Faller, 2009). However under physiological conditions $Cu^{2+}$ has been reported to have between nanomolar and picomolar dissociation constants compared with the micromolar dissociation constants reported for $Zn^{2+}$ (Faller, 2009; Sarell et al., 2009). The significantly higher affinity of Aβ for $Cu^{2+}$, suggests it is more likely to be the metal involved in pathological interactions with Aβ in the synapse.

Following on from my previous efforts to design an assay to screen for Aβ toxicity in cortical neurons (Chapter 4), I now sought to determine what role if any there was for glutamate in Aβ toxicity and further describe the conditions of this toxicity in a neuronal model. In doing so I aimed to incorporate both

glutamate and $Cu^{2+}$ in to my assays, as described in preceding paragraphs. Finally, upon the successful development of the assay I sought to evaluate the toxicities of three well-described forms of Aβ, each reported to have differing mechanisms mediating their toxicity.

Firstly, I aimed to test dityrosine-linked dimers of Aβ. These dimers are believed to play a significant role in AD. Initial evidence of their involvement came from experiments in which oligomeric forms of Aβ were isolated from AD brains by size exclusion chromatography. It was found that the inhibition of LTP, enhanced LTD and reduced spine density in rodents was specific to the dimeric fractions, not monomeric (Shankar et al., 2008). Inherent problems with these preparations were subsequently encountered in attempts by colleagues and other laboratories to confirm the sequence identity of this 8 kDa oligomeric species that was immunoreacitve to both Aβ40 and 42-specific antibodies. To overcome the uncertainty of the Aβ species, synthetic alkyl- and disulfide-linked Aβ40 dimers have been previously studied but do not represent physiologically relevant dimer in the AD brain (Shankar et al., 2008; Kok et al., 2009). To this end colleagues synthesized dityrosine-linked Aβ40 dimer, based on results showing the Aβ42 dimers to be elevated in blood from AD subjects (Villemagne et al., 2010). A recent publication studying synthetic dityrosine-linked dimers has demonstrated increased stable bioactive aggregates relative to monomer, which confirms a preceding publication by colleagues also demonstrating an increase in stable aggregates which, in turn results in increased toxicity (Kok et al., 2013; O'Malley et al., 2014b).

The second peptide of interest has a mutation in the Aβ sequence that has been reported to act by increasing production rather than toxicity and therefore provides an ideal control for the assay. In 2009 Di Fede and colleagues described an alanine to valine substitution at position 673 in APP (A673V) causing a very early onset AD in a single Italian patient and MCI in their sibling. The recessive mutation only caused disease when homozygous with heterozygous family members unaffected (Di Fede et al., 2009). Following this discovery an A673T mutation was detected from in an Icelandic cohort of 1795 individuals, providing the first evidence of a protective mutation in AD (Jonsson

et al., 2012). The importance of mutations at this location was the subsequent consequences to APP processing. The mutation site is located immediately adjacent to β-cleavage site of APP by BACE, which results in reduced Aβ production by approximately 2.5-fold in the Icelandic A673T mutation and a comparable increase in Aβ levels in the plasma in patients with the Italian A673V mutation. Importantly, these findings taken together provide substantial genetic support to the involvement of Aβ in AD.

The final peptide to be investigated was the amino-terminally truncated, pyroglutamate-modified (Aβ3pE-42) form of Aβ, which was strongly associated with Alzheimer's disease and has been reported to exhibit equivalent (Tekirian et al., 1999; Shirotani et al., 2002; Youssef et al., 2008) or even greater toxicity (Schlenzig et al., 2009; Galante et al., 2012; Nussbaum et al., 2013) than full-length Aβ1-42 (Aβ42). Altered $Cu^{2+}$ binding and increased Aβ oligomerisation have been attributed to the pyroglutamate modification of the glutamate at position 3 of the Aβ sequence (D'Arrigo et al., 2009; Drew et al., 2010). In recent unpublished findings by colleagues, Aβ3pE-42 has been shown to increase reactive oxygen species at the cell membrane in primary neurons above that of the full-length Aβ42 (personal communication, Dr. Adam Gunn). The subsequent findings in this work that Aβ42 caused greater cytosolic reactive oxygen species compared to Aβ3pE-42 indicated that the two isoforms caused oxidative stress by different mechanisms. Following on from this observation, I sought to determine if the effect of these peptides on $Ca^{2+}$ flux differed from full-length Aβ42.

## 5.2 Results

### 5.2.1 Optimisation of glutamate toxicity in cortical neurons

Resting concentrations of glutamate in the extracellular fluid of the brain and cerebrospinal fluid in rabbits are normally around 3 - 4 µM and 10 µM, respectively (Hamberger and Nyström, 1984). Levels of glutamate in the rodent brain are similar with these concentrations and have been determined to be between 3 – 6 µM (Reisi et al., 2009; Hascup et al., 2011). In establishing the parameters to investigate an Aβ-specific effect on cell death in the presence of glutamate and $Cu^{2+}$, I needed to determine what are sub toxic levels of glutamate that would be physiologically relevant (Figure 5-1) In these experiments mouse cortical neurons, cultured for 9 DIV to ensure necessary NMDAR expression, were exposed to increasing concentrations of glutamate for 48 h. Cell viability assays demonstrated significant toxicity was caused in neurons exposed to the two highest concentrations tested, 7.5 and 10 µM glutamate. Based on these findings it was decided to proceed with the lowest and highest non-toxic concentrations of glutamate; i.e. 1 and 5 µM, which provided a concentration range to test with Aβ.

### 5.2.2 Aβ42, glutamate or $Cu^{2+}$ in combination are toxic to neurons

As was demonstrated in chapter 4 (section 4.2.1), Aβ treatment of cortical neurons required 72 - 96 h to induce significant toxicity, as measured by cell viability assays. In the subsequent experiments I investigated the effect of Aβ, glutamate and/or $Cu^{2+}$, either independently or in combination, following treatment of cortical neurons for 48 h. In determining the concentrations of $Cu^{2+}$ to be tested the stoichiometric relationship with Aβ was considered. The ratio of Aβ: $Cu^{2+}$ of greater than 0.6:1 has been shown to form histine-bridged dimers by EPR spectroscopy and this markedly affects toxicity at equimolar or super-stoichiometric concentrations of copper (Smith et al., 2006). Each of the 48 h

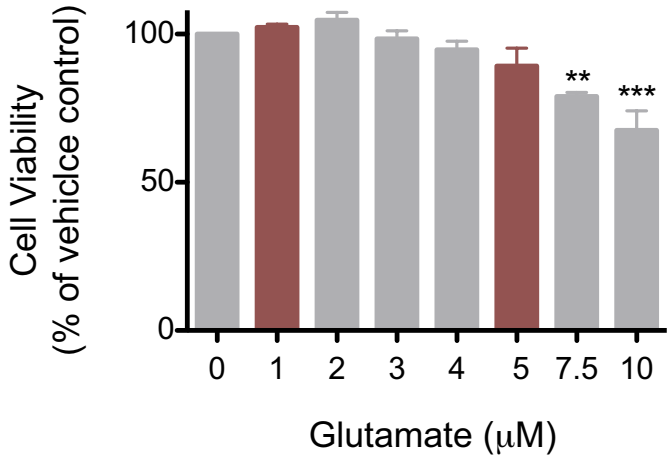



**Figure 5-1. Determining non-toxic levels of glutamate to cortical neurons.** Mouse primary cortical neurons were cultured for 9 DIV and then exposed to increasing levels of glutamate (0 - 10 μM) for 48 h. Cell viability was subsequently determined by CCK-8 assay with results expressed normalised to vehicle treated neurons (0 μM). 1 μM and 5 μM glutamate were selected for subsequent experiments as the lowest and highest concentrations, respectively, that did not significantly decrease cell viability (red bars). Data is expressed as the mean of ± SE; n = 3, **p < 0.01, ***p < 0.001 compared to vehicle treated values by one-way ANOVA using Dunnett's post hoc test.

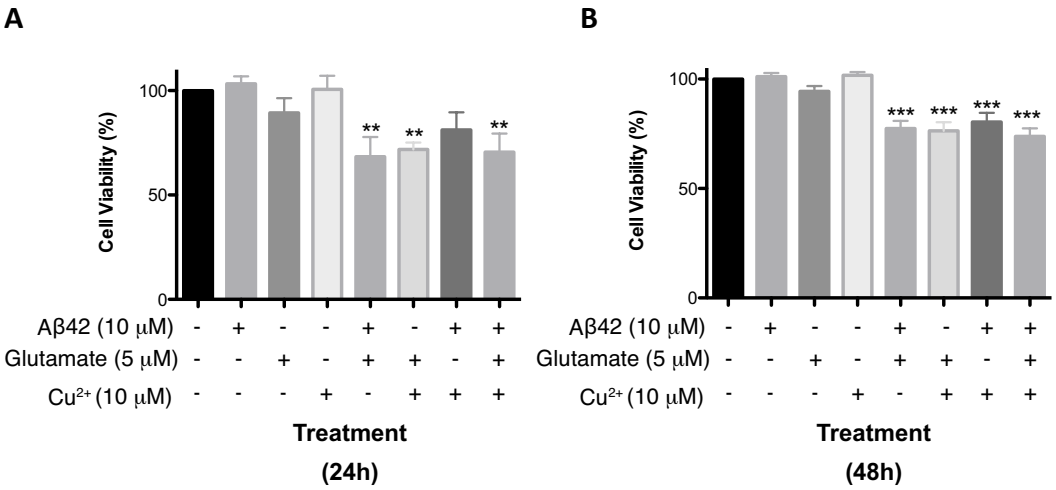



**Figure 5-2. Non-toxic levels of Aβ42, glutamate and/or $Cu^{2+}$, in combination, induce cell death in cortical neurons.** Treatment with combinations of Aβ42, glutamate and $Cu^{2+}$ resulted in significant reduction in cell viability in cortical neurons treated for 48 h compared with neurons exposed to individual treatments. Cell viability was measured by CCK8 assay and values expressed normalised to an untreated vehicle control. Data represent mean ± SE; n = 7; ***p < 0.001; compared to glutamate-treated neurons.

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treatments, in isolation, had no effect on cell viability relative to the vehicle-treated controls but when they were added in combination; Aβ/ glutamate, glutamate/$Cu^{2+}$, Aβ/$Cu^{2+}$ and Aβ/glutamate/$Cu^{2+}$ were significantly toxic to neurons, decreasing cell viability to 77, 80, 74 and 76%, respectively ($p < 0.001$, $n = 7$) (Figure 5-2B). In experiments performed for the shorter 24h period significant toxicity was also seen in Aβ/ glutamate, glutamate/$Cu^{2+}$ and Aβ/glutamate/$Cu^{2+}$ treatments (Figure 5-2A) but not for Aβ/$Cu^{2+}$ treated neurons. The toxicity in neurons that were not treated with either $Cu^{2+}$ or glutamate most likely results from the resting concentration of 5 μM glutamate in neurons and an estimated $Cu^{2+}$ concentration of 15 μM in the synaptic cleft (Hartter and Barnea, 1988). Colleagues have also detected $Cu^{2+}$ concentrations of 1.92 μM ± 0.33 in the cortical neuronal media (White et al., 2004) and ICP-MS anaylsis of cell lysates performed on cell lysates from my experiments detected $Cu^{2+}$ at 2.29 μM ± 0.04 ($n = 35$).

### 5.2.3 Toxicity induced by Aβ42, glutamate and $Cu^{2+}$ is an NMDAR-mediated event.

The newly discovered increase in Aβ toxicity appeared to be dependent on glutamate, which raised the question, whether the cell death is mediated through the glutamatergic system and more specifically the NMDAR, implicating an excitotoxic mechanism in the observed cell death. To test this hypothesis cortical neurons were exposed to the same treatments described in section 5.2.2 but in the presence or absence of the NMDAR antagonist, MK-801 to demonstrate specificity (Figure 5-3) Aβ/ glutamate, Aβ/$Cu^{2+}$ and Aβ/glutamate/$Cu^{2+}$ were all significantly toxic to neurons, decreasing cell viability to 65, 77 and 76%, respectively ($p < 0.01$, $n = 3 - 4$). In each instance the cell viability of neurons pretreated with MK-801 were not significantly different to vehicle or Aβ-only treated neurons. In the experiments testing glutamate and Aβ (Figure 5-3A), neurons were more susceptible to glutamate-induced toxicity than

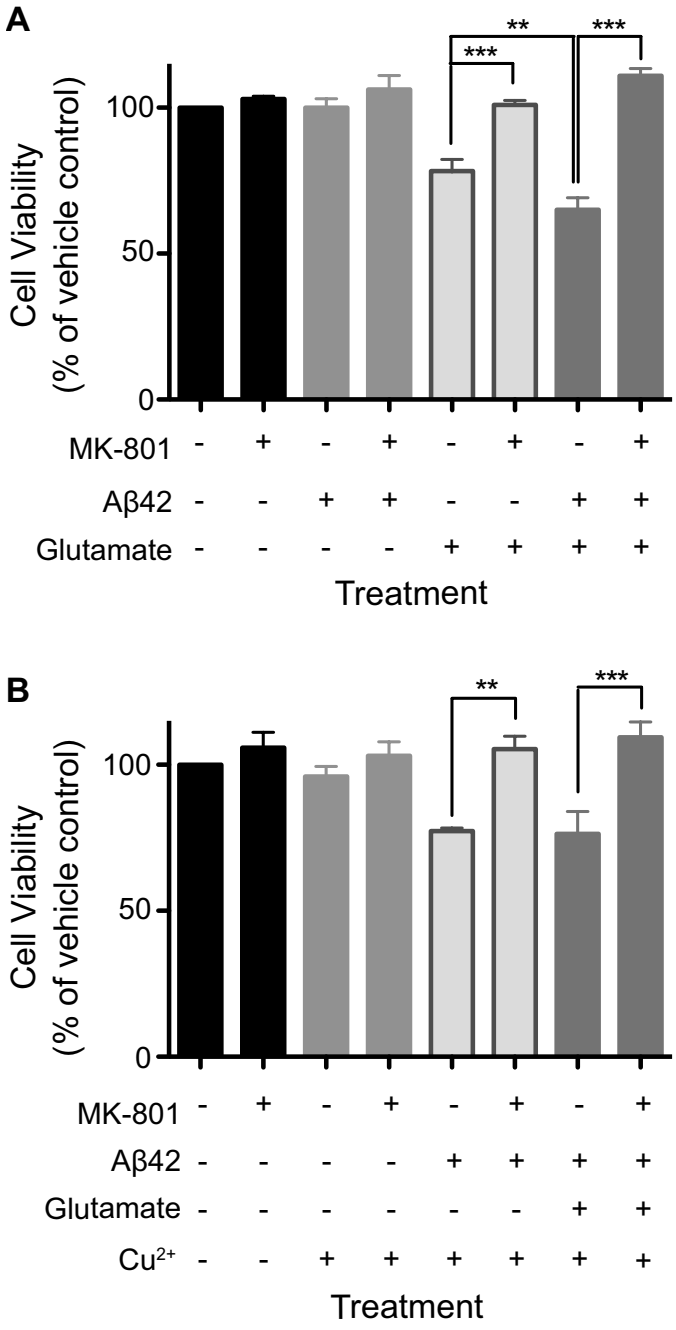


**Figure 5-3. Toxicity resulting from treatment with combination of Aβ, glutamate and/or $Cu^{2+}$ is NMDAR-mediated**. Cortical neurons were treated with Aβ (10 μM), glutamate (5 μM) and/or $Cu^{2}$ (10 μM) in isolation or in combination with or without the NMDAR-antagonist, MK-801 (10 μM) (***A*** and ***B***). The resulting cell death was NMDAR-mediated as was demonstrated by its prevention with MK-801 to . No significant difference was observed between MK-801 treated neurons and those treated with Aβ, glutamate or $Cu^{2+}$, in isolation. Cell viability was measured by CCK8 assay and values expressed normalised to an untreated vehicle control. Data represent mean ± SE; n = 4 (**A**) and 3 (**B**); **p < 0.01, ***p < 0.001; compared to MK-801-treated values by unpaired *t*-tests.

previously observed (Figure 5-2). The combination of glutamate and Aβ, however, still induced significant toxicity beyond that of glutamate-only treated neurons (Figure 5-3A).

### 5.2.4 Activation of toxic signalling pathways in neurons by Aβ42, glutamate and $Cu^{2+}$.

Previously I have shown that acute glutamate-induced excitotoxicity acts through a protein-signaling cascade mediated by calcium (section 3.2.7). This pathway begins with the activation and uncontrolled constitutive expression of calcineurin upon cleavage by calpain, a calcium-dependent cysteine protease. This in turn results in activation of GSK3 alpha and beta by dephosphorylation, with the latter known to mediate cell death through the hyperphosphorylation of Tau (Hooper et al., 2007). In these experiments I studied the total protein and/ or the phosphorylation levels of calcineurin, Tau, GSK3 alpha and beta in order to determine whether the toxicity due to glutamate, $Cu^{2+}$ and Aβ acted through this pathway. Western analysis showed activation of Calcineurin (Figure 5-4), which agreed with cell viability experiments previously performed, in which combined treatments with Aβ, glutamate and $Cu^{2+}$ were toxic to neurons (Figure 5-3). Levels of calcineurin were reduced due to its activation following treatment with glutamate, Aβ/glutamate and Aβ/ $Cu^{2+}$, respectively. There was also observed a significant decrease in calcineurin levels in neurons treated with Aβ/glutamate/$Cu^{2+}$. In studying the consequences of toxic signaling by western blots I did not detect a statistically significant Aβ-specific decrease in calcineurin levels, in agreement with previous experiments using cell viability as a readout (Figure 5-2). Subsequent western blot analysis was unable to demonstrate the involvement of Tau, GSK3 alpha and beta, in the toxic signaling by independent or combined treatment with Aβ, glutamate and $Cu^{2+}$. This would have been observed if a reduction in the phosphorylated form of the proteins were detected (Figure 5-4, B-E).

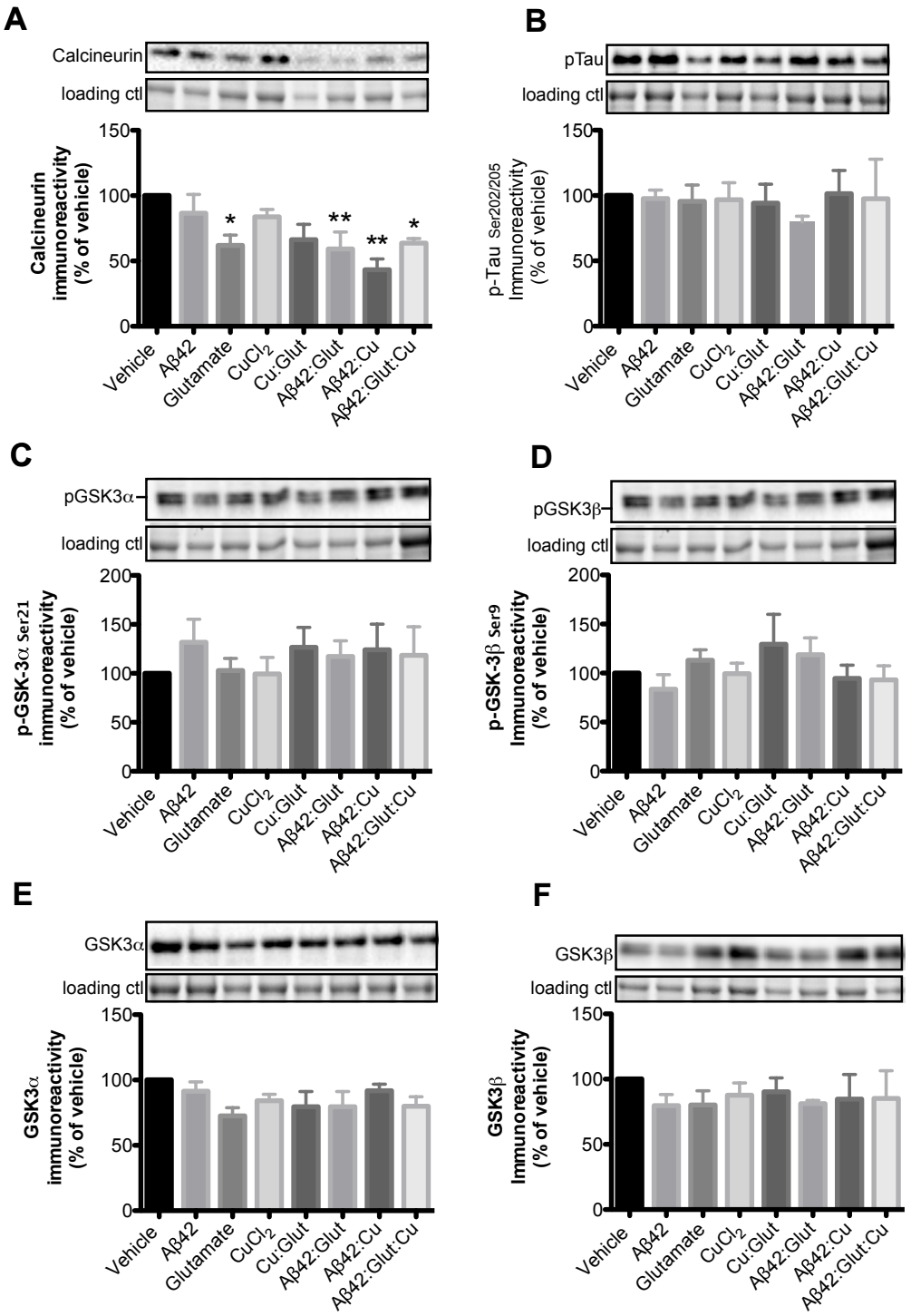


**Figure 5-4 Expression levels of proteins involved in excitotoxic signalling in neurons.**
***A-F***, Western blots (top panels) of calcineurin, phosphorylated Tau (pTau), phosphorylated GSK3α/β (pGSK3α/β), total GSK3α/β respectively, normalised to loading controls (middle panels) by in gel staining (Gürtler et al., 2013). Cortical mouse neurons (D.I.V. 9) were treated with Aβ42 (10 µM), glutamate (5 µM) and $Cu^{2+}$(10 µM) for 24 h prior to protein analysis. Analysis of western blots from 5 independent experiments was performed and displayed as bar graphs (***A-F***, bottom). Data represent mean ± SE; n = 5, *p < 0.05, **p < 0.01 compared to vehicle treated samples. Protein levels in all remaining samples were not significantly different to the vehicle treated samples.

### 5.2.5 $Ca^{2+}$ Flux as a Functional Readout of Excitotoxicity.

In sections 4.2.3 and 4.2.4 of chapter 4, the assay employed was unable to detect a specific Aβ-induced effect on $Ca^{2+}$ flux. However, the subsequent discovery that Aβ-induced toxicity is increased in the presence of physiological levels of glutamate and $Cu^{2+}$, led to the following experiments which tested whether these findings could be recapitulated in a $Ca^{2+}$ flux assay. This assay could not only provide a higher throughput of samples, as it's performed in a 96-well rather than a 48-well format, but more importantly, it is highly sensitive and performed in real time. As in the toxicity assays (Figure 5-3) cortical neurons were cultured for 9 DIV to ensure expression of NMDARs. Prior to exposure to Aβ treatments, neurons were preloaded with Fluo4 dye (Life Technologies) containing probenecid, which is used to prevent extrusion of the dye by inhibiting organic anion transporters in the cell membrane (Di Virgilio et al., 1990). Treatments were brought to RT immediately before addition to avoid temperature-induced flux in the neurons. To establish base line levels of fluorescence in the cortical neurons, 10 reads were taken before addition of samples and 10 reads immediately following. The $Ca^{2+}$ flux was calculated by subtracting the average of the first 10 baseline reads ($F_0$) from the first read immediately after treatment ($F_1$) and then divided by the average baseline reads ($F_0$). To observe Aβ-specific effects on $Ca^{2+}$ flux, data was expressed normalised to glutamate–only treated cells (Figure 5-5). There was no observable effect on $Ca^{2+}$ flux above the vehicle control in neurons treated with Aβ and $Cu^{2+}$, in isolation. The effects of Aβ on $Ca^{2+}$ flux, in the presence of non-toxic levels of glutamate and $Cu^{2+}$, were in direct agreement with the cell viability assays (Figure 5-3) Treatment with Aβ and Aβ/$Cu^{2+}$ caused a significant increase to glutamate-induced $Ca^{2+}$ flux that was not attributed to an additive effect of the individual components. $Ca^{2+}$ flux in neurons exposed to Aβ/glutamate was increased to 119% ± 2.5 (n = 9) and Aβ/glutamate/$Cu^{2+}$ to 123% ± 3.5 (n = 7) whereas the relative flux for Aβ was 6.9% ± 3.1 (n = 9) and $Cu^{2+}$ was 0.6% ± 3.5 (n = 9). These results demonstrate for the first time that I was able to detect an Aβ-

specific effect on $Ca^{2+}$ flux in neurons and that this increase parallels the results of the toxicity assays.

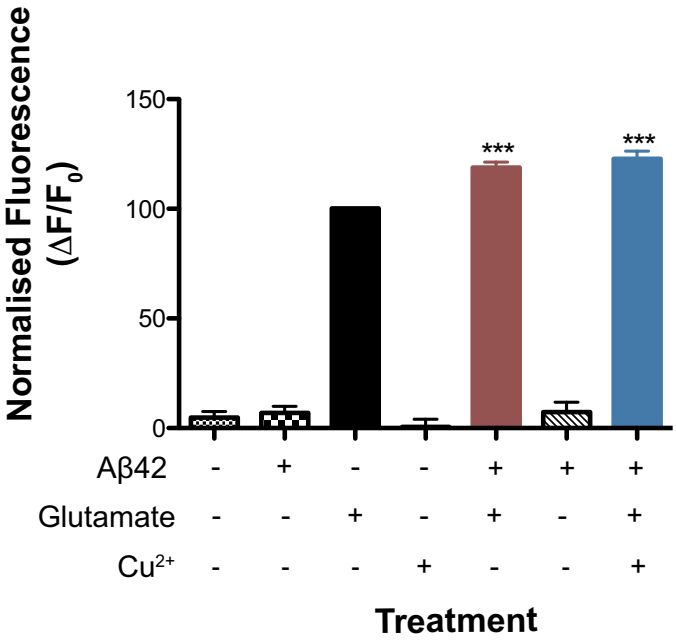


**Figure 5-5. Aβ specifically increases glutamate-induced $Ca^{2+}$ flux +/- $Cu^{2+}$.** Primary cortical neurons were treated with Aβ (10 μM), glutamate (5 μM) and $Cu^{2+}$ (10 μM) independently or in combination after cells were preloaded with Fluo4 dye for 1 h. The resulting effect on $Ca^{2+}$ flux was measured before and immediately after the addition of samples to neurons by detecting the increase in intracellular fluorescence (ΔF) relative to background fluorescence ($F_0$). Aβ causes a significant increase in $Ca^{2+}$ flux when neurons were treated with glutamate in the presence of Aβ42 alone (red bars) or Aβ42 and $Cu^{2+}$ (blue bars). Contaminating $Cu^{2+}$ in culture media most likely accounts for the significant increases in glutamate and Aβ. Values were normalised to glutamate and represent mean ± SE; n = 7 - 9; *** $p < 0.001$ compared to glutamate-treated samples; one-way ANOVA using Dunnett's post hoc test.

### 5.2.6 Aβ42 increases glutamate-induced $Ca^{2+}$ flux in a concentration- dependent manner

Additional support of a specific Aβ effect on $Ca^{2+}$ flux in cortical neurons was observed when further experiments to optimise Aβ concentrations were performed. These experiments aimed to determine the minimum concentration of Aβ required to induce a detectable increase of $Ca^{2+}$ flux in glutamate-treated neurons (Figure 5-6) As in the preceding experiments (section 5.2.5), cortical neurons, preloaded with Fluo4 dye, were exposed to treatments once baseline fluorescence readings had been taken. Calcium flux was measured in neurons following treatment with Aβ concentrations of 1, 5 and 10 µM (red bars). The effects of additional $Cu^{2+}$ (10 µM) to this $Ca^{2+}$ flux were also observed (blue bars). A concentration-dependent effect of Aβ was observed as neurons only demonstrated a significant increase in $Ca^{2+}$ flux when treated with 5 µM Aβ (123% ± 4.4, n = 4) and 10 µM Aβ (125 % ± 7.0, n = 4) or with 5 µM Aβ/$Cu^{2+}$ (122% ± 10.6, n = 3) and 10 µM Aβ/$Cu^{2+}$ (120 % ± 3.2, n = 3). Again this data indicated that additional $Cu^{2+}$ was not required for the increase in $Ca^{2+}$ flux when added in conjunction with Aβ. This may indicate there is sufficient concentrations of $Cu^{2+}$ present in the synaptic cleft where glutamatergic $Ca^{2+}$ flux occurs from background $Cu^{2+}$ in the media, as previously discussed (section 5.2.2) or that $Cu^{2+}$ is not involved in the increase in $Ca^{2+}$ flux mediated by glutamate and Aβ. When the concentration of Aβ was reduced to 1 µM there was no longer an observable change in $Ca^{2+}$ flux to glutamate treated cells (98% ± 2.6, n = 3) as was also observed when additional $Cu^{2+}$ was present (101% ± 3.4, n = 4).

### 5.2.7 The Aβ42-specific increase in $Ca^{2+}$ flux is metal-dependent

Previously it has been shown that $Cu^{2+}$ and $Zn^{2+}$ affects the oligomerisation of Aβ (Bush et al., 1994a; Atwood et al., 1998; 2000) and recent findings have implicated $Cu^{2+}$ in the increased production and decreased clearance of Aβ (Singh et al., 2013). These features of Aβ, together with research that has described a role for $Cu^{2+}$ in the NMDAR-mediated toxicity of Aβ (You et al., 2012)

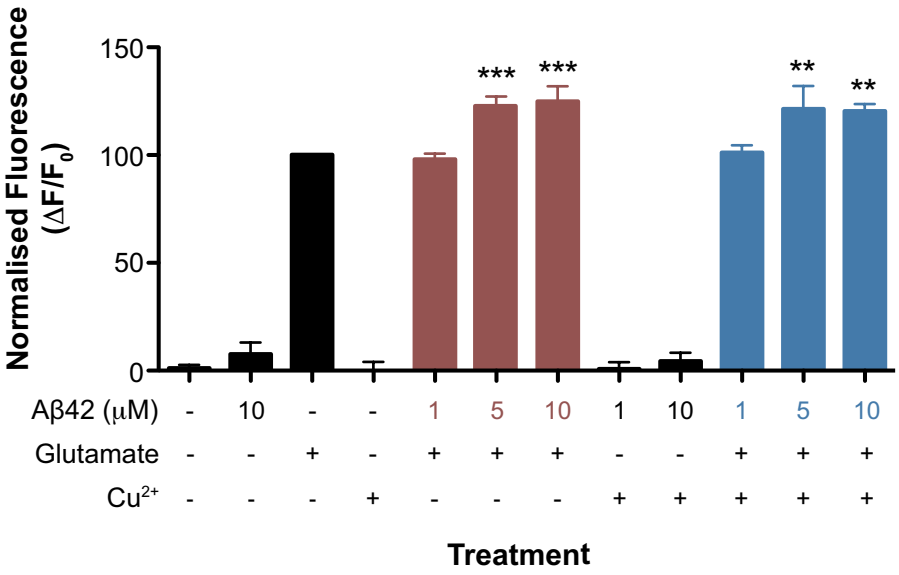


**Figure 5-6. The specific Aβ42 increase in glutamate-induced $Ca^{2+}$ flux is concentration-dependent.** Primary cortical neurons were treated with Aβ (1, 5 and 10 μM), glutamate (5 μM) and $Cu^{2+}$ (10 μM) independently or in combination after cells were preloaded with Fluo4 dye for 1 h. The resulting effect on $Ca^{2+}$ flux was measured before and immediately after addition of samples to neurons by detecting the increase in intracellular fluorescence (ΔF) relative to background fluorescence ($F_0$). Treatment with 5 & 10 μM Aβ, but not 1 μM significantly increases $Ca^{2+}$ flux in conjunction with glutamate (red bars) or glutamate and $Cu^{2+}$ (blue bars). Contaminating $Cu^{2+}$ in culture media most likely accounts for the significant increases in glutamate and Aβ treated neurons (red bars) without additional $Cu^{2+}$ added. Values were normalised to glutamate and data represent mean ± SE; n = 3 - 5; **p < 0.01; ***p < 0.001; compared to Aβ42 (1 μM)/glutamate (red) or Aβ42 (1 μM)/glutamate/$Cu^{2+}$ (blue) samples by one-way ANOVA using Dunnett's post hoc test.

formed the basis of the next experiments. To determine the contribution of metals in the observed increases in $Ca^{2+}$ flux, namely $Cu^{2+}$ and $Zn^{2+}$ given their high abundance in the brain and synaptic clefts (Brown et al., 1997; Smart, 2004), the non discriminant divalent cation chelator, Diamsar, was employed. Cortical neurons were exposed to Aβ at 1 and 10 μM, concentrations that do not affect flux and significantly increase flux, respectively (Figure 5-6). As observed in the preceding section, Aβ and Aβ/$Cu^{2+}$ induced significant increases in glutamate-treated neurons when metals were present (111% ± 4.3 and 114% ± 3.6, respectively)(Figure 5-7A). In corresponding neurons metals were depleted by treatment with Diamsar for 1 h prior to dye loading and then the addition of the treatments (Figure 5-7B). The depletion of metals from the neuronal media had a significant effect on $Ca^{2+}$ flux in glutamate-treated neurons exposed to Aβ or Aβ/$Cu^{2+}$. Whether with or without $Cu^{2+}$, treatment of 10 μM Aβ was no longer sufficient to induce a significant increase in $Ca^{2+}$ flux (Figure 5-7B). Although the flux in neurons depleted of metals and then treated with glutamate and Aβ/$Cu^{2+}$ were not significantly increased compared to glutamate only, it is a trend toward an increase (110% ± 6.4, n =3). This suggests the involvement of $Cu^{2+}$, as this trend is not observed when glutamate-treated neurons were exposed to Aβ-only (98% ± 3.6, n = 3). As $Cu^{2+}$ is only added back on to the neurons for a matter of seconds before measurements were taken these samples cannot be considered to have $Cu^{2+}$ reintroduced as would be case if it was added to media prior. This could potentially explain why a significant increase is not reintroduced in the Aβ/$Cu^{2+}$ treatments. This raises the possibility that it is the levels of $Cu^{2+}$ present in the cultures and not the additional $Cu^{2+}$ supplemented in at 10 μM, which are required for the Aβ-induced $Ca^{2+}$ flux of glutamate-treated neurons. As described in Chapter 3, in these experiments using the metal chelator there was no observable difference between $Ca^{2+}$ flux in neurons treated with Diamsar compared with untreated neurons.

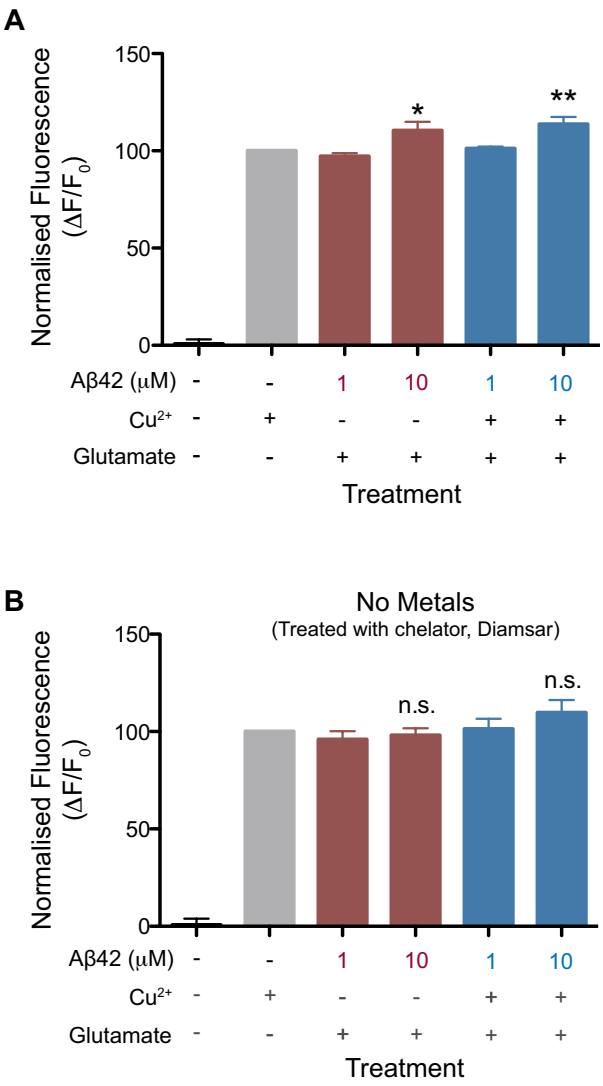


**Figure 5-7. The Aβ-specific increase in $Ca^{2+}$ flux is metal-dependent.** Primary cortical neurons were treated with Aβ (1 and 10 μM), glutamate (5 μM) and $Cu^{2+}$ (10 μM) independently or in combination after cells were preloaded with Fluo4 dye for 1 h. The resulting effect on $Ca^{2+}$ flux was measured before and immediately after addition of samples to neurons by detecting the increase in intracellular fluorescence (ΔF) relative to background fluorescence ($F_0$). ***A,*** Treatment with 10 μM Aβ, but not 1 μM significantly increases $Ca^{2+}$ flux in conjunction with glutamate (red bars) or glutamate and $Cu^{2+}$ (blue bars). ***B,*** Metals were removed from neurons by treatment for 1 h with the metal chelator, Diamsar (10 μM) before dye loading. In the absence of metals, Aβ no longer significantly increased glutamate-induced $Ca^{2+}$ flux in neurons, with or without $Cu^{2+}$. Values were normalised to glutamate and data represent mean ± SE; n = 3; *p < 0.05; **p < 0.01; n.s. not significant compared to Aβ42 (1 μM)/glutamate (red bar) or Aβ42 (1 μM)/glutamate/$Cu^{2+}$ samples (blue bar) and also compared to glutamate-only samples (black bar) by one-way ANOVA using Dunnett's post hoc test.

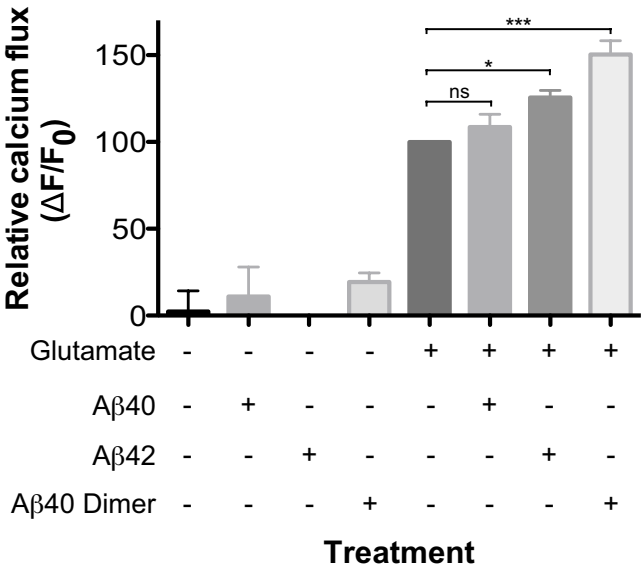


**Figure 5-8. The dimeric form of Aβ40 potently increases $Ca^{2+}$ flux**. The monomeric Aβ40 was compared to monomeric Aβ42 and the dityrosine cross-linked dimeric form of Aβ40 for their effect on glutamate-induced $Ca^{2+}$ flux. As in the preceding figures, $Ca^{2+}$ flux in neurons was measured by way of fluorescence immediately following addition of Aβ (10 μM) or glutamate (5 μM), in isolation or combination and expressed as the change in fluorescence (ΔF) relative to background fluorescence ($F_0$). Data represent mean ± SE; n = 3 - 4; *p < 0.05; ***p < 0.001; ns, not significant; compared by one-way ANOVA using Dunnett's post hoc test.

### 5.2.8 The Dimeric form of Aβ

As a first demonstration of the $Ca^{2+}$ flux assay's uitlity for screening Aβ toxicity, the glutamate-induced $Ca^{2+}$ flux in cortical neurons was measured for the effects of synthetic Aβ40 and Aβ42 monomers and the dityrosine-linked dimers (Figure 5-8). Results from Aβ40 monomer-treated neurons agreed with its relative low toxicity (Yan and Wang, 2007) as no significant effect on glutamate-induced $Ca^{2+}$ flux was observed (108.6% ± 7.4, n= 3) whereas treatment with Aβ42 caused a significant increase (126% ± 4.0, n = 3) compared with glutamate treated neurons. A potent and significant increase was detected by treatment with the dityrosine-linked Aβ40 dimer above the glutamate alone treated neurons (150.3% ± 7.9, n = 4).

### 5.2.9 Aβ42-A2V: The Italian Mutation

The aggregation experiments by Di Fede et al. (2009), describing the recessive A673V mutation in APP (position 2 of Aβ42) showed significantly faster aggregation kinetics compared to wild type Aβ as well as causing increased toxicity *in* vitro. This indicated the mutation may result in biophysical modifications to the peptide that affects toxicity of Aβ (Di Fede et al., 2009). To test this hypothesis the effect of the Aβ42-A673V peptide on $Ca^{2+}$ flux was compared to wild-type Aβ42 (Figure 5-9). Treatment of neurons with the peptides alone did not significantly affect $Ca^{2+}$ flux compared to vehicle but in conjunction with glutamate both peptides caused a significant increase compared with glutamate-only treated neurons (Aβ42; 123% ± 5.4, n = 4 and 125% ± 6.6, n = 5). The finding that the A673V mutation had no further effect on $Ca^{2+}$ flux above that of the Aβ42 wild-type peptide suggested the significant effects observed *in vivo* may not due to an increase of the intrinsic toxic properties of the peptide.

### 5.2.10 Pyroglutamate modified Aβ3-42

In my final experiments the toxicity of Aβ and the effects of the Aβ3pE-42 peptide were tested for their effects on $Ca^{2+}$ flux in an effort to identify a causal

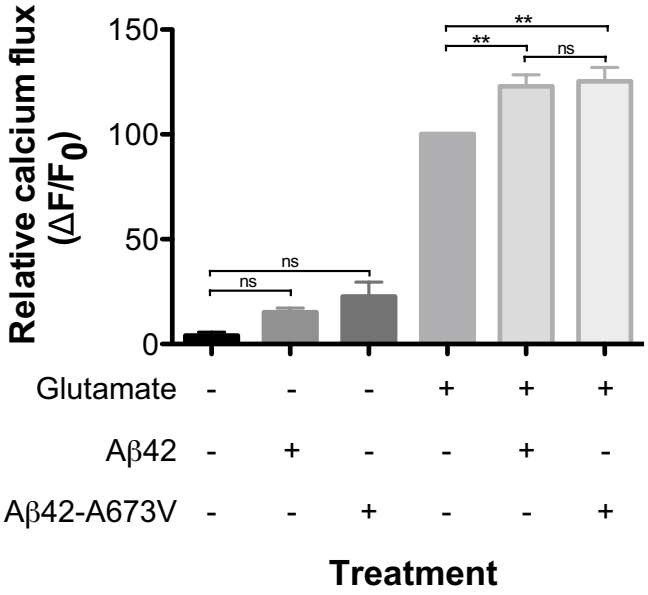


**Figure 5-9. The effects of the Aβ42-A673V mutation on $Ca^{2+}$ flux.** As in preceding figures, $Ca^{2+}$ flux in neurons was measured by way of fluorescence immediately following addition of Aβ42 (10 μM) or glutamate (5 μM), in isolation or combination and expressed as the change in fluorescence (ΔF) relative to background fluorescence ($F_0$). No significant difference to the increase in $Ca^{2+}$ flux was detected specific to the recessive mutation (light grey bar) when compared to wild type Aβ42. Data represent mean ± SE; n = 4 - 5; **p < 0.01; ns, not significant by one-way ANOVA using Dunnett's post hoc test.

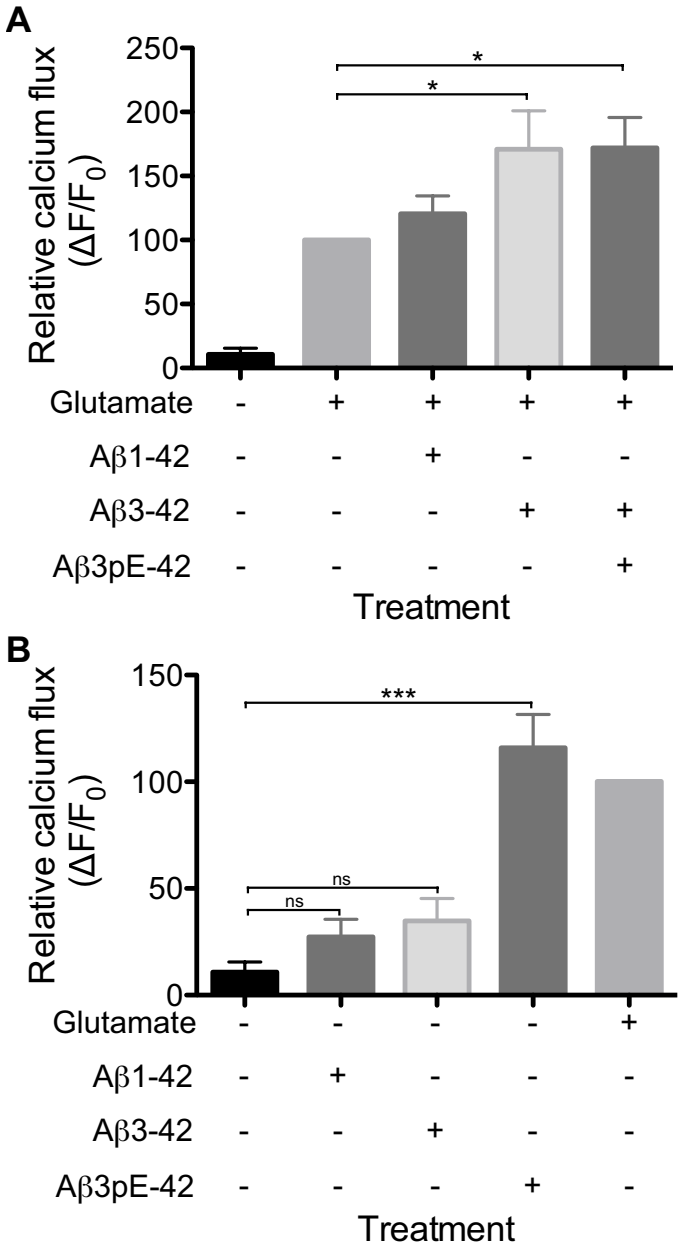


**Figure 5-10. The effects of the Pyroglutamate-modified Aβ3-42 on $Ca^{2+}$ flux with or without glutamate.** ***A,*** As in preceding figures, $Ca^{2+}$ flux in neurons was measured by way of fluorescence immediately following addition of Aβ42 (10 μM) or glutamate (5 μM), in isolation or combination and expressed as the change in fluorescence (ΔF) relative to background fluorescence ($F_0$). Both the Aβ3-42 (light grey bar) and Aβ3pE-42 (dark grey bar) significantly increase $Ca^{2+}$ flux above neurons treated only with glutamate (grey bar) but not Aβ1-42. ***B,*** Aβ3pE-42 induced a significant $Ca^{2+}$ flux in the absence of glutamate (dark grey bar) in contrast to Aβ1-42 and Aβ3-42. Values from independent experiments were normalised to neurons treated with glutamate (1 μM). Data represent mean ± SE; n = 3 - 7; *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant; compared by one-way ANOVA to vehicle treated neurons using Dunnett's post hoc test.

link between the specific properties of the peptide and its cellular toxicity. To control for the pyroglutamate modification to this truncated form of Aβ the Aβ3-42 peptide was tested in parallel (Figure 5-10). In the presence of glutamate both Aβ3-42 and its pyroglutamylated form significantly increased $Ca^{2+}$ flux relative to glutamate-only treated neurons (171% ± 30.0, n = 3 and 172% ± 23.9, n = 5, respectively) (Figure 5-10A). Despite the substantial increase in flux the considerable standard error between the samples prohibits any significant increase above the flux from the wild-type Aβ1-42 in glutamate-treated neurons (120% ± 14.0, n = 6). The more significant finding from these experiments was observed when I tested these peptides in the absence of glutamate (Figure 5-10B). Unlike any preparation of Aβ examined previously (chapter 4, section 4.2.3 and section chapter 5, sections 5.2.8 and 5.2.9), the Aβ3pE-42 peptide induced a substantial increase in $Ca^{2+}$ flux when treated alone (116% ± 15.6, n = 7) compared to vehicle (10% ± 4.8, n = 7) after results were normalised to neurons treated with glutamate (1 μM). This was in contrast to the Aβ1-42 and Aβ3-42 form, which did not affect $Ca^{2+}$ flux by itself (27% ± 8.3, n = 7 and 35% ± 10.5, n = 3).

## 5.3 Discussion

The experiments presented here demonstrate further characterization of Aβ toxicity in primary mouse neurons. These results were in agreement with the findings of Mattson et al., that Aβ induced specific cell death in the presence of physiological, non-toxic levels of glutamate (Mattson et al., 1992). In addition they describe the requirement of metals in this NMDAR-mediated pathway. As discussed in the preceding chapter (section 4.1) a significant shortcoming of currents assays employed to study Aβ toxicity is the duration required for a significant effect following treatment. This is overcome with LTP assays, which are generally considered the gold standard, but efforts to screen different Aβ preparations are severely hampered by the considerably low throughput nature of the assay. In exploiting the increased toxicity of Aβ in glutamate-treated cells I

have developed a high-throughput assay measuring $Ca^{2+}$ flux in primary cortical neurons in a 96-well format. This assay provides a rapid functional readout that parallels the Aβ-specific toxicity observed in corresponding cell viability assays.

The discovery of Aβ-specific toxicity in the presence of glutamate has significant implications for the field of Aβ research and in particular research investigating the ADDLs preparations. As previously discussed (section 4.3) the Ham's F12 media with or without glutamine contains 100 μM glutamate (section 7.1), consequently upon treatment with ADDLs, an equivalent concentration of glutamate is being added to the cells. In published research demonstrating the effects of ADDLs on $Ca^{2+}$ flux this concentration of ADDLs and therefore glutamate, has ranged from 500 nM (De Felice et al., 2007) to 5 μM (Alberdi et al., 2010). This range of glutamate concentrations was shown to be at sub toxic levels to primary neurons (Figure 5-1). In agreement with my findings, the presence of glutamate during this treatment is sufficient for Aβ to induce significant toxicity as measured by cell viability assays (Figure 5-2) and this specific effect could be observed through the measurement of $Ca^{2+}$ flux (Figure 5-5).

It has been reported that Aβ can impair synaptic plasticity by affecting LTD mediated by either mGluR or NMDAR activity (Li et al., 2009). It was concluded that this effect was due to a "toxic spill over" of synaptically released glutamate acting on extrasynaptic NR2B-containing NMDARs (Li et al., 2011). The results from the experiments undertaken here provide evidence that this is not the mechanism by which Aβ is mediating its toxicity in cortical cultures. The toxicity observed, following treatment with Aβ in the presence of glutamate, was entirely inhibited by the NMDAR antagonist, MK-801 (Figure 5-3). Not only does this negate the involvement of mGluRs but also extrasynaptic receptors, which are not inhibited by MK-801. The NMDAR antagonist is an open channel blocker (Huettner and Bean, 1988) that is routinely used to isolate extrasynaptic receptors by acting on active receptors at the synapse by acting as a 'pre-block' (Tovar and Westbrook, 1999; Hardingham et al., 2002).

Supporting evidence that the toxicity is mediated through the NMDARs was shown by western blot analysis. A significant decrease, and therefore

activation, of calcineurin was observed following independent or combined treatment with Aβ, glutamate and/or $Cu^{2+}$. I have previously shown calcineurin to be involved in excitotoxicity resulting from acute exposure to glutamate (section 3.2.7) and this data was in agreement with these findings. Activation of the GSK3 alpha and beta, as well as phosphorylation of Tau was studied being key proteins I previously identified in excitotoxic signaling upon acute glutamate exposure (section 3.2.7). Contrary to the findings from these experiments no significant changes to GSK3 activation and Tau phosphorylation were observed under the chronic conditions with the low glutamate used in these experiments with Aβ, glutamate and $Cu^{2+}$, i.e. 10 μM for 24 h as opposed to 40 μM for 1 h. The inherent variability of western blotting, which significantly affects reproducibility and therefore sensitivity of the assay, has meant that under these substantially reduced excitotoxic conditions it was not possible to detect subtle changes in this signaling pathway. To overcome this limitation my research subsequently employed a more sensitive readout, $Ca^{2+}$ flux.

Further evidence supporting Aβ toxicity being mediated through the synaptic NMDARs was demonstrated in my subsequent experiments. These demonstrated that the same conditions that caused toxicity in primary neurons, as measured by cell viability assays (Figure 5-2), also induced an appreciable effect on $Ca^{2+}$ flux specific to Aβ (Figure 5-5). The $Ca^{2+}$ flux being mediated by NMDAR as demonstrated by early experiments by Koh and Choi et al. describing excitotoxicity (reviewed in Choi, 1992). This research showed the majority of cell death associated with brief glutamate exposure to be NMDAR-mediated.

In additional experiments to characterise the effects on $Ca^{2+}$ flux, Aβ was shown to act in a concentration-dependent manner (Figure 5-6). In previous publications the oligomeric species of Aβ preparations has been estimated at 1% of total Aβ (Laurén et al., 2009) and subsequent concentrations were expressed accordingly, i.e. 1 μM was expressed as 10 nM Aβ (Um et al., 2012). As preparations in these experiments were tested immediately and were not allowed to aggregate, 1% oligomeric Aβ would be a conservative estimate of the concentrations used in my experiments. In taking these broad estimates into consideration it can be inferred that a concentration of 50 nM Aβ or greater was

required to significantly affect $Ca^{2+}$ flux. Although expressing Aβ concentrations in such a manner is somewhat dubious, these concentrations fall more inline with those previously described in LTP experiments with ADDLs (Lambert et al., 1998; Walsh et al., 2002).

Along with the effect of Aβ concentration on Ca flux, a role of metals was also demonstrated. In the microenvironment of the synaptic cleft Aβ peptides are secreted along with $Zn^{2+}$ from the presynapse and $Cu^{2+}$ from the post synapse during neuronal excitation (Kamenetz et al., 2003; Schlief, 2005; Paoletti et al., 2009). These increased Aβ levels in the synapse, whether due to overproduction or decreased clearance, are thought to result in pathological consequences (reviewed in Bush, 2013). In the experiments presented here I have demonstrated that the increase in glutamate-induced $Ca^{2+}$ flux due to Aβ can be inhibited by the use of the transition metal chelator, Diamsar (Figure 5-7). This finding is contrary to previous findings demonstrating Aβ-induced toxicity in the absence of $Cu^{2+}$ (You et al., 2012). It is important to note the considerably stronger binding affinities of $Cu^{2+}$ than $Zn^{2+}$ indicate it to be a more favorable candidate in binding Aβ and affecting $Ca^{2+}$ flux. The strong binding affinities of $Cu^{2+}$ mean its concentrations present in neurobasal media are sufficient for Aβ binding and almost certainly explain the absence of effect when additional $Cu^{2+}$ was introduced in to the $Ca^{2+}$ flux assays (Figures 5-5, 5-6 and 5-7).

Having described key parameters of the $Ca^{2+}$ flux required for Aβ-specific toxicity in the cortical cultures the subsequent experiments with the Aβ40 monomer and a synthetic dityrosine linked dimer form provided the first validation of the assay. Previous experimental evidence with these peptides has demonstrated Aβ40 monomers to be relatively inert (Dahlgren, 2002; Yan and Wang, 2007) and the dimer to induce potent effects on LTP (Shankar et al., 2008; O'Malley et al., 2014a) and significantly increase toxicity (O'nuallain et al., 2010; Kok et al., 2013). The two additional c-terminal amino acids in Aβ42 increase the peptide's hydrophobicity, which has been attributed to its propensity to rapidly aggregate and dramatically increase toxicity, up to 40-fold that of the monomer (Dahlgren, 2002). Structural analysis of c-terminus of Aβ42 has concluded these two amino acids stabilize the neurotoxic oligomers in a non–β-sheet secondary

structure thus prolonging its toxic oligomeric form (Ahmed et al., 2010). Recent findings with the dityrosine-linked dimeric form of Aβ40 are in agreement with this and attribute the increased toxicity of this form of Aβ to its delayed aggregation to fibrils (Kok et al., 2013; O'Malley et al., 2014a). The potent effect of these dimeric forms of Aβ40 on $Ca^{2+}$ flux assays indicates significant pathological consequences of chronic exposure to these oligomeric species of Aβ (Figure 5-8). A substantial increase in the flux caused by the Aβ40 dimer relative to Aβ42 was also observed and is indicative of the relative concentrations of the toxic species in the respective Aβ preparations.

Genetic mutations in Aβ can affect toxicity *in vivo* by modifications to the peptide's biophysical properties that lead to increased Aβ production, degradation or aggregation. In a comparable mechanism to the Aβ40 dimers, described above, a mutation at position 22 of Aβ (APPE693Δ) has been reported that significantly increases oligomerisation but not fibrillisation (Tomiyama et al., 2008). As previously described (section 5.1) another genetic mutation in Aβ resulting in AD, the A673V mutation in APP, was discovered in one Italian family. This mutation, rather than altering the toxic properties of the peptide, significantly increased processing of APP resulting in the early onset of AD. The pathogenic effects of this mutation most likely result from the overproduction of Aβ. Results obtained from $Ca^{2+}$ flux assays were in agreement with this conclusion, as the Aβ-A673V mutation significantly increased $Ca^{2+}$ flux but this increase did not significantly differ from the wild type Aβ42 peptide (Figure 5-9).

The final experiments utilising the $Ca^{2+}$ flux assay resulted in the unexpected finding that a form of Aβ peptide could induce flux in the absence of glutamate. Data from testing the Aβ3pE-42 demonstrated a significant increase in $Ca^{2+}$ flux in the presence of glutamate but this effect was not specific, with the unmodified Aβ3-42 causing similar increases (Figure 5-10). As the effect of the truncated peptides did not differ significantly to the effect of the wild type Aβ1-42, it suggested a related glutamatergic-mediated mechanism. However, when the effect of Aβ3pE-42 was tested in isolation, a potent increase was observed compared with neurons treated with vehicle and the unmodified Aβ peptides. This indicated an action at the membrane that was not mediated through active

glutamatergic receptors. One such mechanism in which Aβ3pE-42 could affect these changes is through perturbations of the lipid membranes (Kayed et al., 2004; Demuro et al., 2005). In this work the Aβ-specific increase in $[Ca^{2+}]_i$ was shown to be immediate, precluding second messenger-mediated events and showed no evidenced of pore formation. Taken together this research supported increased membrane permeability as the more likely mechanism by which $[Ca^{2+}]_i$ was increased. Such a mechanism for Aβ3pE-42 is further supported by recent findings that this modified Aβ peptide disrupts lysosomal membrane integrity in neurons (De Kimpe et al., 2012) and generates higher levels of membrane leakage in astrocytes relative to Aβ1-42 (Russo et al., 2002). In my findings, the data also favours perturbation of the cell membrane as its mechanism, as the $Ca^{2+}$ flux observed was considerable and immediate and is most likely a result of the peptide's increased neuronal binding and lipid peroxidation demonstrated in colleagues' recent work (personal communication, Dr. Gunn).

Glutamate-induced $Ca^{2+}$ signaling plays a vital role in neural development; function and plasticity and as such activation of this pathway is strictly regulated. The NMDAR is a key receptor in this process, accommodating cell survival signaling and maintaining physiological concentrations of intracellular $Ca^{2+}$. Under pathological conditions, chronic exposure or excessive concentrations of glutamate results in excitotoxic cell death. In the work I have presented here I have described conditions in which normal levels of glutamate and metals become toxic in the presence of Aβ. The increases in $Ca^{2+}$ flux resulting from this combination with glutamate, standard Aβ preparations and metals, although not potent, were sufficient to cause moderate neuronal death indicating a time course more in accordance with the degenerative nature of AD. When considered as a whole these findings strongly implicate dysregulation of glutamatergic signaling through NMDARS as a mechanism by which Aβ peptides cause the synaptic loss and neuronal death associated with AD. In addition, these experiments have described the conditions for a high-throughput primary neuron-based assay that will aid in the identification of distinct toxic species of Aβ responsible for neurodegeneration in AD.

# 6 Discussion

In this final chapter I will address the findings from the research presented in the thesis in a therapeutic context. Initially, the neuroprotective mechanisms of intracellular calcium and the role of metals in modulating $Ca^{2+}$ are discussed. In describing a novel mechanism for the treatment of acute and chronic neurodegeneration by the use of metal ionophores, namely PBT2, the findings of each chapter are discussed as a whole to demonstrate the potential benefits from this work. The chapter concludes with a proposed model encompassing these findings.

## 6.1 Modulating Intracellular Calcium Levels to Protect Against Excitotoxicity.

In the neuron the concentration of intracellular $Ca^{2+}$ controls a wide array of vital functions through allosteric interactions affecting such processes as neurotransmitter release, gene expression and protein activity. $Ca^{2+}$ can mediate signal transduction from activation of ionotropic receptors such as NMDARs or acting as a second messenger. Pathological increases in intracellular calcium levels result in reduced function and neuronal viability observed in both acute (Dugan and Choi, 1999; Pohl et al., 1999) and chronic (Ferrante et al., 1993; Zhang et al., 2008; Ittner et al., 2010; Um et al., 2012; You et al., 2012) neurodegenerative diseases. Under physiological conditions this toxicity is prevented though the strict regulation of intracellular $Ca^{2+}$ concentrations with receptors located on the cell membrane controlling $Ca^{2+}$ influx and intracellular stores being mediated by receptors on cellular organelles such as the endoplasmic reticulum (Figure 6-1). Many of these receptors have been implicated in neurodegenerative diseases, which have made them appealing targets for therapeutic intervention. To this end antagonists have been designed to attenuate flux of $Ca^{2+}$ from the millimolar levels in the synaptic cleft in to the

cytosol, where $Ca^{2+}$ is at a resting concentration in the nanomolar range (Figure 6-1). Intracellular stores also play a significant role in resting levels of intracellular $Ca^{2+}$ and given the micromolar concentrations in the endoplasmic reticulum, have been targeted. Results from trials with these therapeutics have been varied showing efficacy in mouse models (Horn et al., 2001; Anekonda et al., 2011; Peng et al., 2012) but being largely ineffective in clinical trials of ischemia and AD (Lipton, 2004; Ginsberg, 2008).

The data presented in this thesis support a novel mechanism to protect against the excitotoxic cell death resulting from such significant increases in

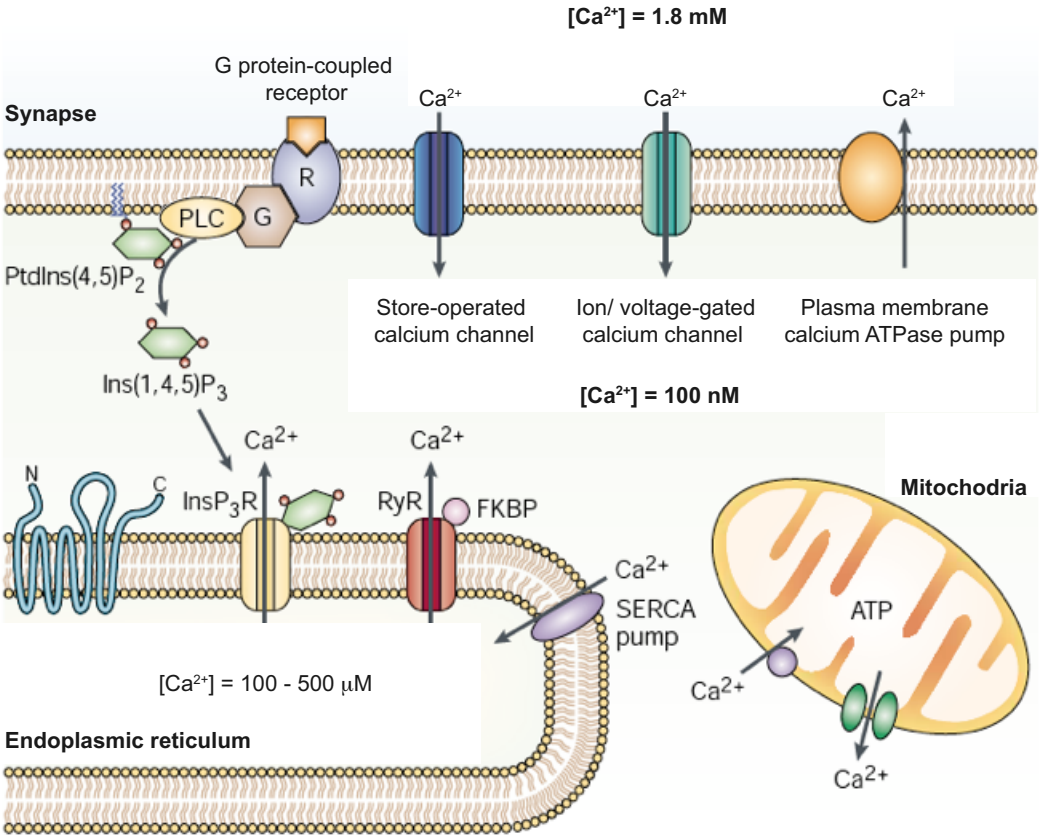


**Figure 6-1 Cellular mechanisms for intracellular calcium homoeostasis.** Neurons can regulate the entry of calcium ($Ca^{2+}$) from millimolar levels in the synaptic cleft by store-operated channels, voltage-gated channels following depolarisation and through the ion-gated channels such as NMDARs. At rest cytosolic concentrations of $Ca^{2+}$ are maintained between 50 and 300 nM. Synaptic activity can raise cytosolic $Ca^{2+}$ to micromolar levels through activation of the cell surface receptors or by release of intracellular $Ca^{2+}$ stores from endoplasmic reticulum (ER) stores by activation of the inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptors (InsP3Rs) and ryanodine receptors (RyRs). The excitotoxic cell death that has been the focus of this thesis occurs by dramatic increases in the intracellular $Ca^{2+}$ levels following chronic stimulation of NMDARs by excessive levels of glutamate in the synapse (Adapted from LaFerla, 2002)

intracellular $Ca^{2+}$. The model employed in this work has demonstrated the ability of transitional metals, namely $Zn^{2+}$, to protect against acute excitotoxicity induced by maximal concentrations of glutamate in cortical neurons. Using the zinc ionophore PBT2, a therapeutic currently in clinical trials to prevent chronic neurodegeneration, it was possible to prevent cell death by moderately increasing intracellular $Ca^{2+}$ levels (Figure 6-2). This represents a key finding in this thesis and strongly suggests the potential efficacy in treatment of acute neurodegeneration in diseases such as ischaemic stroke and traumatic brain injury by protecting against further excitotoxicity resulting from a positive feedback mechanism (Lipton and Rosenberg, 1994). In further support of such a role are the favourable results obtained from animal models and clinical trials with therapeutics sharing similarly moderate chelating properties as PBT2 (Diener et al., 2008; BarKalifa et al., 2009; Wang et al., 2010a).

## 6.2 Slow Excitotoxicity in Chronic Neurodegeneration

PBT2 has shown success in phase II clinical trials in the chronic neurodegenerative diseases; HD and AD with cognitive improvement in both instances. The mechanisms of action of initially proposed for PBT2 have included disaggregation of Aβ fibrils and inhibiting reactive oxygen species formed by Aβ:Cu and more recently stabilizing non toxic forms of Aβ (Cherny et al., 2001; Adlard et al., 2008; Bush, 2008; Ryan et al., 2015). The data I have presented demonstrates not only a mechanism for its use in acute neurodegeneration but also a novel mechanism for its therapeutic efficacy in both HD and AD. The proposed mechanism by which PBT2 could protect against the onset of both of these chronic neurodegenerative diseases is by preventing the gradual neuronal loss resulting from 'slow' excitotoxicity.

In attempts to explain the gradual neuronal loss associated with these chronic diseases two distinct forms of excitotoxicity were proposed (Beal, 1992a). The two forms of excitotoxicity are accordingly named; acute and slow, directly reflecting the progression of the diseases in which they've been implicated. It is believed that acute neurodegenerative diseases such as

ischaemic stroke and TBI result from excess glutamate in the synaptic cleft. This is caused by either excessive release of glutamate from the presynapse, defects in its catabolism or in its reuptake by glutamate transporters on supporting astrocytes (Albin and Greenamyre, 1992; Lipton and Rosenberg, 1994; Bridges et al., 2012). However in slow excitotoxicity two possibilities have been suggested to account for the gradual onset of symptoms; either abnormalities in signaling by glutamatergic receptors or impaired cellular energy metabolism (Albin and Greenamyre, 1992; Beal, 1992b). The data presented in the final results section of this thesis provides evidence of the former possibility by demonstrating increased intracellular $Ca^{2+}$ following exposure to Aβ. Unlike the neuroprotective increase in $Ca^{2+}$ resulting from pretreatment with PBT2, this chronic exposure to increased levels of $Ca^{2+}$ was toxic and resulted in gradual cell death. This effect was only observed in the presence of glutamate indicating the involvement of excitatory neurons. This is in agreement with findings that Aβ enhances excitatory activity in glutamatergic networks but does not increase intracellular $Ca^{2+}$ in inhibitory synapses (Brorson et al., 1995). The enhanced neuronal sensitivity to non toxic levels of glutamate caused by Aβ is perpetuated through positive feedback, which shifts APP processing to favour β-secretase production and increase Aβ in the synaptic cleft (Lesné, 2005).

## 6.3 Proposed model for metals and Aβ in neurodegeneration

In healthy neurons, upon stimulation, $ZnT_3$ and ATP7A release $Zn^{2+}$ and $Cu^{2+}$ in to the synaptic cleft, respectively where they can achieve micro to millimolar concentrations. In conjunction, low nanomolar levels of Aβ are constitutively expressed in to the synaptic cleft following β-secretase cleavage of APP. Despite their high concentrations, in normal signaling free metal levels in the synaptic cleft remain low by buffering and reuptake mechanisms that involve astrocytes and metallothionein proteins. In this environment physiological levels of extracellular Aβ are removed by the protease action of neprilysin and insulin degrading enzymes. Following activation of NMDARs cell survival mechanisms

are triggered that involve the phosphoinositide-3-kinase (PI3-K) - Akt kinase pathway. Continued activation of NMDARs in the hippocampal region of the brain leads to learning and memory formation through the process of long-term potentiation. These protective pathways can be activated by moderate increases in intracellular levels of $Ca^{2+}$, which can be brought about by increasing zinc levels in the cytosol, for example, by using a class of metal chaperones called metal protein attenuated compounds (MPACs), of which PBT2 is a lead compound (Figure 6-2). Under acute excitotoxic conditions such as in ischaemic stroke or traumatic brain injury increase glutamate release and/or a failure to clear or catabolise glutamate results in overactivation of the NMDAR. This results in massive $Ca^{2+}$ influx to the cytosol, which causes overactivation of the $Ca^{2+}$ -

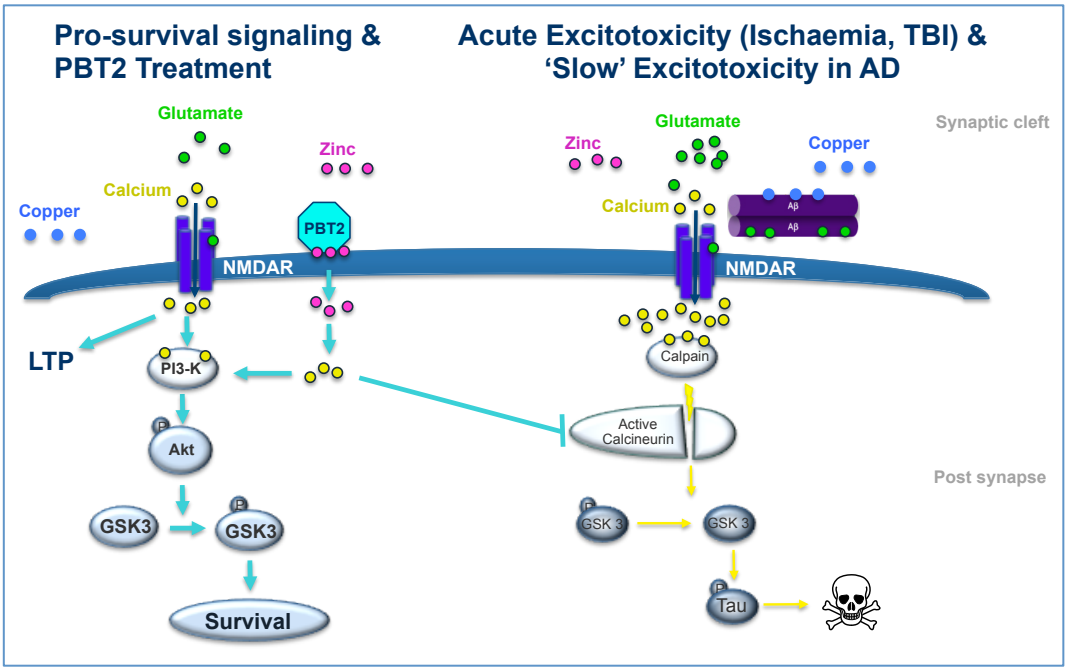


**Figure 6-2 Prevention of excitotoxicity in neurodegenerative diseases.** Under physiological conditions NMDAR activity at the post synapse is involved in synaptic formation underlying long term potentiation (LTP) and pro-survival signalling mediated by phosphoinositide-3-kinase (PI3-K) - Akt kinase cascade and inhibition of GSK-3 activity by its phosphorylation. Under acute excitotoxic conditions thought to underlie ischaemia and traumatic brain injury (TBI), exposure to excessive levels of glutamate activates the $Ca^{2+}$ -dependent protease, calpain that in turn causes the unregulated phosphatase activity of calcineurin by cleavage. Cell death follows through the resulting modifications to GSK-3 and Tau. Pre-treatment with PBT2, a therapeutic that acts as a zinc ionophore, protects against this acute excitotoxicity by moderately increasing intracellular $Ca^{2+}$ levels that activate survival pathways and inhibit calcineurin-mediated cell death. In AD overproduction of Aβ or its reduced clearance causes increased sensitivity to non-toxic levels of glutamate. This effect is dependent on Cu, which exists at millimolar concentrations in the cleft of active synapses, and on the presence of oligomeric forms of Aβ that are increased in the presence of $Zn^{2+}$ and $Cu^{2+}$. Pre-treatment of prodromal AD patients with PBT2 may prevent the subsequent neuronal loss through the same mechanism it is effective in acute excitotoxicity.

dependent protease, calpain and the unregulated constitutive activation of calcineurin upon its cleavage. This results in cell death mediated by GSK3 activation and Tau phosphorylation. This process is inhibited by pretreatment with PBT2, which prevents calpain-cleavage and the activation of calcineurin and subsequent GSK activation through its dephosphorylation. PBT2 treatment also activates cell survival pathways preconditioning neurons to subsequent glutamate-induced excitotoxic insults.

In Alzheimer's disease genetic or environmental risk factors result in increased levels of Aβ due to its overproduction or impaired clearance. In the synaptic cleft the stable Aβ oligomers are formed due to the presence of high levels of $Cu^{2+}$/$Zn^{2+}$. The oligomeric species of Aβ are more resistant to degradation than their monomeric counterparts and cause toxic glutamate activation of NMDARs, potentially by the formation of ternary complexes with $Cu^{2+}$ and glutamate. This results in moderate neuronal loss by increased glutamate-facilitated $Ca^{2+}$ flux in to the neuron and is mediated through calpain and calcineurin activation as described previously. Unlike acute excitotoxicity the levels of neuronal death are significantly less in accordance with the gradual onset of the disease. PBT2 treatment potentially acts through two mechanisms; firstly it binds to the high levels of zinc and copper in the synaptic cleft preventing metal-induced aggregation and, in conjunction, removes bound metals from Aβ. This facilitates the dissolution of Aβ from toxic oligomers to monomers making them more susceptible to clearance. Secondly, in the novel mechanism proposed in this thesis, PBT2 preconditions neurons through its ionophoric increase in intracellular zinc that causes moderate increases in intracellular $Ca^{2+}$. As in acute neurodegeneration PBT2 inhibits calcineurin-mediated cell death and activates pro-survival signaling through the PIP3 – Akt pathway (Figure 6-2). In order to obtain the full efficacy of the both of these mechanisms for the drug's actions the selection criteria for patient's in future clinical trials would require revision. To exploit the drugs' preconditioning against excitotoxicity rather than studying the effects of PBT2 in people with early to moderate AD, these future trials should be targeted at patients presenting with prodromal AD with the prospect of realising significant prevention of the disease.

# 7 Supplementary Information

## 7.1 F12 media, not neurobasal media, contains glutamine/ glutamate.

* Ham's F12 media contains 1 mM glutamine and 100 µM contaminating glutamate (L-Glutamic acid).

Ham's F12 media without glutamine from Life technologies (previously Gibco and Biosources), PromoCell and Sigma still contains 100 mM glutamate (14.7 mg/mL).

Life Technologies Media Formulation for Ham's F12 Media used for ADDLs preparations of Aβ;

### 11765 - Ham's F-12 Nutrient Mix

**Catalog Number(s)**

11765047 , 11765054 , 11765062 , 11765070

| Components | Molecular Weight | Concentration (mg/L) | mM |
|---|---|---|---|
| **Amino Acids** | | | |
| Glycine | 75.0 | 7.5 | 0.1 |
| L-Alanine | 89.0 | 8.9 | 0.099999994 |
| L-Arginine hydrochloride | 211.0 | 211.0 | 1.0 |
| L-Asparagine-H2O | 150.0 | 15.01 | 0.10006667 |
| L-Aspartic acid | 133.0 | 13.3 | 0.1 |
| L-Cysteine hydrochloride-H2O | 176.0 | 35.12 | 0.19954544 |
| L-Glutamic Acid | 147.0 | 14.7 | 0.1 |
| L-Glutamine | 146.0 | 146.0 | 1.0 |
| L-Histidine hydrochloride-H2O | 210.0 | 21.0 | 0.1 |
| L-Isoleucine | 131.0 | 4.0 | 0.030534351 |
| L-Leucine | 131.0 | 13.1 | 0.1 |
| L-Lysine hydrochloride | 183.0 | 36.5 | 0.19945355 |
| L-Methionine | 149.0 | 4.5 | 0.030201342 |
| L-Phenylalanine | 165.0 | 5.0 | 0.030303031 |
| L-Proline | 115.0 | 34.5 | 0.3 |
| L-Serine | 105.0 | 10.5 | 0.1 |
| L-Threonine | 119.0 | 11.9 | 0.099999994 |
| L-Tryptophan | 204.0 | 2.04 | 0.01 |

Life Technologies Media Formulation for Neurobasal Media used for preparations of all Aβ other than ADDLs in this thesis;

* Neurobasal media does not contain glutamine or glutamate.

## NEUROBASAL™ Medium (1X) liquid

Neurobasal™ Media are basal media formulated to meet the neuronal cells special requirements.

They allow for long-term maintenance of the normal phenotype and growth of neuronal cells, and maintain pure populations of neuronal cells without the need of an astrocyte feeder layer.

**Catalog Number(s)**

21103049

| Components | Molecular Weight | Concentration (mg/L) | mM |
|---|---|---|---|
| **Amino Acids** | | | |
| Glycine | 75.0 | 30.0 | 0.4 |
| L-Alanine | 89.0 | 2.0 | 0.02247191 |
| L-Arginine hydrochloride | 211.0 | 84.0 | 0.39810428 |
| L-Asparagine-H2O | 150.0 | 0.83 | 0.0055333334 |
| L-Cysteine | 121.0 | 31.5 | 0.2603306 |
| L-Histidine hydrochloride-H2O | 210.0 | 42.0 | 0.2 |
| L-Isoleucine | 131.0 | 105.0 | 0.8015267 |
| L-Leucine | 131.0 | 105.0 | 0.8015267 |
| L-Lysine hydrochloride | 183.0 | 146.0 | 0.7978142 |
| L-Methionine | 149.0 | 30.0 | 0.20134228 |
| L-Phenylalanine | 165.0 | 66.0 | 0.4 |
| L-Proline | 115.0 | 7.76 | 0.06747826 |
| L-Serine | 105.0 | 42.0 | 0.4 |
| L-Threonine | 119.0 | 95.0 | 0.79831934 |
| L-Tryptophan | 204.0 | 16.0 | 0.078431375 |

## 7.2 Manuscript from research conducted in Chapter 3

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# PBT2 inhibits glutamate-induced excitotoxicity in neurons through metal-mediated preconditioning

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ABSTRACT

Excitotoxicity is the pathological process by which neuronal death occurs as a result of excessive stimulation of receptors at the excitatory synapse such as the NMDA receptor (NMDAR). Excitotoxicity has been implicated in the acute neurological damage from ischemia and traumatic brain injury and in the chronic neurodegeneration in Alzheimer's disease (AD) and Huntington's disease (HD). As a result NMDAR antagonists have become an attractive therapeutic strategy for the potential treatment of multiple neurodegenerative diseases. However NMDAR signaling is dichotomous in nature, with excessive increases in neuronal intracellular calcium through excessive NMDAR activity being lethal but moderate increases to intracellular calcium levels during normal synaptic function providing neuroprotection. Subsequently indiscriminant inhibition of this receptor is best avoided as was concluded from previous clinical trials of NMDAR antagonists. We show that the metal chaperone, PBT2, currently in clinical trials for HD, is able to protect against glutamate-induced excitotoxicity mediated through NMDARs. This was achieved by PBT2 inducing $Zn^{2+}$-dependent increases in intracellular $Ca^{2+}$ levels resulting in preconditioning of neurons and inhibition of $Ca^{2+}$-induced neurotoxic signaling cascade involving calpain-activated cleavage of calcineurin. Our study demonstrates that modulating intracellular $Ca^{2+}$ levels by a zinc ionophore is a valid therapeutic strategy to protect against the effects of excitotoxicity thought to underlie both acute and chronic neurodegenerative diseases.



### Introduction

N-methyl-D-aspartate receptors (NMDARs) are ionotropic channels gated by the excitatory amino acid, glutamate. They play an essential role in synaptic plasticity, enhancing synaptic signal strength through long term potentiation (LTP), a process thought to underlie learning and memory (Bliss and Collingridge, 1993). At the synapse, NMDARs mediate neuroprotective-signaling pathways including the regulation of calcineurin activity and inhibition of glycogen synthase kinase (GSK3). Under pathological conditions the prolonged and enhanced exposure of NMDARs to glutamate results in an excessive flux of calcium ($Ca^{2+}$) into the cell. This triggers a range of responses resulting in cell death, including increased oxidative stress, inappropriate activation of proteases such as calpain, dysregulation of $Ca^{2+}$-related pathways, mitochondrial damage and an apoptotic cascade. This process, termed excitotoxicity (Olney, 1969), contributes significantly to the acute neurodegeneration in ischemia and traumatic brain injury (TBI) (Dugan and Choi, 1999; Pohl et al., 1999) and to the chronic neurodegeneration in Huntington's disease (HD) (Ferrante et al., 1993; Zhang et al., 2008) and Alzheimer's disease (AD) (Ittner et al., 2010; Um et al., 2012; You et al., 2012).

A significant body of research has been dedicated to devising strategies to inhibit excitotoxicity, either by blocking the channels through which $Ca^{2+}$ enters the cell or by inhibiting the degenerative cell signaling pathways that arise as a consequence of excessive $Ca^{2+}$ influx (reviewed in Pivovarova and Andrews, 2010). However, there is a fundamental limitation of NMDAR antagonists as therapeutic agents, as indiscriminate blocking of $Ca^{2+}$ flux through the NMDAR can also

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inhibit activity of the receptor that is necessary for normal synaptic function. In order to avoid the negative effects of NMDAR antagonists a strategy must be employed whereby excitotoxic $Ca^{2+}$ flux is prevented while maintaining synaptic signaling.

The deregulation of synaptic zinc ($Zn^{2+}$), as with $Ca^{2+}$, has been observed in acute and chronic neurodegeneration (reviewed in Corona et al., 2011; Vogler and Busciglio, 2014) and its modulation has been investigated as a therapeutic target for the treatment of both AD and HD. To this end a metal chaperone (PBT2; Prana Biotechnology, Australia) has recently been shown to induce $Ca^{2+}$-mediated neuroprotective signaling cascades involving calcineurin, the calcineurin substrates cAMP response element binding (CREB) protein and $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) and GSK3 inhibition by chaperoning zinc from extracellular pools into cells (Adlard et al., 2013; Crouch et al., 2011). PBT2 has been shown to rescue disease phenotypes in transgenic mouse models of both HD and AD (Adlard et al., 2008), and is currently undergoing clinical evaluation in HD. Excitotoxicity has been implicated in the pathogenesis of both HD and AD raising the question as to whether protection against excitotoxicity is a common mechanism of action resulting in these therapeutic benefits. Here, we evaluate the ability of PBT2 to ameliorate glutamate-induced excitotoxicity in cortical neuronal cultures and provide data supporting a novel neuroprotective action of the drug. PBT2 pretreatment induces a moderate increase in intracellular calcium, which preconditions the neurons to subsequent excitotoxic exposure.

## Materials and methods

### *Materials*

PBT2 was provided by Prana Biotechnology Limited. 6-Cyano-7-nitroquinozaline-2,3-dione (CNQX), Dizocilpine (MK-801) and Memantine Hydrochloride were purchased from Sigma Aldrich (Australia) and dissolved in $H_2O$. Zinc Chloride ($ZnCl_2$) (Sigma Aldrich, Australia) was used dissolved in $H_2O$. Diamsar, (1,8-diamino-3,6,10,13,16,19-hexaaza-bicyclo(6,6,6)eicosane) was prepared as described previously (Bottomley et al., 1994).

### *Primary neuronal cultures*

Mouse cortical neuronal cultures were prepared as previously described (Barnham et al., 2003). Briefly, embryonic day 14 (E14) C57BL/6 mouse cortices were removed, dissected free of meninges, and dissociated in 0.025% (w/v) trypsin in Krebs' buffer. The dissociated cells were triturated using a fine pipette tip, pelleted, resuspended in plating medium (minimum Eagle's medium, 10% fetal calf serum, 5% horse serum), and counted. Cortical neuronal cells were plated into poly-D-lysine-coated 48-well plates for excitotoxicity assays and 96-well plates for $Ca^{2+}$ flux assays at a density of 150,000 cells/well in plating medium. Following 2 h at 37 °C with 5% $CO_2$ the plating medium was replaced with fresh neurobasal medium containing B27 supplements, gentamicin, and 0.5 mM Glutamax (all tissue culture reagents were purchased from Invitrogen unless otherwise stated).

### *Excitotoxicity assays*

Neurons were cultured for 9 days in vitro (DIV) in neurobasal medium plus B27 supplements (Life Technologies) with media changes at 6 days before commencing treatment. All drugs and glutamate were diluted in water. Neuronal cultures were pretreated with PBT2 (3 μM) or Memantine (10 μM) for 6 h and (MK-801) (10 μM) for 20 min prior to 1 h excitotoxic exposure to glutamate (40 μM). Cell viability was measured using a Cell Counting Kit 8 (Dojindo, Japan) following 18 h in fresh neurobasal media. Specificity was investigated by depleting metals by the addition of Diamsar (10 μM), a cell impermeable metal chelator, to neurobasal media ≥1 h prior to treatments. $ZnCl_2$ was dissolved in water and added to cells with all treatments including PBT2, after the depletion of metals by treatment with Diamsar-containing neurobasal media (1 h).

### *Calcium flux assays*

Neurons were cultured as described above and then loaded with Fluo4 dye (Life Technologies) according to the manufacturer's recommendations. Briefly, cells were incubated for 30 min in complete Fluo-4 loading solution (containing loading buffer, Fluo-4 dye reagent and probenecid) at 37 °C followed by a 30 min incubation at RT. To measure fluorescence the inner 72 wells of 96-well plates were measured taking 19 s for each cycle. To establish background ($F_0$) measurements were averaged for the 10 cycles preceding injection of glutamate/glycine (100 μM/10 μM). The 11th cycle, the first post-injection, was used for fluorescence ($F_1$), which was measured by a Fluostar plate reader (excitation at 490 nm, emission at 520 nm) with values expressed as the difference in fluorescence over initial fluorescence ($\Delta F_1/F_0$). As with the excitotoxicity assays all drug treatments in the calcium flux assays were performed in an identical manner. To investigate specificity, metals were depleted by the addition of Diamsar (10 μM) to neurobasal media ≥1 h prior to treatments. $ZnCl_2$ was dissolved in water and added to cells with all treatments including PBT2, after the depletion of metals by treatment with Diamsar-containing neurobasal media (1 h).

### *Multielectrode array experiments*

Neocortex was dissected from C57BL/6 pups (postnatal days 1–3) under sterile conditions. Cortices were cut into pieces of about 1 $mm^3$, prior to dissociation using papain and trituration. Cells were plated at 5000 cells/$mm^2$ on standard 8 × 8 titanium arrayed MEAs (Multi Channel Systems, Reutlingen, Germany) coated with poly-ethylene-imine (PEI) and laminin. Cultures were maintained for 2–3 weeks prior to recording, in the following medium: high glucose DMEM with 10% Horse Serum, 0.5 mM Glutamax, 1 mM sodium pyruvate, and 2.5 μg/ml insulin (All from Life Technologies, Australia). Cultures were kept for 21 days in an incubator at 37 °C, 65% relative humidity, 5% $CO_2$, and 9% $O_2$.

The MC_RACK software and the MEA-2100-60-System (Multi Channel Systems, Reutlingen, Germany) were used to record and analyze data. The MC_RACK software was used to detect and record unit activity. Data was gathered from neuronal cultures pre-treated with or without PBT2 (3 μM) for 5 min pre- and post-exposure to glutamate (100 μM) and glycine (10 μM). A −20 μV threshold was set for the detection of spike and an inter spike interval detection threshold set to greater than 10 ms to avoid contamination with burst firing. Data was analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Channels that had a baseline greater than −10 μV were denoted as noisy channels, and were excluded from the data set.

### *Western blot analysis*

Western blots were performed on total cell extracts (20 μg/lane) from mouse cortical neuronal cultures described above. Cultures in 48-well plates (Nunc) were lysed in 120 μL/well of lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 0.1% Triton-X) and 1% protease inhibitor mix set 1 (Calbiochem). Primary antibodies were as follows: polyclonal rabbit anti-GSK3α, monoclonal mouse anti-phospho (Ser21)-GSK-3α, monoclonal rabbit anti-GSK3β, polyclonal rabbit anti-phospho (Ser21/Ser9) GSK3α/β (all at 1:5000 dilution, Cell Signaling Technology) and polyclonal rabbit anti-Calcineurin A (1:1000 dilution, Sapphire Bioscience). Secondary antibodies were from mouse or rabbit and conjugated to Horseradish Peroxidase (GE Healthcare, UK). Proteins were visualized using Immobilon chemiluminescent HRP substrate (Millipore, Australia) on Amersham Hyperfilm XP (GE Healthcare, UK). Western blot data were quantified by densitometric analysis in three different blots per experiment.

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*Statistical analysis*

Densitometric analysis was performed using ImageJ software (Rasband, 1997). Cell viability, $Ca^{2+}$ flux, MEA and densitometry results are expressed as ± standard error mean (SEM). Prism data analysis software (GraphPad) was used to compare treatments by one-way analysis of variance (ANOVA) with Dunnett's post hoc test.

**Results**

*The metal chaperone activity of PBT2 is required for its protection against glutamate-induced excitotoxicity*

Based on previous work demonstrating PBT2's efficacy in preventing Aβ42 inhibition of LTP (Adlard et al., 2008) and recent findings implicating excitotoxicity in both AD & HD (Fan and Raymond, 2007; Um et al., 2012) our initial experiments tested the capability of the drug to confer protection against glutamate-induced toxicity in cortical neurons. To achieve this, experimental conditions were optimized to induce approximately 50% cell death upon a 1-hour treatment with glutamate (40 μM), as neurons at this cell viability showed maximal protection. Following a 6 hour pretreatment of neurons with increasing concentrations of PBT2 (1, 3 and 7.5 μM), its maximum protection against excitotoxicity was conferred where no effect on excitotoxicity by PBT2 was observed at 1 h. This was in contrast to MK-801 and Memantine, both direct NMDAR channel blockers, that demonstrated full protection in 1 h (Figs. 1A and B). Pre-treatment with PBT2 protected against excitotoxic insult restoring cell viability of glutamate-treated neurons (56 ± 6%) in a dose-dependent manner (glutamate + PBT2 at 0, 3 and 7.5 μM = 59 ± 6%, 87 ± 5% and 88 ± 2%, respectively, $n = 6$, $p < 0.001$) (Fig. 1B). These levels were comparable to the non-competitive NMDA antagonists, MK-801 and Memantine (89 ± 4% and 90 ± 2%, $n = 6$, $p < 0.001$) (Fig. 1A). To test whether the protective effect of PBT2 against excitotoxicity was dependent on its metal chaperone activity, metals were depleted from the cell culture media by a 1-hour treatment with $(NH_2)_2$sar (Diamsar) (10 μM) prior to incubation with PBT2 and then glutamate (Fig. 1C). Diamsar is a high affinity metal chelator, unable to permeate the cell membrane (Crouch et al., 2011). Upon sequestration of metals by Diamsar, glutamate-treated cells (44 ± 6%) were no longer protected by PBT2 (1 μM, 49 ± 9%, 3 μM, 49 ± 9% and 7.5 μM, 53 ± 8%) demonstrating the requirement of metals as co-factors for PBT2 to mediate protection. As expected the NMDA antagonists have no requirement of metals to mediate their effect (MK-801, 86 ± 12% and Memantine, 86 ± 5%, $n = 4$, $p < 0.01$, $p < 0.001$, respectively) (Fig. 1C). To further illustrate the role of metals in PBT2 protection $Zn^{2+}$ was titrated back into the metal-depleted media and restoration of protection by PBT2 against excitotoxicity was observed ($Zn^{2+}$ at 7 μM, 50 ± 7% to 72 ± 8%, $n = 5$, $p < 0.05$) (Fig. 1D). Given that previous ICP-MS analysis had detected 3–5 μM $Zn^{2+}$ in neurobasal media, the 7 μM $Zn^{2+}$ supplemented back into the metal-depleted media was sufficient to overcome chelation by 10 μM Diamsar. In these experiments PBT2 coordinates the extracellular metals, namely $Zn^{2+}$ and increases its ability to cross cellular membranes, therefore acting as an ionophore or metal chaperone. Once

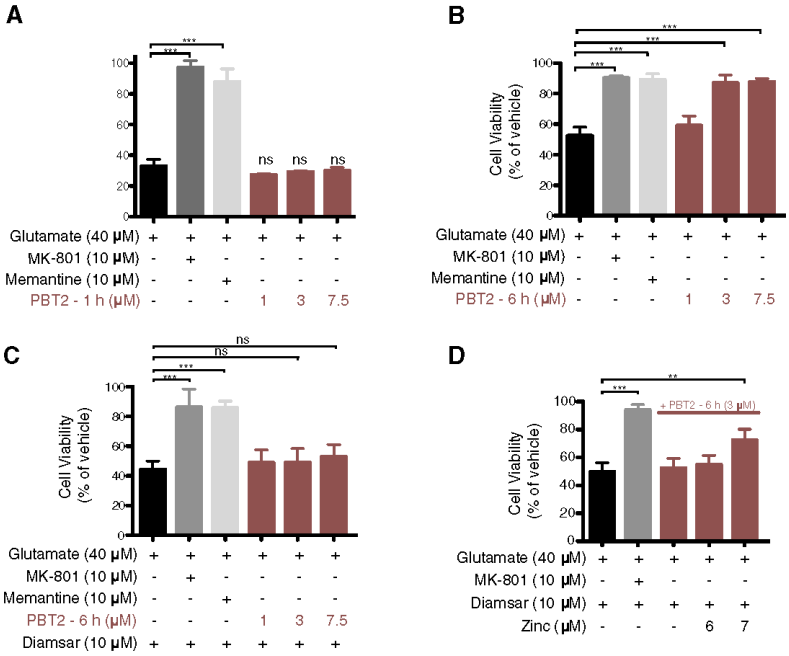


**Fig. 1.** PBT2 protects against glutamate-induced excitotoxicity by its metal chaperone activity. Mouse primary cortical neurons were treated with the NMDA-specific antagonists, MK-801 (dark gray) and Memantine (light gray) for 1 h and PBT2 at increasing concentrations for 6 h prior to exposure to glutamate (40 μM). Cell viability was measured and results were normalized to the untreated vehicle. (A) The non-competitive antagonists, MK-801 and Memantine prevent excitotoxicity where as 1 h pre-treatment with PBT2 is not sufficient to effect cell viability. (B) 6 h pre-treatment with PBT2 prevents excitotoxicity in a concentration-dependent manner equivalent to the levels of the non-competitive antagonists, MK-801 and Memantine. (C) Removal of metals from neuronal media by pre-treatment with the chelator, Diamsar abolishes PBT2 protection against excitotoxicity. (D) Titrating zinc into metal-depleted media restores PBT2 protection against excitotoxicity. Data represent mean ± SE; $n = 3–6$; $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant compared to glutamate treated values by one-way ANOVA using Dunnett's post hoc test.

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inside the cell the metal/drug complex dissociates making the metal bioavailable and able to activate neuroprotective signaling cascades (Adlard et al., 2008; Crouch et al., 2011). In this work we have demonstrated a $Zn^{2+}$ requirement for this effect by depleting the metals with Diamsar and reintroducing only $Zn^{2+}$.

*PBT2 significantly reduces NMDAR-mediated $Ca^{2+}$ flux*

MK-801 protects against excitotoxicity by directly acting on NMDA receptors preventing the excessive influx of $Ca^{2+}$ required for NMDA-mediated cell death (Stout et al., 1998). To investigate whether PBT2 protects by this mechanism we measured intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) flux in cultured mouse cortical neurons using the Fluo-4 dye. Neurons were pre-treated with vehicle, MK-801 (10 μM), and PBT2 (10 μM) for 1 h (Fig. 2A) and 6 h (Fig. 2B) prior to dye loading and treatment of cells with a concentration of glutamate and the co-agonist, glycine (100 μM/10 μM) to ensure maximum $Ca^{2+}$ flux (light gray line). Background levels of fluorescence were established prior to the injection of glutamate/glycine treatment. By treating for 1 h we sought to determine whether PBT2 immediately antagonized the NMDA receptor in a similar mechanism to MK-801. Upon a 1-hour treatment with MK-801, glutamate-induced $Ca^{2+}$ flux was significantly attenuated to 48.5% (51.5 ± 7.5%, n = 4, $p < 0.001$). The remaining $Ca^{2+}$ flux can be attributed to recovery from MK-801 blockade during extended agonist exposure (McKay et al., 2013) and to a lesser extent, extrasynaptic NMDARs, non-NMDA ionotropic receptors and metabotropic receptors. Treatment for the same duration with PBT2 however resulted in a reduction in $Ca^{2+}$ flux of only 16% (84 ± 4.5%, n = 4, $p < 0.001$) (Fig. 2A). When neurons were pre-treated for 6 h with MK-801, glutamate-induced $Ca^{2+}$ flux remained significantly attenuated (65.3 ± 3.3%, n = 4, $p < 0.001$) however the longer treatment with PBT2 resulted in a significant reduction in $Ca^{2+}$ flux of 31.2% (68.8 ± 5.6%, n = 5, $p < 0.001$) (Fig. 2B). In these experiments a 6 h pretreatment with PBT2 was required for maximal attenuation of $Ca^{2+}$ flux with little effect at 1 h (Figs. 2A and B, respectively).

*PBT2 requires zinc to reduce NMDAR-mediated $Ca^{2+}$ flux*

The results obtained from the excitotoxicity experiments demonstrated a requirement of metal for the protective effect of PBT2 on cell viability of neurons. To determine whether metals present in the media were also a requirement for the observed effect of PBT2 on $Ca^{2+}$ flux, experiments were performed in the presence and absence of metals by using the metal chelator, Diamsar. The reduction in $Ca^{2+}$ flux previously seen following a 6-hour treatment with PBT2 (Fig. 2B) is ablated by the removal of metals (87.3 ± 9.3%, n = 4, $p < 0.001$) (Fig. 3A). Although experiments with ionotropic receptor agonists support the role of NMDAR mediating glutamate-induced excitotoxic cell death (Choi, 1992) glutamate activation of AMPA and Kainate receptors could still contribute to $Ca^{2+}$ flux. To ensure that the effects of PBT2 were mediated solely by NMDARs, neurons were concomitantly treated with the AMPA/Kainate receptor antagonist, CNQX, which showed no effect on the action of PBT2 (Figs. 3B and C). We then tested whether the reintroduction of zinc to metal-depleted neurons would restore the ability of PBT2 to modulate $Ca^{2+}$ flux. The concentration of $Zn^{2+}$ shown to restore PBT2's ability to rescue glutamate-induced excitotoxicity (7 μM) (Fig. 1C) in metal-depleted media was used during PBT2 pre-treatment of cells (Fig. 3C). The additional $Zn^{2+}$ in conjunction with $Zn^{2+}$ in the Neurobasal media (3–5 μM by ICP-MS, data not shown) was sufficient to overcome chelation by 10 μM Diamsar and fully restored the ability of PBT2 to modulate $Ca^{2+}$ flux. The relative $Ca^{2+}$ flux in PBT2-treated neurons, in the absence of metals (94 ± 5%, n = 6, $p < 0.001$; Fig. 3B), was significantly inhibited to 52 ± 8%, respectively, n = 4, $p < 0.001$ (Fig. 3C). This was shown to be an NMDAR-mediated event by blocking AMPA & Kainate receptors with concomitant treatment with CNQX (Figs. 3B and C).

*Preconditioning by PBT2 treatment is neuroprotective against excitotoxicity*

The phenomenon of neuroprotection by preconditioning has been well documented in ischemic models (Katsura et al., 2001; Kirino et al., 1991; Murry et al., 1986) in which glutamate-induced

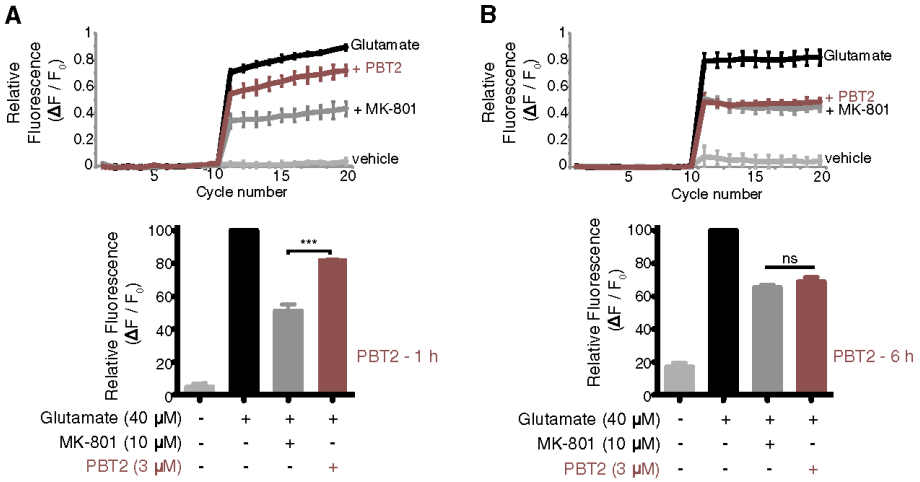


**Fig. 2.** The time-dependent effect of PBT2 on $Ca^{2+}$ flux through the NMDA receptor. Intracellular $Ca^{2+}$ levels in cortical neurons preloaded with the $Ca^{2+}$ indicator Fluo4, were measured upon stimulation by glutamate/glycine (100 μM/10 μM) (black) and reading fluorescence at 490 nm excitation and 520 nm emission. The top panels of (A) and (B) show representative traces with measurements taken at cycle number 11, t = 209 s ($F_1$) and the average background fluorescence measured from cycles 1–10, t = 0–190 s ($F_0$), data is expressed in bottom panels as bar graphs where $\Delta F = F_1 - F_0$. (A) Prior to Fluo4 dye loading neurons were treated for 1 h with PBT2 (red) or a non-competitive NMDA antagonist, MK-801 (dark gray). (B) Neurons were also pre-treated with PBT2 for 6 h, the time required for PBT2 to protect against glutamate induced excitotoxicity, and MK-801 for 1 h. Data represent mean ± SE; n = 4; ***$p < 0.001$; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

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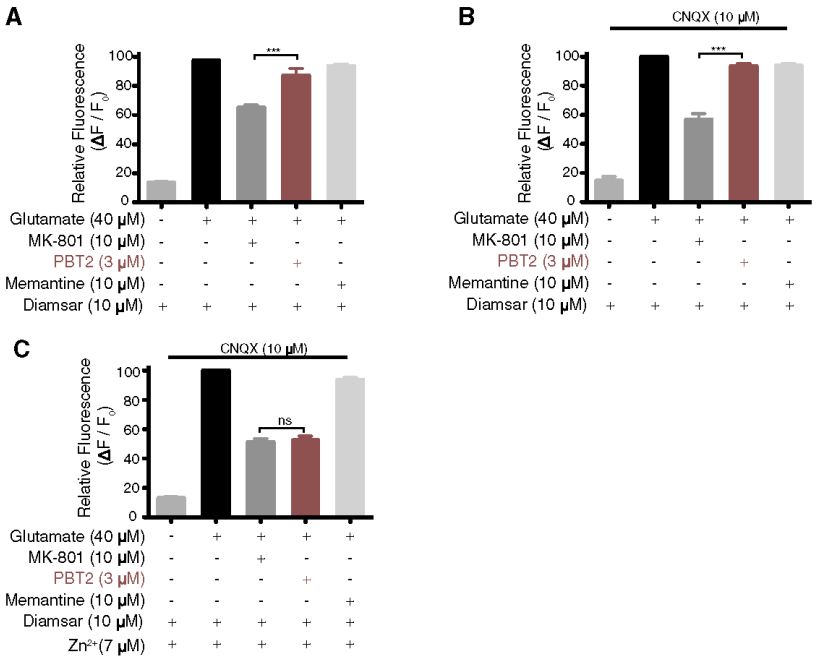


**Fig. 3.** Zinc is required for the inhibition of $Ca^{2+}$ flux by PBT2. Intracellular $Ca^{2+}$ levels in cortical neurons preloaded with the $Ca^{2+}$ indicator Fluo4, were measured upon stimulation by glutamate (100 μM/10 μM) (black) and reading fluorescence at 490 nm excitation and 520 nm emission. Measurements were taken at cycle number 11, t = 209 s ($F_1$) and background fluorescence averaged from cycles 1–10, t = 0–190 s, ($F_0$), data is expressed as bar graphs where $\Delta F = F_1 - F_0$. (A) Prior to Fluo4 loading neurons were treated with PBT2 (3 μM) (red) or the non-competitive NMDA antagonist, MK-801 (10 μM) (dark gray) for 6 h in media depleted of metals by Diamsar (10 μM). (B) To demonstrate NMDA-specificity neurons were co-treated with the competitive AMPA/Kainate antagonist, CNQX (10 μM). (C) Neurons were treated with CNQX (10 μM) and PBT2 (3 μM) or MK-801 (10 μM) for 6 h in media depleted of metals by Diamsar (10 μM) then exogenous $Zn^{2+}$ (7 μM) was added. Data represent mean ± SE; n = 4–6; ***$p < 0.001$; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

excitotoxicity underlies neuronal death. Additionally, sublethal levels of intracellular zinc have been shown to be neuroprotective in rats (Lee et al., 2008) and cause an increase in $[Ca^{2+}]_i$ by release from the endoplasmic reticulum (ER) (Qin et al., 2011). Given that moderate increases in $[Ca^{2+}]_i$ can precondition neurons (Bickler and Fahlman, 2004; Bliss and Collingridge, 1993) and the strong chaperone action of PBT2 on $Zn^{2+}$ we sought to determine whether PBT2 treatment would have a metal-dependent effect on $[Ca^{2+}]_i$ levels in our neuronal model. These data were obtained from observing the $[Ca^{2+}]_i$ levels in the $Ca^{2+}$ flux experiments (Figs. 2 and 3) prior to treatment with glutamate/glycine. A significant increase (113.0 ± 3.9%, n = 5, $p < 0.001$) in $[Ca^{2+}]_i$ was observed in neurons pretreated with PBT2 for 6 h but not in vehicle-treated (99.05 ± 1.5%, n = 5) or neurons treated with the NMDAR antagonists; MK-801 for 1 h (97.8 ± 2.2%, n = 5) and Memantine for 6 h (97.7 ± 3.2%, n = 5) (Fig. 4A). The metal-dependent effect of PBT2 was again demonstrated by the depletion of metals by supplementing media with Diamsar at an excess concentration of 10 μM. Levels of $Zn^{2+}$ in neurobasal media have been determined to be between 3 and 5 μM by ICP-MS (data not shown) and therefore chelated by treatment with 10 μM Diamsar prior to and during drug treatment. The metal chelation ablated the increase in $[Ca^{2+}]_i$ levels due to PBT2 (Fig. 4B). When $Zn^{2+}$ was supplemented back into metal-depleted media treated with PBT2 it was necessary to use 7 μM to overcome chelation by 10 μM Diamsar. This resulted in an approximate concentration of $Zn^{2+}$ up to 2 μM, despite these levels being lower than in Neurobasal media not treated with the chelator, it was still sufficient to significantly increase $[Ca^{2+}]_i$ levels (120.6 ± 4.6%, n = 4, $p < 0.001$) compared with neurons treated with vehicle (99.13 ± 1.4%, n = 4), MK-801 (99.42 ± 1.8%, n = 4) and Memantine (100.4 ± 3.7%, n = 5) (Fig. 4C). In experiments that tested the effect of treatment time, neurons treated with PBT2 for 1 h did show a small but significant increase in $[Ca^{2+}]_i$ levels (103.9 ± 3.4%, n = 6, $p < 0.05$). This significant difference was ablated by prior metal depletion of the media as was observed in the longer 6 h treatment with PBT2.

### *The effect of PBT2-induced preconditioning on network activity*

To further investigate PBT2 treatment we employed 60-channel MEAs to observe network-wide extracellular activity. Results were obtained by measuring the spike activity of three separate cortical neuron preparations cultured on MEAs. The results showed a significant decrease in excitotoxic network activity induced by glutamate/glycine following pretreatment with PBT2 for 6 h (Figs. 5A and B). In further support of PBT2 preconditioning neurons, a small but significant increase in network activity was observed in neurons treated with PBT2 alone compared with vehicle, as measured by the number of spikes (Fig. 5B). This increase in activity corroborates the increases in $[Ca^{2+}]_i$ levels that were observed previously (Figs. 4A and C) and also past findings demonstrating the requirement of prolonged and marked elevation of electrical activity for preconditioning (Olney, 1969; Tauskela et al., 2008).

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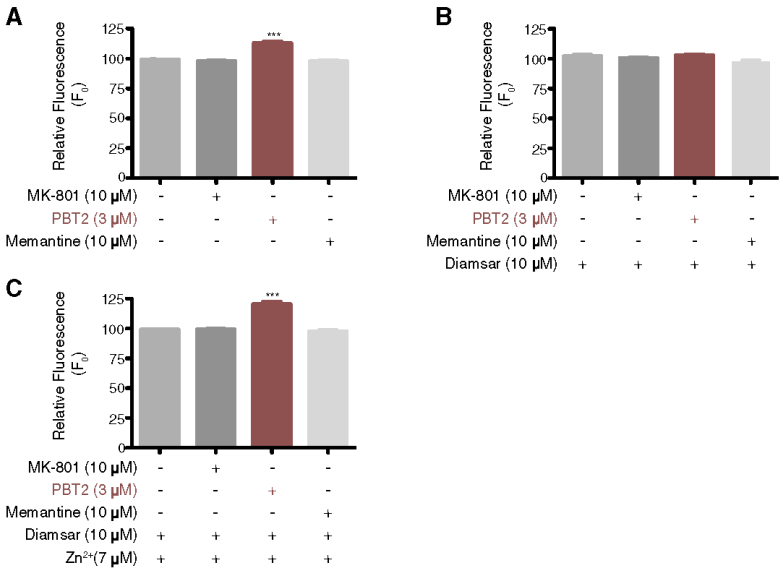


**Fig. 4.** PBT2 preconditioning protects against excitotoxicity by increasing intracellular $Ca^{2+}$ levels. (A) Following pre-treatment with PBT2 (3 μM) (red), the non-competitive NMDA antagonists; MK-801 (10 μM) (dark gray) or Memantine (10 μM for 6 h) (light gray) cortical neurons were loaded with the $Ca^{2+}$ indicator Fluo4 for 1 h and fluorescence was measured at 490 nm excitation and 520 nm emission. Mean fluorescence was expressed as a bar graph with values normalized to untreated vehicle. (B) Metals were depleted from neuronal media by pre- and co-treatment with Diamsar (10 μM). (C) Exogenous $Zn^{2+}$ (7 μM) was supplemented into Diamsar-treated neurons during treatment. Data represent mean ± SE; n = 4–6; ***$p < 0.001$; ns, not significant compared to MK-801 treated values by one-way ANOVA using Dunnett's post hoc test.

*PBT2 restores normal levels of protein expression and phosphorylation under excitotoxic conditions*

Calcineurin, a $Ca^{2+}$-dependent protein phosphatase, and glycogen synthase kinase 3 (GSK3) both mediate survival in primary neurons from rodents (Dugan and Choi, 1999; Endo et al., 2006; Pohl et al., 1999; Wu et al., 2007). Phosphorylation of GSK3 at its N-terminus (Ser-21 for GSK3α and Ser-9 for GSK3β) prevents phosphorylation of downstream microtubule-associated proteins, namely Tau and subsequent apoptosis (Ferrante et al., 1993; Li et al., 2000; Zhang et al., 2008). The calcineurin-mediated signaling pathway is regulated by calpain, a $Ca^{2+}$-dependent cysteine protease, and this protease is up-regulated during glutamate-induced excitotoxicity in which it irreversibly activates calcineurin by cleavage (Ittner et al., 2010; Um et al., 2012; Wu et al., 2007; You et al., 2012). Previous work in cultured SH-SY5Y human neuroblastoma cells has demonstrated that an increase of GSK3 phosphorylation by PBT2 is a result of inhibition of calcineurin (Crouch et al., 2011; Pivovarova and Andrews, 2010). To determine whether PBT2 activates cell survival pathways by preventing the cleavage of calcineurin and inhibiting GSK3α and GSK3β activity by phosphorylation, we analyzed their respective protein expression following pre-treatment with drugs and exposure to excitotoxic glutamate in mouse cortical neurons. PBT2 pre-treatment inhibited activation/cleavage of calcineurin significantly increasing protein levels in glutamate treated neurons when normalized to vehicle (from 21 ± 7% to 81 ± 9%, $n = 3$, $p < 0.05$, $p < 0.01$ for PBT2) (Fig. 6A). Further evidence of neuroprotection by PBT2 mediated by GSK3 was seen by its ability to increase phosphorylation of both GSK3α (from 8 ± 4% to 85 ± 19%, $n = 3$, $p < 0.01$ for PBT2) and GSK3β (from 55 ± 9% to 92 ± 8%, $n = 3$, $p < 0.001$ for PBT2, respectively) (Figs. 6B and C).

## Discussion

A role for PBT2's action in glutamatergic signaling has previously been indicated by its ability to inhibit the Aβ-induced inhibition of LTP in rodents (Adlard et al., 2008; Bottomley et al., 1994). PBT2 was originally identified by compound screens for its ability to inhibit toxic extracellular Aβ–metal interactions, a mechanism thought to involve the sequestration of Zn from the extracellular Aβ aggregates and drug-mediated intracellular metal delivery (Adlard et al., 2008; Barnham et al., 2003). However in the work presented here we provide evidence of an alternative mechanism of action of the drug, one that supports the findings in which PBT2 has shown efficacy in animal models of AD and clinical trials of HD.

The data we present here demonstrate a novel mechanism of action of PBT2; namely the ability to protect against glutamate-induced excitotoxicity in a metal-dependent manner. As evidence of this, the protective effect of PBT2 was ablated when the metal chelator, Diamsar, removed metals in the cell culture media. The removal of metals had no effect on the actions of NMDA-specific antagonists, MK-801 and Memantine (Fig. 1). NMDA receptors play a vital role in the normal function of the nervous system including their role in cognition, learning and memory through LTP. Clinical trials of NMDA antagonists such as MK-801 in stroke, Huntington's disease and traumatic brain injury failed to show safety resulting in psychotomimetic effects including hallucinations, agitation, sensory disturbance and catatonia (Lees et al., 2000; Muir, 2006; Rasband, 1997). These severe side effects have been attributed to the slow "off-rates" of the drugs leading to prolonged blockage of the normal signaling by NMDA receptors. Memantine is an NMDA antagonist that has EU and FDA approval for moderate to severe AD. The drug is clinically tolerated reportedly due to a faster "off-rate" (Adlard et al., 2008; Lipton, 2006) Unlike MK-801, Memantine has

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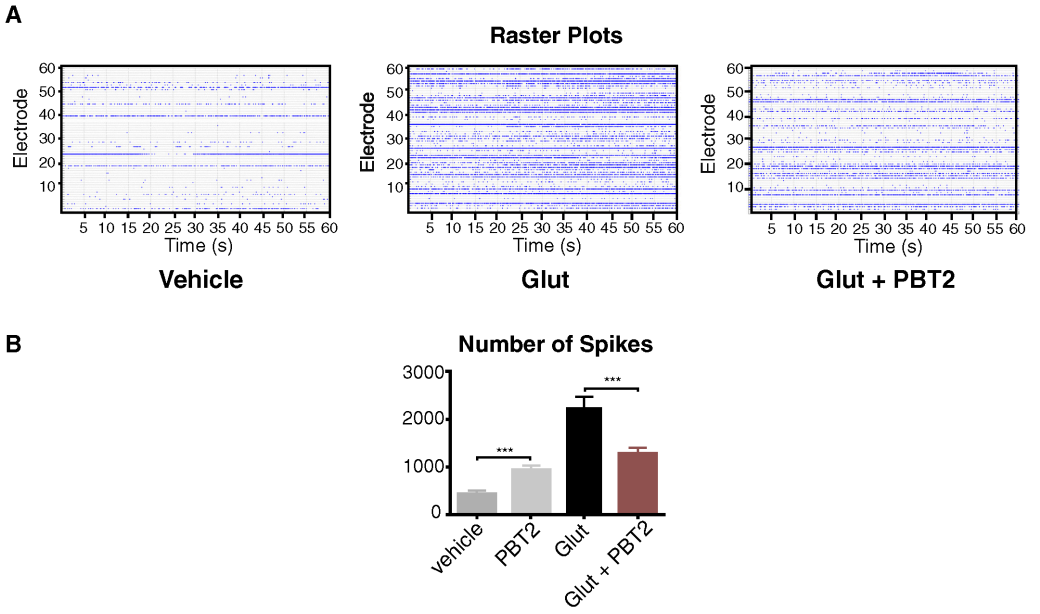


**Fig. 5.** PBT2 induces moderate synaptic activity and reduces excitotoxic network excitation following glutamate exposure. (A) Example of Raster plots of a single MEA experiment with cortical neurons treated with vehicle, glutamate/glycine (100 μM/10 μM) (glut) or pre-treated for 6 h with PBT2 (3 μM) prior to treatment with glutamate/glycine (glut + PBT2). The raster plots show neural activity by recording spikes at each of the 60 electrodes (blue dots) as a function of time with each dot representing a single action potential. (B) Graphical representation of data obtained from the above MEA experiments. The number of spikes was recorded over the period of 5 min pre- and post-application of glutamate/glycine for each treatment. Data represent mean ± SE; n = 3; ***$p < 0.001$ compared to vehicle and glutamate treated values, respectively, by one-way ANOVA using Dunnett's post hoc test.

been shown to act selectively through extrasynaptic NMDARs (Stout et al., 1998; Talantova et al., 2013). This mechanism is supported by our findings in which glutamate-induced $Ca^{2+}$ flux, mediated through NMDARs at the post-synapse, is not altered in neurons pretreated with Memantine (Figs. 2 and 3).

In the subsequent calcium flux assays we demonstrated a significant metal-dependent reduction in calcium flux by PBT2 to levels seen in neurons treated with NMDAR antagonist, MK-801 (Fig. 3A). In these experiments metals were chelated using Diamsar, which has a high affinity for Zn but could potentially bind $Ca^{2+}$ albeit with much lower affinity. To control for this, analysis of fluorescence data established that co-treatment with Diamsar had no effect on $[Ca^{2+}]_i$ levels and subsequently no effect on the observed changes in $Ca^{2+}$ flux between samples (Figs. 2B and 3A). Non-specific effects of PBT2 on $Ca^{2+}$ in this experiment were also negated by the observation that when metals were removed $Ca^{2+}$ flux was not significantly different to vehicle (Figs. 3A and B). It was also important to determine whether the intracellular flux of calcium was mediated solely by NMDARs or also by the two remaining classes of glutamatergic receptors; AMPA and Kainate receptors. Co-administration with the competitive AMPA/Kainate receptor antagonist, CNQX, demonstrated no effect on calcium flux in untreated and treated neurons indicating the $Ca^{2+}$ flux to be NMDAR-mediated (Figs. 3A and B).

Preconditioning by pre-exposure to subtoxic levels of glutamate and NMDA (Chuang et al., 1992; Lee et al., 2008; Rocha et al., 1999) or brief ischemic insults (Kirino et al., 1991; Murry et al., 1986; Qin et al., 2011) is neuroprotective against excitotoxic trauma and has been well documented in the literature. Further work describing this phenomenon has demonstrated the involvement of cellular pathways mediated by NMDARs. Preconditioning with low doses of NMDA induces key proteins in neuroprotective pathways including the phosphatidylinositol 3 kinase-protein kinase B (Akt) signaling to GSK3β inhibition (Soriano et al., 2006). These survival signals can also be activated by using $Ca^{2+}$ ionophores to moderately increase $[Ca^{2+}]_i$ conferring long-term tolerance of ischemia or other stresses and this occurs independently of NMDAR-mediated $Ca^{2+}$ flux (Bickler and Fahlman, 2004). A central finding of this work is that PBT2 causes a moderate but significant increase in $[Ca^{2+}]_i$ levels following treatment (Fig. 4). In this data PBT2 treatment did not protect against excitotoxicity by attenuating total $[Ca^{2+}]_i$ mediated by NMDARs as with MK-801 (Figs. 2 and 3), rather it reduced flux by increasing initial levels of $[Ca^{2+}]_i$ and in turn preconditioned neurons against excitotoxic exposure to glutamate (discussed below). This is because it is the total net flux of $Ca^{2+}$ into the cell rather than total levels of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) that correlates with the extent of excitotoxic cell death (Abdel-Hamid and Baimbridge, 1997; Dugan and Choi, 1999; Soriano et al., 2006).

There are several potential mechanisms by which PBT2 could result in the zinc-dependent increase in $[Ca^{2+}]_i$. It can rise by crossing the cell membrane through NMDARs, AMPAR, store-operated $Ca^{2+}$ channels (SOCCs), voltage-gated $Ca^{2+}$ channels (VGCCs), and ionotropic glutamate receptors (N-methyl-D-aspartate receptors, NMDARs; a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, AMPARs) or it can be released from intracellular stores in the endoplasmic reticulum (Corona et al., 2011). Another obvious candidate recently described involves the post-synaptic $Zn^{2+}$-sensing receptor (ZnR)/G-protein coupled receptor (GPR39) and as with many metabotropic receptors the resulting increase $[Ca^{2+}]_i$ is mediated through phospholipase C and subsequent generation of inositol 1,4,5 triphosphate (IP3) (Chorin et al., 2011; Hershfinkel et al., 2001). In our work we did not see an increase in $[Ca^{2+}]_i$ in neurons other than those treated with PBT2 in normal,

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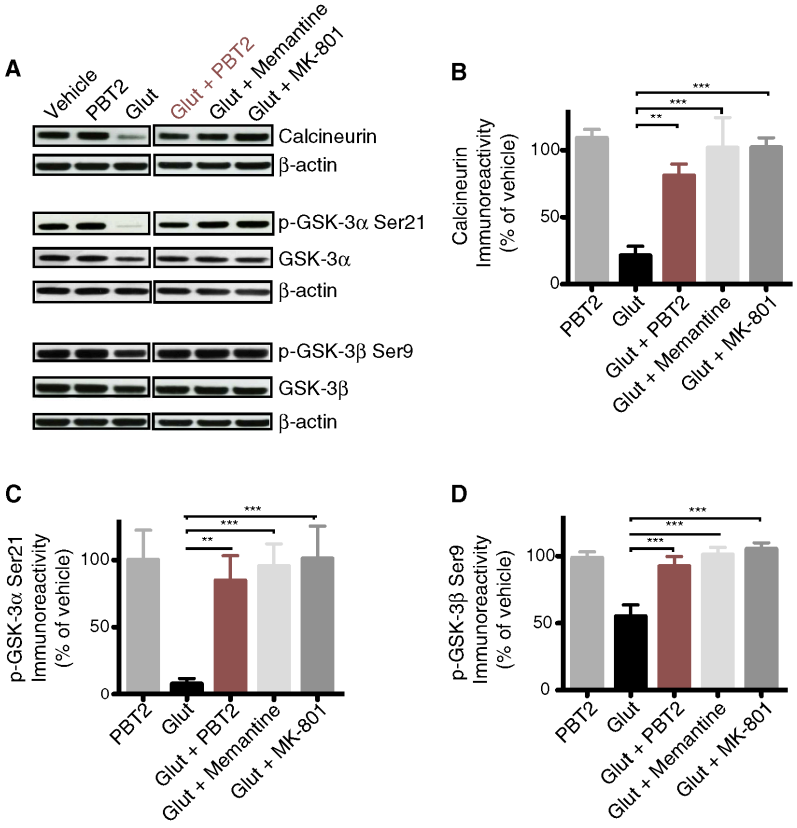


**Fig. 6.** PBT2 inhibits calcineurin and GSK3 activation. A. Western blot analysis of calcineurin, p-GSK3α/β, total GSK3α/β and the control protein β-actin in cortical mouse neurons (D.I.V. 9). Neurons were pre-treated with PBT2 (3 μM) (red) and with the NMDA-specific antagonists; Memantine (10 μM) for 6 h or MK-801 (3 μM) for 1 h prior to treatment with glutamate (glut) (black) for 1 h. Protein was harvested after an 18 h incubation in fresh media. (B–D) Densitometry analysis of western blot data is shown in panel (A), and PBT2 treatment inhibited calcineurin activity by preventing its calpain-mediated cleavage demonstrated by restored protein levels (B). PBT2-treated neurons (red) showed significantly increased levels of inactive phosphorylated GSK-3α (C), to a lesser extent GSK-3β (D) compared with glutamate-treated samples (black). All samples were normalized to the β-actin to control for loading. Western blots are representative of 3 or more separate experiments. Data represent mean ± SE; $^{**}p < 0.01$, $^{***}p < 0.001$ compared to glutamate treated values.

metal-depleted and metal-depleted/$Zn^{2+}$ conditions (Fig. 4). This observation in conjunction with the metal-dependent nature of the drug's action makes it is less likely that PBT2 acts directly on an extracellular receptor to increase $[Ca^{2+}]_i$ instead acting through its ionophoric ability to increase intracellular zinc triggering the release of $Ca^{2+}$ from the ER via the inositol 1,4,5-trisphosphate (IP3R) and ryanodine (RyR) receptors. In support of this mechanism cytosolic levels of $Zn^{2+}$ have been shown to modulate ER $Ca^{2+}$ stores (Qin et al., 2011).

In work delineating the effects mediated through synaptic and extrasynaptic NMDARs by Hardingham and colleagues, elevated levels of electrical activity were shown to precondition neurons against apoptosis from serum deprivation, excitotoxicity or oxidative stress (Hardingham et al., 2002; Papadia et al., 2008; Tauskela et al., 2008). Our results demonstrating an increase in $[Ca^{2+}]_i$ indicate that PBT2 is effecting synaptic activity to a moderate extent (Fig. 4). In our subsequent experiments studying the effect of PBT2 on network activity with MEAs we confirmed that PBT2 indeed can reduce excitotoxic electrical signaling due to glutamate/glycine as was originally hypothesized. An important finding that provided further evidence of preconditioning as a mechanism of action of PBT2 was a moderate but significant increase in electrical activity across the MEAs due to PBT2 treatment.

As discussed prior, moderate increases in $[Ca^{2+}]_i$ (Bickler and Fahlman, 2004) result in preconditioning of neurons by activation of the neuroprotective pathways involving Akt, CMK, & GSK3. Previous research has demonstrated the capability of structurally diverse metal chaperone compounds to activate neuroprotective signaling pathways (Adlard et al., 2008; Crouch et al., 2009, 2011; Donnelly et al., 2008; White et al., 2006). The restoration of normal calcineurin levels indicates that PBT2 acts on a key pathway associated with neurodegeneration involving the calcium-activated cysteine protease, calpain (Bradley et al., 2012). The cleavage and subsequent constitutive unregulated activation of one of calpain's downstream targets, calcineurin, has been shown to induce dephosphorylation and activation of GSK3 α and β. GSK3 dysregulation has been implicated in AD (Avila et al., 2004) and other relevant neurodegenerative diseases including HD (Carmichael et al., 2002) and frontotemporal dementia with parkinsonism (Engel et al., 2006). In this study we have shown that PBT2 protects against glutamate-induced excitotoxicity by increasing $[Ca^{2+}]_i$, inhibiting

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cleavage and therefore activating calcineurin and restored its normal protein levels (Fig. 6B). Further evidence of the drug acting to inhibit neurotoxic signaling was observed by the increase of phosphorylation of both GSK3 isomers (Figs. 6C and D).

## Conclusions

In this work we report a new mode of action for the metal chaperone, PBT2. Our data shows that PBT2 is able to protect against excitotoxic insults through a zinc-dependent increase in intracellular $Ca^{2+}$. This inhibition of excitotoxicity may explain the drug's ability to improve cognition in early clinical trials of AD (Lannfelt et al., 2008) and HD.

*Abbreviations*

| | |
|---|---|
| $Ca^{2+}$ | calcium |
| $Zn^{2+}$ | zinc |
| NMDAR | N-methyl-D-aspartate receptor |
| AD | Alzheimer's disease |
| HD | Huntington's disease |
| LTP | long term potentiation |
| TBI | traumatic brain injury |
| CREB | cAMP response element binding protein |
| CaMK | $Ca^{2+}$/calmodulin-dependent protein kinase |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| MK-801 | Dizocilpine |
| Akt | protein kinase B |
| GSK3 | glycogen synthase kinase 3 |
| Diamsar | 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo(6,6,6)eicosane |
| $[Ca^{2+}]_i$ | intracellular calcium |
| ANOVA | analysis of variance |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |

## Competing interests

Dr Barnham is a shareholder and paid scientific consultant for Prana Biotechnology Pty Ltd.

## Authors' contributions

Scientific concept: TJ and KJB. Experimental design: TJ, PSD, SP, AFH, and KJB. Experiments: TJ, NS, and XL. Manuscript preparation: TJ. Manuscript edit: all authors. All authors read and approved the final manuscript.

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