

EVALUATION OF GENE REGULATION AND THERAPEUTIC DRUGS RELATED TO
ALZHEIMER'S DISEASE IN DEGENERATING PRIMARY CEREBROCORTICAL CULTURES

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Abstract

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EVALUATION OF GENE REGULATION AND THERAPEUTIC DRUGS RELATED TO ALZHEIMER'S DISEASE IN DEGENERATING PRIMARY CEREBROCORTICAL CULTURES

Alzheimer's disease (AD) is a neurological disorder defined by the presence of plaques comprised mostly of amyloid- β ($A\beta$), and neurofibrillary tangles consisting of hyperphosphorylated microtubule associated protein tau (MAPT). AD is also characterized by widespread synapse loss and degeneration followed by death of neurons in the brain. Inflammatory processes, such as glial activation, are also implicated. In order to study mechanisms of neurodegeneration and evaluate potential therapeutic agents that could slow or reverse this process, a tissue culture system was developed based on primary embryonic cerebrocortical neurons. This culture system was observed to exhibit time-dependent neurodegeneration, glial proliferation, and synaptic marker loss consistent with AD-affected brains.

The regulatory promoter regions of several genes implicated in AD, including the $A\beta$ precursor protein (*APP*), β -amyloid cleaving enzyme (*BACE1*), and *MAPT*, were studied in this culture model. The *MAPT* gene promoter activity followed the pattern of neuronal maturation and degeneration quite closely, increasing in the initial phase of the tissue culture, then reducing markedly during neurodegeneration while *APP* and *BACE1* gene promoters remained active. Deletion series of these promoters were tested to give an initial indication of the active regions of the gene promoter regions. Furthermore, the effects of exogenous $A\beta$ and overexpression of p25, which are two

possible pathogenic mechanisms of gene regulation in AD, were studied. Response to A β varied between the promoters and by length of the A β fragment used.

Overexpression of p25 increased *MAPT*, but not *APP* or *BACE1*, promoter activity.

This neurodegeneration model was also used to study the putative neuroprotective action of the NMDA receptor antagonist memantine. Treatment with memantine prevented loss of synaptic markers and preserved neuronal morphology, while having no apparent effect on glial activation. The protective action on synaptic markers was also observed with two other structurally distinct NMDA receptor antagonists, suggesting that the effects of memantine are produced by its action on the NMDA receptor. It is concluded that this tissue culture model will be useful for the study of gene regulation and therapeutic agents for neurodegeneration, and that the efficacy of memantine may result from preservation of synaptic connections in the brain.

Debomoy K. Lahiri, Ph.D., Chair

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Abbreviations

A β	Amyloid- β
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
APH-1	Anterior pharynx defective-1
APLP2	APP-like protein 2
APP	Amyloid precursor protein
<i>APP</i>	Amyloid precursor protein gene
ATP	Adenosine triphosphate
BACE1	β -amyloid cleaving enzyme 1
<i>BACE1</i>	β -amyloid cleaving enzyme 1 gene
BSA	Bovine serum albumin
C1q	Complement component 1q
CPP	(\pm)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
Cy-3	Cyanine-3
CTG	Cell Titer-Glo
DPBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
ECL	Enhanced chemiluminescence
FITC	Fluorescein isothiocyanate
HACU	High-affinity choline uptake
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidase
LDH	Lactate dehydrogenase
MAPT	Microtubule associated protein tau
<i>MAPT</i>	Microtubule associated protein tau gene
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NMDA	N-methyl-D-aspartate
NR1/2/3	NMDA receptor subunit 1/2/3
NSE	Neuron specific enolase
PAGE	Polyacrylamide gel electrophoresis
<i>PSEN1</i>	Presenilin 1 gene
PEN-2	Presenilin enhancer-2
SDS	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP-25	Synaptosomal associated protein of 25kDa
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly, which affects approximately 5.4 million Americans, a number that is expected to grow to over 13 million by 2050 (Thies and Bleiler, 2011). AD is defined by neurofibrillary tangles of hyperphosphorylated microtubule associated tau (MAPT) protein and senile plaques consisting principally of the 38 to 43 amino acid amyloid- β ($A\beta$) fragment of the amyloid- β precursor protein (APP). These lesions progress in a predictable manner starting in the hippocampus and progressing through other regions of the cortex over time (Braak and Braak, 1997b). Also apparent in post-mortem brain tissues is a loss of synaptic density in the cortex. Although not part of the definitive criteria for AD, this change in synaptic density has been found to correlate with cognitive decline more strongly than many other pathological features including plaque or tangle density (DeKosky and Scheff, 1990). While there are many possible causes of synapse loss, there is strong evidence to support the role of disrupted APP metabolism, NMDA receptor mediated excitotoxicity, oxidative stress, and inflammation, much of which may be linked to glial activation (Tilleux and Hermans, 2007). This project is aimed to address the hypothesis that several important aspects of AD can be modeled and studied *in vitro* in terms of neurodegeneration, gene regulation, and pharmacological effects.

Alzheimer's disease overview

The first published observations of AD were first made by Alois Alzheimer in 1906 after a *post-mortem* examination of a 51-year-old woman that presented with delusions, possible hallucinations, agitation, and severe memory loss (Alzheimer et al., 1995). The lesions now known as neurofibrillary tangles and amyloid plaque were observed, as was gross atrophy of the brain. Clinically, AD remains a diagnosis of exclusion and definitive confirmation of AD can only be made at autopsy based on the presence of amyloid plaque and neurofibrillary tangles (Braak and Braak, 1997a), although much progress has been made in the search for diagnostic biomarkers (Barber, 2010). AD is principally identified in the clinic as a disturbance of episodic memory and executive function in the absence in the absence of other possible causes (Lahiri et al., 2004). A small proportion of cases are inherited as an autosomal dominant trait, as a result of mutations in the *APP*, presenilin 1 (*PSEN1*), or presenilin 2 (*PSEN2*) genes, however the preponderance of cases are idiopathic (Thies and Bleiler, 2011). AD, and perhaps other neurodegenerative disorders, may be the result of complex gene-environment interactions, in which a non-pathological genotype or set of genotypes can become dysregulated in such a way as to initiate a pathological condition through epigenetic mechanisms. This has been proposed as the “Latent Early-life Associated Regulation” (LEARn) model (Lahiri et al., 2009b). Outside of the heritable forms, no clear cause of AD has been determined, though the pathology in the brain is distinct from other neurodegenerative disorders.

Atrophy of the cerebral cortex is apparent, with cortical volume reducing in an uneven and accelerating pattern. This starts in the temporal cortex at the early stages of mild cognitive impairment, and spreads to the frontal and parietal cortices during the course of AD, while other areas such as the occipital cortex are relatively spared (McDonald et al., 2009). Microscopically, widespread neuron loss has been observed in *post-mortem* brain tissue, but most profoundly affected are cholinergic neurons terminating in the hippocampus (Davies and Maloney, 1976). It has been determined that the amyloid deposits first observed by Alzheimer are composed principally of the short 39 to 43 amino acid β -amyloid ($A\beta$) peptide (Masters et al., 1985). In its native conformation, $A\beta$ is primarily α -helical in structure, however in these plaques, there is a conformational shift to a self-aggregating, primarily β -sheet structure. This conformational transition is thought to be central to plaque development. $A\beta$ is increased in the brain in AD for reasons not currently understood, but possible causes include overproduction, impaired proteolytic (e.g., proteosomal) degradation, and reduced outflow from the brain to the periphery.

Memory consolidation is thought to be highly dependent on cholinergic activity in the hippocampus, however there is some controversy in the recent literature concerning exactly what aspects of memory require cholinergic input, as the type of lesion (chemical or mechanical) to basal forebrain cholinergic system appears to affect memory deficits in animal models (Niewiadomska et al., 2009). Controversies aside, it logically follows that the principle treatment for AD is cholinesterase inhibition, which acts to prolong the action of acetylcholine in the synapse. This strategy has produced

generally only palliative results, with some cognitive improvement but similar rates of decline in patients treated with a cholinesterase inhibitor and those receiving placebo (Rogers et al., 1998).

NMDA receptor and the synapse in Alzheimer's disease

The n-methyl-d-aspartate (NMDA) receptor is important to a wide variety of central nervous system functions, both normal and pathological. Normal functions of the NMDAr include roles in long-term potentiation and depression in the hippocampus, processes which are vital to memory consolidation. Much attention has also been given to the NMDAr with respect to pathological conditions, including implications as broad as ischemic and traumatic brain injury (Han et al., 2009), neurodegenerative disease (Hardingham and Bading, 2010) and addiction (Ma et al., 2009). Given its involvement in such a broad range of physiological and pathogenic processes, a detailed understanding of this receptor is highly important.

Inhibition of the NMDA receptor has been shown to protect cultured neuronal cells from a variety of insults, including low potassium and staurosporine (Jantas and Lason, 2009), in addition to hypoxia, MPP+, and NO mediated damage (Volbracht et al., 2006). However, these studies typically focus on rescue or protection of the whole neuron, which is a logical and convenient experimental endpoint. However, there is evidence to support the assertion that survival of the whole cell is an oversimplification of the pathogenic state modeled *in vitro*. Protection of the synapse is of great importance, though this has been given relatively little attention. The prevailing

assumption appears to have been that if a neuron survives at all, it will survive intact and generally function properly, but there is evidence to suggest that this may not be the case. To illustrate this point, in brains affected by Alzheimer's disease (AD), apparently viable neuronal cells have been observed to contain neurofibrillary tangles, a severe disruption of neuronal morphology and capacity for synaptic communication. Statistical models using known rates of neuronal loss and tangle formation predict that many of these neurons likely survive in this state for years and perhaps decades (Morsch et al., 1999). This observation has two important implications, first that life or death of whole neurons may be an inadequate experimental endpoint in some situations, and second that a pool of neurons may exist in the AD brain that are viable but dysfunctional and rescue of these neurons may represent a valid therapeutic goal.

It has been observed using an *in vivo* model of neurite outgrowth and synaptogenesis that activation of the NMDA receptor can drastically reduce axon length and branching. Conversely, it was shown in the same model that chronic administration of an NMDA receptor antagonist increases axon sprouting, branching, and synapse formation (Colonnese et al., 2005). Similarly, in a cortex-specific NR1 knockout mouse, thalamocortical axons were found to have similar numbers of branch points, but dramatically longer axonal arbor segment lengths than wild type mice (Lee et al., 2005). While this might suggest the NMDA receptor as a promising target for the treatment of AD or other disorders where neurite pruning is observed, the enhanced axonal sprouting in these thalamocortical projections also included greater numbers of axon terminals outside the normal target area (cortical "whisker barrels"). Thus, partial

inhibition of the NMDA receptor may provide greater clinical benefits through enhanced axonal branching and synaptic terminal formation while complete inhibition could lead to aberrant neuronal structure.

Synapse loss in AD

A strong correlation has been observed between synapse loss and severity of dementia in AD (DeKosky et al., 1996; Terry et al., 1991). Cholinergic terminals appear to be especially vulnerable to the various toxic events that have been observed in the AD brain as compared to glutamatergic and monoaminergic terminals in *post mortem* brain tissue (Davies and Maloney, 1976). It was noted in these examinations that the extent of cholinergic marker loss appeared to correlate strongly with neurofibrillary tangle density. Furthermore, it has been observed that in amyloid plaques, synapses are completely lost (DeKosky et al., 1996). Several lines of evidence have converged on the concept that in AD, synapse loss is a very early event, which may occur before widespread amyloidosis or tauopathy. It has been hypothesized that synapse loss may explain the earliest symptoms of AD, such as subtle personality changes, aphasia, and difficulty with simple arithmetic (Terry, 2000). Early observations comparing middle aged to elderly brains, and elderly with senile dementia indicate that in normal aging dendritic trees increase in length and branching, while this change is absent in cases of dementia (Buell and Coleman, 1979). More recently, it has also been observed that in the earliest stages of mild cognitive impairment or mild AD, a reduction in synapse number is apparent but independent of amyloid plaque or neurofibrillary tangle

pathologies (Scheff et al., 2007). And finally, in transgenic mouse models, it has been demonstrated that there is a significant reduction in dendritic spine density very early, before the appearance of AD associated pathological changes (Lanz et al., 2003), suggesting that structural changes to neurons may be underway long before other pathological indicators or even overt cognitive deficits.

Glial cells in Alzheimer's disease

Glia were long thought to play only accessory roles in the brain, primarily providing nutritional and structural support for neurons, and their role in neuronal signaling was thought to be limited to inactivation of neurotransmitters released by neurons and providing the myelin coat which increases conduction speed along axons. However, glial cells are now known to be involved in a number of other important processes and thought to participate in neuron signaling and modulate neuron structure. For example, while the central nervous system is relatively immune-privileged, glial cells mediate the innate immune function in the brain. Glial cells are known to produce components of the complement system as well as express complement receptors, and may recruit peripheral immune cells in the event of infection. Complement component 1q (C1q) has been observed in amyloid plaque and is increased in brain areas with heavy plaque burden (Matsuoka et al., 2001), suggesting a mechanism for the recruitment of activated glia to plaque-rich areas. There is some evidence that such glial recruitment may, under normal circumstances act to clear amyloid plaque (Wyss-Coray et al., 2003).

Conversely, activation of glial cells also results in increased inflammatory signaling (Salminen et al., 2009). Increased inflammatory signaling has been observed in the AD brain and this is thought to be a contributor to synapse and neuron loss (Haus-Wegrzyniak et al., 2002). In addition to increased inflammatory signaling, activated glia are known to exhibit reverse glutamate transport. Contrary to their usual role in terminating glutamatergic transmission through uptake of synaptically released glutamate, activated glia may release glutamate through a non-vesicular mechanism involving reverse flow through the glutamate transporter (Gouix et al., 2009). Given the widespread activation of glia in the AD brain, this relatively recent discovery suggests a potentially important mechanism through which memantine could produce its clinical efficacy, and may ultimately suggest new drug targets upstream of the glutamate receptor. If glial over-activation can be reduced, then perhaps many aspects of AD pathology could be addressed simultaneously.

The NMDA receptor antagonist memantine in the treatment of Alzheimer's disease

Memantine is an uncompetitive NMDA receptor antagonist with a lower affinity and a faster off-rate compared to other ion channel binding drugs that have failed in the clinic (Rammes et al., 2008). The designation 'uncompetitive' is often used to emphasize the distinction between the mode of action of memantine as compared to typical noncompetitive antagonists. Like a noncompetitive antagonist, memantine binds at a site other than the binding site of the endogenous ligand, but differs in that it requires receptor activation to reach its binding site. Additionally, memantine binding is voltage-

dependent, such that it is released from its binding site with sufficient membrane depolarization (Parsons et al., 1995). This allows memantine to counteract NMDA receptor hyperactivation while avoiding adverse side-effects exhibited by some mechanistically similar but more potent drugs. Memantine has been observed to produce a paradoxical dose-response curve with respect to NMDA receptor blockade in which receptors were blocked more efficiently in the presence of higher concentrations of the activating ligand NMDA (Chen et al., 1992), indicating that receptor activation is required for pharmacological activity. Since its channel blocking action is dependent on activation of the receptor, it cannot completely block NMDA receptor activity. Furthermore, memantine has been shown to preferentially inhibit extrasynaptic NMDA receptors (Leveille et al., 2008). Blocking extrasynaptic NMDA receptors while leaving synaptic NMDA receptor mediated transmission unaffected is likely key to the effectiveness of this drug. For example, it has been determined that while extrasynaptic NMDA receptors can mediate excitotoxicity (Leveille et al., 2008), synaptic NMDA receptor activity can enhance intrinsic antioxidant activity by activation of the thioredoxin system (Papadia et al., 2008).

Memantine is typically administered with a dosing regimen of 20mg/day, which produces a steady state plasma level of approximately 1 μ M. This plasma concentration is near the K_i of memantine at the NMDA receptor, however the brain concentration is predicted to be slightly lower, around 0.8 μ M, based on *in vivo* microdialysis studies (Hesselink et al., 1999). Memantine has demonstrated clinical efficacy in several clinical trials (Parsons et al., 2007) and has a unique mechanism of action among the five drugs

currently approved by the United States Food and Drug Administration for the treatment of AD in that all others approved for this purpose belong to the cholinesterase inhibitor class of drugs. Memantine is known to bind to several other receptor types present in the brain, including the 5HT-3 serotonin receptor, the $\alpha 7$ nicotinic receptor, and the $\alpha 4/\beta 2$ nicotinic receptors, however a strong argument can be made that the NMDA receptor is in fact the therapeutic target (Rammes et al., 2008).

Cholinesterase inhibitors in the treatment of Alzheimer's disease

Cholinesterase inhibiting drugs were the earliest therapeutic agents used for the treatment of AD, and four of the five drugs currently approved by the United States Food and Drug Administration belong to this class. Use of cholinesterase inhibiting drugs is founded in the cholinergic hypothesis of AD, which proposes that the proximal cause of the cognitive and memory deficits caused by AD is the selective vulnerability of cholinergic terminals (Davies and Maloney, 1976). Though they are the most widely used class of drugs to date, cholinesterase inhibitors have provided relatively modest improvement. Donepezil, for example, provided up to 3-point improvements on the 70-point ADAS-cog scale relative to placebo, though decline rates were not affected and the benefits were fully reversible after washout (Rogers et al., 1998). Some evidence does exist that suggest that cholinesterase inhibition can slow disease progression in some types of dementia, such as dementia with Lewy bodies (Touchon et al., 2006), though this represents a small proportion of all dementia cases.

With respect to disease modification, it has been shown *in vitro* that cholinesterase inhibitors can modify APP processing and A β production in cultured neuron-like cells (Lahiri et al., 1997; Lahiri et al., 1998). It is interesting that under the conditions used in these experiments, these tissue culture models are not known to produce acetylcholine. The reduction in A β secretion observed as the result of cholinesterase inhibitor treatment is interesting for several reasons. First, this effect is consistent with the therapeutic goal of reducing amyloid, which is thought to be directly involved in the cognitive symptoms of AD. The reduction of A β in cultured cells suggests that this model is adequate for the evaluation of potential treatments for AD. Second, the reduction of A β by cholinesterase inhibiting drugs in the apparent absence of acetylcholine suggests that the clinical efficacy of these drugs may not be simply the result of inhibition of cholinesterase enzymes. Rather, these observations present the possibility that the clinical efficacy of these drugs is the result of effects aside from, or in addition to, cholinesterase inhibition. Further investigation of these effects in this kind of system where the presumed primary effect of cholinesterase inhibition can be ruled out could enhance our knowledge of the mechanisms of these drugs and inform future drug development efforts a great deal.

Treatment modalities under development

Of all treatments for AD developed to date, none have been proven to satisfactorily alter the course of AD. Thus, a proliferation of alternative strategies has come under investigation in recent years. The sources of these potential treatments

range from ancient herbal remedies to the most recent developments in molecular biology. Treatments under investigation include compounds such as curcumin, which has long been used in traditional asian medicine. At the other end of the spectrum, strategies are under exploration to target the much more recently discovered micro RNA (miRNA) system in order to modulate AD-related gene expression via modification of mRNA stability. Both of these examples have shown promise in pre-clinical settings but have suffered problems with delivery. Curcumin was shown to inhibit plaque formation *in vitro* (Ono et al., 2004) and in animal models (Yang et al., 2005), although prohibitively high doses (1 to 8 g/day) were required to reach serum concentrations that were effective in inhibiting plaque formation in model systems (Cheng et al., 2001). Some similarities are to be found with miRNA-based strategies. For example, the miRNA designated miR-101 was shown to reduce APP levels in cultured cells (Long and Lahiri, 2011), but there is not currently a delivery system available that could target an miRNA molecule to specific cell populations *in vivo*. Some of these difficulties may be overcome by technological advances in drug delivery, such as packaging curcumin in soluble liposomes or nanoparticles to increase bioavailability, which has shown promising initial results in animals (Ray et al., 2011a). Similarly, recent technological innovations have given some hope that the problems with delivery of miRNA, particularly targeting specific tissues, may be overcome. Some specific tissues have been targeted by adding chemical modifications to miRNA molecules, such as conjugation to lipid moiety to target the liver or adipose tissue (Czech et al., 2011), but no such modification has been discovered that will target delivery of miRNA oligonucleotides to the brain. While these

potential treatments differ greatly in their mode of action, they both show much promise for disease modification in AD, but suffer from drug delivery problems that may be overcome with recent technological advances in this area.

Given the generally unsatisfactory clinical results of existing therapeutics, there are continued efforts to identify new drug targets. While the existing cholinesterase inhibitors have provided only limited clinical improvements, there is adequate justification to continue development of new drugs of this class. For example, there are ongoing efforts to develop inhibitors selective for the butyrylcholinesterase enzyme, which is increased in AD (Greig et al., 2002). What is also interesting with respect to cholinesterase-based drug development is posiphen, the cholinergically inactive stereoisomer of the AChE inhibitor phenserine. While the cholinergically active phenserine produced some decreases in APP and A β production in animal models, dosing was limited by cholinergic side-effects. In contrast, posiphen could be administered at much higher doses without such side-effects, and produced greater reductions in APP and A β levels (Lahiri et al., 2007). This suggests that the effects on APP processing observed with this class of drugs may be due, at least in part, to off-target effects, and warrants further exploration of this class of drugs both in terms of development of new cholinesterase inhibitors and further examination of the potential non-cholinergic effects of existing drugs. It has been previously observed that a subset of cholinesterase inhibitors increase, decrease, or do not affect APP processing in a manner that is apparently independent of their primary mode of action (Lahiri et al., 1997). This work is ongoing, including investigations of more recent ChEIs such as

rivastigmine, which may also alter APP processing by non-cholinergic mechanisms (Bailey et al., in press).

Still other potential drug targets and compounds are being pursued that represent diverse potential treatment mechanisms (Lahiri et al., 2003). Some are aimed at treating the downstream products of AD, such as the antioxidant s-allyl cysteine (SAC) was shown to counteract and protect neuronal cells from oxidative damage, which is widespread in AD. In transgenic model animals, the addition of SAC to the diet increased synaptic markers that are reduced in AD, suggesting that dietary antioxidants can alter neuron viability and synaptic structure *in vivo* (Ray et al., 2011b). Similar strategies of feeding model animals antioxidant-rich foods have also been attempted with some success, but effects on AD pathology vary. For example, transgenic model animals fed antioxidant-rich pomegranate juice showed better performance in water maze learning and reduced A β ₄₂ in the hippocampus (Hartman et al., 2006). In contrast, the addition of blueberries, which are also antioxidant-rich, to the diet of transgenic model animals improved memory performance but did so without altering A β levels (Joseph et al., 2003). Our current understanding of how antioxidant-rich diets may work is very limited, thus the differences in response, e.g., to pomegranate and blueberry enriched diets, cannot be fully explained, the ultimate results of improved memory, or preserved synaptic markers, in model animals seems promising.

In the decade between 1993 and 2003, five drugs were brought to market for the treatment of AD. These include tacrine (1993), donepezil (1997), galantamine (2000), rivastigmine (2001), and memantine (2003). During this time, on average, one

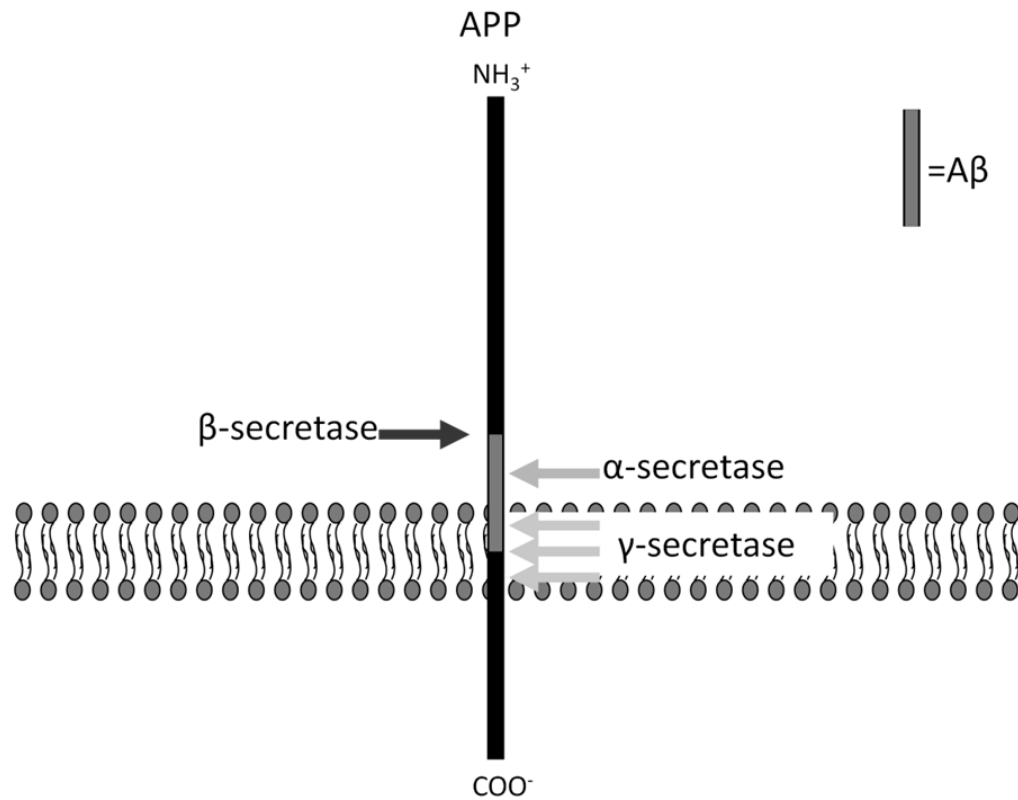
new drug successfully completed clinical trials and received FDA approval for use in the treatment of AD every two years, indicating steady progress in drug development. Unfortunately, while many promising compounds with varied mechanisms of action have reached late-stage clinical trials in the last eight years, none of these have received approval for widespread clinical use. This has led some to suspect that the clinical trials are themselves flawed, producing negative results for effective compounds (Schneider and Lahiri, 2009). This may be due to a number of design flaws including poorly optimized dosing, group assignment problems, and error in patient assessments, among others (Becker and Greig, 2008). These issues notwithstanding, there is still a need to identify new drug targets in the preclinical setting, which may be accomplished through the development of new model systems, or through continued evaluation of established therapeutic agents.

AD is a disease that involves accumulation of excess proteins in the brain, particularly A β and tau protein. Whether this is the result of overproduction or impaired clearance is still under debate, but regardless of the mechanism, it has been suggested that treatments aimed at reducing the amount of the protein produced may alter the course of the disease and produce better clinical outcomes. A polymorphism in the *APP* gene promoter region that alters transcriptional activity is associated with AD risk (Lahiri et al., 2005b), suggesting that development of AD can be altered by reducing transcriptional activity of the *APP* gene.

APP pathways

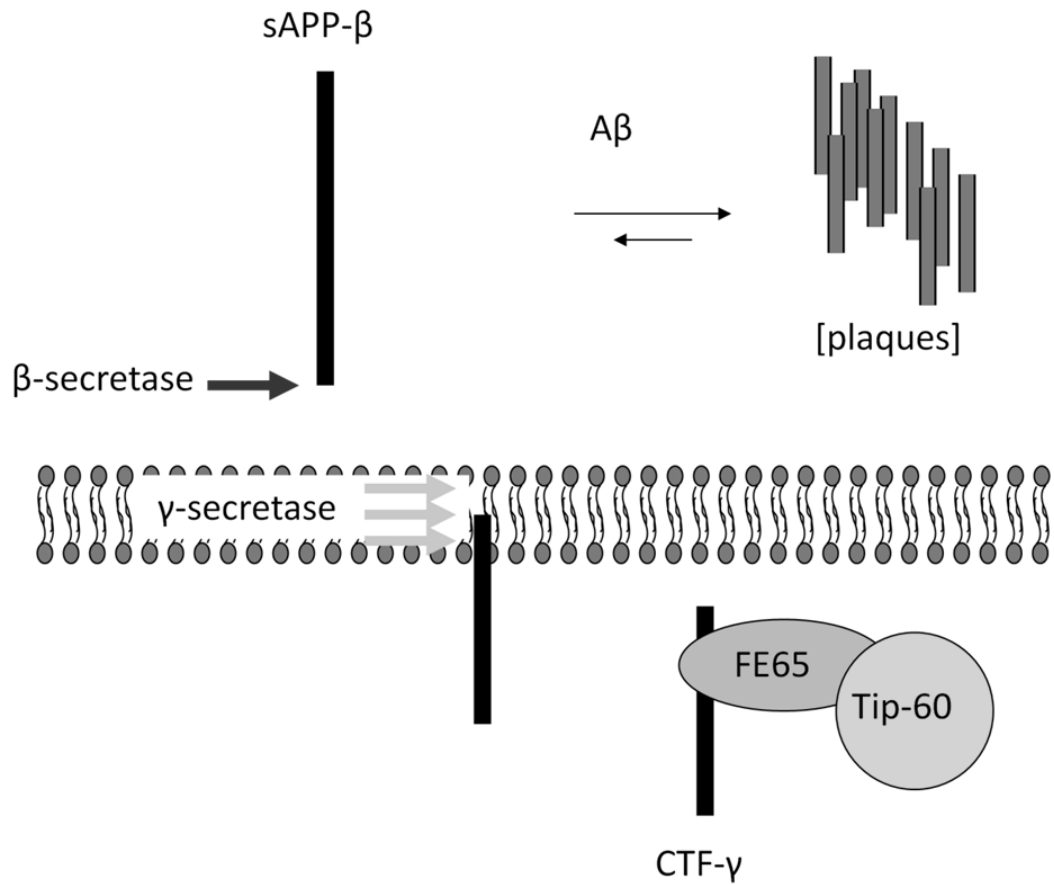
The amyloid precursor protein (APP) is a type I transmembrane protein (figure 1), the function of which is not completely understood. Initially, the 40 amino acid A β peptide was isolated and identified as the primary constituent of amyloid plaque (Glennner and Wong, 1984), which was soon followed by identification of a partial mRNA that contained a sequence predicted to encode 28 amino acids of A β as part of a larger protein. Very quickly, a full-length mRNA was sequenced and predicted to encode the 695 amino acid precursor or A β (Kang et al., 1987), which was subsequently determined to be identical to the previously identified protease nexin-II (Van Nostrand et al., 1989). While the majority of APP in neural tissues is 695 amino acids in length (APP₆₉₅), several other alternately spliced transcripts have been identified, primarily derived from alternate splicing of exons 7 and 8, producing APP of either 751 or 770 amino acids in length (Sandbrink et al., 1994). Exons 7 and 8 encode domains with homologies to the Kunitz type protease inhibitor and oxytocin, respectively. APP₇₅₁ is missing exon 8, while APP₆₉₅ is missing both exons 7 and 8.

Figure 1: The amyloid precursor protein



The amyloid precursor protein (APP) is a type I transmembrane protein, typically of 695, 751 or 770 amino acids in length. These several forms of APP result from alternate splicing of a Kunitz-type protease inhibitor domain and an oxytocin-2 homology domain in the extracellular N-terminal domain. In addition to these two, APP contains several other potential functional domains and post-translational modification sites for glycosylation and phosphorylation, however its function is still incompletely understood. The most well-classified post-translational modification is proteolytic cleavage by a group of enzymes termed the “secretases”. Three sites in or around the partially membrane-embedded amyloid-β domain of APP are recognition sites for α-secretase, β-secretase, or γ-secretase.

Figure 2: Secretase processing of the amyloid precursor protein



Typically, APP is proteolytically by either a combination of for α -secretase and γ -secretase, or β -secretase and γ -secretase. These are termed the “ α pathway” or “ β pathway” as cleavage by either of these two enzymes seems to preclude cleavage by the other, and γ -secretase is common to both pathways. The α pathway (not shown) cleaves within the $A\beta$ domain, producing sAPP α and the small p3 fragment is produced by subsequent γ -cleavage. The p3 fragment is readily degraded, and sAPP α is either innocuous or may have neurotrophic effects. The β pathway, as shown here, cleaves APP at the β secretase site, releasing sAPP β , and subsequent cleavage by γ -secretase releases $A\beta$. While the effects of sAPP β are less well understood, $A\beta$ has been well established as a highly aggregation-prone, toxic peptide that is damaging to neurons through multiple mechanisms.

APP is proteolytically cleaved at three sites designated the α -secretase, β -secretase, and γ -secretase sites (figure 2). Specific enzymes responsible for these cleavages have been identified for the β -secretase and γ -secretase sites, however some uncertainty remains regarding which specific enzyme is responsible for cleavage of APP at the α -secretase site. The beta amyloid cleaving enzyme (BACE1) cleaves the β -secretase site and a large complex of proteins produce the γ -secretase activity. This complex includes presenilin-1, presenilin-2, anterior pharinx defective-1 (APH-1), and presenilin enhancer-2 (PEN-2), and possibly several other proteins (Marlow et al., 2003). The α -secretase activity is probably carried out by one of the a disintegrin and metalloproteinase (ADAM) family members, most likely ADAM10 or ADAM17, which both can cleave APP at the α -secretase site, though subcellular localization data favor ADAM10 for this role *in vivo*. The products of α -secretase cleavage include sAPP α , which may have neuroprotective properties (Thornton et al., 2006), the secreted p3 peptide, which is quickly degraded, and the intracellular CTF- γ , which participates in nuclear gene regulation through its interactions with a multimeric complex that includes the histone acetyltransferase TIP60 (Cao and Sudhof, 2001). The β -secretase pathway produces the same CTF- γ , sAPP β , and the A β peptide, which is transported to the nucleus by the A β death inducing protein (A β DIP) (Lakshmana et al., 2005), a process which may be stimulated by oxidative stress (Bailey et al., Gene, 2011, in press).

The A β peptide is thought to regulate a variety of other cellular processes through multiple pathways. One important example is A β -mediated activation of cyclin dependent kinase 5 (CDK5), and its downstream targets. CDK5 is activated and localized

to the cell membrane by its p35 subunit under physiological conditions. Activation of CDK5 can result in reactivation of the cell cycle, leading to apoptosis in post-mitotic neurons (Lopes et al., 2010). A β is thought to over-activate CDK5 through its effect of increasing intracellular calcium concentration (Kawahara et al., 2011), which activates the protease calpain, which in turn cleaves p35 into p25. Once cleaved into p25, this regulatory subunit of CDK5 is released from the membrane and is more resistant to further proteolysis, thus CDK5 bound to p25 becomes persistently active and its activity is no longer localized to the cell membrane (Patrick et al., 1999). In addition to cell cycle reactivation, CDK5 activity results in cytoskeletal disruption, including phosphorylation of the tau protein (Monaco, III, 2004). It has been proposed that this A β -mediated p25 generation and subsequent CDK5 activation could be targeted in several ways in order to treat AD. For example, certain drugs that can prevent formation of the CDK5-p25 complex were shown to reduce neuron apoptosis (Camins et al., 2006). Targeting A β itself in order to prevent activation of this pathway has also been proposed.

Immunotherapies are under development that are aimed at removal of A β from the brain. This strategy could reduce apoptotic signaling of the CDK5-p25 complex, and represents a possible mechanism for the neuroprotective effects of immunotherapies seen in animal models (Biscaro et al., 2009).

Three major potential therapeutic targets have been identified in the amyloid pathway that could mitigate A β production and potentially modify the course of AD. First, it has been proposed that reduced production of APP could limit A β release. This approach may appear prone to detrimental side-effects since APP is potentially involved

in a wide range of functions, many of which are incompletely understood. However, *APP* knockout mice appear to be quite normal (Zheng et al., 1996) which suggests that this may indeed be a viable approach. This is perhaps due to the presence of a highly homologous protein, APP-like protein 2 (APLP2), which may be functionally redundant since the double APP and APLP2 knockout is embryonically lethal (von Koch et al., 1997).

A second approach to treating AD by targeting APP metabolism is inhibition of the BACE1 enzyme. This could be this could represent the most direct route for reducing A β production, as β -secretase cleavage of APP is the rate-limiting step in A β synthesis. A number of BACE1 inhibiting peptides and peptidomimetics have been developed that bind the active site and inhibit BACE1 *in vitro*, however the compounds developed to date have had very poor bioavailability. Progress has been made toward the development of smaller compounds that inhibit BACE1, and these have provided some hope that a BACE1 inhibitor that can cross the blood-brain barrier may be developed (Ghosh et al., 2008; Hills and Vacca, 2007), however no such compound has yet reached the stage of clinical trials.

A third possible approach would be modulation or inhibition of γ -secretase activity, which is the final step in A β production. In contrast to efforts directed at BACE1, a γ -secretase inhibitor was developed at Eli Lilly (LY450139) progressed to phase III clinical trials (Henley et al., 2009) before the trial was ended due to poor outcomes. Some have proposed that, since the γ -secretase complex cleaves multiple substrates, modulation of its activity rather than nonselective inhibition would be preferable (Sambamurti et al., 2011). If it is possible to interfere with γ -secretase cleavage of APP

specifically while leaving other substrate interactions intact, such a compound could be expected to produce better outcomes. Alternatively, it would be desirable to shift the variable cleavage activity of γ -secretase toward production of shorter, less aggregation prone forms of A β . Modulation of the γ -secretase complex by either of these mechanisms could result in a reduction in brain levels of A β without interruption of other signaling systems.

While for the most part, drugs that are specifically designed to target these aspects of the APP processing pathways are unavailable, it seems worthwhile to evaluate existing therapeutics that have demonstrated some capacity to modify A β production. These types of effects have been observed in tissue culture experiments examining the effects of various cholinesterase inhibitors (Lahiri et al., 2000) as well as memantine (Alley et al., 2010). By examining the effects of these compounds further, it is expected that indirect mechanisms for modulation of amyloid processing could be discovered and exploited for the treatment of AD.

Tau dysregulation in Alzheimer's disease

Concurrent with plaque deposition is neurofibrillary tangle formation. These tangles are the product of excess phosphorylation of the microtubule associated protein tau, and the result of this hyperphosphorylation is aggregation of tau which leads to dystrophic neurites and disrupted axoplasmic transport (Iqbal et al., 2009; LaPointe et al., 2009). While tau tangles may be a more proximal cause of neuron loss, some data indicate that tau hyperphosphorylation may be a downstream effect of excess A β

(Huang and Jiang, 2009), hence amyloid receives the majority of attention with respect to of AD pathogenesis.

The physiological function of tau protein involves microtubule assembly. This function is regulated by phosphorylation, such that unphosphorylated tau protein promotes microtubule assembly, while phosphorylated tau protein is released from the microtubule complex, destabilizing the microtubule (Lindwall and Cole, 1984).

Conversion to pathological activities occurs when excess phosphorylated tau is present in the cytoplasm and begins to self-aggregate into neurofibrillary tangles (Iqbal et al., 2005). Intracellular tau tangles are known to disrupt axoplasmic transport, and thereby synaptic function, adversely affecting neuronal function and viability. Thus, it appears that production of tau beyond what is necessary for microtubule stabilization leads to excess phosphorylated tau in the neuronal cytoplasm and subsequently, neurofibrillary tangle formation. Genetic polymorphisms that increase tau protein production are associated with increased risk of developing AD (Myers et al., 2005a; Sun and Jia, 2009b). In a transgenic animal model, reducing endogenous tau protein levels through genetic manipulations reduced the memory impairment caused by A β overproduction (Roberson et al., 2007). Thus, reducing the amount of tau protein produced in neurons is therefore a potentially effective therapeutic strategy for AD.

Pharmacological reduction of tau protein has been attempted, and several compounds were discovered that can reduce tau levels in cultured neuronal cell lines (Dickey et al., 2006). However, the compounds effective at reducing tau levels were chemotherapeutic agents, which have generally unfavorable side-effect profiles that make them unsuitable

for long-term treatment. These compounds did reduce transcription of the *MAPT* gene, suggesting that transcriptional regulation may be plausible. In order to achieve this goal of treating AD through reducing tau protein levels, a more complete understanding of *MAPT* gene regulation will be necessary, in order to identify targets and produce drugs with less severe side-effects. Therefore, the *MAPT* gene promoter region was examined using reporter gene assays, in order to increase our understanding of *MAPT* regulation.

Selection of experimental models in neurodegenerative disease research

In any biological research, selection of the proper model is of vital importance. In general, the available options include human patients, animal models, various tissue culture models, or purely *in vitro* preparations. Each model has unique advantages and limitations. Data derived from *in vivo* human subjects are often the most definitive, such as in studies of drug efficacy. The major limitations of this model are the restrictions in what are considered acceptable experimental manipulations (pharmacological, surgical, or otherwise) and in the available experimental endpoints available are also restrictive. While less invasive techniques, such as positron emission imaging, have expanded the types of observations possible in human subjects, endpoints of many studies are limited to accessible tissues such as blood and cerebrospinal fluid. Fine dissection of metabolic pathways or observations of effects of most manipulations at the cellular level is often not possible.

Animal models, mammalian models in particular, have the great advantage of possessing many important similarities to humans ranging from molecular to gross

anatomical characteristics. Any specific tissue of interest can be examined *post mortem*, and techniques are now available to monitor some parameters *in vivo*, such as neurotransmitter levels or electrophysiological events in the brain, providing much more information on the effects of any given manipulation. Behavior can also be measured and manipulated in order to provide data on memory, for example. Despite these advantages, animal models are also inadequate for some types of basic research, such as determination of the pharmacological properties of compounds. For example, it is difficult, or even impossible, to determine receptor binding in the brain in an intact animal given issues such as liver metabolism of many compounds, and distribution of a compound throughout the body and across the blood-brain barrier.

For use in studies related to specific neurodegenerative diseases, genetic modifications have been made to produce an array of transgenic and knockout mouse models that can mimic aspects of pathologies observed in human disease. With respect to models of Alzheimer's disease, dozens of transgenic mouse models have been developed, each of which exhibits one or several of the major features of AD. Most of these models have focused on the development of amyloid plaque by one of several mechanisms. The major categories of transgenic models that have been developed have utilized *APP*, presenilin (*PSEN1*), and *MAPT* transgenes. These models typically model one aspect, or a subset of features, of AD, but a complete animal model is still lacking. *APP* and presenilin transgenic animals typically produce large amounts of $A\beta_{1-42}$. Due to the use of human variants of these genes that are associated with familial AD. While these genetic manipulations do result in the formation of amyloid plaque, neurofibrillary

tangles, which are also a definitive characteristic of AD, are absent. Furthermore, there is wide variation between models in the ultimate result of genetic manipulations aimed at recapitulating A β plaque formation. For example, in two of the most well-characterized models, TG2576 and PDAPP, A β plaques form but there is no apparent neurodegeneration (Leskovjan et al., 2009; Stein and Johnson, 2002; Wati et al., 2009). Conversely, in an older model that overexpresses A β , neurodegeneration was reported (LaFerla et al., 1995). In an *APP* and *PSEN1* double transgenic mice, neurodegeneration was also observed, but only in forebrain monoaminergic neurons, which may have limited relevance in AD (Liu et al., 2008), highlighting the difficulty in producing disease-specific models. In a different model that includes transgenic human *APP* and mutated endogenous *PSEN1*, neuron loss was apparent in the CA1 and CA2 regions of the hippocampus (Casas et al., 2004). Neurodegeneration in these regions may be highly relevant to AD, however the combination of genotypes required to produce this effect in mice is unlikely to occur in humans, and thus the pathogenic mechanisms at work in this, as well as other transgenic models, may have important limitations in modeling human disease.

Tissue culture models are highly useful when observations on the effects of pharmacological or other manipulations on a single tissue or cell type are desired. Perhaps the greatest advantage is direct access to the cells for microscopic analysis of live cells or for investigations of pharmacological agents, which can be applied directly to the cells at defined concentrations via the culture medium. With specific relevance to AD, it is possible to evaluate secreted molecules with limited clearance relative to *in vivo*

tissues. For example, the A β peptide accumulates as the result of various factors, such as reduced vascular clearance or impaired proteolysis. In A β plaque producing animal models it can be difficult to differentiate between deposition that occurs before or during an experimental manipulation, or whether changes in A β are the result of altered production or clearance (Alley et al., 2010). Furthermore, acute effects on A β may be undetectable in the presence of the significant A β present early in the lifespan of these animals. In cultured cells, amyloid is not deposited in plaque, and amyloid levels are reset to nearly zero with every media change, allowing observations of acute effects on A β production.

An additional advantage to tissue culture models is in morphological measurements. In a tissue culture plate, cells are essentially compressed into two dimensions, allowing morphology of the entire cell to be readily observed in a single plane. Additionally, the direct access to cells afforded by culture models allows for genetic manipulations, such as siRNA, transfection and reporter gene assays to assess gene promoter activity, or overexpression techniques that are not readily applied in whole animals. Despite lacking the anatomical structure present in an intact animal, tissue cultures have been successfully used to evaluate the effects of pharmacological agents on growth factor production in glial cells (Caumont et al., 2006), apoptosis (Jantas and Lason, 2009), and APP processing (Lahiri, 1994), and many other phenomena. Given the history of fruitful research in these areas of neurobiology relevant to AD, it is expected that development and application of additional cell models will be fruitful in the study of AD therapeutics and development of new drug targets.

Research plan

In order to study the interaction of the NMDA receptor with APP metabolism and synaptic markers, a well-characterized tissue culture model is needed. Given the unknown variables intrinsic to animal models, including drug distribution and metabolism in whole animals, and the important differences between development of AD-related pathology and the induced pathologies in AD model animals, tissue culture was chosen as the model for this project. Cultured cells are amenable to drug treatments, DNA transfection experiments, and may provide data concerning neurodegeneration on a much more rapid timeframe than aging model animals. Tissue culture models are further removed from human disease relative to animal models, which may limit the conclusions that can be drawn from such models with respect to human disease. However, tissue culture models allow for observations to be made in a much simpler system that allows greater control of experimental conditions. Primary neuronal cultures were selected because these are a better approximation of the cell populations present *in vivo* than the available cell lines, and they are known to express many relevant proteins. Embryonic rat cortical cultures were used because they are obtainable in much greater quantities and much more easily dissociated relative to adult neurons, allowing greater opportunities for data collection per batch of cells. The tissue culture technique selected follows closely that of Brewer et al. (1993), using the serum-free Neurobasal medium with B27 serum replacement combination (Brewer et al., 1993), which further reduces variations in the environment of the cells that is brought on by lot-to-lot variation in the serum used in other culture media.

Prior to other experiments, it is necessary to characterize the time-dependent expression of the proteins of interest. With regard to synaptic proteins, it is expected that, while axon terminal proteins such as synaptophysin and SNAP-25 are present at this embryonic stage (Becher et al., 1999), the terminals will be lost during the process of tissue dissociation and will require some time for the terminals and these proteins to regenerate. Similar cultures from fetal mouse cortices have been demonstrated to require at least ten days *in vitro* to develop active NMDA receptors (Mizuta et al., 1998). Likewise, proliferation of glial cells was shown during the initial characterization of this tissue culture technique to be inhibited, however these data were collected only at day 4 *in vitro* (Brewer et al., 1993), and much anecdotal evidence suggests that at later time points, glial cells proliferate, becoming the majority cell population in long-term (e.g., 2-3 week) cultures. The hypothesis will be tested that this mixed cell type tissue culture model will show glial proliferation and activation, which will lead to synapse and neuron loss. Understanding any time-dependent shift in cell population in the tissue culture model is of high importance in planning experiments and interpretation of any results.

The specific proteins to be tracked in these cultures over time include neuronal and glial markers, neuron-specific enolase- γ (NSE), and glial acidic fibrillary protein (GFAP), respectively, the synaptic vesicle protein synaptophysin, the synaptic terminal membrane protein SNAP-25, and the NMDA receptor NR1 subunit. NSE and GFAP are constitutive “housekeeping” proteins in neurons and glia, respectively, which will allow an estimation of the relative amounts of protein of neuronal and glial origin in the lysates of these cultures. Synaptophysin and SNAP-25 are included because both

proteins have been found to be reduced in AD (Masliah et al., 2001; Shimohama et al., 1997). Reductions in SNAP-25 are somewhat less widely acknowledged, perhaps because changes in this protein are more subtle than synaptophysin (Shimohama et al., 1997). In primary tissue culture, it is assumed that expression of these proteins will vary over time, and in order to evaluate strategies aimed at synapse preservation, it is important to know when these proteins appear in these cultures, as well as when they peak and begin to decline. And finally, the NR1 subunit of the NMDA receptor will be monitored with time in order to provide a more detailed understanding of the timing of NMDAR expression. The active NMDAR is composed of a combination of NR1, NR2, and NR3 subunits. Several interchangeable NR2 subunits are transcribed from several discrete genes, while the NR1 subunit is transcribed from a single gene. While relative expression levels of the various NR2 and NR3 subunits may have important implications, the NR1 subunit was chosen in order to simplify data collection to a single protein required for receptor function.

Acetylcholinesterase inhibition remains the predominant treatment given to AD patients. Several of these drugs have been evaluated in primary cortical tissue cultures obtained from fetal rats, and these models have provided interesting insight into the activities of these drugs. For example, treatment of primary embryonic cortical tissue cultures with the acetylcholinesterase inhibitor donepezil was shown to protect against oxygen-glucose deprivation (Akasofu et al., 2003) and modulate NMDA receptor activity (Moriguchi et al., 2005). In the adult brain, the cerebral cortex contains cholinergic terminals, but no endogenous cholinergic cell bodies. Thus, dissociated cerebrocortical

neurons seem unlikely to differentiate into a cholinergic phenotype, though this has not been directly tested. The artificial environment of a tissue culture plate will inevitably omit factors that guide neuronal differentiation, or the artificial medium may contain components to which these neurons are not normally exposed. Given these unknowns and the observed effects of acetylcholinesterase inhibiting drugs, it is therefore worthwhile to investigate the presence of cholinergic markers in these cultures. To this end, high affinity choline uptake (HACU) and choline acetyltransferase (ChAT) activity will be measured over time in these cells. These markers are specific to cholinergic neurons, and the radioisotope-based assays used to detect them (Ray et al., 2009) are highly sensitive.

Aberrant protein expression has been implicated in AD. Specific proteins implicated in AD are encoded in the *BACE1*, *APP*, and *MAPT* genes. The upstream regulatory (“promoter”) region of most genes include binding sites for a wide variety of proteins involved in transcriptional activity, therefore numerous signaling pathways may be involved in the regulation of any given gene. Thus, the promoter regions of these genes may represent a wealth of unexplored potential drug targets for disease modifying interventions. These promoters have been inadequately studied for this purpose, therefore they will be further characterized in this neurodegeneration model. The hypotheses will be tested that activities of these gene promoters varies with time-dependent neurodegeneration, that various subregions of the promoters will display varied positive and negative influences on transcriptional activity of the promoter, and that suspected transcription modifying agents (p25 and A β proteins) will affect

promoter activity. This will be carried out by transfection of primary cultures with plasmids containing luciferase reporter genes under control of segments of the *BACE1*, *MAPT*, and *APP* genes under varied conditions (neurodegenerating or non-neurodegenerating) and treatments (exogenous A β or p25 overexpression) followed by the appropriate reporter gene assay.

After time-dependent expression of these proteins has been characterized, the tissue culture model will be applied to test the hypothesis that AD-related therapeutic agents act, at least in part, through preservation or enhancement of synaptic markers. Particular interest will be given to the NMDA receptor antagonist memantine, which is the most recently approved drug for treatment of AD and the first that is not a cholinesterase inhibitor. This drug is of particular interest with respect to slowing disease progression because it is thought to work by a neuroprotective mechanism. Theoretically, cholinesterase inhibition may compensate for the loss of cholinergic terminals seen in AD, but does not address the continued loss of cholinergic neurons. Thus, it would be expected that they will eventually fail to provide any clinical benefit in later stages of AD when the cholinergic system is almost completely degenerated. Neuroprotection by memantine may be expected to provide a disease-modifying effect by preserving the remaining cholinergic terminals. It is therefore expected that memantine will preserve synapses in tissue cultures that model some aspects of AD.

Materials and Resources

Tissue culture medium

Cells were maintained and treated in Neurobasal medium with serum-free B27 supplement (Brewer et al., 1993), 500 μ M glutamine, and 20ng/ml basic fibroblast growth factor (Invitrogen). Neurobasal medium contains all essential amino acids, glucose, vitamins and the necessary buffering agents to maintain physiological pH under a 5% CO₂ atmosphere. B27 contains several important serum components, including hormones, and several antioxidants, e.g., catalase, and a high concentration of bovine serum albumin (BSA), which is prominent on Western blots of media samples, and to a lesser extent, blots of the lysates.

Tissue culture plates

Cells were seeded into tissue culture treated polystyrene multiwell plates (Corning) coated with 70,000-150,000Da molecular weight poly-D-lysine (Sigma-Aldrich) at a density of about 2,200 cells/mm² in all formats used. This is equivalent to 2x10⁶ cells/well in the 6-well plates used, or 7.5x10⁵ cells/well in 12-well plates.

Drugs

The pharmacological manipulation used in most experiments to inhibit NMDA receptors was memantine (provided as a gift from Forest Laboratories). This compound was first synthesized at Eli Lilly as an intermediate in the production of compounds for evaluation as anti-diabetic drugs (GERZON et al., 1963). It was later developed by Merz

and approved in the United States for treatment of AD in 2003. Memantine is an uncompetitive NMDA receptor antagonist that binds within the calcium channel of the receptor (Parsons et al., 1999), which was initially established by its displacement of radiolabeled MK-801 in postmortem tissue (Kornhuber et al., 1989).

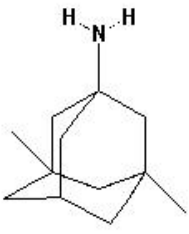
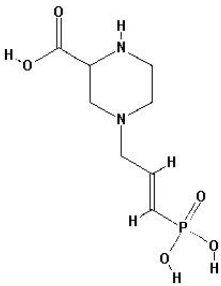
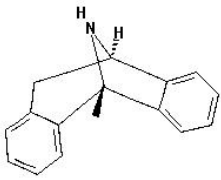
To confirm the specificity of the effects of memantine, two other NMDA receptor antagonists MK-801 and CPP (both from Sigma-Aldrich), were also tested in some experiments. MK-801 is an uncompetitive antagonist with a mechanism similar to that of memantine, i.e., a channel blocking drug, but with a higher affinity for the NMDA receptor (Huettner and Bean, 1988). CPP differs from memantine and MK-801 in its mechanism of action in that this drug inhibits binding glutamate to the receptor (Lehmann et al., 1987). Structures of the drugs used are shown in table 1.

Western immunoblot

Criterion XT Western blot system

The Criterion XT Western Blot System (Bio-Rad) is a precast SDS-PAGE system that offers several types of gels, of which the 12% polyacrylamide, 26-lane variety were used here. Many of the Western blot experiments described here produced a large number of samples that were best resolved on the same gel, e.g., time course experiments with $n \geq 4$ for each of 5 time points. In these cases, this was the method used unless loading volume (maximum 15 μ l per well) became limiting.

Table 1: AD and research compounds

<i>Compound name (trade or alternate name)</i>	<i>IUPAC name</i>	<i>Molecular weight (Da)</i>	<i>Structure</i>	<i>Mechanism</i>
Memantine hydrochloride (Namenda®)	3,5-dimethyladamantan-1-amine hydrochloride	215.14		Ion channel blocking uncompetitive NMDA receptor antagonist
CPP (Cypene)	4-[(E)-3-phosphonoprop-2-enyl]piperazine-2-carboxylic acid	250.19		Competitive antagonist at NMDA receptor glutamate binding site
MK-801 (Dizocilpine)	(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[<i>a,d</i>]cyclohepten-5,10-imine	221.30		Ion channel blocking uncompetitive NMDA receptor antagonist

Structure data adapted from the public PubChem database maintained by the National Center for Biotechnology Information (<http://pubchem.ncbi.nlm.nih.gov/>), accessed April 16, 2011.

Mini Protean II Western blot system

In experiments in which loading volume of the Criterion XT gels became limiting, specifically blots intended to measure secreted proteins, the Mini Protean II system (Bio-Rad) was used. Western blots produced by this system allow a maximum of 15 samples, but at volumes up to 35 μ l, allowing up to 28 μ l of sample with 7 μ l of 5x Laemmli sample buffer, as compared to 12 μ l of sample and 3 μ l of sample buffer with the Criterion XT system.

Antibodies

Primary antibodies and suppliers are listed by application in table 2. Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Pierce (Rockford, IL; anti-mouse and anti-goat) or from Santa Cruz (anti-rabbit). ELISA kits were obtained from Immuno-Biological Laboratories (Minneapolis, MN). The kits contained microwell plates pre-coated with capture antibodies, and the HRP labeled detection antibodies were provided in the same kit. For immunocytochemistry, secondary antibodies and avidin conjugates were all obtained from Jackson ImmunoResearch (West Grove, PA).

Table 2: Antibody source, dilutions, application

Western blot

<i>Target</i>	<i>Host</i>	<i>Epitope/antigen</i>	<i>Source</i>	<i>Dilution</i>	<i>Secondary</i>
APP	Mouse	N-terminus, amino acids 66- 81	Chemicon	1:3000	goat-anti- mouse-HRP (1:3000)
GFAP	Goat	Full-length protein	Sigma- Aldrich	1:2000	goat-anti- rabbit-HRP (1:3000)
NSE	Rabbit	Full-length protein	Abcam	1:500	goat-anti- rabbit-HRP (1:3000)
SNAP-25	Mouse	Full-length protein	Chemicon	1:2000	goat-anti- mouse-HRP (1:3000)
Synaptophysin	Goat	C-terminal 20 amino acids	Santa Cruz	1:500	rabbit-anti- goat-HRP (1:3000)

ELISA

<i>ELISA target</i>	<i>Capture antibody epitope</i>	<i>Labeled antibody epitope</i>	<i>Assay detection range</i>
A β ₁₋₄₀	A β ₃₅₋₄₀	A β ₁₁₋₂₈	4pM –231pM
A β ₁₋₄₂	A β ₁₁₋₂₈	A β ₁₁₋₂₈	3pM –178pM

Immunocytochemistry

<i>Epitope</i>	<i>Source</i>	<i>Dilution</i>	<i>Secondary</i>
GFAP	Sigma-Aldrich	1:100	rabbit-anti-goat- biotin (1:1500) + streptavidin-Cy3 (1:1500)
SNAP-25	Chemicon	1:100	Donkey-anti-mouse- biotin (1:300) + streptavidin-FITC (1:300)
Tau	rPeptide	1:2000	Donkey-anti-mouse- biotin (1:300) + streptavidin-FITC (1:300)

Plasmids

hAPP1.2

A 1250 bp fragment of the APP promoter region cloned previously (Lahiri and Robakis, 1991) between PstI and BamHI restriction endonuclease sites consisting of 1164 bp of the 5' upstream regulatory region and 86 bp of the 5' untranslated region of the human *APP* gene was cloned into the multiple cloning site of the promoterless pGL3-Basic plasmid (Promega). This plasmid contains the firefly luciferase reporter gene under control of the promoter sequence inserted at the multiple cloning site (figure 15).

hBACE1P2

A 3339 bp fragment of the BACE1 promoter region, described previously (Ge et al., 2004), between two BglII restriction endonuclease sites containing 3032 bp of the *BACE1* gene upstream regulatory region and 307 bp of the 5' untranslated region was cloned into the multiple cloning site of the promoterless pGL3-Basic plasmid. As above, this promoter sequence drives transcription of the firefly luciferase gene (figure 15).

Imaging instruments

For visualization of cells labeled in the immunocytochemistry experiments, a Leica DM2500 epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) mounted with a SPOT RT3 camera (Diagnostic Instruments, Sterling Heights, MI). All images were collected at 200x magnification.

Experimental Methods

Primary tissue culture

Animals

All procedures involving animals were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee and conform to NIH guidelines.

Timed pregnancy embryonic day 16 (E16) Sprague-Dawley rats were delivered to the facility by Harlan (Indianapolis, IN) the morning of the tissue harvest. The dam was typically about four months of age upon delivery. On average, 12 pups per dam were obtained, which produced approximately 90×10^6 cells at the time plates were seeded.

Dissection and plating

Primary embryonic rat cerebrocortical cultures were performed using slight modifications from previously published procedures (Brewer et al., 1993). The dam was sacrificed in a CO₂ chamber and decapitated. The uteri were removed through an incision in the abdomen and the pups transferred to ice-cold Hanks Buffered Salt Solution (Invitrogen, Carlsbad, CA) supplemented with 1mM pyruvate (Sigma-Aldrich, St. Louis, MO) and 1x antibiotic cocktail (HBSS; Invitrogen). After rinsing, the heads were moved to fresh HBSS for dissection. Under a dissecting microscope, the overlying tissues and vasculature were removed from the surface of the brain with fine forceps and whole cortices were separated from subcortical tissue and collected in a separate tube of HBSS on ice. After all cortices were collected, the overlying HBSS was removed and

the tissue was homogenized by trituration with a fire-polished Pasteur pipet in 2-3ml Neurobasal medium supplemented with B27 and 5ng/ml human recombinant basic fibroblast growth factor (Invitrogen). Cells were counted and seeded into poly-D-lysine (Sigma-Aldrich) coated 6-or 12- well plates for western blot experiments or 96-well plates for transfection experiments (Corning, Lowell, MA) at a density of 2,200 cells/mm² (equivalent to 2x10⁶ cells per well in 6-well plates).

Tissue culture maintenance

Cultures were maintained in a sterile tissue culture incubator under a 5% CO₂ atmosphere at 37°C. Medium was changed after 3 hours to remove cell debris and subsequently every 4 days. The typical feeding, treatment, and sample collection schemes are described in figure 3. At the conclusion of the experiments, media samples were collected and cells were rinsed once in Dulbecco's phosphate buffered saline (DPBS; Invitrogen) to remove media protein and then collected from the culture plates by scraping with a rubber-tipped spatula in DPBS. Cell pellets were collected by centrifugation and lysed by sonication in lysis buffer containing a protease inhibitor cocktail (Roche).

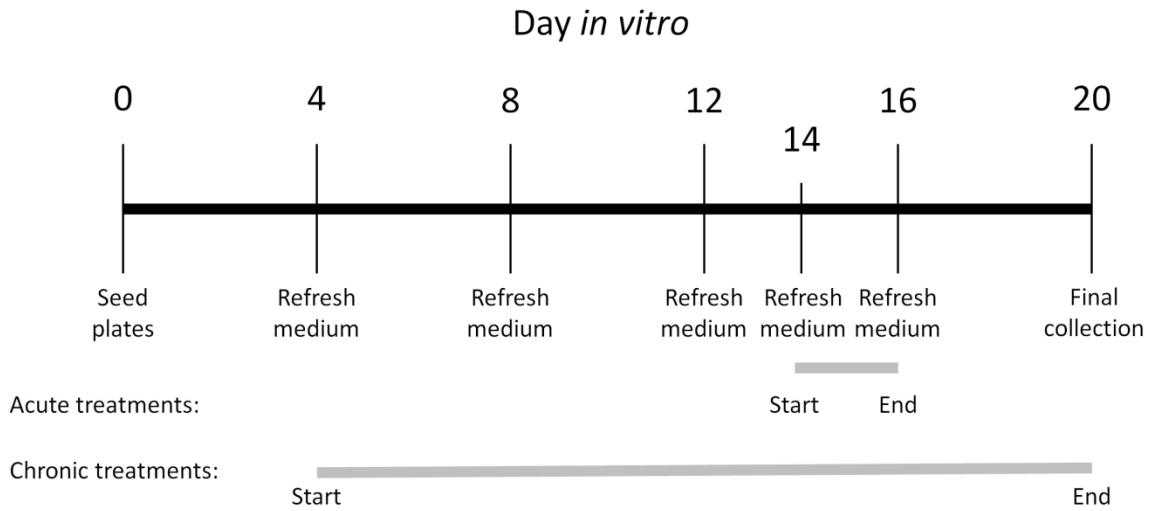
Viability and toxicity assays

Cell Titer-Glo

To compare cell viability, relative ATP concentration was measured using the luciferase based Cell Titer-Glo kit (CTG; Promega, Madison, WI). Luciferase-based ATP detection

was initially developed as a means to detect bacterial infection in blood and urine but later adapted for use in screening of potential chemotherapeutic agents in cultured cells (Kangas et al., 1984). Here, the assay is used to detect differences in cell density between wells. An alternate MTS-based assay was used for this purpose in initial cultures, however sensitivity of this colorimetric assay was insufficient in several cases. After cells were collected from the culture plates by scraping in DPBS, and prior to centrifugation and lysis, 30 μ l aliquots of these cell suspensions were transferred to an opaque white 96-well plate and 30 μ l of the CTG assay solution was added and the plate was placed on an orbital shaker for 10 minutes to induce lysis of the cells. In the time course experiments, samples from different collection times were quickly stored at -80°C until all samples had been collected, then all were thawed and assayed at the same time. The luminescent signal was quantified using a Glowmax luminometer (Promega).

Figure 3: Typical tissue culture timeline



This diagram represents the timing of various manipulations carried out over time in primary fetal neuronal cultures. Dissociated cortical cells were seeded into multi-well plates at day 0 and fed every fourth day until experiments were terminated by day 20. In the case of chronic treatments, drugs were refreshed each time the medium was refreshed. Acute treatments were initiated at day 14 and cells were removed from the plate and media samples collected at day 16. In the case of chronic treatments, memantine was added at day 4 to multiple multi-well tissue culture plates, and one plate was removed and samples collected every fourth day, while medium was refreshed in all remaining culture plates.

Lactate dehydrogenase assay

Cellular toxicity was assessed by quantification of lactate dehydrogenase (LDH) released by cells into the medium. The commercially available TOX-7 kit (Sigma-Aldrich) was used for this purpose as previously described (Alley et al., 2010). LDH is a cytosolic enzyme that is part of the glycolysis pathway that is released into the medium proportionately to the number damaged and dead cells in a culture plate (Legrand et al., 1992). LDH content of 30 μ l aliquots of the conditioned media samples were assayed per manufacturer's instructions in a microwell plate format, and OD values were measured using a GENios plate reader (Tecan, Männedorf, Sweden). To quantify the amount of LDH released by the cells, samples were compared to a standard curve of known LDH content (Roche Diagnostics, Indianapolis, IN).

Western blot

Bradford assay and sample preparation

To prepare Western blots of cell lysate samples, cells were lysed in IP buffer containing nonionic detergents and the Complete protease inhibitor cocktail (Roche, Indianapolis, IN) by sonication. Protein content of these samples was estimated using the Bradford technique (Bradford, 1976), wherein an aliquot of the protein sample was diluted in water and mixed with a preparation of Coomassie Brilliant Blue G-250 dye, the peak absorbance of which shifts from 465 nm to 595 nm when bound to protein (Bio-Rad). BSA was used to generate a standard curve to allow calculation of the protein content of each sample, and sample volumes were adjusted in each assay to fall within

the linear range of the standard curve. These data were used to prepare samples for Western blot with equal amounts of protein per lane. When possible, 20µg of protein was loaded per lane, however loading volumes of the gels were often limiting, in which case the maximum was loaded of the most dilute sample, and equivalent amounts of protein were loaded in all other wells. Samples were prepared in 1x Laemmli sample buffer (Laemmli, 1970) and heated at 95°C for 5 minutes in an iCycler thermal cycler (Bio-Rad).

Media samples were prepared with equal volume in each well. Protein adjustments were not made as most of the protein present in these samples is derived from the medium, with secreted proteins making a relatively small contribution to the total. Unless otherwise specified, the maximum volume of media was used (28µl for 15-lane gels, 12µl for 26-lane gels), and denatured by heating with 1x Laemmli sample buffer (Laemmli, 1970) using the same procedures as with the lysate samples.

Electrophoresis conditions

Samples were loaded onto SDS-PAGE gels (described above) and 180 V was applied for the time required for the bromophenol blue tracking dye to pass completely through the gel, usually between 55 and 65 minutes. The gels were then removed to a tray of transfer buffer containing 20% methanol and allowed to equilibrate for 10-15 minutes. Proteins were then transferred to 0.22µm poresize PVDF membrane (Bio-Rad). Protein transfer was checked for uniformity using 0.1% (w/v) Ponceau S stain (Salinovich and Montelaro, 1986) in 5% v/v acetic acid (Sigma-Aldrich). Stain not bound to protein

was washed off with 5% acetic acid and the blot was documented using a flat-bed scanner. Protein-bound stain was then removed by several washes of tris buffered saline (TBS).

Antibody incubation conditions

After the Ponceau S stain was washed off, primary antibodies were applied in a solution of 5% nonfat dry milk reconstituted in tris buffered saline (Amresco) with 0.05% Tween-20 (TBST; Sigma-Aldrich) at antibody-specific dilutions listed in table 2. Primary antibodies were applied over night at 4°C, and the blots were washed 4 times for 5 minutes each in TBST before application of secondary antibodies. The appropriate host-specific horseradish peroxidase secondary antibody was also prepared in 5% nonfat dry milk in TBST, generally at a 1:3000 dilution. The blots were incubated with the secondary antibody at room temperature for one hour then washed in several changes of TBST for at least 30 minutes.

Detection

Proteins were then detected using enhanced chemiluminescence (Schneppenheim and Rautenberg, 1987) (ECL; GE Healthcare). The detection reagents were applied to the blot for 60 seconds then the blot was placed in a transparent plastic sheet protector and exposed to ECL film (GE Healthcare) for the required amount of time specific to each antibody. Exposure times of between 15 and 60 seconds were

required for most proteins, depending on the relative abundance of the protein in the sample.

Immunocytochemistry

After treatment, cells were washed with DPBS and fixed in 4% paraformaldehyde in phosphate buffer for 10 minutes. Nonspecific binding was then blocked using 10% horse serum in DPBS for 30 minutes. Primary antibodies were applied at an antibody-specific dilution as indicated in table 2 in 1% horse serum for 1 hour and washed 3 times for 5 minutes each with DPBS. Host-appropriate secondary antibodies were applied in 1% horse serum for 1 hour and washed again. Secondary antibodies were conjugated to fluorescein isothiocyanate (FITC), cyanine-3 (Cy-3), or biotin. Biotinylated secondary antibodies were subsequently incubated with either Cy-3 or FITC conjugated streptavidin. Biotin and FITC conjugates were used at 1:300 dilutions, while Cy-3 secondary conjugates were used at a 1:1500 dilution. All secondary agents and streptavidin conjugates were from Jackson IR.

Cholinergic markers

High Affinity Choline Uptake

High affinity choline uptake (HACU) was determined by incubating cultured cells in Krebs's ringer solution containing 300nM radiolabeled [³H]-choline (Perkin-Elmer), or approximately 70,000 CPM. Choline uptake specific to the choline transporter is defined here as uptake that is inhibited by 145 μM hemicholinium-3 (Simon and Kuhar, 1975).

After incubating cells at 37°C for 15 minutes, the assay buffer was removed by aspiration and cells were rinsed twice with ice cold 0.9% saline and then lysed in Mammalian Protein Extraction Reagent (M-PER) buffer (Pierce, Rockford, IL). Relative levels of choline uptake were then determined by scintillation counting of the lysates.

Choline Acetyltransferase activity

Activity of the choline acetyltransferase enzyme (ChAT) was measured in the lysates of cell lysed in M-PER buffer using an established protocol (Ray et al., 2009). 4 μ l of lysate was incubated with 10 μ l of an assay buffer that consists of phosphate buffered saline (pH 7.4) plus 0.14 μ M EDTA and 0.11mM [14 C]-acetylcoenzyme A (approximately 20,000 CPM per reaction; Perkin-Elmer), and 0.086 μ M eserine sulfate to prevent breakdown of the [14 C]-acetylcholine product. The reaction is then stopped by the addition of 25 μ l 0.5 M HCl on ice. The [14 C]-acetylcholine product is then extracted from the reaction mix by the addition of 100 μ l of 3-heptanone with 30 mg/ml tetraphenyl boron. Samples are vortexed for 10 seconds, followed by centrifugation at 12,000 x g. 70 μ l of the organic phase containing the reaction product is then transferred to a scintillation vial with counting cocktail and read in a scintillation counter. ChAT enzyme activities in the samples are proportional to the scintillation counts.

Transfection and Reporter Assays

Transfection

Cultured cells grown in 96-well plates were transfected using the Transfectin cationic lipid-based transfection reagent (Bio-Rad) per the manufacturer's recommended protocol. Each well is transfected in a total of 50 μ l Neurobasal medium. A total of 300 ng of plasmid per well DNA is used, which includes 285 ng of the pGL3 (Promega) based experimental plasmid containing a firefly luciferase reporter gene under control of the promoter of interest, plus 15 ng of the control plasmid pRL-SV40 (Promega), which contains a *renilla* luciferase reporter gene under control of the constitutive SV40 promoter. DNA is added to a microcentrifuge tube and brought to 25 μ l per well with Neurobasal medium. In a separate tube, 0.75 μ l per well of the Transfectin reagent is brought up to 25 μ l per well with Neurobasal medium. The contents of the two tubes are then combined to allow DNA-lipid complexes to form at room temperature for 20 minutes. This mixture is then added at 50 μ l per well, and the plate is returned to the incubator for 48 hours before the reporter gene assay.

Luciferase reporter gene assays

Firefly and *renilla* luciferase reporter gene assays are performed 48 hours after transfection when reporter gene expression is at a maximum in most types of cells using the Dual Luciferase detection kit (Promega). First, the medium is removed and the cells are lysed in 30 μ l of the provided lysis buffer. Then, 30 μ l of the firefly luciferase detection reagent solution is added using the auto-injector system on a Glowmax

luminometer (Promega) and the luminescent signal is read for 10 seconds per well. After the firefly luciferase activity has been recorded in each well, the process is repeated, adding 30 μ l per well of a second reagent solution that contains the *renilla* luciferase reagent and inhibits the firefly luciferase reaction. The data are presented as the ratio of firefly/*renilla* luciferase activities, using the constitutive *renilla* expression to correct the data for inevitable well-to-well differences in transfection efficiency.

Section 1: Development of a primary neuronal tissue culture model

Introduction

A tissue culture based model of neurodegeneration would be of great value to neurodegenerative disease research, especially investigations of AD, which involves widespread loss of cortical and subcortical neurons. Although a number of animal models have been developed that mimic aspects of AD, there are several advantages to tissue culture models. Importantly, tissue culture allows direct access to neurons for drug exposures at defined concentrations, DNA transfection, and morphological observations. Current *in vivo* models of AD-related neurodegeneration are based mostly on the assumption that A β deposition is the primary pathogenic event in AD, while evidence exists for hypercholesterolemia (Ullrich et al., 2010), infectious disease (Miklossy, 2008), and toxin-mediated epigenetic changes (Wu et al., 2008), among others, are all possible upstream events leading to amyloidosis for which there already exist treatment and preventative options. Rather than focus on a single aspect of AD, tissue culture models allow investigations of a wide variety of neurodegenerative mechanisms with a much shorter time between initiation of an experiment and the final endpoints.

There is convincing evidence for the involvement of glial cells as mediators of neuron degeneration (Ryu and McLarnon, 2009; Salmina, 2009). It was observed in AD affected brains that oxidative stress mediated by glial activation occurs very early in the course of AD, perhaps even before plaque formation is apparent (Nunomura et al., 2001). Additionally, some inflammatory signals that are elevated in AD can increase

glutamate release from astrocytes, contributing to excitotoxicity and neuron damage (Domercq et al., 2006). Most primary tissue culture model development has focused on maintaining active, healthy neurons over long periods of time. This would certainly be beneficial, for example, to study neuron physiology under non-degenerating conditions (Brewer et al., 2008). This is often accomplished by restricting glial growth by the addition of mitotic inhibitors at an early phase of the tissue culture (Kolodny et al., 1985). What is more desirable for AD research is a mixed neuronal and glial culture system that exhibits predictable time-dependent neuron degeneration. In such a model, it may be possible to study mechanisms of neurodegeneration and effects of potential therapeutics aimed at preventing or slowing neurodegeneration.

Currently, no such model has been clearly established. Initial observations of embryonic cerebrocortical cultures maintained in the Neurobasal medium with B27 serum-free medium system suggested that this may provide such a model. Initial data reported on the development of embryonic cerebrocortical cultures are somewhat limited, and extend only to 4 days *in vitro* (Brewer, 1995). It was hypothesized that primary neurons grown under conditions of unrestricted glial growth will exhibit neurodegeneration by glial-mediated mechanisms which are relevant to AD. Primary E16 cerebrocortical cultures were therefore monitored over time for various parameters relevant to AD, including neuronal and synaptic markers, glial activation, and glutamate levels, to determine the timing of neurodegeneration in these cultures in order to guide future experiments.

Results

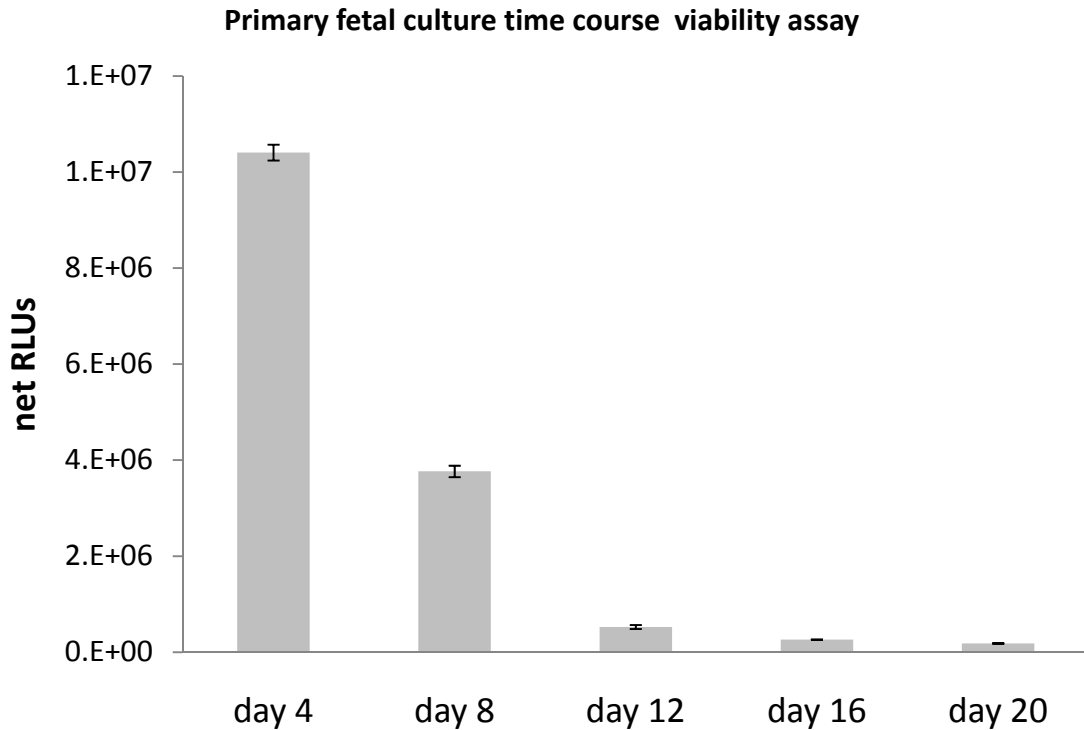
Cell viability time course

To determine the viability of cells in this culture system over time, lysates were collected at four day intervals and frozen quickly at -80°C and stored until the final collection had been frozen at day 20. Samples were then thawed and aliquots were subjected to the Cell Titer-Glo assay. A dramatic drop in this viability measure was observed between day 4 and day 8, and again between day 8 and day 12, and then declined at a slower rate for the remaining two time points (figure 4). The early decline between day 4 and day 8 is perhaps to be expected, as damaged cells or dissociated fragments (e.g., synaptosomes) may survive in culture for a short time and die off over the first few days. Subsequent declines in viability are more surprising. Particularly between day 8 and day 12 at which time there is a readily observable increase in neurite length and apparent synaptogenesis (see below).

Morphological characterization

In an early experiment, untreated neuronal cultures were photographed every three days under bright field microscopy. Neurite length was estimated using a modified version of a previously described technique (Ronn et al., 2000). Estimated average neurite length increased from day 4 to peak at day 9-12, then reduced at the final two time points as neurons decreased in number and cell debris appeared to increase (figure 5).

Figure 4: Cell Titer-Glo time course



Cells were lysed in the tissue culture plate and quickly stored at -80°C every fourth day from day 4 to day 20. When all samples had been collected, samples were thawed simultaneously, such that each sample underwent a single freeze-thaw cycle, and relative cell viability was measured by the Cell Titer-Glo assay (Promega). This assay produces a luminescent signal relative to the ATP concentration in the sample, therefore intact cells are not required, and this assay has been observed to perform well in lysed and frozen samples. A precipitous drop in cell viability was observed between day 4 and day 8, then again between day 8 and day 12. This may represent the delayed loss of cells damaged during the initial tissue dissociation process.

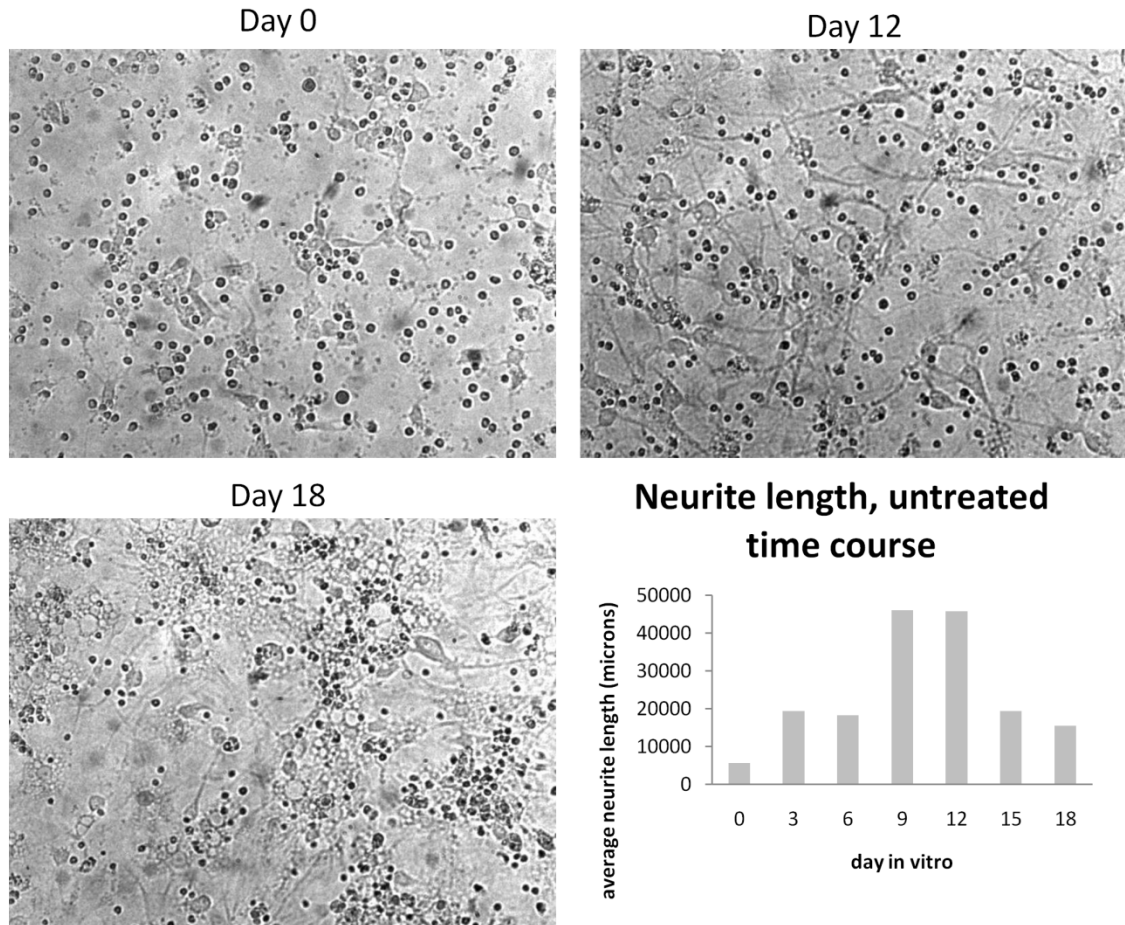
To better visualize neurons in these cultures separately from other cell types that may be present, samples were fixed in the culture plate and subjected to immunocytochemistry for detection of tau protein. Only the later time points, days 12, 16, and 20 were evaluated because this appeared to be the declining phase of neurite length in previous experiments, which is potentially interesting as a neurodegeneration model. A drastic reduction in total tau immunoreactivity was observed by ICC between days 12 and 16 *in vitro* (figure 6a). This reduction continued at day 20 *in vitro*. At day 12, there are many tau-positive cell bodies with a number of neurites, however by day 16 only cell bodies with no significant neuritic extensions are labeled.

GFAP immunoreactivity follows a very different course than tau (figure 6b). At the three time points investigated here, GFAP is lowest at day 12 and increases from day 12 to day 16 and from day 16 to day 20. While glial proliferation cannot be ruled out by these data, the main difference with time appears to be the size of individual glial cells. GFAP-positive glia appear to expand and form a nearly confluent sheet over the tissue culture plate by day 20 *in vitro*.

Protein marker characterization

Lysates of cells maintained in culture for various durations were subjected to Western blotting to determine relative amounts of several proteins. NSE levels increased from day 4 to day 8, and further increased to their peak values at day 12 (figure 7). At the two subsequent time points, NSE declined to undetectable levels at

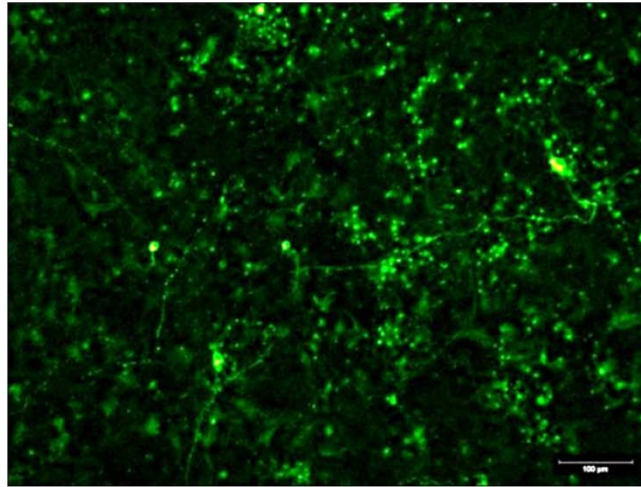
Figure 5: Neurite length time course



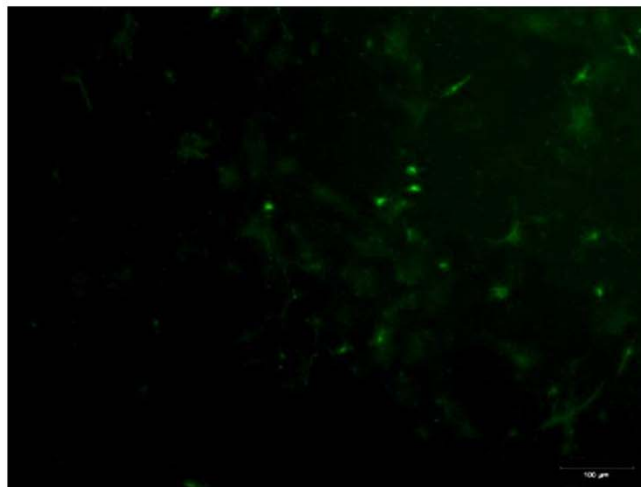
Cultured cortical cells were observed and photographed with a Polaroid MicroCam under a bright-field microscope and neurite length was estimated using a modification of the technique developed by Ronn et al. (2000). Average neurite length was observed to increase starting at day 3 and peak around day 9 to day 12 then decline at later time points.

Figure 6a: Tau ICC time course

Day 12



Day 16



Day 20

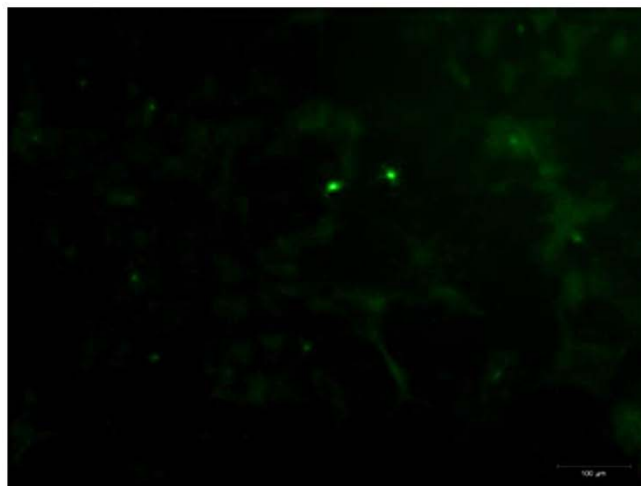
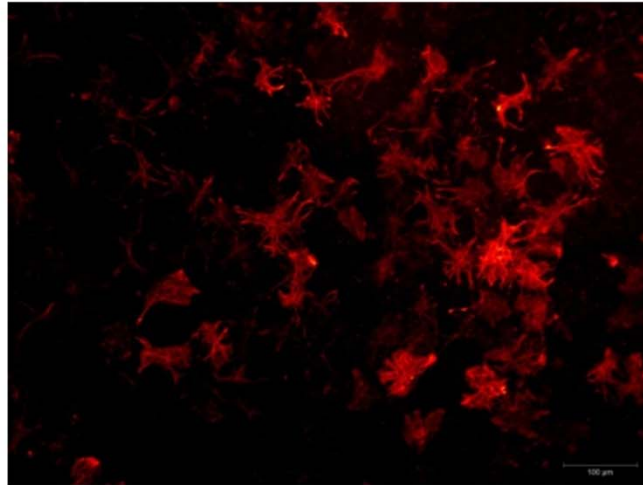
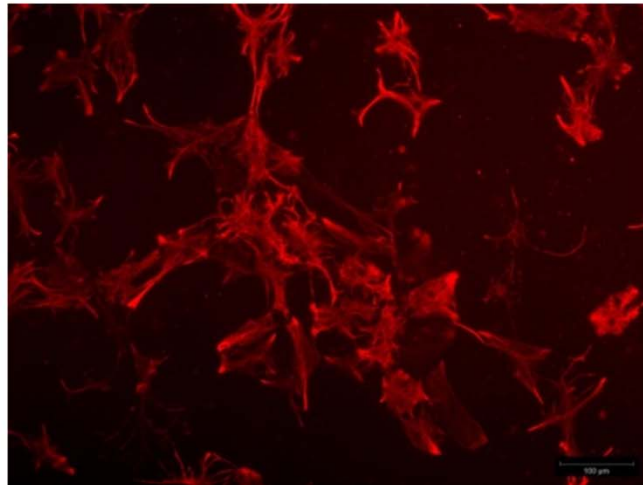


Figure 6b: GFAP ICC time course

Day 12



Day 16



Day 20

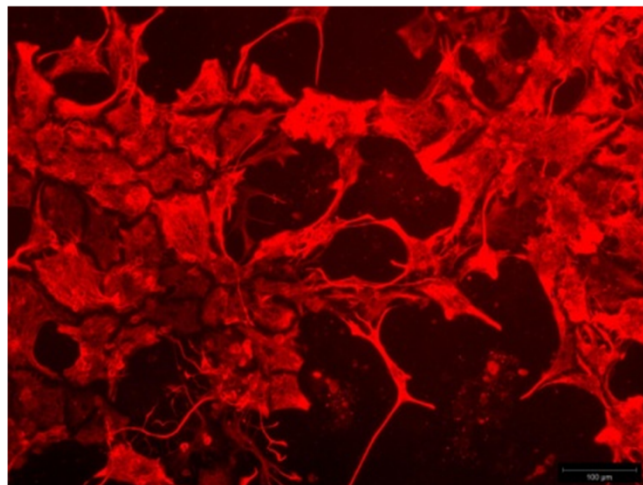


Figure 6 (a, b): Tau and GFAP time course

At the last three time points in culture, cells were fixed and labeled with anti-tau or anti-GFAP antibodies to visualize neurons and glia, respectively, with immunofluorescent labeling. A clear decrease in tau labeling was observed between day 12 and day 16, and perhaps a continued decrement in labeling between day 16 and 20. Conversely, GFAP labeling appeared to increase steadily over the course of these three time points. Both of these observations are consistent with neuron damage mediated by glial activation.

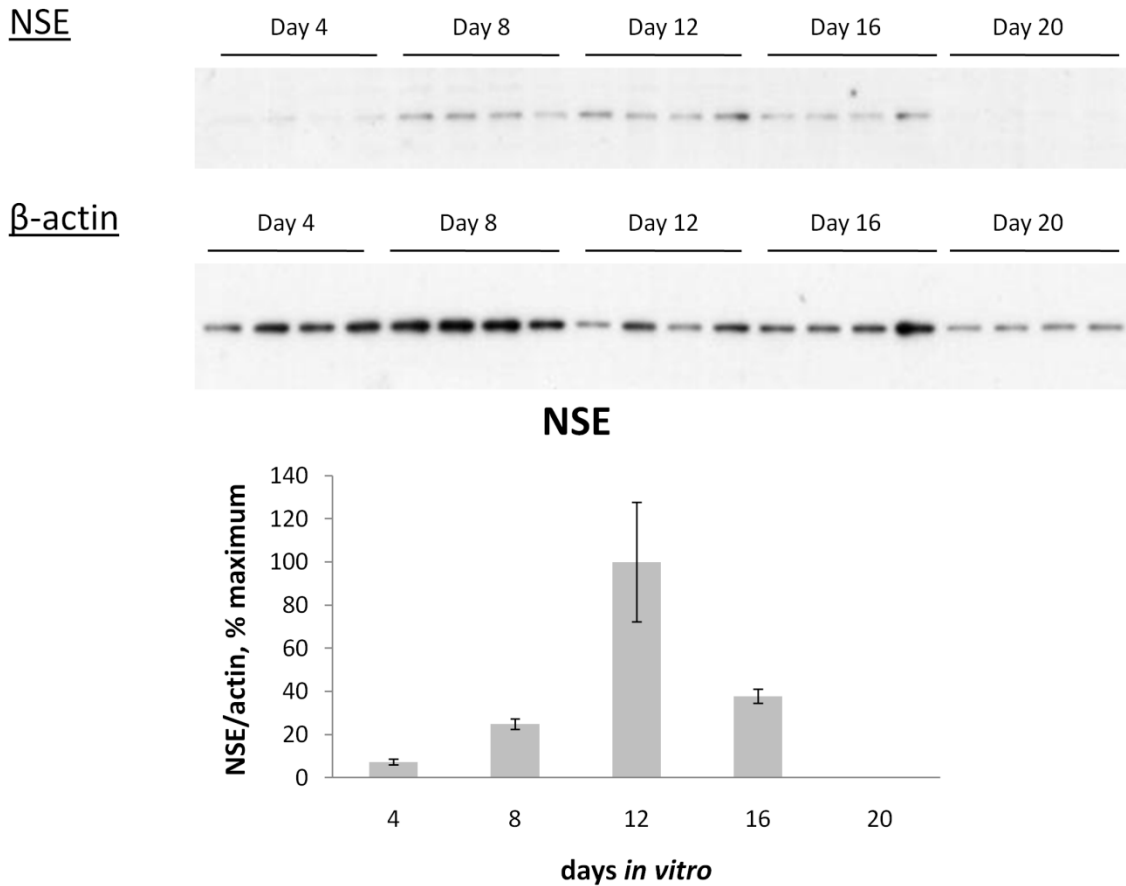
day 20. These data appear to corroborate well with the ICC data indicating a decrease in tau-positive neurons in these cultures.

GFAP is measured by Western blot in the lysates of these cells appears to corroborate well with the ICC data. While GFAP is undetectable at days 4 and 8, it is detectable starting at day 12 and persisted through the end of the experiment (figure 8). In fact, it appears to increase exponentially during the final three time points. Whether this is the result of increased glial cell volume, or glia producing GFAP in greater proportion to total protein production is unclear since the glia do not appear to reach complete confluence within 20 days *in vitro*.

The NR1 subunit of the NMDA receptor was present in quantities detectable by Western blot up to day 16 of the time course experiment, and at day 20 it was not detectable (figure 9). It is interesting that the highest levels were detected at the earliest two time points (day 4 and day 8) given that data obtained in similar cultures has determined that NMDA receptors do not become active until around day 10 *in vitro* (Mizuta et al., 1998). Why expression of this subunit begins so long before the receptors become active is unclear, however it is apparent that peak expression does occur immediately before the decline in NSE was observed, and both markers are absent at day 20, suggesting a severe depletion of neurons at this time point.

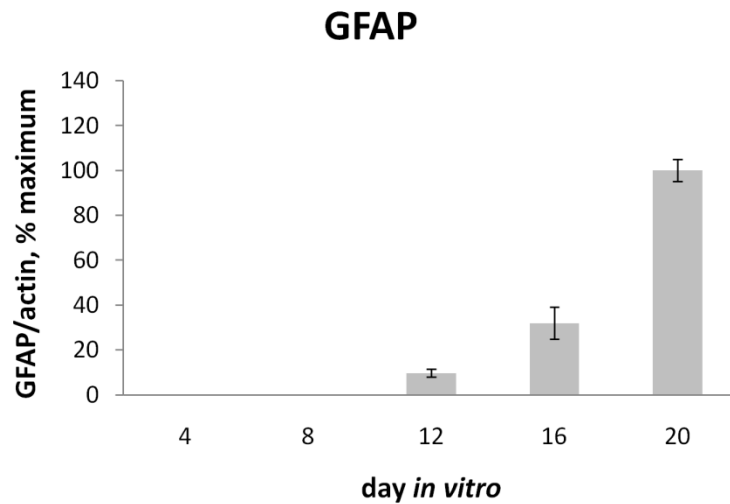
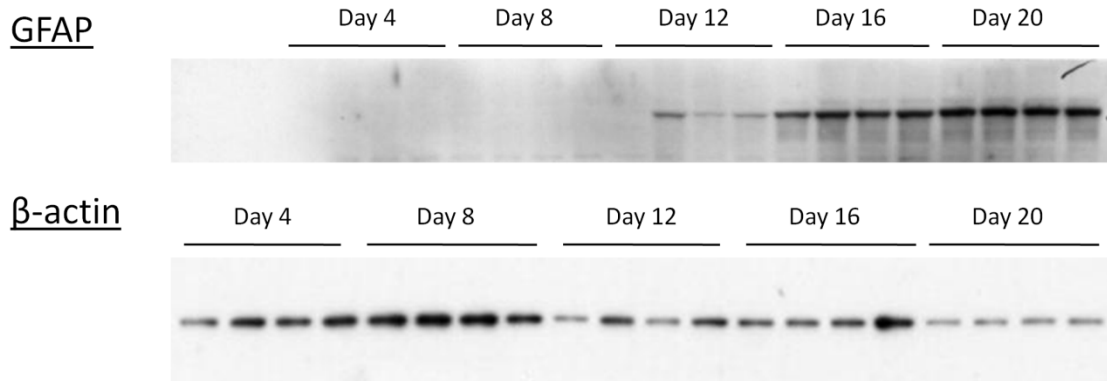
SNAP-25 was not detectable at day 4, but increased dramatically between day 4 and day 12, but returned to undetectable levels at day 16 and day 20 (figure 10). The precipitous nature of the decline in SNAP-25 suggests a rapid onset of toxicity that coincides with the increase in GFAP levels and glial activation seen in both Western blot

Figure 7: Neuron-specific enolase untreated time course Western Blot



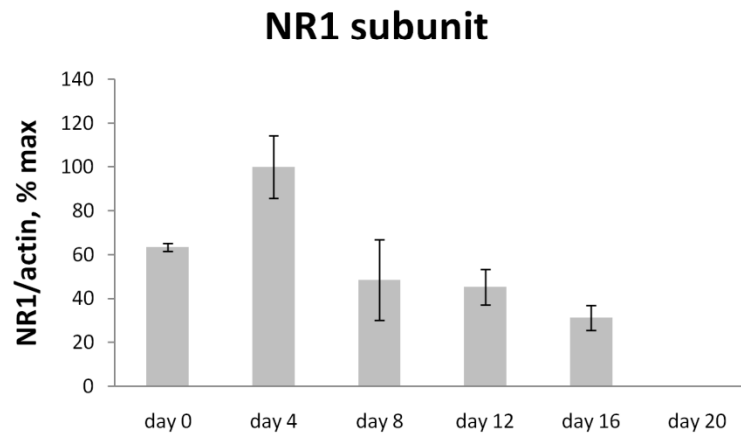
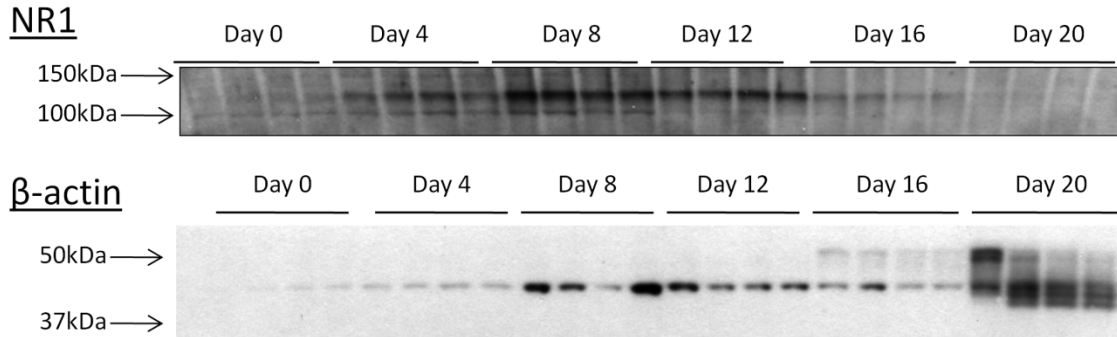
10 μ g of cell lysates collected at four day intervals up to day 20 were subjected to Western blotting with immunodetection for NSE. Four wells of a 12-well plate were lysed and removed from the plate at each time point, and β -actin was used as an internal control. There were apparent differences in actin between groups of samples, so the data are presented as a ratio of NSE/actin. NSE increased to peak at day 12 then decreased to undetectable levels by day 20. These results suggest that the environment in the culture plate is amenable to neuronal maturation at least during the first 12 days, then changes to an inhospitable environment at later time points.

Figure 8: Glial fibrillary acidic protein untreated time course Western blot



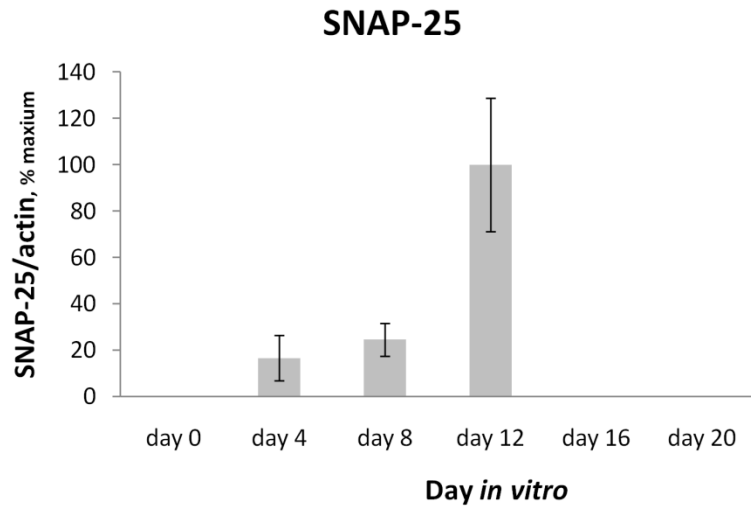
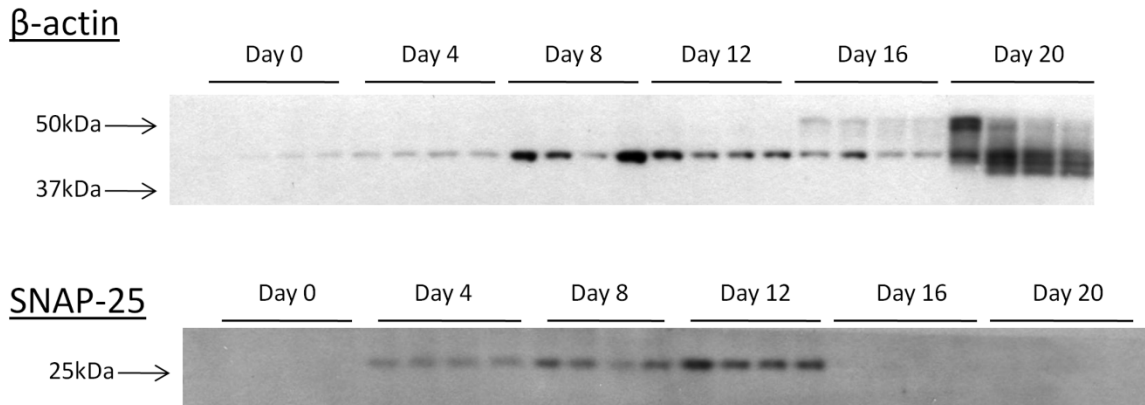
10µg of cell lysates collected at four day intervals up to day 20 were subjected to Western blotting with immunodetection for GFAP. Four wells of a 12-well plate were lysed and removed from the plate at each time point, and β-actin was used as an internal control. There were apparent differences in actin between groups of samples, so the data are presented as a ratio of GFAP/actin. GFAP was undetectable at day 4 and day 8, but increases were observed between days 12 and 16, and the maximum GFAP levels observed were at day 20. These results suggest an onset of glial activation, and perhaps proliferation, around day 12. This continues at least through day 20 when the experiment was terminated.

Figure 9: N-methyl-D-aspartate NR1 subunit untreated time course Western blot



10 μ g of cell lysates collected at four day intervals up to day 20 were subjected to Western blotting with immunodetection for the NR1 subunit of the NMDA receptor. Of the several NMDA receptor subunits that could have been measured, NR1 is of particular interest as it is the only single subunit that is not at least partially interchangeable with another. Four wells of a 12-well plate were lysed and removed from the plate at each time point, and β -actin was used as an internal control. There were apparent differences in actin between groups of samples, so the data are presented as a ratio of NR1/actin. The NR1 subunit was present at all time points examined except at day 20 when the neuronal marker NSE is undetectable. This suggests that the mechanism of neuronal loss in these cultures could involve the NMDA receptor.

Figure 10: Synaptosomal associated protein of 25kDa (SNAP-25) untreated time course Western blot



10 μ g of cell lysates collected at four day intervals up to day 20 were subjected to Western blotting with immunodetection for SNAP-25. Four wells of a 12-well plate were lysed and removed from the plate at each time point, and β -actin was used as an internal control. There were apparent differences in actin between groups of samples, so the data are presented as a ratio of SNAP-25/actin. SNAP-25 was not detectable at day 0 but increased from day 4 to day 12, then was subsequently reduced to undetectable levels.

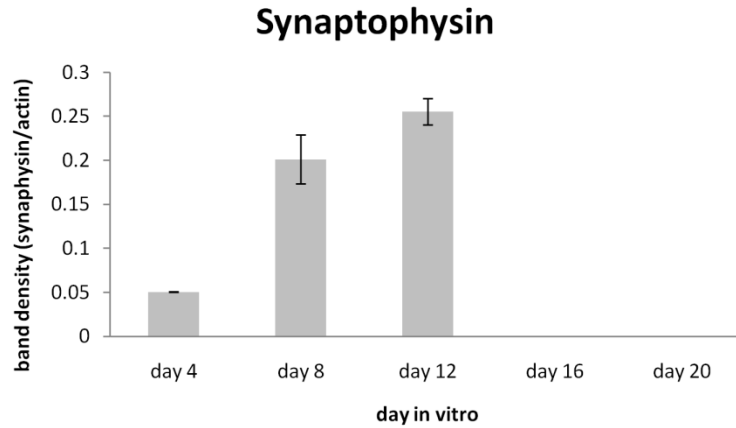
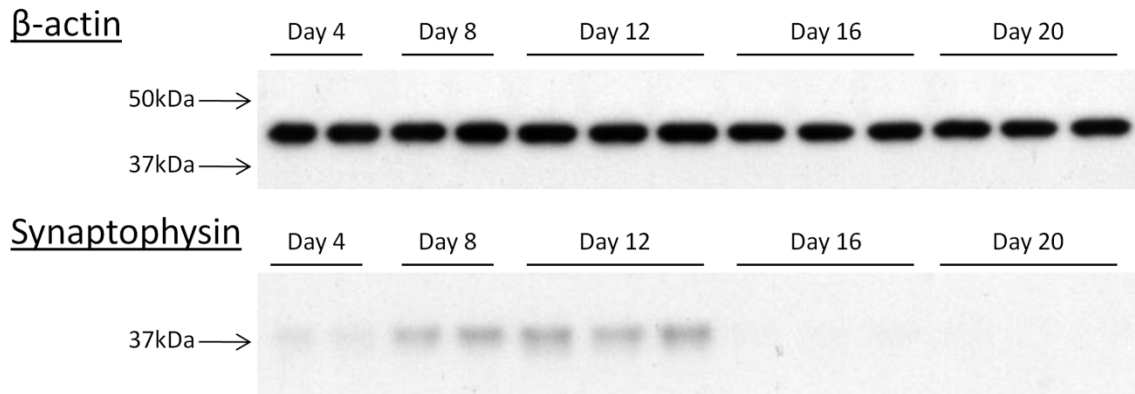
and ICC experiments. It is also interesting to note that the decline of SNAP-25 to undetectable levels precedes the loss of NSE by 4 days, indicating that there is severe loss of synapses at some time before the complete loss of neuronal proteins, suggesting a potential window for rescue of neurons and perhaps restoration of synaptic contacts between neurons.

Synaptophysin increased dramatically between day 4 and day 12, but dropped precipitously to undetectable levels between day 12 and day 16 (figure 11), with a pattern similar to that of SNAP-25. This suggests that all presynaptic proteins may be affected similarly and that this loss is not specific to a single protein. In other words, it is likely there is presynaptic terminal loss rather than down-regulation of any single protein in these cultures.

Time-dependent changes in APP secretion

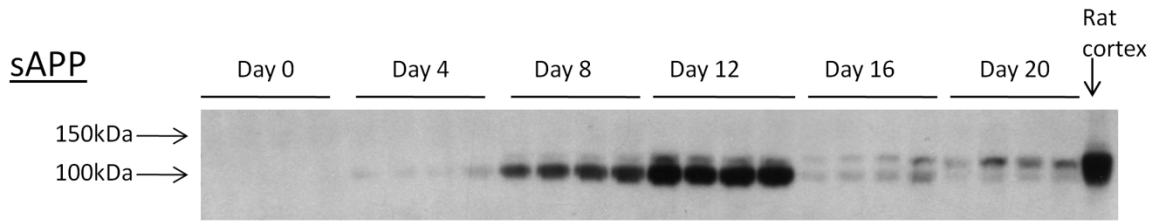
The time-dependent pattern of APP secretion was first determined by subjecting media samples collected from these cultures at 4-day intervals (figure 12). Total secreted APP levels increased until day 12, but were then reduced at day 16, simultaneous with synaptic marker loss. Secreted APP appeared as a characteristic pair of bands of approximately 100kDa and 130kDa that both increased from day 4 to day 12. At later time points, intensity of the lower APP band declined while the top band became the predominant form at day 20. These changes that occur as the relative cell populations in the culture plate shift from neuron-rich to glia-rich suggest that the

Figure 11: Synaptophysin untreated time course Western blot

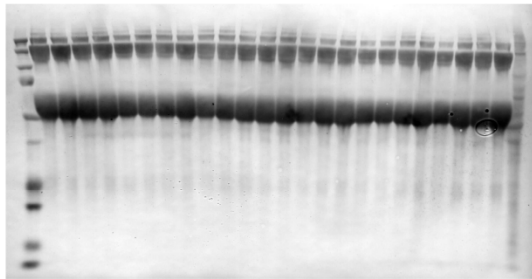


10 μ g of cell lysates collected at four day intervals up to day 20 were subjected to Western blotting with immunodetection for synaptophysin. Three wells of a 6-well plate were lysed and removed from the plate at each time point, and β -actin was used as an internal control. Data are presented as a ratio of synaptophysin/actin. Synaptophysin increased from day 4 to day 12, and was then reduced to undetectable levels at subsequent time points.

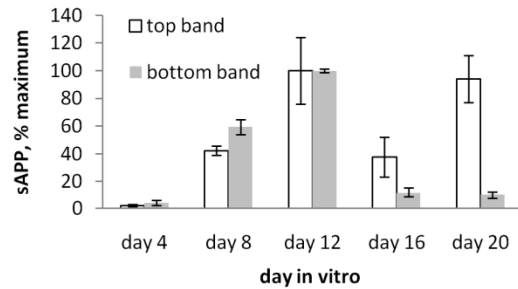
Figure 12: sAPP time course



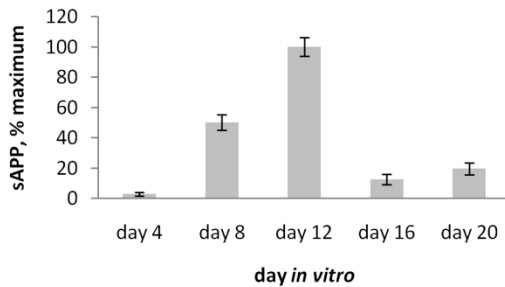
Ponceau-S stain



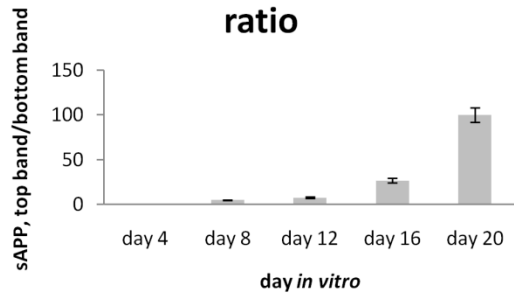
sAPP, top vs. bottom bands



sAPP, combined bands



sAPP, top/bottom band ratio



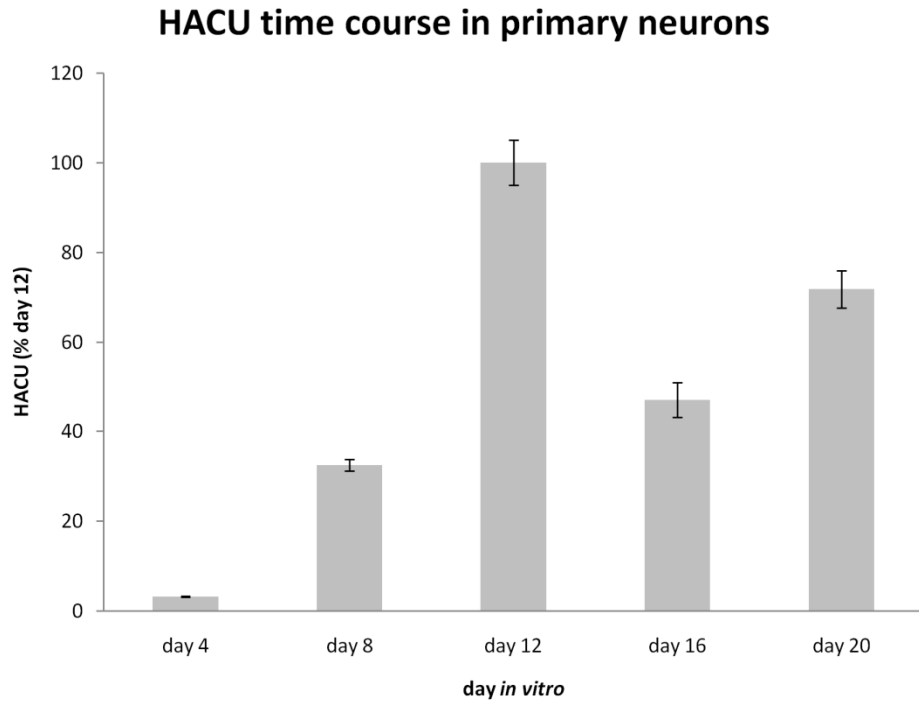
Media samples were collected every fourth day up to day 20. 10 μ l of medium was loaded per well and the blot was probed with the 22C11 monoclonal anti-APP antibody. β -actin was not detectable on this blot, so the Ponceau S stained membrane is shown to demonstrate equal protein loading. The day 0 samples were collected approximately 30 minutes after the plates were seeded, and this was insufficient time for detectable quantities of sAPP to accumulate in the medium.

higher molecular weight form of secreted APP is primarily of glial origin, while the lighter form is primarily neuronal in origin.

Cholinergic neuronal markers

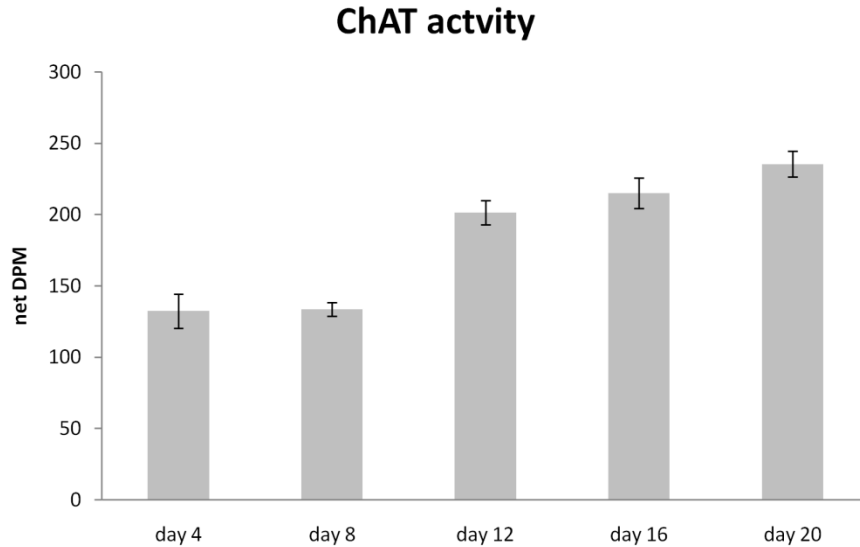
High affinity choline uptake (HACU), defined as the hemicholinium-sensitive portion of total ^3H -choline uptake, peaked at day 12 *in vitro* (figure 13), simultaneously with the peak in the synaptic terminal marker SNAP-25. Given that HACU occurs at the synaptic terminal in neurons, it would be expected that HACU would decline precipitously between day 12 and day 16, following the trend of SNAP-25 closely, if cholinergic neurons were present. However, this was not observed. Rather, hemicholinium-sensitive choline uptake persisted throughout all time points tested, suggesting non-neuronal uptake. Hemicholinium-sensitive choline transport has been observed in rat astrocytes (Inazu et al., 2005) that differs from the neuronal transporter in kinetic parameters and sodium dependence (Apparsundaram et al., 2000) suggesting an alternate transporter in glial cells. Sodium sensitivity was not tested and kinetics were not measured, therefore transport in these cells cannot be compared to the published data, however glial choline uptake seems likely.

Figure 13: High affinity choline uptake, untreated time course



Uptake of [^{14}C]choline was measured at the time points indicated in untreated primary cortical neurons. Levels of choline uptake were normalized to input radioactivity (i.e., the total amount of ^{14}C added to the assay) at various days, which varied only slightly ($6.82 \times 10^5 \pm 4 \times 10^4$ CPM, mean \pm SEM). Choline uptake increased in the initial phase of the time course, and peaked at day 12, around 9% uptake of total choline in the assay.

Figure 14: choline acetyltransferase activity, untreated time course



Choline acetyltransferase activity was measured in lysates of untreated primary cells collected at the time points indicated. Samples were lysed in M-PER buffer and lysates were stored at -80°C until the completion of the time course. This assay measures the transfer of radiolabeled acetyl groups transferred from $[^3\text{H}]$ acetyl-CoA to choline to form $[^3\text{H}]$ acetylcholine. ChAT activity was very low, averaging only 0.6% conversion of input $[^3\text{H}]$ acetyl-CoA to ACh. A gradual increase was observed over the course of the experiment, even at the later two time points where neuronal protein was observed to be in decline, suggesting that this assay may measure other products in addition to ACh, such as acetylcarnitine.

To investigate the capacity for acetylcholine synthesis in these cultures, a choline acetyltransferase (ChAT) activity assay was performed (figure 14). The pattern of ChAT activity was expected to mirror our other cholinergic activity marker, HACU, however, this was not observed. ChAT activity was present only in very low levels but increased steadily throughout the time course, even up to day 20 where synaptic markers are absent and severe neurite pruning has taken place. This suggests either a non-neuronal source of ChAT activity, or another enzyme producing a product that is detected by this assay at low quantities. This assay is based on incorporation of the ^{14}C -labeled acetyl group of acetyl-coenzyme A into acetylcholine, however it is possible that this acetyl-coenzyme A could potentially act as a substrate for several other enzymes, including carnitine acetyltransferase (Goodman et al., 1984), producing this unexpected pattern.

Discussion

The study of many pathological and physiological phenomena is greatly aided by the availability of well-characterized tissue culture systems. Such experimental models provide a simplified system in which the effects of various manipulations can be observed without many of the complications present in whole animals, e.g., the blood-brain barrier, are absent but an organotypic, heterogeneous cell population is maintained. Additionally, primary tissue cultures are of particular interest as an alternative to common tumor-derived tissue culture models since they may contain true, fully-differentiated neurons. Neurons in the cultures used here were shown to differentiate and then degenerate over the course of 2 to 3 weeks, which is far more rapid than any available animal model of neurodegeneration, and they exhibit some features that are relevant to the study of AD.

Recent work has shown that embryonic neurons do form synapses *in vitro*, although these experiments used slightly different conditions, including serum-containing medium during the initial plating and cells derived from E19 pups, (Grabrucker et al., 2009). The time-dependent increases in synaptic markers observed in the model used here appear to agree with this finding. Grabrucker et al. also observed stable neuronal population up to 21 days *in vitro*, which is contrary to the results presented here. Furthermore, it has been claimed that the Neurobasal plus B27 supplement system inhibits glial growth, although the data have been reported only up to day 4 *in vitro* (Brewer et al., 1993), or not shown (Grabrucker et al., 2009). These primary cultures are clearly not static in their relative populations of neuronal and glial

cells, and have demonstrated dynamic changes in neuronal, glial, and synaptic protein expression. It is clear that in any experiment using these cells, the timing of any manipulation is highly important. The timing of the expression of the several proteins of interest in these experiments was therefore investigated.

Neurite development and decline is highly dynamic in these tissue cultures. Neurites are completely pruned during tissue dissociation, but then re-grow to peak around day 12, coincident with synaptic protein levels. The processes of neurite outgrowth and synaptogenesis (or synapse pruning) are thought to be central to memory formation and are impaired in AD (DeKosky et al., 1996). In these cultures, both neurite outgrowth, and potentially synaptogenesis, can be observed at earlier time points, while neurite and synaptic pruning can be observed at later time points. Although it may be difficult to perfect the timing of experiments using these cultures, these observations suggest that this could be a highly valuable research tool that could help expand our understanding of neuron differentiation and deterioration.

The glial marker used in these experiments (GFAP), is undetectable at the earliest time points, which is consistent with previously reported data (Brewer et al., 1993), but GFAP increases dramatically starting around day 12-16 *in vitro*, which has not been previously reported. GFAP has been linked to glial activation, both in astrocytes and microglia, which suggests that this tissue culture system may in some ways mimic glial activation. Glial activation, as determined by increased levels of GFAP, is important in neurodegenerative diseases, such as AD (Simpson et al., 2010), and traumatic brain

injury (Honda et al., 2010; Li et al., 2009b), suggesting that this tissue culture system has broad potential for investigations regarding diverse types of neurodegeneration.

Regarding AD specifically, the cholinergic markers ChAT and HACU were measured over the progression of these tissue cultures. Studies in cultured cerebrocortical cells from embryonic rats have demonstrated the efficacy of cholinesterase inhibiting drugs in protecting cerebrocortical neurons against a variety of insults, including glutamate toxicity (Takada-Takatori et al., 2006) and hypoxia (Akasofu et al., 2008). It is generally presumed that cultured cells originating from the cerebral cortex do not contain cholinergic neurons, although this has not been thoroughly tested in the tissue culture system used here. Given the uncertainties of placing incompletely differentiated neurons into the foreign environment of the tissue culture plate in artificial medium, in addition to these previously observed cholinergic drug effects, it is worthwhile to investigate the presence of cholinergic neurons in this culture model. To this end, highly sensitive radionuclide-based methods were employed. HACU and ChAT activity, two definitive measures of cholinergic neurons, were measured over time in these cultures. Both assays showed detectable signal that varied with time. These activities are localized *in vivo* to cholinergic synaptic terminals, and therefore would be expected to follow the expression pattern of synaptic terminal protein markers measured by Western blot, but this was not observed. Rather, the observed levels of these cholinergic markers appear to be synapse-independent, persisting until day 20 when synaptic markers are absent. It is likely that the observed signals in these assays are related to non-neuronal activities, for example, hemicholinium-sensitive choline

uptake has been observed in cultured cortical astrocytes (Inazu et al., 2005).

Alternatively, the apparent cholinergic activity could be related to non-specific activities, which has been observed in the ChAT assay, wherein [¹⁴C]-acetylated carnitine has been demonstrated to contaminate the assay in some tissues (Goodman et al., 1984). As it appears unlikely that cultured cortical cells from fetal rats possess cholinergic activities, these data provide strong evidence to support non-cholinergic activities for the cholinergic drugs that have shown protective effects in similar culture models.

Aside from the apparent lack of cholinergic cells in the neuronal population of these cultures observed here, it is not yet clear what neurotransmitter phenotypes are present. In other culture models based on dissociated embryonic cerebrocortical cells, it has been found that glutamate and GABA neurons predominate in ratios similar to those found in the intact cerebral cortex (Gotz et al., 1995). While these cells were obtained from a similar source (embryonic day 16 rats), there were some differences between the conditions used in this study and those used here. These include the use of a glial feeder layer and serum-containing medium, both of which could plausibly influence the differentiation of immature neurons. Thus, while it is reasonable to expect that the neurons in the cultures reported here were predominantly glutamatergic and GABAergic, this should be examined further under the specific conditions used here.

The pattern of sAPP bands on Western blots of the media conditioned by these cells contained a typical doublet, though the relative intensities of the two bands varied over time. The low molecular weight sAPP band appears earliest in the time course at day 4, followed by a higher molecular weight sAPP band that appears at day 8. The low

molecular weight band increases and peaks at day 12, after which it is decreased drastically at subsequent time points. This pattern matches closely those of NSE and the synaptic markers in the matching lysates. Conversely, the high molecular weight sAPP band follows more closely the pattern of GFAP. This prevents the attractive possibility that sAPP can be used as an indicator of the relative neuronal and glial populations in a mixed culture. The division between sAPP neuronal and glial origin does not appear to be complete, as the low molecular weight band is still visible at day 20 when neuronal and synaptic markers are no longer detectable. Thus, while the comparison of relative levels of these two bands may be useful, some caution is warranted against overinterpretation of such results.

Both NSE and the synaptic markers SNAP-25 and synaptophysin appear to peak and decline at fairly predictable time intervals. It is very interesting that the synaptic proteins measured here appear to decline in advance of NSE. This could be quite important with respect to AD, in which there is synaptic loss, as well as apparent neuron loss. It is known that synapse loss occurs early in AD, while it is possible that complete loss of neurons occurs at a later stage of the disease (Morsch et al., 1999). This suggests a window of opportunity for AD and perhaps other neurodegenerative disorders during which clinical manifestations brought on by synapse loss could be corrected by pharmacological or other interventions between synaptic terminal pruning and neuron death.

This tissue culture model has now been successfully used to investigate the mechanisms of several investigational compounds and drugs used to treat AD. For

example, rivastigmine was shown to protect neurons during the degenerating phase of this primary tissue culture model, as well as maintain levels of synaptic proteins at peak levels while neurons in vehicle-treated cultures degenerated almost completely (Bailey and Lahiri, 2010). Similarly, this culture model was used to demonstrate that memantine can increase the viability of cultured neurons, as well as modulate APP cleavage and A β production, suggesting a possible mechanism for the clinical efficacy of this drug (Alley et al., 2010). Thus, although this model is time- and resource-intensive relative to other tissue culture models, such as immortal cell lines, it is still much less so than whole animal models, and can provide unique insights into neurodegeneration and neuroprotective strategies.

It is clear that these cultures exhibit synapse pruning and neurodegeneration in a time-dependent fashion. However, the exact cause of this degeneration is unclear. Using the same media conditions employed here, neurons have been maintained for at least 35 days *in vitro* (Yang et al., 2010). One major difference between the culture method of Yang et al. and those used here is the exclusion of non-neuronal cells from the culture. It is a common practice in culturing primary neurons to inhibit or eliminate glial proliferation by the addition of mitotic inhibitors, such as Cytosine arabinofuranoside (Ara-C) (Bains and Heidenreich, 2009; Schnitzler et al., 2008). This increases the lifespan of neurons in culture, although it precludes any observation of neuron-glia interactions in cultured cells, and thus mitotic inhibitors were not used here. It has been proposed that in AD and other disorders, cytokines released by glial cells can lead to neurodegeneration (Mrak and Griffin, 2005). While the exact series of events

leading to neurodegeneration in this model is currently unclear, in combination with increasing glutamate levels, glia-derived cytokines are likely candidates. These cultures have been observed to generate A β (Alley et al., 2010), and glia have been observed to stimulate NMDA and tumor necrosis factor- α (TNF α) receptors in cultured neurons in response to A β exposure (Floden et al., 2005). This presents a plausible mechanism for neurodegeneration in these cultures which, if found to be correct, could both indicate the relevance of this model to various diseases and allow further interpretation of data collected in this model.

While several facets of this tissue culture model offer potential parallels with AD, it is important to note that important parts of AD pathology are missing from this model. For example, the definitive pathological findings in the AD brain, amyloid plaque and neurofibrillary tangles, are absent in these tissue cultures. Of particular interest, these cultures do show profound synaptic marker loss, which is known to occur very early in the course of AD, even before these definitive pathological signs of AD appear (Scheff et al., 2007), and synapse loss is strongly associated with cognitive decline (DeKosky et al., 1996; Terry et al., 1991). Therefore, while plaque and tangle formation is absent in this model, it may be suitable for investigation of synaptic protein regulation and synaptic integrity. These earliest changes in the AD brain should be considered as prime targets for intervention in order to prevent synapse loss and neuron death before these changes progress to disabling levels. Given the synaptic marker loss and neurodegeneration demonstrated in these cultures, this tissue culture model may be a

useful tool in investigating possible mechanisms of preventing or restoring synaptic markers in higher-order models or human subjects.

Section 2: Gene regulation studies in primary cerebrocortical cultures

Introduction

It has been observed that in AD that several proteins are dysregulated. The three of these most directly implicated in AD are *APP*, *BACE1*, and *MAPT*. The *APP* gene is localized to chromosome 21q21, and as its name implies, it is the precursor of the A β peptide that forms toxic aggregates in the AD affected brain. Greater *APP* expression has also been observed in AD affected brain tissues relative to control brain (Matsui et al., 2007), and polymorphisms in the *APP* gene promoter region that are associated with greater expression also associate with greater risk of developing AD (Lahiri et al., 2005b; Theuns et al., 2006). The *APP* gene resides on chromosome 21, and a third copy of the *APP* gene in Down's syndrome is sufficient to cause early onset AD by the age of 40 in nearly all cases (Armstrong, 1994). Thus there is ample evidence that excessive production of APP in the brain can play a causative role in AD pathogenesis.

The *BACE1* gene is located on chromosome 11q23, and the BACE1 enzyme is the rate-limiting step in A β production, and was found to be increased in brain tissue affected by AD (Holsinger et al., 2002). Consistent with protein levels, BACE1 enzyme activity is also increased in AD brain tissue relative to control (Stockley et al., 2006). A mechanism has been proposed by which early life exposure to lead has been shown to result in persistent changes in methylation-mediated epigenetic regulation of the *BACE1* gene, among others. In animals exposed to lead early in life, these changes manifested later in life as increased BACE1 enzymatic activity and increased A β and AD-like pathology (Wu et al., 2008). In other animal models, it has been shown that BACE1

activity increases prior to amyloid deposition (Heneka et al., 2005), and that through genetic manipulations, relatively slight (~12%) reductions in BACE1 activity can result in pronounced reductions in A β plaque deposition (McConlogue et al., 2007).

The *MAPT* gene is located on chromosome 17q21. It was an early discovery that the tau protein is regulated by phosphorylation, such that unphosphorylated tau promotes microtubule assembly, and phosphorylation reduces this activity (Lindwall and Cole, 1984). Subsequently it was determined that genetic variants that increase *MAPT* gene expression levels are associated with AD risk (Myers et al., 2005b; Sun and Jia, 2009a). While data that suggest heightened levels of tau production as a pathogenic mechanism in AD are lacking, in animal models, it has been observed that A β plaque formation is upstream of tau tangle formation, and genetic manipulations that produce even modest decreases in tau levels also reduce tau phosphorylation and AD-like memory deficits induced by A β overproduction (Roberson et al., 2007).

These studies suggest that dysregulation of protein production at the level of these gene promoters constitute potential pathogenic mechanisms, or at least contributing factors. Therefore, possible treatment options for AD include targets that may modulate transcriptional activity of the *APP*, *BACE1*, and *MAPT* genes via their upstream promoter elements. In order to pursue treatment modalities that target promoter activities, a more complete understanding of their regulation is required. While it is understood that these genes may be dysregulated in AD, it remains unclear whether their dysregulation is a causative agent or a downstream effect in the development of AD pathology.

In order to advance our understanding toward modulation of these genes at the point of promoter activation level, activities of fusion constructs containing luciferase reporter genes under control of *APP*, *BACE1* and *MAPT* gene promoters were tested in primary cerebrocortical cultures under both degenerating and non-degenerating conditions. Activities of these gene promoters were assessed by transfection with reporter constructs at various time points, starting from the initial differentiation stage to the final degeneration stage. For the *BACE1* and *MAPT* promoters, promoter regions of varied length were tested under non-degenerating conditions in order to determine which parts of the upstream sequence are transcriptionally active. And finally, the effects of hypothetical modulators of these gene promoters, A β and p25, were added in the presence of the promoter construct. It was hypothesized that promoter activities will vary with time as neuron differentiation occurs at early time points, followed by neurodegeneration at later time points. Furthermore, it was expected that fragments of various length of the promoter regions studied here will vary in their activities such that the locations of sequences with high transcriptional activity, or inhibitory sequences, can be approximated.

In more specific terms, it is hypothesized that *APP* promoter activity, given the role of APP in synaptogenesis (Mucke et al., 1994), will follow the previously observed pattern of presynaptic marker levels (figures 10-11), and APP secretion (figure 12), increasing to a maximum at day 12 then reduced at subsequent time points. Neurons and glia express several splice variants of APP in different ratios, but all of these are transcribed from the same gene controlled by a single promoter. Thus, some activity will

remain with later-stage, principally glial cultures. Similarly, *BACE1* is expressed primarily in neurons (Heneka et al., 2005), and so it was expected that *BACE1* promoter activity will follow neuronal marker expression. *BACE1* is not exclusively neuronal, and can also be expressed by activated astrocytes (Hartlage-Rubsamen et al., 2003), so transcriptional activity of the *BACE1* gene promoter was expected to persist during the later time points when neurons have degenerated and glia have become activated, but at lower levels. *MAPT* expression is different from *APP* and *BACE1* in that *MAPT* is expressed almost exclusively in neurons. Thus it was expected that *MAPT* expression would follow neuronal marker expression as they increase and then subside, with little or no glial contribution, at the later time points.

Of these three gene promoters, deletion series have been generated from *BACE1* and *MAPT*. Transcription factor binding sites can be predicted *in silico*, but until the activity of each is confirmed to either stimulate or inhibit transcriptional activity, no prediction of their aggregate activity is possible. Thus, an informative method to determine the transcriptional “hot spots” is the deletion series. From such a data set, a general understanding of where the major stimulatory and inhibitory activities lie within smaller sections of the upstream promoter sequence can be obtained. With these data in hand, the search for active transcription factor binding sites will hopefully be simplified by narrowing the search to a few key highly active regions within the promoter region. It was hypothesized that promoter fragments of different lengths will produce varied levels of transcriptional activity, with the more proximal segments exerting the greatest influence over transcriptional activity.

A β and p25 are two factors thought to alter transcription of genes related to AD. A β may alter gene expression by acting directly on regulatory DNA sequences (Ohyagi et al., 2005), while p25, the calpain-cleaved activated form of p35, acts through cyclin dependent kinase 5 (CDK5) to activate the myocyte enhancing factor 2 (MEF2) (Gong et al., 2003) and signal transducer and activator of transcription 3 (STAT3) (Fu et al., 2004) transcription factors, among others, which may be involved in AD. To determine the potential effects A β and p25 may have on transcription of AD-related genes, primary cultures were transfected with reporter plasmids containing the largest *APP*, *BACE1* or *MAPT* promoter fragments available at a time point when the neurons have matured, but not started to degenerate. The cells were either co-transfected with a p25 expression vector, or treated with 1 μ M of A β ₁₋₄₀, A β ₁₋₄₂, or A β ₁₋₂₈ after transfection. These experiments take advantage of aspects of primary neuronal culture to study the action of A β on these gene promoters. Transfection of these cells with the gene promoter of interest fused with a luciferase reporter gene allows relatively rapid and highly sensitive detection of any regulatory influence a subsequent treatment may have. Furthermore, specific fragments of A β can be applied to these transfected cells, allowing the study of potential differences in function between various peptides, e.g., between A β ₁₋₄₀ and A β ₁₋₄₂. This may be of particular importance given that different forms of A β have been observed to form distinct pools in the brain (Steinerman et al., 2008), and that the γ -secretase complex can alter the ratio of A β ₁₋₄₀ to A β ₁₋₄₂ produced under certain circumstances (Yin et al., 2007). This implies that A β may have distinct functions depending on the length of the peptide produced, and the simplified environment of

the tissue culture allows the effects of these different peptides to be evaluated individually.

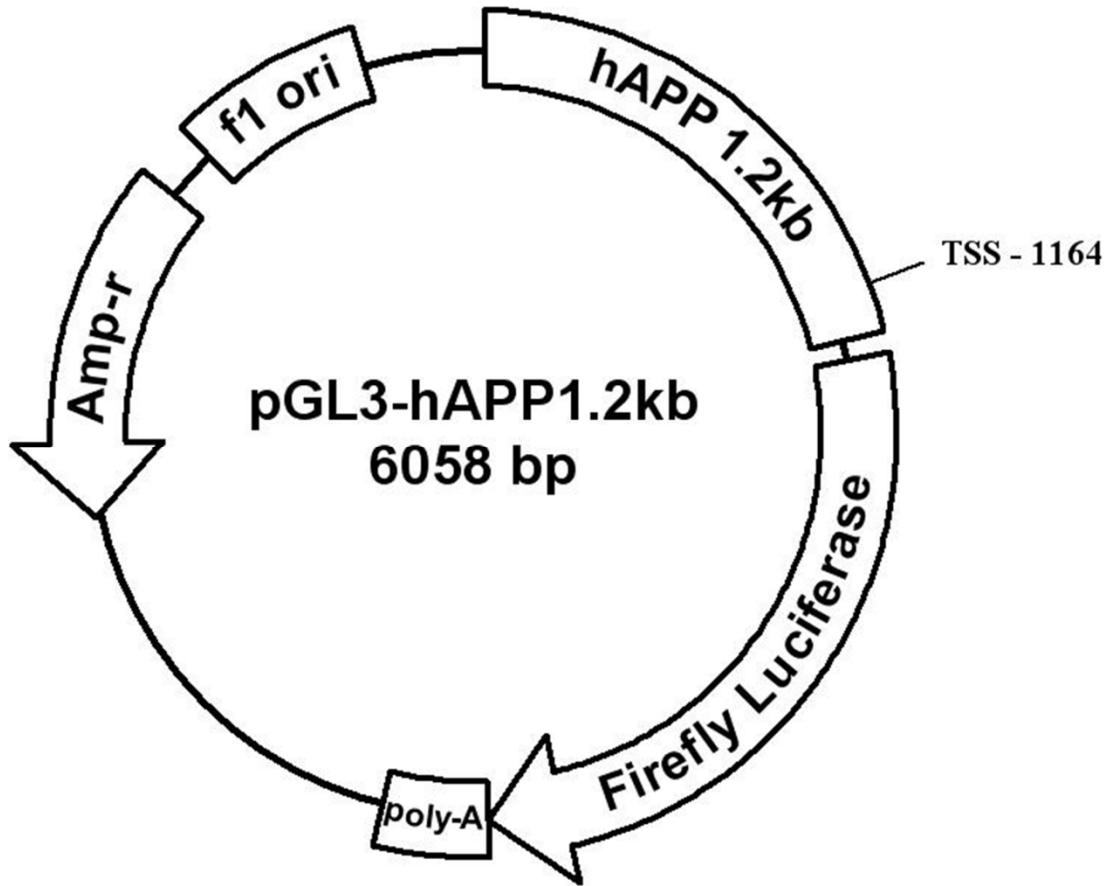
A β was shown to increase p53 promoter activity (Ohyagi et al., 2005), but whether the response to A β binding is similar in other promoter regions is currently unknown. We have shown that A β can be internalized, transported to the nucleus, and can bind to DNA (Lahiri et al., 2009a), although the functional consequences of A β binding to DNA are not clear and many other activities have been proposed by which A β could alter transcription of various genes. To the extent that the response of the p53 gene promoter is representative of other promoter regions, it could be hypothesized that A β treatment will increase the activity of any promoter with an A β binding site. Alternatively, binding sites within the *APP* and *BACE1* genes were observed, which suggests the attractive counter-hypothesis that A β would inhibit transcription when bound to a gene promoter, providing a negative feedback loop to limit its own production. p25 accumulation has been observed in AD affected brain tissues and is likely to contribute to neurodegeneration (Patrick et al., 1999). CDK5 activated by p25 may affect transcription through phosphorylation of a wide variety of substrates, making predictions regarding these specific promoters difficult, but it seems reasonable to hypothesize that p25 overexpression will stimulate *MAPT*, *BACE1*, and *APP* transcription, given its link to neurodegeneration.

Results

Gene promoter activities over time

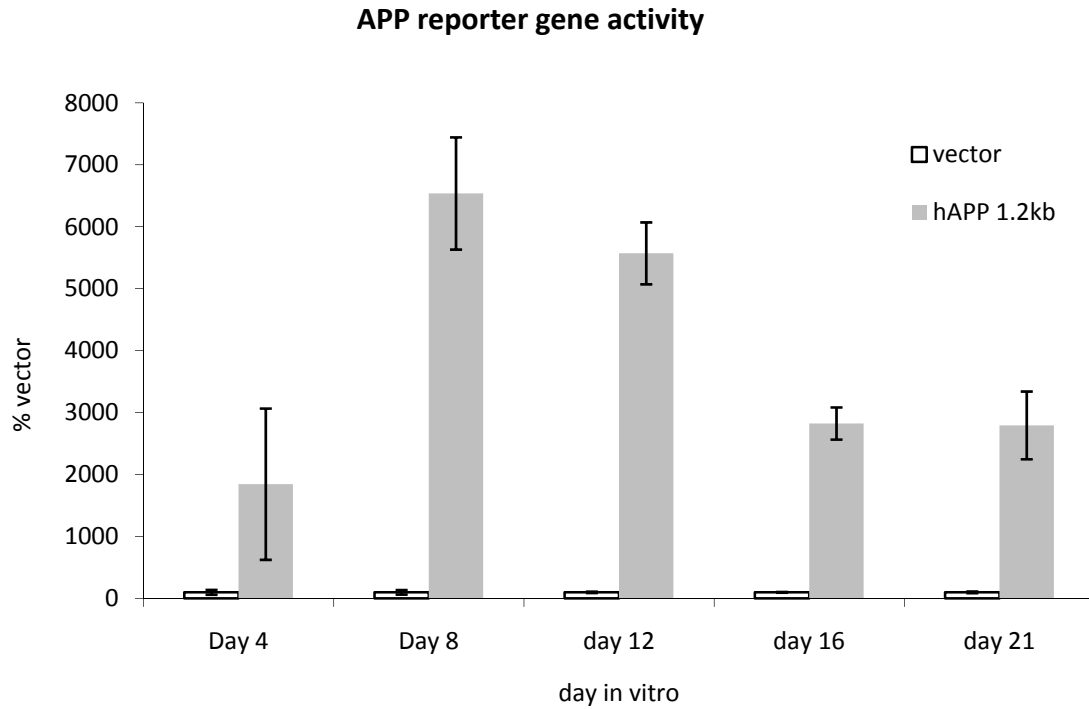
Cultured cells transfected with reporter constructs containing upstream regulatory elements of the human *APP*, *BACE1*, and *MAPT* genes showed variations in transcriptional activity in these two genes with time. A representative plasmid map is shown in figure 15. The *APP* promoter region was active at all points tested, peaking early at day 8 in vitro then declining (figure 16). Interestingly, *APP* promoter activity was stable between day 16 and day 20, when neuronal and synaptic markers are declining or undetectable, suggesting a significant contribution of glial cells to the APP produced by these cultures. Similarly, *BACE1* promoter activity was lowest, approximately equal to vector, at day 4, and also peaked at day 8 (figure 17). Subsequently, *BACE1* promoter activity declined at every time point tested, perhaps indicating a lesser contribution of BACE1 protein in these cultures by the glial component. *MAPT* promoter activity was also detectable from the earliest time points and peaked at day 12 (figure 18) when protein levels of NSE, synaptic proteins, and neurite length are highest. Subsequently, *MAPT* promoter activity drops to very low levels at day 16 and day 20, mirroring neuronal decline over time.

Figure 15: pGL3 Basic hAPP1.2 plasmid map



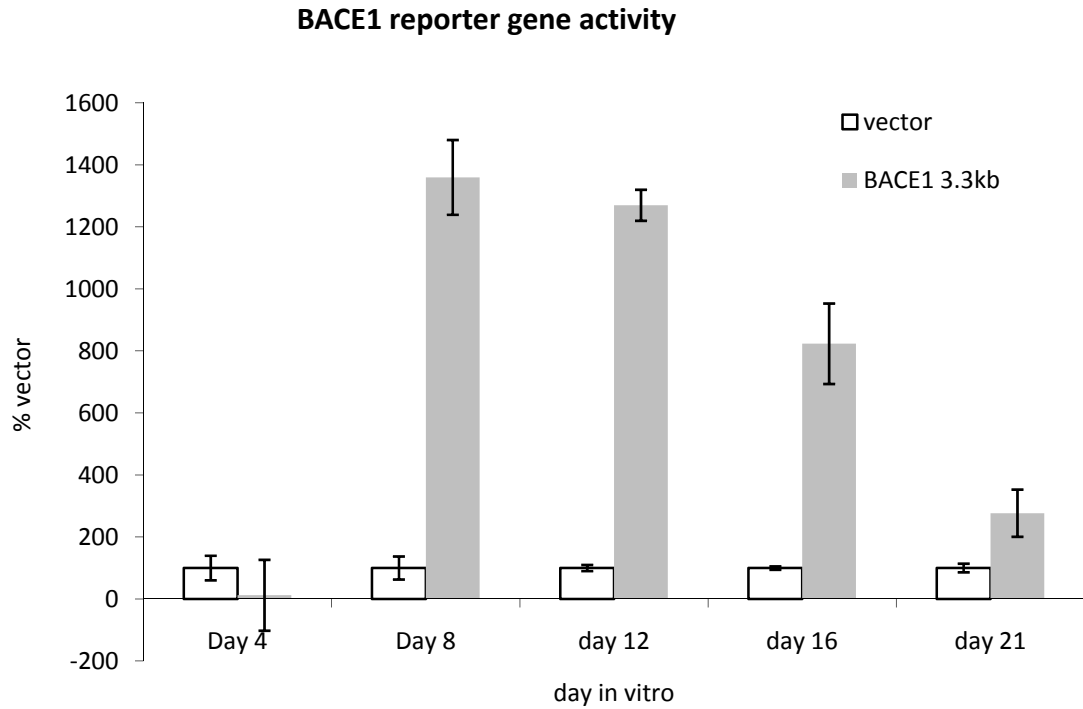
A representative plasmid map of the pGL3-Basic based constructs. In this example, a 1250bp PstI/BamHI fragment of the *APP* promoter was cloned into a promoterless pGL3-Basic plasmid. This insert includes 1164bp of the human *APP* upstream regulatory region and 86bp of the 5' untranslated region. In other plasmids, the promoter fragments are different, but all other elements of the plasmid remain constant.

Figure 16: *APP* promoter activity untreated time course



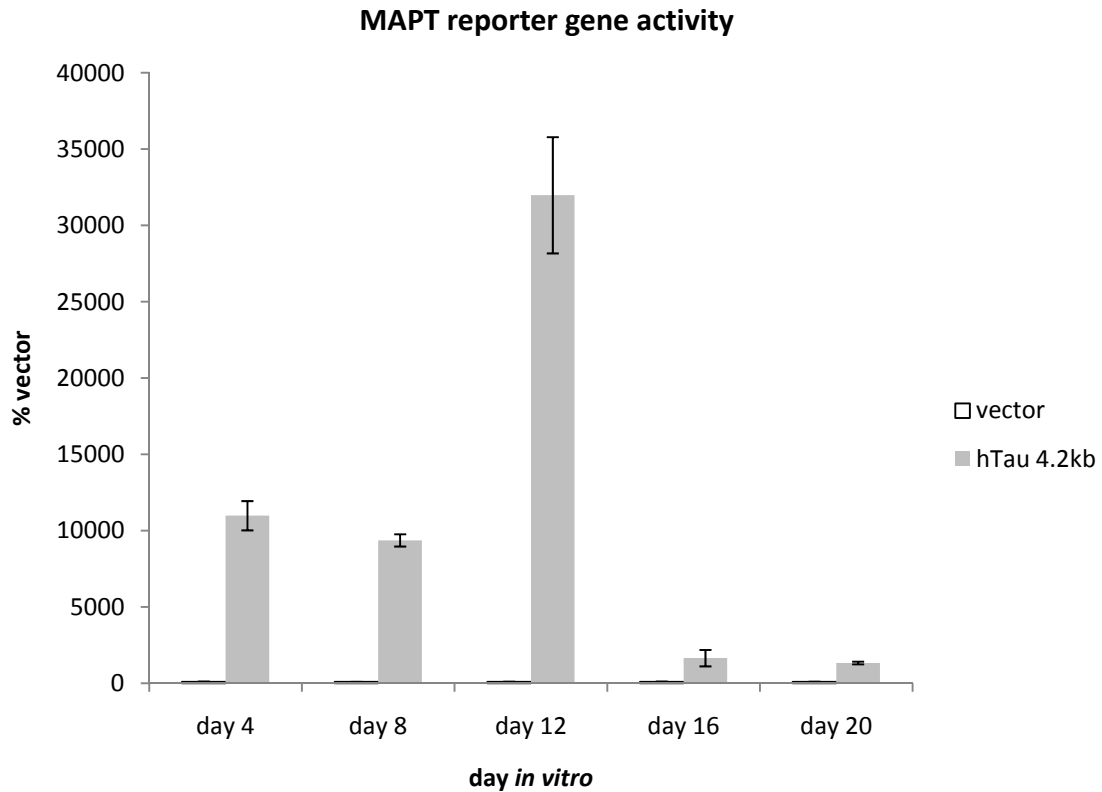
Activity of the *APP* upstream promoter region was measured at various time points. Plasmid DNA (pGL3-Basic) containing the firefly luciferase reporter gene under control of 1.2kb of the *APP*, or promoter region was transfected into primary cultures at the various time points indicated. A reference plasmid containing the *renilla* luciferase gene under control of the SV-40 promoter was co-transfected with the experimental plasmid or a promoterless empty vector plasmid, and data were calculated as relative to *renilla* luciferase activity. *APP* promoter activity was low at day 4, then peaked quickly at day 8, and gradually declined at subsequent time points as neurons began to degenerate.

Figure 17: *BACE1* promoter activity untreated time course



Activity of the *BACE1* promoter region was measured at various time points. Plasmid DNA (pGL3-Basic) containing the firefly luciferase reporter gene under control of 3.3kb of the *BACE1* promoter was transfected into primary cultures at the various time points indicated. A reference plasmid containing the *renilla* luciferase gene under control of the SV-40 promoter was co-transfected with the experimental plasmid or a promoterless empty vector plasmid, and data were calculated as relative to *renilla* luciferase activity. *BACE1* promoter activity was virtually undetectable at day 4, then peaked quickly at day 8, and gradually declined at subsequent time points.

Figure 18: *MAPT* promoter activity untreated time course



Activity of the *MAPT* promoter region was measured at various time points. Plasmid DNA (pGL4.14) containing the firefly luciferase reporter gene under control of 4.2kb of the *MAPT* promoter region was transfected into primary cultures at the various time points indicated. A reference plasmid containing the *renilla* luciferase gene under control of the SV-40 promoter was co-transfected with the experimental plasmid or a promoterless empty vector plasmid, and data were calculated as relative to *renilla* luciferase activity. *MAPT* promoter activity was readily detectable at early time points and came to a sharp peak at day 12 when neuronal and synaptic proteins peak, then declined to nearly undetectable levels when the neurons began to degenerate.

BACE1 and *MAPT* deletion series

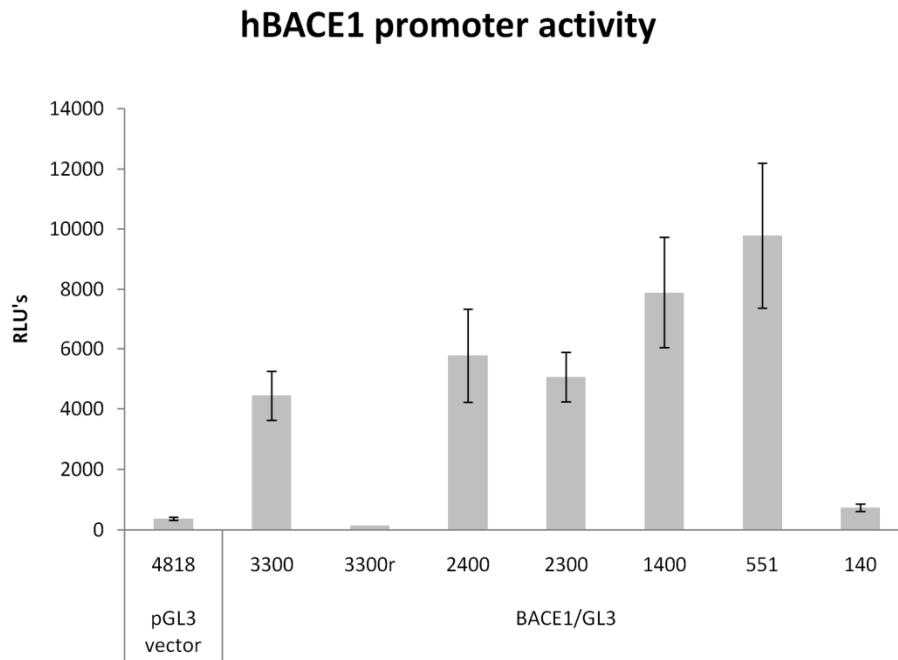
A deletion series of pGL3-Basic based plasmids containing successively truncated fragments of the *BACE1* upstream regulatory region were transfected into cultured primary cells at day 14 *in vitro*. While some slight variation was seen between the longer fragments, the largest differences were observed with the shorter fragments. The 551bp fragment demonstrated the highest transcriptional activity, while longer fragments were less active, suggesting the presence of sequences that are inhibitory in these cells (figure 19). Transcriptional activity of the smallest 140bp fragment was only slightly higher than the empty vector, suggesting a strong positive regulatory element within the ~300bp difference between these two shortest fragments.

A similar strategy was applied to investigation of the *MAPT* promoter, in which reporter plasmids containing three different overlapping fragments of the upstream regulatory region were transfected into cultured primary cells (figure 20). The strongest signal was observed with the 596bp fragment, and successively lower transcriptional activity was observed with larger promoter fragments. This suggests the presence of inhibitory sequences increasing with distance from the *MAPT* gene transcription start site.

Effects of A β treatment on gene promoter activity

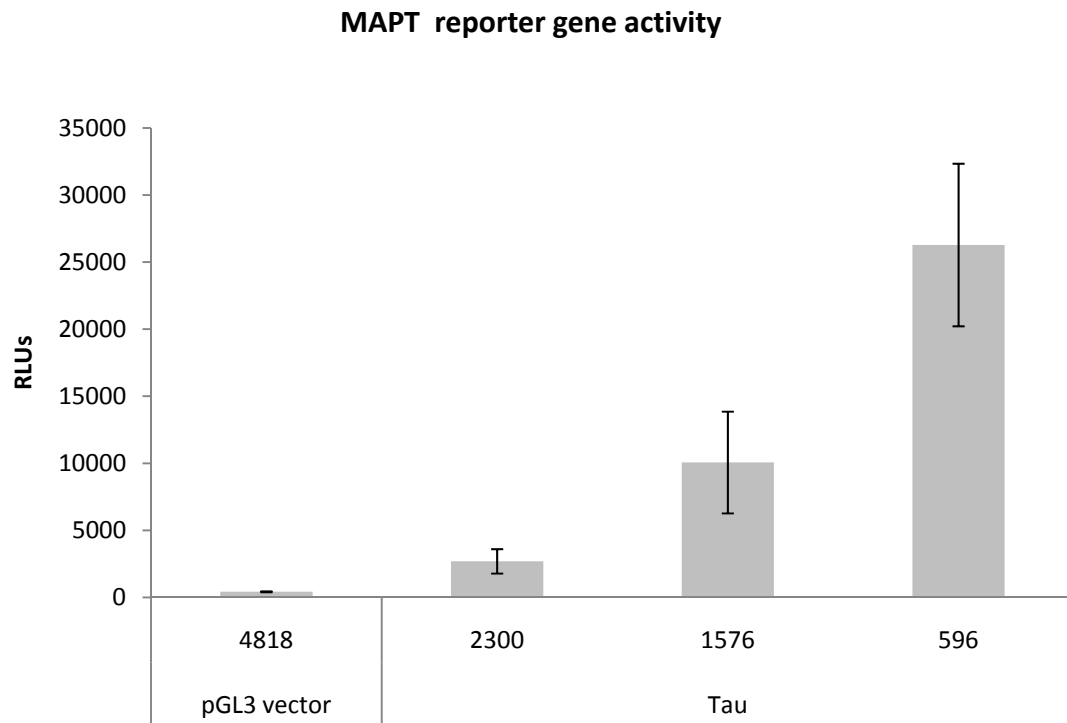
To test the effect of A β on transcriptional activity of three of the genes most directly implicated in AD, reporter constructs containing fragments of the upstream regulatory regions of the *BACE1*, *APP*, and *MAPT* genes were transfected into cultured

Figure 19: *BACE1* promoter activity



A series of pGL3-Basic based deletion constructs containing fragments of the human *BACE1* upstream regulatory region were transfected into primary cultures at day 14 *in vitro* and luciferase reporter gene activity was measured at day 16. Promoter activity varied with length of the insert, and highest activity was observed with the 551bp fragment, suggesting the presence of inhibitory elements upstream of this region.

Figure 20: *MAPT* promoter activity



A series of pGL3-Basic based deletion constructs containing fragments of the human *MAPT* upstream regulatory region were transfected into primary cultures at day 14 *in vitro* and luciferase reporter gene activity was measured at day 16. Promoter activity varied with length of the insert, and highest activity was observed with the 596bp fragment, suggesting the presence of inhibitory elements upstream of this region.

primary cells. After transfection, these cells were then treated with 1 μ M A β , a concentration below the toxicity threshold, for two days prior to the reporter gene assay.

Relative to vehicle, A β_{1-40} increased transcriptional activity of both the 3.3kb and 2.4kb *BACE1* promoter fragments (figure 21b). Conversely, in the 3.3kb fragment, A β_{1-42} reduced transcriptional activity dramatically. This activity of A β_{1-42} was not observed in the 2.4kb promoter fragment (figure 21c), suggesting the presence of some sequence between 2.4kb and 3.3kb upstream of the *BACE1* gene transcription start site involved in transcriptional suppression of the *BACE1* gene as a response to A β_{1-42} . This effect was also observed with the 3.3kb fragment treated with the A β_{42-1} reverse sequence peptide.

Similar treatments with 1 μ M A β were carried out in cells transfected with the *APP* promoter construct (figure 22). In these cells, no effect of A β_{1-40} , A β_{1-42} , or A β_{42-1} treatment was observed. In cells transfected with a pGL4.14 based plasmid containing 2.3kb of the *MAPT* gene upstream regulatory region, neither A β_{1-40} nor A β_{1-42} had a significant influence on transcriptional activity of the *MAPT* promoter (figure 23). In contrast, the reverse A β_{40-1} peptide reduced promoter activity to nearly undetectable levels. This would typically be a sign of toxicity or poor transfection efficiency, however the activity of this plasmid in other cells in the same culture plate would suggest good transfection efficiency. Additionally, there was no apparent difference in LDH secretion between A β_{40-1} treated cells and vehicle treated cells.

Figure 21: *BACE1* promoter activity

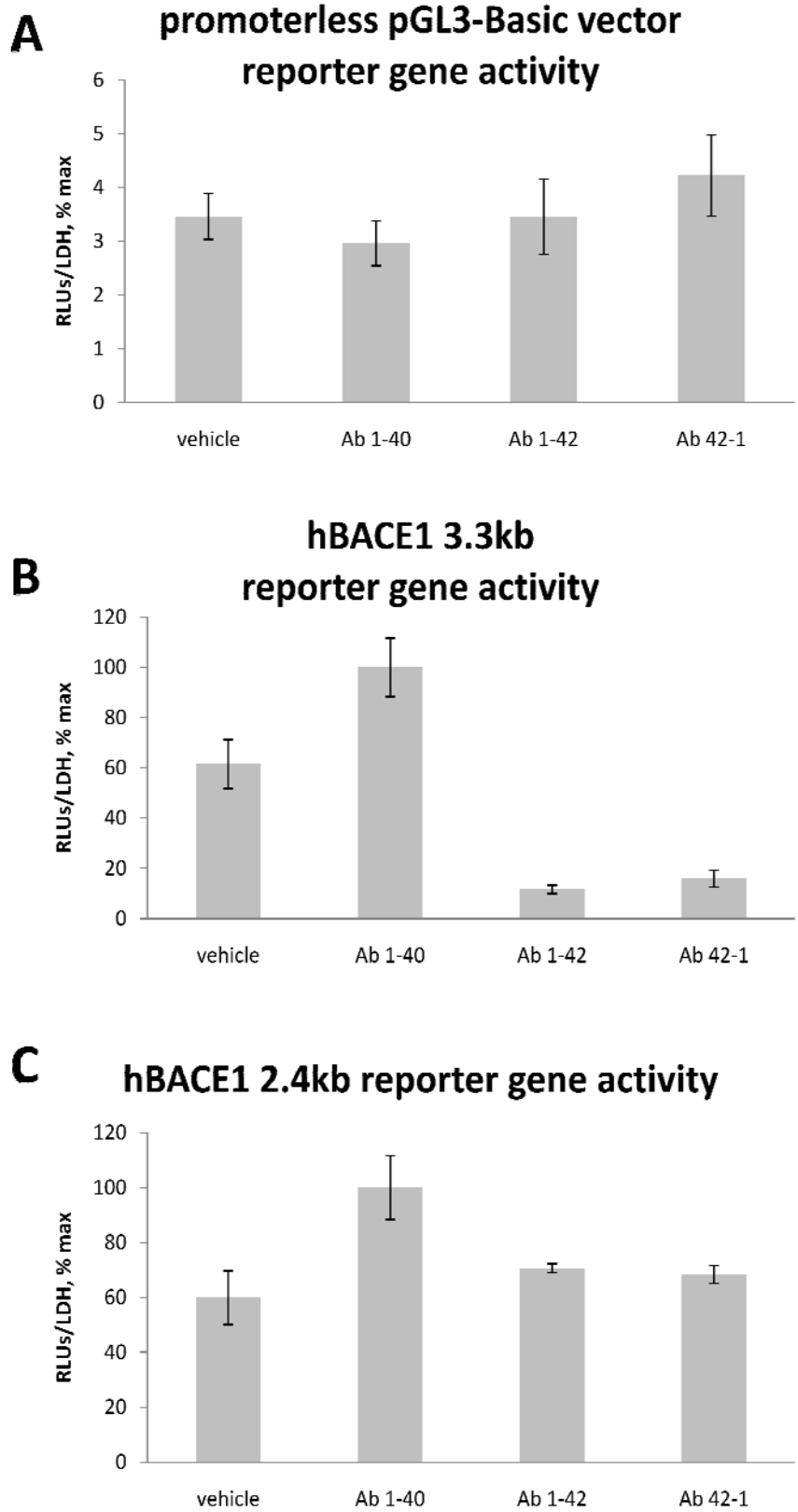
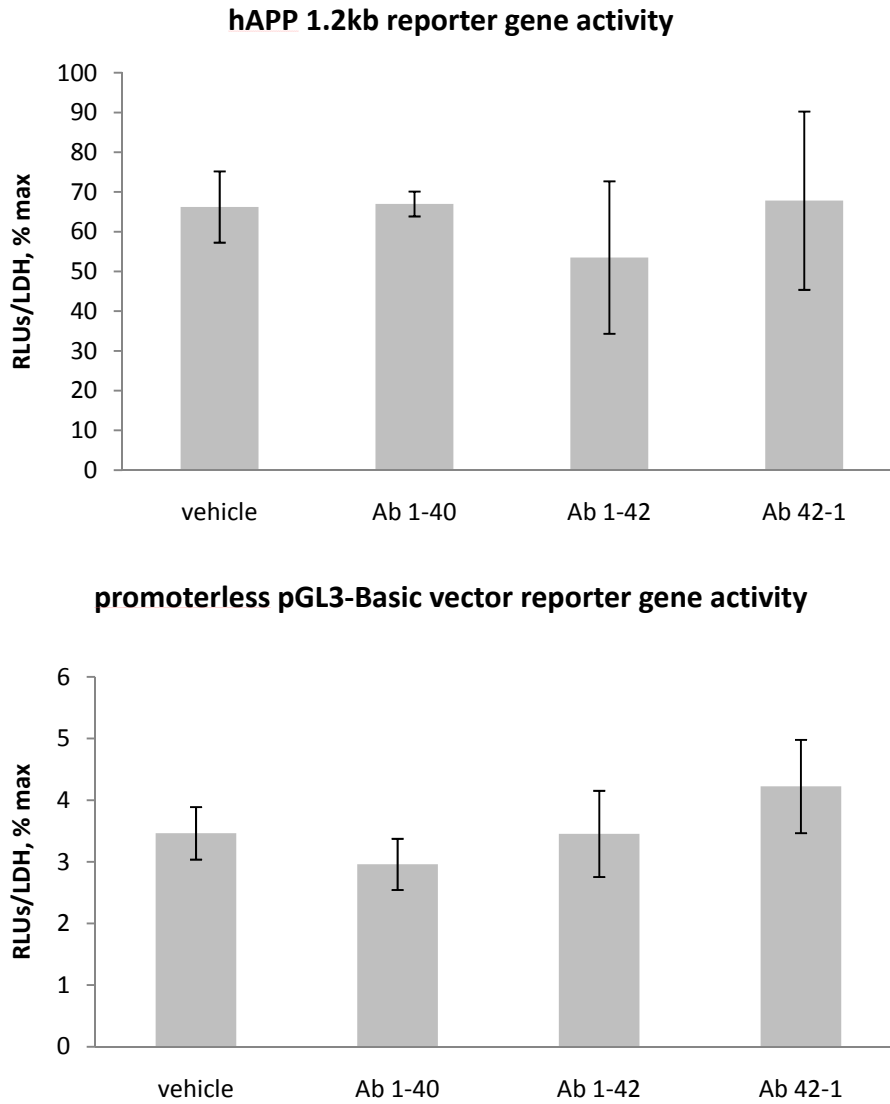


Figure 21: *BACE1* promoter activity

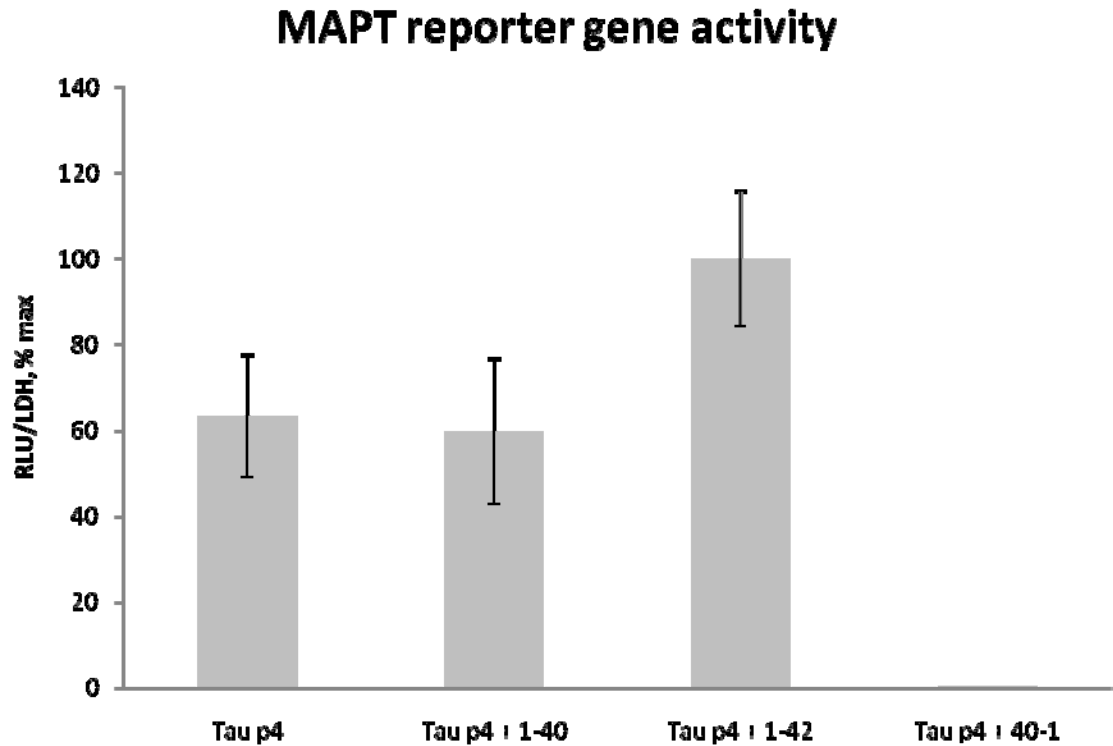
Two of the previously tested pGL3-Basic based deletion constructs containing 3.3kb or 2.4kb fragments of the human *BACE1* upstream regulatory region, or the empty vector, were transfected into primary cultures at day 14 *in vitro* and the cells were treated with 1 μ M A β ₁₋₄₀, A β ₁₋₄₂, or a A β ₄₂₋₁ reverse peptide as a negative control. Luciferase reporter gene activity was measured at day 16. Significant (p<0.05) increases in promoter activity were observed for both constructs with A β ₁₋₄₀ treatment, relative to vehicle, indicating a common stimulatory response element in both. An inhibitory effect was observed with A β ₁₋₄₂ treatment in the longer 3.3kb promoter fragment, suggesting the presence of an A β ₁₋₄₂ specific inhibitory region between 2.4kb and 3.3kb upstream of the transcription start site. However, a similar response was observed with the reverse A β ₄₂₋₁ peptide, suggesting a non-specific mechanism for this inhibitory activity.

Figure 22: *APP* promoter activity



A pGL3-Basic based construct containing a 1.2kb fragment of the human *APP* upstream regulatory region, or the empty vector, was transfected into primary cultures at day 14 *in vitro* and the cells were treated with $1\mu\text{M}$ $\text{A}\beta_{1-40}$, $\text{A}\beta_{1-42}$, or a $\text{A}\beta_{42-1}$ reverse peptide as a negative control. Luciferase reporter gene activity was measured at day 16. No significant effect on promoter activity was observed with any treatment.

Figure 23: *MAPT* promoter activity



A pGL4.14 based construct containing a 2.3kb fragment of the human *MAPT* upstream regulatory region, or the empty vector, was transfected into primary cultures at day 14 *in vitro* and the cells were treated with 1 μ M A β ₁₋₄₀, A β ₁₋₄₂, or a A β ₄₀₋₁ reverse peptide as a negative control. Luciferase reporter gene activity was measured at day 16. A significant decrease in transcriptional activity was observed with A β ₄₀₋₁ treatment ($p < 0.05$), but no other treatment effects were observed.

Effect of p25 overexpression on *APP*, *BACE1* and *MAPT* gene promoter activity

The influence of the proapoptotic protein p25 on *APP*, *BACE1*, and *MAPT* promoter activity was investigated by co-transfection of a p25-GFP fusion protein developed previously (Wen et al., 2008), which was designed to express the active fragment of the p35 protein (figure 24). There was no significant effect of p25 overexpression on *APP* or *BACE1* promoter activities, however a significant increase in *MAPT* promoter activity was observed ($p < 0.05$).

Figure 24: APP promoter activity

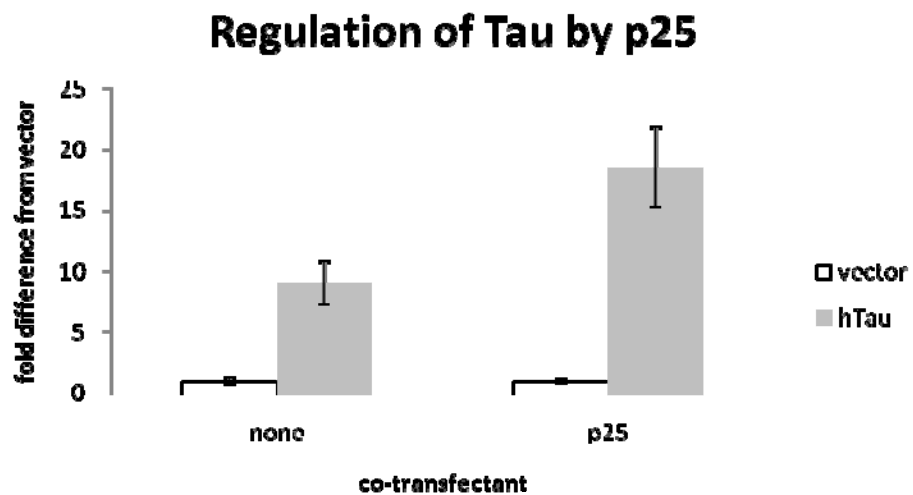
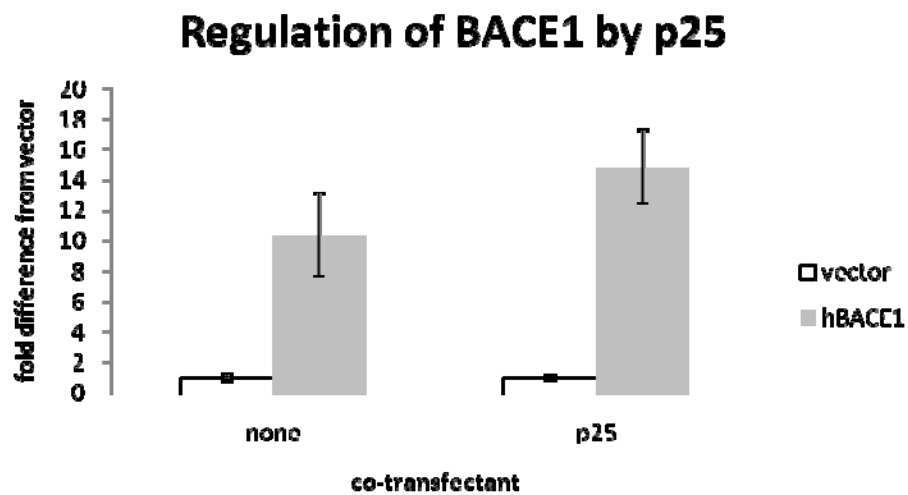
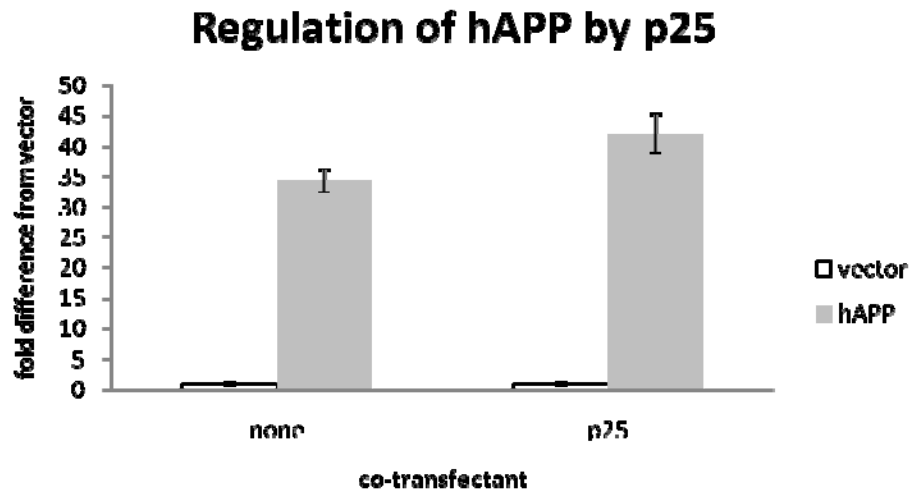


Figure 24: *APP* promoter activity

Three pGL3-Basic based constructs containing fragments of the upstream regulatory region of *APP* (1.2kb), *BACE1* (3.3kb), *MAPT* (2.3kb) , or the empty vector were transfected into primary cultured cells at day 14 *in vitro*. Cells were simultaneously transfected with a pcDNA based p25 expression construct in order to determine the effect of p25 overexpression on transcriptional activity of these three genes. Cells that were not transfected with the p25 construct were co-transfected with an equal amount of pBLCAT3 DNA to avoid transfection efficiency differences. Overexpression of p25 did not significantly influence either the *APP* or *BACE1* promoter activities, but it did result in a significant increase in Tau promoter activity ($p < 0.05$).

Discussion

Differences in promoter activities were elicited by several types of manipulation. Differences in promoter activity were observed between different lengths of each gene promoter region investigated. Differences in promoter activity with time *in vitro*, which most likely correspond to changes in neuronal maturity and viability, as well as changes in relative cell populations (e.g., neuronal vs. glial populations) over time were observed. And finally, changes in gene promoter activity were observed in response to both an exogenously applied signaling molecule ($A\beta$), and an intrinsically overexpressed signaling molecule (p25). The observed response to overexpressed p25 is particularly interesting in this system, since this requires either a very strong interaction, or transfection of two plasmids into the same cell with relatively high frequency, to overcome signal produced in cells transfected with only the luciferase-containing plasmid. Primary neuronal cells have been notoriously difficult to transfect efficiently, and transfection efficiency is dependent upon a number of factors, including the substrate on which the cells are grown (Wiesenhofer and Humpel, 2000). However, using the luciferase reporter gene system and a sensitive luminometer, primary cortical cells transfected with an efficient lipid-mediated transfection system produced readily detectable signal (Bailey et al., 2007; Wen et al., 2008).

Successive deletions of *BACE1* and *MAPT* gene promoter regions studied were compared for relative activity levels in cultured primary cortical cells. This type of evaluation of a gene promoter region can provide only general, but very useful, information about the general structure of the promoter region that can be used to

guide subsequent investigations. In the *BACE1* promoter, successively smaller promoter fragments produced increasing signal, progressing from the largest 3.3kb promoter fragment up to the 551bp construct. The observed pattern of promoter activities suggests that the most transcriptionally active sequences are concentrated in a relatively short upstream region, though sequences several kilobases upstream of the transcription start site also contribute to transcriptional activity of the *BACE1* gene. Additionally, the lack of transcriptional activity in the reverse-orientation 3.3kb promoter construct indicates that the transcriptional activity of this sequence is directional in nature. Down-regulation of the *BACE1* gene promoter activity is one potential strategy for preventing or treating AD, and these data provide information that could lead to drug target identification in the future. Transcriptional inhibition could be achieved, for example, by stimulating activity of an inhibitory transcription factor that might bind upstream of the sequence contained in the 551bp construct. Conversely, reduced *BACE1* transcription could also be achieved through inhibition of stimulatory factors that regulate the highly active 551bp promoter fragment that produced the highest level of transcriptional activity in this assay. The type of research suggested, aimed at identifying specific proteins that bind within the proximal promoter and 5' untranslated region of the *APP* promoter, has been reported (Lahiri et al., 2005a). This study resulted in the identification of several potential nuclear protein binding sites within the 5' untranslated region of the *APP* gene that may confer tissue specificity, and represent novel drug targets that could be used to modify the course of AD. Given the large number of putative transcription factor binding sites in the *BACE1* promoter region

(Sambamurti et al., 2004), further studies of this kind seem promising for the identification of new drug targets.

These data could be useful to guide future experiments aimed at identification of therapeutic targets in AD. This approach would start with *in silico* identification of potential transcription factor binding sites using a growing array of available informatics tools. The activity of promising candidates identified with these tools could then be tested by site-directed mutagenesis of the sequences in the existing plasmids. A number of promising candidate transcription factors that affect both *BACE1* and *APP* have been proposed (Lahiri et al., 2006), and our constantly expanding understanding of transcriptional regulation will almost certainly reveal more in the future. Any change in reporter gene activity that results from mutation of these candidate sequences would implicate a specific transcription factor that could possibly represent a new therapeutic target. Though highly labor-intensive, this approach could be expected to produce a number of specific candidates, or candidate pathways, which could be targeted for treatment of AD. Using similar techniques, the hypoxia-inducible factor HIF-1 has been suggested to have a role in hypoxia mediated increases in *BACE1* expression and A β generation (Sun et al., 2006), and thus may be worthwhile to pursue as a therapeutic target.

Promoter polymorphisms in the *MAPT* gene have been described that affect risk of developing AD (Sun and Jia, 2009), but much less attention has been given to other factors that could influence transcriptional regulation. In a mouse model of AD, knocking out the *MAPT* gene ameliorated memory deficits and AD-like pathology in the

hippocampus (Gomez de et al., 2010), which suggests that reducing tau expression in the brain may be a valid approach to the treatment of AD. The approximately half kilobase upstream of the *MAPT* gene transcription start site appears to be the most active, similar to the *BACE1* gene promoter. Conversely, transcriptional activity decreases much more rapidly with increasing promoter fragment size. This suggests the presence of sequences within the difference between the 596kb and 1576kb promoter fragments, i.e., the kilobase between 0.5kb and 1.5kb upstream of the transcription start site, contain sequences that negatively regulate transcription in these cells. While the concept of targeting the production of tau has not been so thoroughly considered or pursued as the approach of reducing *BACE1* or *APP* protein production, it may be worth testing. The *MAPT* protein becomes involved in AD when it is hyperphosphorylated, however it may be possible that reduced levels of total tau might bring about reduced levels of the phosphorylated form, though this has not yet been tested.

Activities of the *APP*, *BACE1*, and *MAPT* promoters were tested at multiple time points over the lifespan of these primary cortical cultures. When compared to the time-dependent changes in protein markers of glial and neuronal cells in these cultures, some inference can be made regarding the specificity of these promoters to different cell types. For example, the *APP* gene was active at all time points tested, including earlier time points when neuronal cells are more strongly represented, and later time points when these cultures consist primarily of glial cells, suggesting that this promoter is active in both neuronal and glial cells. In contrast to this, the *MAPT* gene promoter was only active at time points when neuronal cells are highly represented. This was

expected, as the tau protein is often used as a neuron-specific marker. *BACE1* gene promoter activity appears to follow a pattern that is intermediate between *APP* and *MAPT* in that it is most active at time points when neuronal and synaptic markers are also at their highest levels, but retains some detectable activity even at 21 days in culture when the cell population in the culture plate is almost purely glial.

These data suggest that the now-commonplace lipid-based transfection method used here is effective in transfecting both neuronal and glial cells. It has been demonstrated that these cultures contain a mixture of cell types, but it is unknown whether this transfection technique preferentially transfects one cell population with greater efficiency than another. It seems reasonable to assume that all subpopulations within these cultures are transfected with equal efficiency, however, it would be worthwhile to test this assumption experimentally. Preliminary experiments have shown that GFP can be expressed in these cells, and a variety of cellular morphologies were observed. Fluorescence of GFP survives after the cells have been fixed in 4% paraformaldehyde, so it should be possible to combine transfection with immunocytochemistry to determine whether there are differences in the transfection rates for glial and neuronal subpopulations in these cultures.

It has been suggested that A β can regulate various aspects of neuronal physiology, and perhaps affect transcription of various genes directly (Bailey et al., 2009; Ohyagi et al., 2005). To test the possible effects of A β on gene regulation, cultured primary cells transfected with the previously tested plasmid constructs containing promoter regions for the *APP*, *BACE1*, and *MAPT* genes were treated with vehicle,

$A\beta_{1-40}$, $A\beta_{1-42}$, or the reverse sequence $A\beta$ peptides prior to the onset of neurodegeneration. Neither the pGL3-Basic or pGL4.14 vector plasmids responded to this treatment, however there were some responses observed in the plasmids containing promoter regions of these genes. Two plasmids from the *BACE1* deletion series were tested, and it was found that transcriptional activity was reduced in the 3.3kb *BACE1* promoter construct by $A\beta_{1-42}$ ($p < 0.05$), but not $A\beta_{1-40}$. It was unexpected that treatment with the reverse $A\beta_{42-1}$ peptide also reduced the activity of the *BACE1* promoter. This would typically suggest a non-specific interaction, however the effects of $A\beta_{1-42}$ and $A\beta_{42-1}$ were not observed in the shorter 2.4kb promoter fragment of this gene. This suggests that the reverse peptide may interact directly with the *BACE1* promoter sequence between 2.4kb and 3.3kb upstream of the transcription start site, or that it interacts with an upstream signaling cascade that regulates *BACE1* transcriptional activity via this region. This reverse peptide requires some further investigation, as it may mimic an as-yet unidentified signaling molecule, which is suggested by its interaction with a specific region of the *BACE1* promoter.

In a somewhat similar fashion, neither $A\beta_{1-40}$ nor $A\beta_{1-42}$ affected transcriptional activity of the *MAPT* promoter, but the reverse $A\beta_{40-1}$ peptide significantly reduced *MAPT* promoter activity to nearly undetectable levels. Of the three promoters reported here, this is the only one that was tested in the newer pGL4 series of plasmids rather than the older pGL3 series. In the same batch of cells, another unrelated pGL4.14-based construct was also tested for $A\beta$ effects and it responded similarly to $A\beta_{40-1}$ treatment in the absence of apparent toxic effects. It is possible that the pGL4.14 plasmid may be

responsive to this reverse peptide and this should be investigated further. While there is no known homolog to the reverse peptide in a mammalian proteome, these plasmids contain sequences necessary for prokaryotic replication as well as sequences of viral origin, so it is plausible that the parent plasmid rather than the insert is responsible for this effect.

A more direct interaction between signaling pathways and gene regulation was also tested by co-transfection with reporter plasmids containing the *APP*, *BACE1*, and *MAPT* promoter sequences with an expression vector containing a p25 cDNA. The p25 protein is a degradation resistant calpain-cleaved fragment of p35 which activates cyclin dependent kinase 5. An increase *BACE1* promoter activity in response to the co-transfected p25 expression vector was previously described in the readily transfectable PC12 cell line (Wen et al., 2008), however this effect was not demonstrated in cultured primary cells. No effect of p25 expression was observed for either *APP* or *BACE1*, however, p25 significantly increased *MAPT* promoter activity. It is surprising that the effect of p25 over expression previously observed in PC12 cells did not replicate in these primary cultures. However, as discussed above, primary cells typically exhibit low transfection efficiency, and this effect depends on a sufficient number of cells being transfected with both plasmids to observe an effect over what is probably a more numerous population of singly-transfected cells. Additionally, the PC12 cell line is known to have aberrant gene and cell cycle regulation by nature of being a tumor-based cell line. It is therefore plausible that the difference between PC12 and primary tissue culture data is due to an interaction of p25 with an abnormal gene regulation system in

PC12. This highlights the importance of verifying results showing differences in transcriptional activity obtained from tumor-based cell lines in primary cells that have intact gene and cell cycle regulatory systems. With the present data, it is not possible to determine whether this discrepancy is due to a unique effect of p25 over-expression in the PC12 cell line, or transfection efficiency issues in primary cells.

Primary cells have typically been a difficult model for transfection experiments, however several plasmids have been transfected into these primary cortical cultures using a relatively simple lipid-based delivery system. Luminescent signal produced by the luciferase reporter gene in these plasmids was readily quantified using a standard luminometer. However, there are caveats to bear in mind when using this type of tissue culture model rather than cell line based models. There are currently no data available regarding the relative transfection efficiencies of neuronal vs. glial cells in these cultures, so it is possible that the activities of the gene promoters examined here may be overrepresented by either glial or neuronal cells. Furthermore, in cells that exhibit low transfection efficiencies, it may become difficult to assess the interaction of two plasmids. If the proportion of cells transfected with the two individual plasmids is low, then the proportion of cells transfected with both plasmids should be expected to be lower. This will infuse significant noise, represented by singly transfected cells, into the final reporter gene assay. Thus, more efficient (e.g., viral) DNA delivery techniques may be required to reliably observe interactions between more weakly interacting genes, while more strongly interacting genes may produce observable effects with the techniques used here.

With regard to modeling AD, artificially increasing p25 expression or A β in the medium mimics specific aspects of the disease. While these manipulations cannot be tested in this model with regard to the ultimate consequences of AD pathology, e.g., memory deficits, these changes are well established in the AD brain and linked to several pathological mechanisms. Therefore these results must be interpreted within the constraints of an individual cell. In other words, only effects at the level of the single cell can be observed here, while higher order tissue level effects (e.g., amyloid plaque formation) or organ level effects (e.g., loss of cholinergic afferents to the hippocampus) are absent. Such larger scale effects can only be observed in an intact tissue or animal model. However, investigation of cell level effects such as those studied here can be informative regarding the mechanisms of these larger scale effects. Typically, pathogenic mechanisms, as well as therapeutic interventions, act at the level of the individual cell in order to produce their larger-scale effects. Thus, while this type of tissue culture is incomplete regarding the effects of A β and p25 in the pathogenesis of AD, or other manipulations that might be carried out in this model, this type of study has implications on larger scales.

Section 3: Mechanistic study of the NMDA receptor antagonist memantine in cerebrocortical cultures

Introduction

Several drugs have been developed and approved for the treatment of AD, although the mechanisms by which they produce their clinical effects are still poorly understood. Four of the five drugs that have been approved for the treatment of probable AD are cholinesterase inhibitors, and the fifth and most recent is the NMDA receptor antagonist memantine. Development of cholinesterase-based treatments has been guided by early observations in the AD brain that the cholinergic system, especially in the hippocampus, is severely affected (Bartus et al., 1982; Davies and Maloney, 1976). Subsequent work led to the hypothesis that glutamate-mediated excitotoxicity is involved in neurodegenerative disease, including AD (Albin and Greenamyre, 1992; Greenamyre, 1986), which provided the rationale for the exploration of NMDA receptor inhibiting drugs such as memantine for use in AD.

Given that the primary culture model described in Section 1 mimics some aspects of AD at the cellular level, particularly synapse loss and glial activation, it may be informative to study the effects of AD-related drugs on neuron viability and synaptic integrity in this system. The drug memantine is of particular interest among these since it is both the newest drug on the market for the treatment of AD, and it is the first that was not designed to target cholinesterase enzymes in the brain. Memantine is thought to work through neuroprotective mechanisms, preventing NMDA receptor-mediated neuron damage, thus providing the potential for disease modification. While the NMDA

receptor mediates post-synaptic responses to pre-synaptic glutamate release, some pre-synaptic effects of NMDA antagonism have been observed. For example, the NMDA receptor antagonist CPP has been shown to alter morphological characteristics of the presynaptic terminal, as visualized by electron microscopy (Medvedev et al., 2010). Additionally, the NMDA receptor antagonist memantine, but not the NMDA receptor antagonist AP-5, was shown to inhibit neurotransmitter release from presynaptic terminals (Lu et al., 2010).

Reductions in presynaptic markers have been observed in AD brains with concomitant increases in the post-synaptic marker PSD95 (Leuba et al., 2008). The increase in the post-synaptic marker may be a compensatory mechanism for the loss of pre-synaptic terminals, which is thought to be largely responsible for the memory deficits in AD. NMDA receptor involvement has been implicated in a number of toxic mechanisms that reduce synapse density *in vivo*. For example, isolation stress reduced synaptophysin levels, which was reversed by NMDA receptor antagonist treatment (Leussis et al., 2008). A β has been shown to stimulate caspase-8 and -3 mediated degradation of both pre- and post-synaptic markers by a mechanism involving the NMDA receptor (Liu et al., 2010). Treatment with the NMDA receptor antagonist MK-801 before or after traumatic brain injury in an animal model reduced memory deficits, and reduced the loss of presynaptic markers (Phillips et al., 1998). And finally, NMDA receptor activity has been implicated in the response to oxidative stress in several paradigms (Evans et al., 2008; Puntel et al., 2005; Sinhorin et al., 2005; Zaja-Milatovic et al., 2009).

Despite these various toxic mechanisms with relevance to AD, the effect of NMDA receptor antagonist treatment on presynaptic markers has not been thoroughly studied with respect to AD. The degenerating tissue culture model described above exhibits several features that are relevant to AD, including presynaptic marker loss, and therefore further study of NMDA receptor antagonists in this system may provide insight into the potential effects of these drugs in the AD-affected brain. It is hypothesized that the NMDA receptor antagonist memantine will preserve synaptic terminal markers, as well as neuronal viability, in the presence of increasing glutamate concentrations and glial activation. Murine cortical astrocytes are known to express active NMDA receptors (Schipke et al., 2001), although the effect of NMDA receptor antagonists on glial receptors has not been well studied, making predictions in this system difficult. In contrast to cortical neurons, astrocytes are not known to be subject to the excitotoxic effects of high glutamate concentrations. Thus, it is expected that memantine treatment will not affect glial activation, but will protect neurons from the deleterious effects of glial activation.

Results

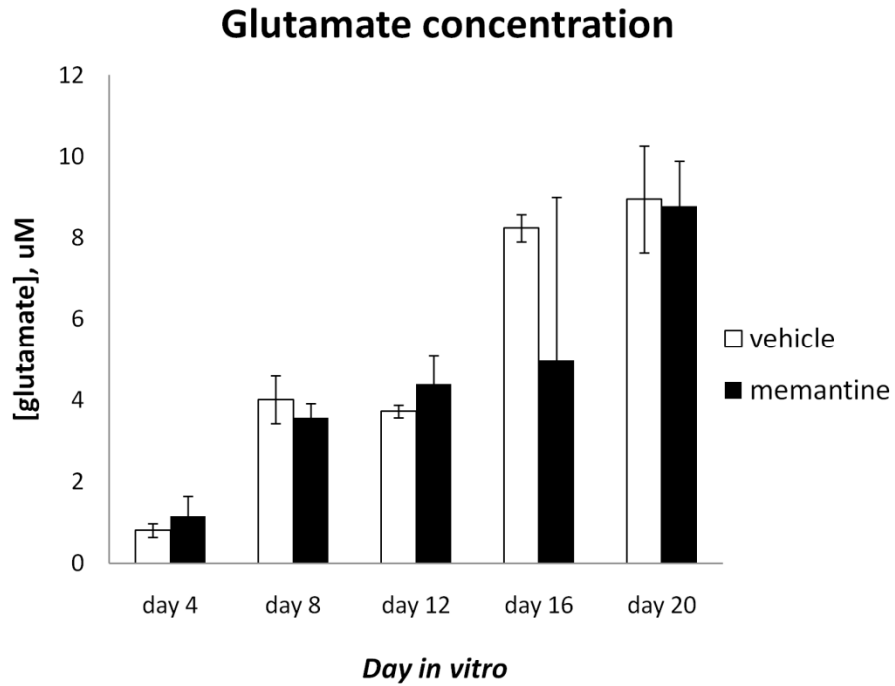
Glutamate concentration time course

To investigate the possible production and release of glutamate in these cultures, glutamate concentrations were measured by HPLC. Glutamate was found to steadily increase in concentration from around 1 μ M at day 4 to about 9 μ M at day 20 (figure 25). Glutamate is not supplied to these cells in the culture medium, however it was expected that some conversion of glutamine to glutamate and some release of glutamate into the culture medium would be observed. Extracellular glutamate is typically regulated very tightly, so the increases at later time points are somewhat surprising. Thus, glutamate-mediated excitotoxicity is a strong candidate for the mechanism of neuronal death in these cultures, which may be of particular interest in modeling AD. Glutamate levels are not affected by memantine treatment and do not follow the pattern of neuronal marker levels, which suggests a primarily glial origin.

Cell viability time course: CTG assay

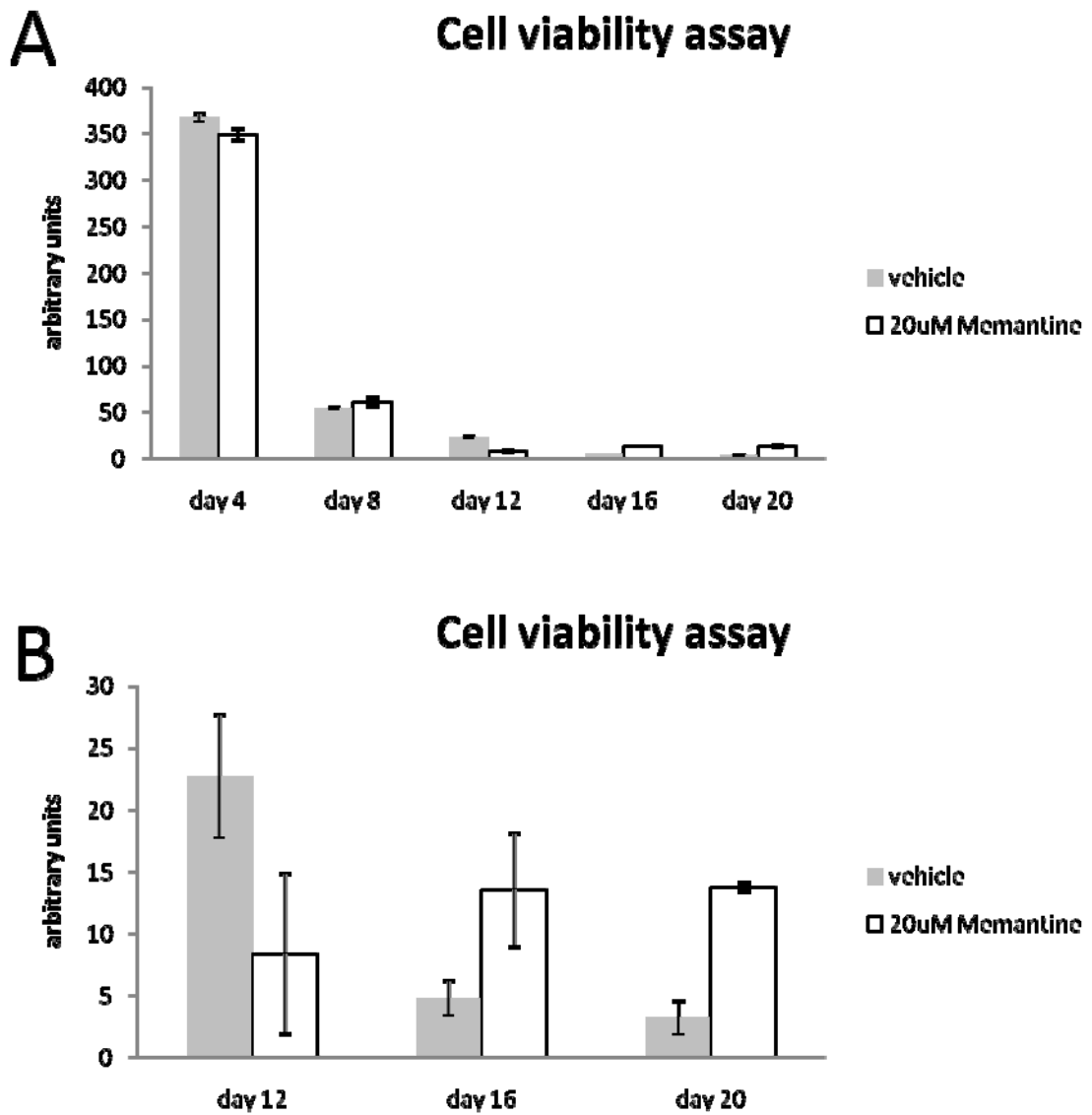
Cells treated chronically with memantine show very similar changes in viability as assessed by the CTG assay during the first several time points observed (figure 26). In both treated and untreated groups, there was a large decline in CTG signal between day 4 and day 8 and gradually declining signal thereafter. Differences between memantine

Figure 25: Glutamate concentration time course



Glutamate concentrations in the media were measured by HPLC. Aliquots of media were cleared of protein by acidification, and supernatants neutralized by the addition of NaOH. Glutamate levels in the media increased steadily over time independent of treatment with 20 μ M memantine. Glutamate concentrations are nearly equal in vehicle and memantine treated samples at day 20 when neuronal proteins are barely detectable in vehicle-treated samples. This suggests a non-neuronal source of glutamate, such as reverse transport, which has been observed in activated astrocytes.

Figure 26: Viability assay, vehicle vs. memantine time course



At the times indicated, cells were lysed and the lysates were quickly frozen and stored at -80°C until the final collection had been frozen on day 20. All lysates were thawed simultaneously and relative ATP concentrations were estimated using the Cell Titer-Glo assay. There was a dramatic drop in signal between day 4 and day 8 and steady but smaller decreases thereafter in untreated cells. The memantine treated cells followed a similar pattern until the latest two time points where ATP levels were higher in treated cells than untreated.

treated and untreated cells began to emerge at day 16 *in vitro*, with memantine producing significant increases in viability relative to vehicle at the final two time points.

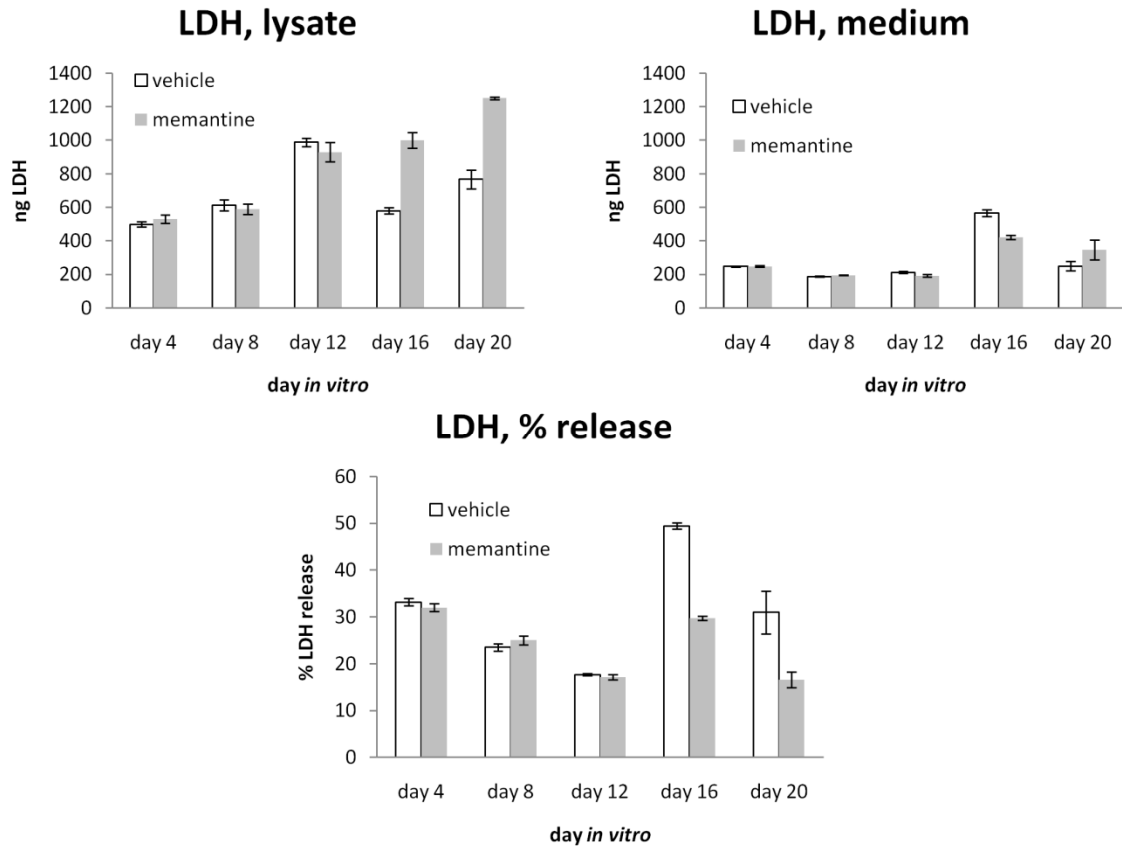
Cell viability and toxicity time course: LDH assay

Media and lysate samples treated with vehicle or memantine were assayed for LDH activity in order to calculate a percent release (figure 27). LDH activity of the lysates of memantine treated cultures steadily increased throughout all time points tested here. LDH activity in vehicle treated cells increased until day 12, after which LDH activity declined by nearly 50%. The percent release of LDH increased at day 16 for both vehicle and treatment groups, but was much higher in vehicle-treated cultures. The calculated percent release declines at day 8 and day 12 while LDH in the lysates are increasing. This might suggest that the low LDH levels measured in the media samples, which appear to be unchanged, are near or below the detection limit of the assay. However, all samples were within the linear range of the standard curve for this assay. An alternate explanation may be increased membrane stability during the early phase of these tissue cultures.

Effect of NMDA channel blocking drugs on LDH release

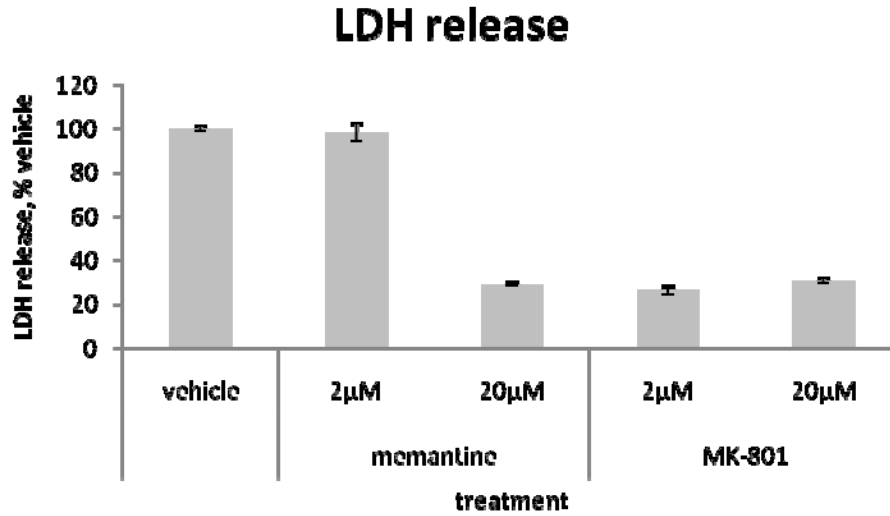
To further investigate the specificity of this protective effect, cultures were treated with either memantine or MK-801, 2 μ M and 20 μ M for both drugs, from day 14

Figure 27: Lactate dehydrogenase, vehicle vs. memantine time course



The total LDH method was applied as an additional measure of cell viability. Small aliquots of lysates (5 μ l) and media (30 μ l) from individual wells were assayed for LDH content, such that the amount of LDH in each aliquot was within the linear range of the assay. The total amount of LDH present in the total volume of each lysate and media sample was calculated from the LDH content of the small aliquot. The amount of LDH retained in the cells (measured in the lysates) is an indication of cell viability, while LDH in the medium is an indication of cell membrane permeabilization, which is interpreted as cell death. Since the number of cells in this culture appears to vary with time, secreted LDH is presented as percent of total, according to the equation: $[\text{media}/(\text{media} + \text{lysate})] \times 100$. LDH in the lysates increases gradually throughout the time course in memantine treated cells, however in vehicle treated cells LDH peaks at day 12, declines at day 16, then continues to increase. Released LDH mirrors changes in retained LDH well. These data are consistent with neuronal loss in untreated cells, but not memantine-treated, and continued cell proliferation.

Figure 28: Lactate dehydrogenase, vehicle vs. memantine



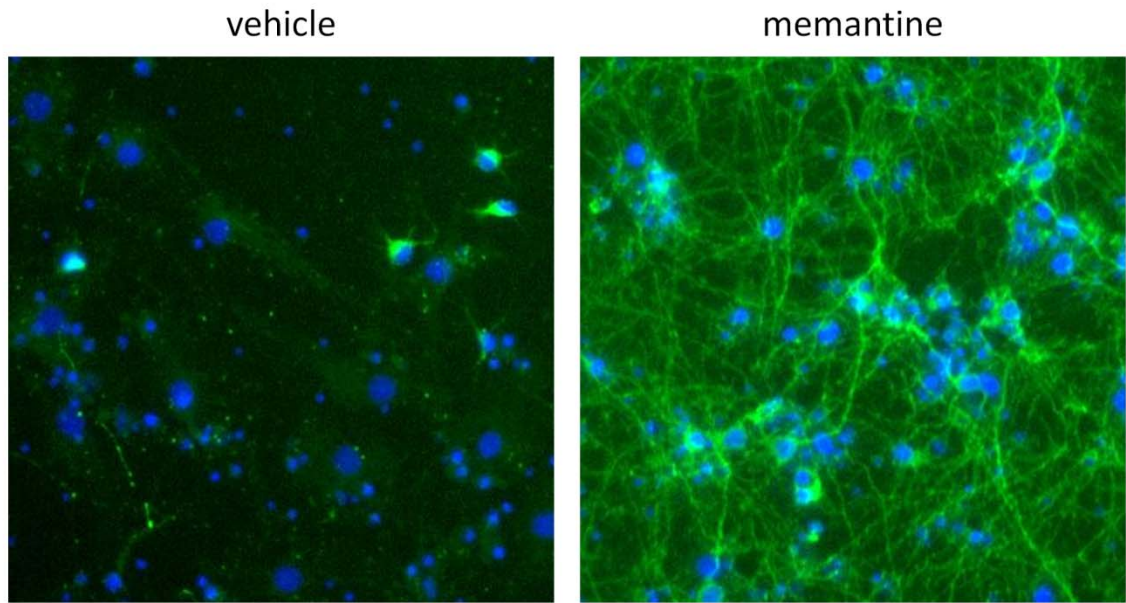
To compare the effect of memantine on LDH release to another NMDA receptor antagonist, cells were treated at a single time point with either memantine or MK-801 at the indicated concentrations. Memantine produced no apparent effect at 2µM, but produced an apparent maximum effect at 20µM. MK-801 produced significant decreases in LDH release at both concentrations, however this is not dose-dependent. This may reflect the higher potency of MK-801 at the NMDA receptor. The LDH-lowering effect of MK-801 suggests that LDH release is dependent on NMDA receptor activation in these cultures.

to day 16 *in vitro* (figure 28). MK-801 is an NMDA channel blocking drug that is mechanistically similar to memantine and was chosen to confirm that the observed reduction in LDH release is dependent on this mechanism. Cultured cells treated with either drug displayed dramatically reduced LDH release at day 16. MK-801 produced an apparently maximum effect at both concentrations, whereas memantine was only effective at the 20 μ M concentration. This difference likely reflects the relative affinities of these drugs for inhibition of the NMDA receptor, with MK-801 inhibiting at much lower concentrations.

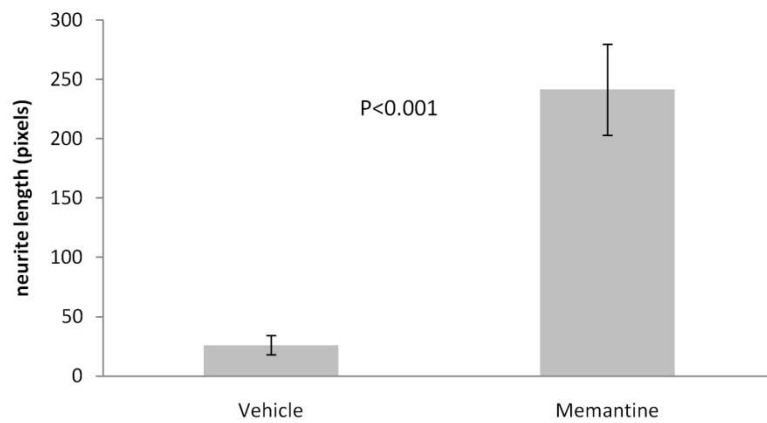
Effect of memantine on neuronal morphology

To examine the effect of NMDA inhibition on neuronal morphology, neurons in cortical cultures were visualized using immunofluorescence techniques (figure 29). Cultures were treated from day 12 to day 16 with 20 μ M memantine and neurons were selectively labeled with an anti-tau antibody (green) and nuclei were labeled with DAPI (blue). These cells demonstrated a dramatic difference in neuronal morphology between vehicle and memantine treated neurons. Memantine significantly increased estimated neurite length approximately ten-fold. These data also suggest a dramatic difference in neuron survival, as few tau-positive cells were visualized in the vehicle treated cultures, while many are present in the memantine-treated group.

Figure 29: Tau ICC, vehicle vs. memantine

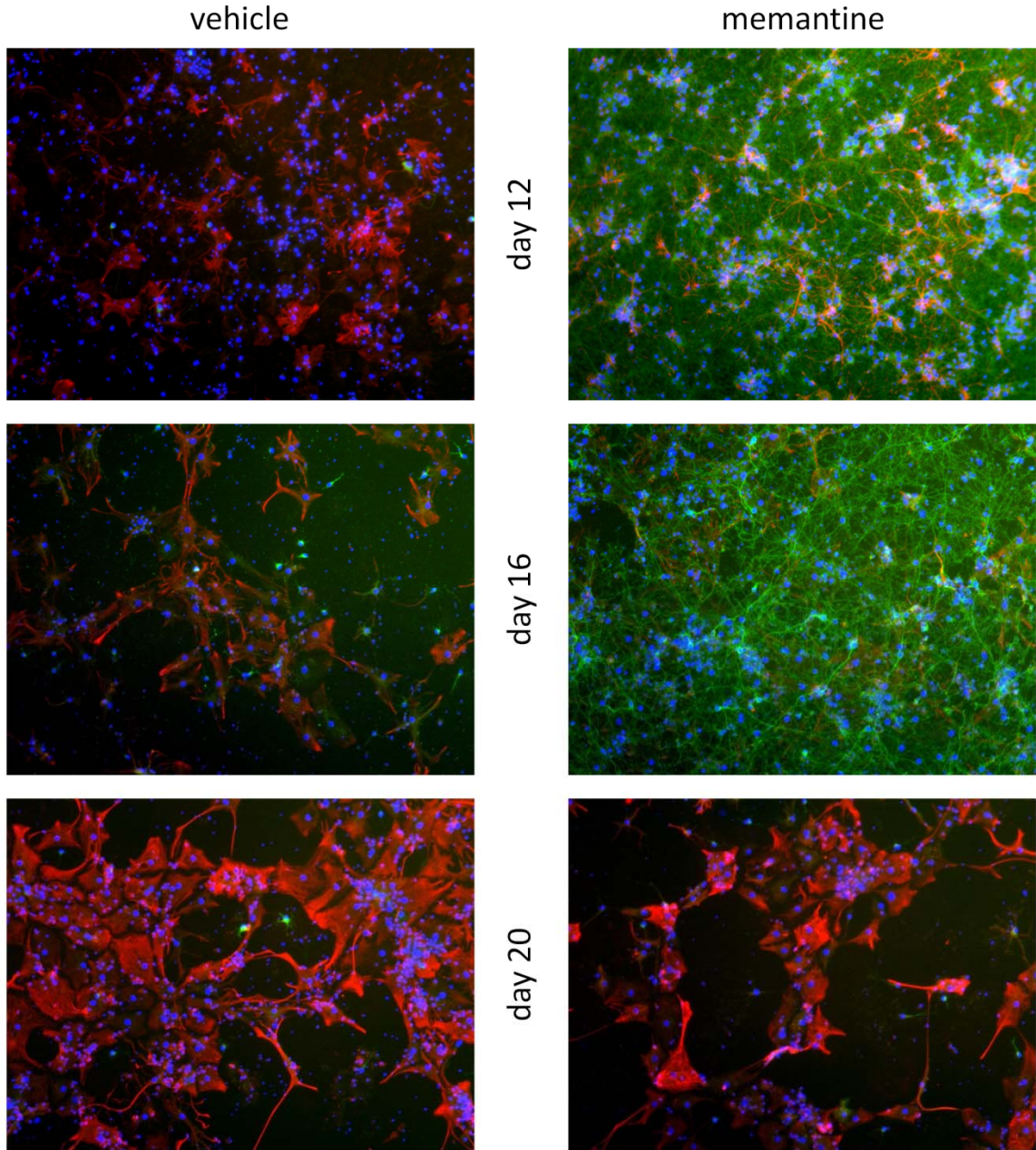


Neurite estimation



To examine the effect of memantine on neuronal morphology, vehicle- and memantine-treated cells were fixed and labeled with an anti-microtubule associated protein tau (anti-tau) antibody and visualized using standard immunocytochemistry techniques. Average neurite length was estimated using a modification of the technique described by Ronn et al. (2000). A large, statistically significant increase in neurite length was observed with memantine treatment.

Figure 30: MAPT and GFAP ICC time course, vehicle vs. memantine



Glia and neurons were visualized by standard immunocytochemistry techniques using anti-GFAP (red) and anti-tau (green) antibodies, respectively. Glia appear to expand over time independent of memantine treatment, however neuronal morphology was preserved, or perhaps even enhanced, by treatment.

Effect of chronic memantine on neuronal morphology and glial proliferation

Cultures treated with memantine or vehicle starting at day 4 were fixed and labeled with anti-GFAP (red) and anti-tau (green) antibodies at day 12, day 16, and day 20 (figure 30). In this batch of cells, a marked difference in tau immunolabeling between vehicle and memantine treated cells is readily apparent. In vehicle treated cultures, a few small cells were positive for tau protein, and only extremely short neurites were observed. Robust neuritic processes were observed at day 12 and day 16 in memantine-treated cultures, but by day 20, tau labeling was similar to vehicle. In both the vehicle treated and memantine treated day 20 cultures, a small number of viable tau-positive cells were present, suggesting that at least a subset of neurons survive the severe neurite pruning that occurs in this model.

Steadily increasing GFAP signal was observed with time. Both the area covered by GFAP immunoreactivity in the culture plate and the size of individual cells appears to increase between day 12 and day 20. Earlier experiments show that GFAP immunoreactivity is extremely limited or undetectable at earlier time points. These changes in glial morphology and GFAP expression are indicative of glial activation in this tissue culture model, which roughly coincides with the loss of synaptic markers and then neurite and NSE loss.

Effect of memantine on SNAP-25 by ICC

The effect of memantine on SNAP-25 immunoreactivity was examined by ICC in a single-labeling experiment (figure 31). Cells were incubated for 48 hours in the presence

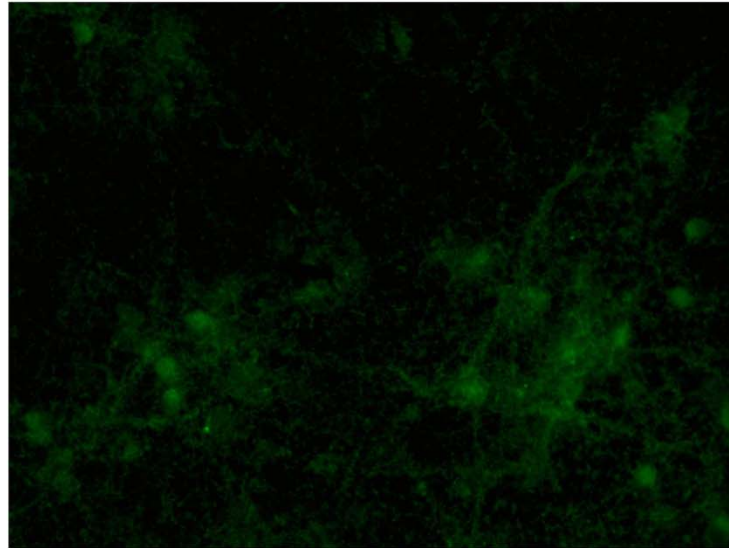
of 20 μ M memantine, then fixed and labeled with an anti-SNAP-25 antibody revealing an apparent difference in the number of synaptic terminals. In these images, the typical SNAP-25 positive puncta are apparent, however resolution is insufficient for quantification. These puncta do appear in a pattern consistent with distribution along axonal processes, as should be expected.

Effect of memantine on NSE levels

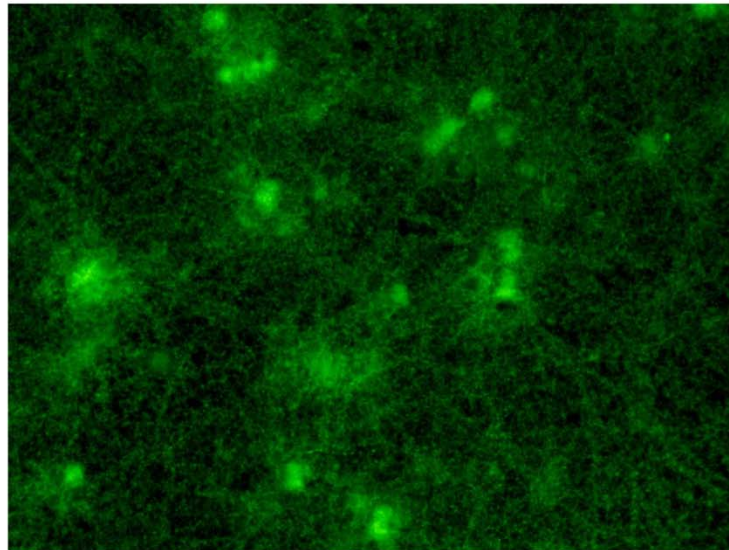
Two parallel time course experiments were carried out using the same lot of cells. One was untreated, and the other was treated with 20 μ M memantine starting at day 3 *in vitro*. To compare neuron survival over time between these paired time course samples, NSE was measured in the lysates (figure 32). All Western blot data are shown as a ratio to β -actin measured in the same blot. NSE levels increased in both sets of samples until day 12, at which time NSE declined in the vehicle group but stabilized, or perhaps increased slightly in the memantine treated group. This could have several implications, including neurogenesis, or increased neurite sprouting (i.e., increased per-neuron cell volume).

Figure 31: SNAP-25 ICC, vehicle vs. memantine

vehicle



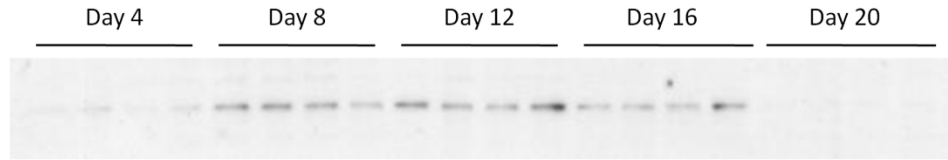
memantine



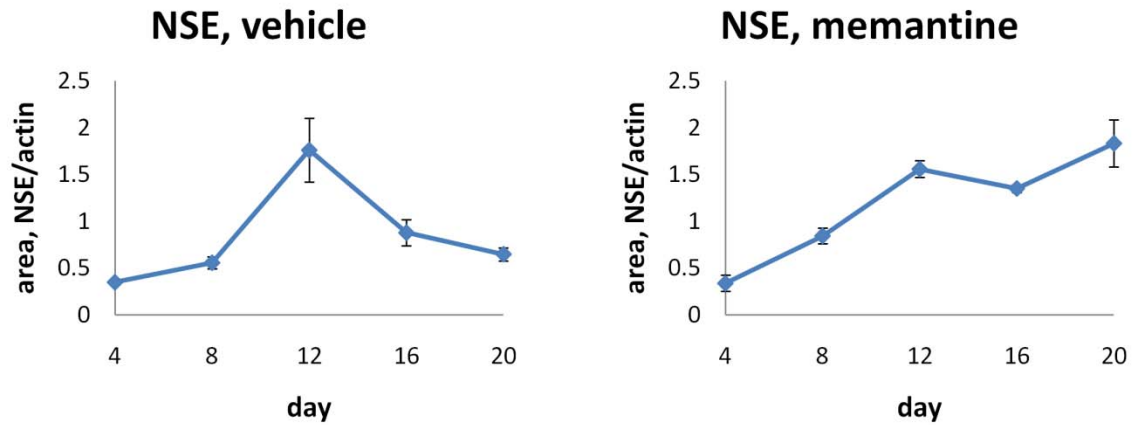
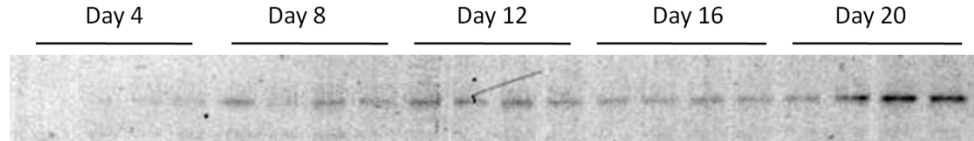
Cells were treated for 48 hours with 20 μ M memantine, then fixed and immunocytochemistry was performed for detection of SNAP-25. Insufficient resolution was available for quantification of SNAP-25 positive puncta, however on visual inspection, SNAP-25 labeling was much stronger in memantine-treated samples.

Figure 32: NSE Western blot, vehicle vs. memantine

NSE: vehicle



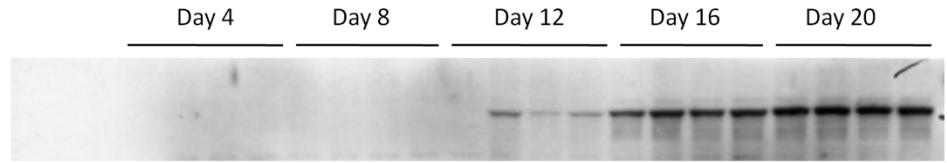
NSE: memantine



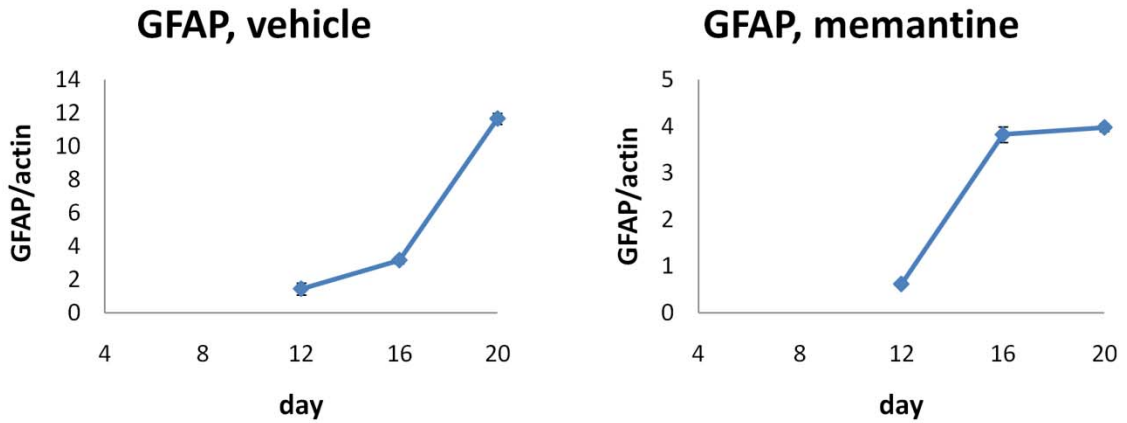
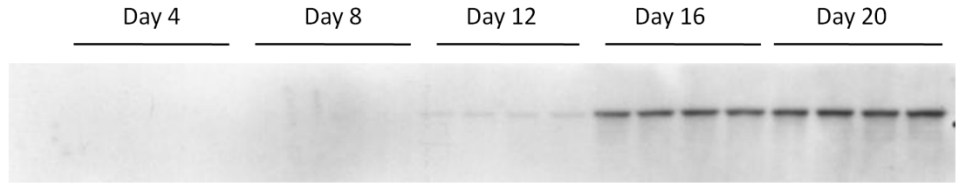
Levels of NSE were compared in vehicle and memantine treated time course experiments. Treatment with 20 μ M memantine started at day 2 *in vitro*. In both time courses, NSE levels were similar until day 12. After peaking at day 12, NSE decreased in vehicle treated cells declined at the two subsequent time points, while levels of this protein were stabilized by memantine treatment.

Figure 33: GFAP Western blot, vehicle vs. memantine

GFAP: vehicle



GFAP: memantine



Levels of GFAP were compared between vehicle and memantine treated cultures. GFAP was undetectable before day 12, then increased dramatically thereafter. This indicates that glia are quiescent in this culture system for less than 12 days, after which glial activation or proliferation begins. Memantine treatment does not appear to influence GFAP levels.

Effect of memantine on GFAP levels

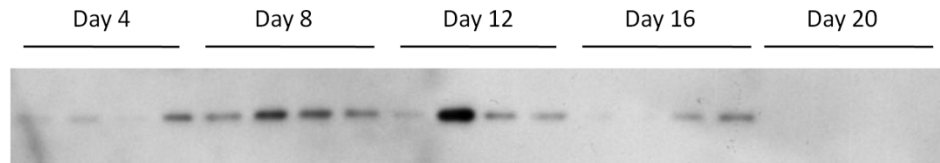
GFAP was also measured in this parallel time course experiment. GFAP levels could be interpreted as either glial proliferation, or activation of glial cells. In these paired sets of samples, GFAP followed a similar pattern (figure 33), with or without memantine treatment. GFAP was not detectable by Western blot until day 12 *in vitro*, and increased in both vehicle- and memantine-treated cells with time. If treatment had any effect, perhaps GFAP increased more rapidly from day 12 to day 16 in the memantine group.

Effect of memantine on SNAP-25 levels

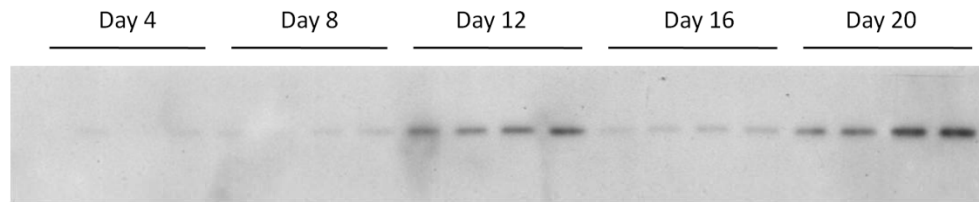
To determine the effect of memantine on synaptic markers, SNAP-25 was measured by western blot in these parallel time course samples (figure 34). In both cases low levels were observed at day 4, which increased to peak at day 12. In the vehicle treated group, SNAP-25 levels then declined at day 16 and were undetectable at day 20. In the memantine treated group, SNAP-25 levels were detectable throughout the experiment, and were as high at day 20 as at day 12. For unknown reasons, levels of this protein were highly variable between samples, especially in the vehicle treated group, however it is still clear that SNAP-25 is present at day 20 in the memantine treated samples, but not in the vehicle group.

Figure 34: SNAP-25 Western blot, vehicle vs. memantine

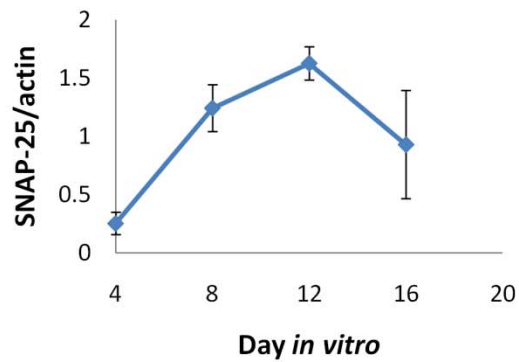
SNAP-25: vehicle



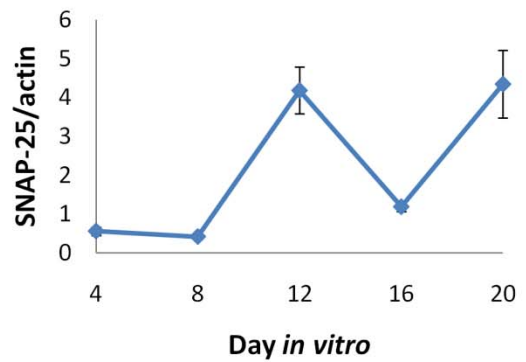
SNAP-25: memantine



SNAP-25, vehicle

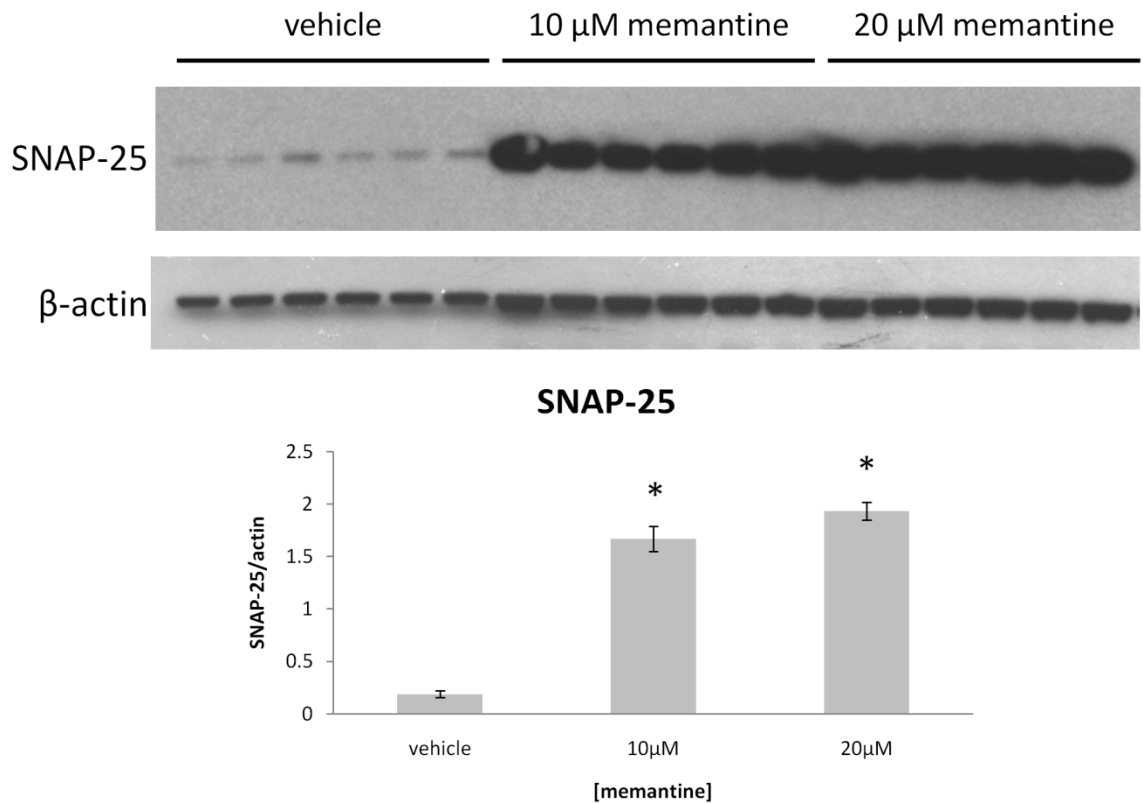


SNAP-25, memantine



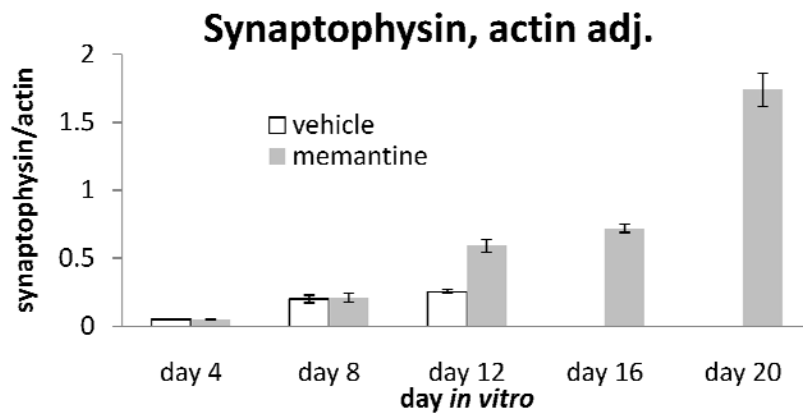
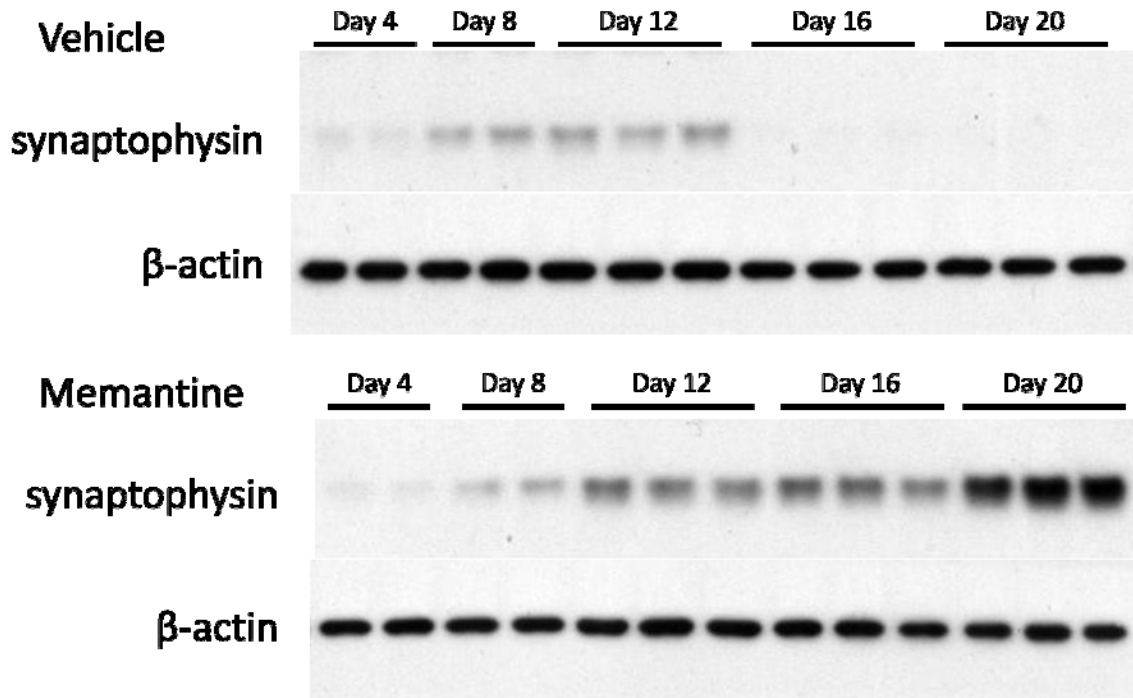
SNAP-25 levels were compared in vehicle and memantine treated cultures. SNAP-25 levels were low in both groups at day 4, suggesting that while neurons are differentiating at this point, synaptic connections have not been widely formed. SNAP-25 peaks at day 12 then declines in the vehicle group to undetectable levels at day 20, but is maintained in memantine treated cells.

Figure 35: SNAP-25 Western blot, vehicle vs. memantine



The effect of two concentrations of memantine on SNAP-25 was examined by western blot in a single time point treatment (day 14-16). SNAP-25 levels were significantly increased in both treatment groups relative to vehicle, but not significantly different from each other. The SNAP-25 signal in samples from cells treated with 10μM or 20μM memantine may be saturated, concealing a possible dose-dependent effect.

Figure 36: Synaptophysin Western blot, vehicle vs. memantine



Memantine (20 μ M) or vehicle treatment started at day 4 and samples were collected at the indicated time intervals. The progression of synaptophysin (syph) levels with time was similar in both treatment groups until they diverged sharply at day 16. Synaptophysin levels continued to rise in the memantine treated group until the experiment was concluded at day 20, however in vehicle treated cells synaptophysin became undetectable after day 12. These results suggest memantine preserves synaptic markers, and may possibly stimulate synaptogenesis in cultured primary cells.

This effect was confirmed in a single time point experiment wherein cells were treated with vehicle, 10 μ M or 20 μ M memantine starting at day 14 and ending at day 16 *in vitro* (figure 35). There was a strong, significant increase in SNAP-25 levels in cultures treated with both memantine concentrations. The exposure time required for detection of SNAP-25 in vehicle treated samples in this instance was long enough to produce a saturating signal in both treatment groups, therefore dose-dependence could not be established.

Effect of memantine on synaptophysin levels

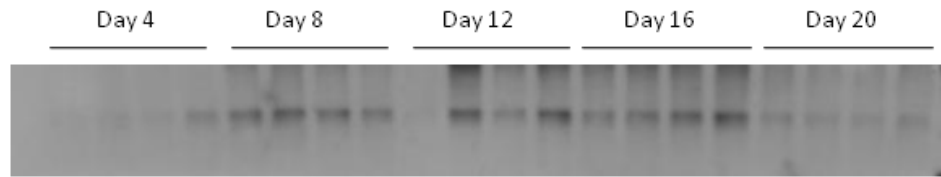
Synaptophysin was also found to be increased by memantine treatment in these cultures (figure 36). Very low synaptophysin levels were observed at day 4, and levels increased in both vehicle and memantine treated cells until day 12. After day 12, memantine treated cells continued to show increases in synaptophysin levels until the experiment ended at day 20. This suggests that the effect of memantine on synaptic markers is not specific to SNAP-25, and that memantine may act to promote synaptogenesis in cultured primary neurons.

Effect of memantine on NR1 levels

NR1 levels were stabilized by memantine treatment around their peak levels at day 12, while vehicle treated cells showed reduced NR1 levels at day 20 (figure 37). It is

Figure 37: NR1 Western blot, vehicle vs. memantine

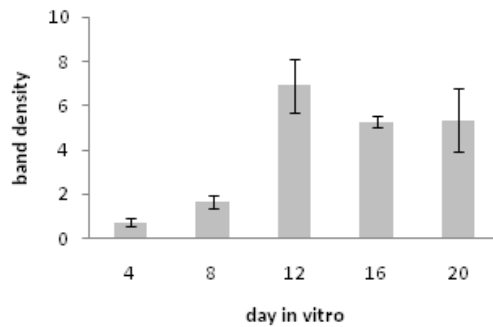
NR1: vehicle



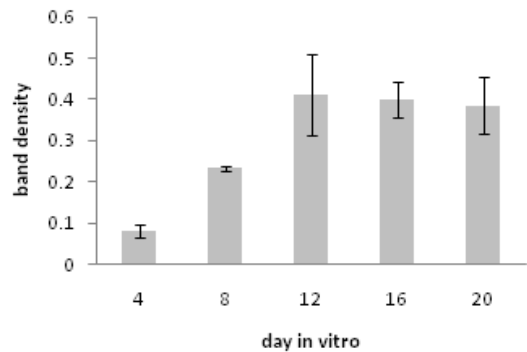
NR1: memantine



Memantine NR1/actin

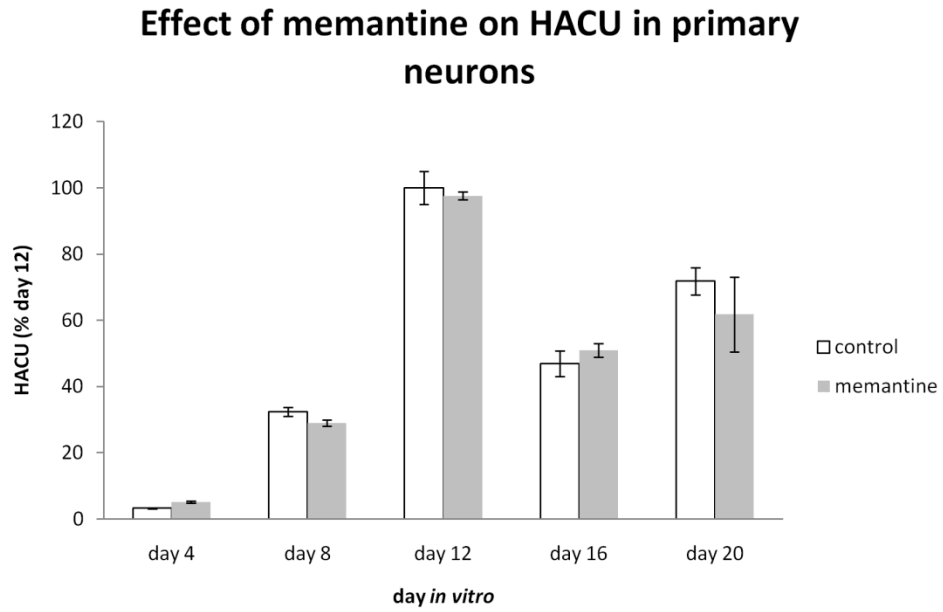


Vehicle NR1/actin



Levels of the NMDA receptor subunit NR1 were monitored in culture over time in vehicle and memantine treated cultures. NR1 levels were low at the beginning of the time course, but peaked around day 12-16, then declined at day 20 in untreated cells. In memantine treated cells, NR1 levels were maintained at day 20, suggesting that post-synaptic elements of the synapse are also preserved.

Figure 38: HACU, vehicle vs. memantine



High affinity choline uptake was measured over time in both vehicle and memantine treated cells. Surprisingly, no effect was observed with memantine treatment at any time point, suggesting that the observed uptake is not related to synaptic density.

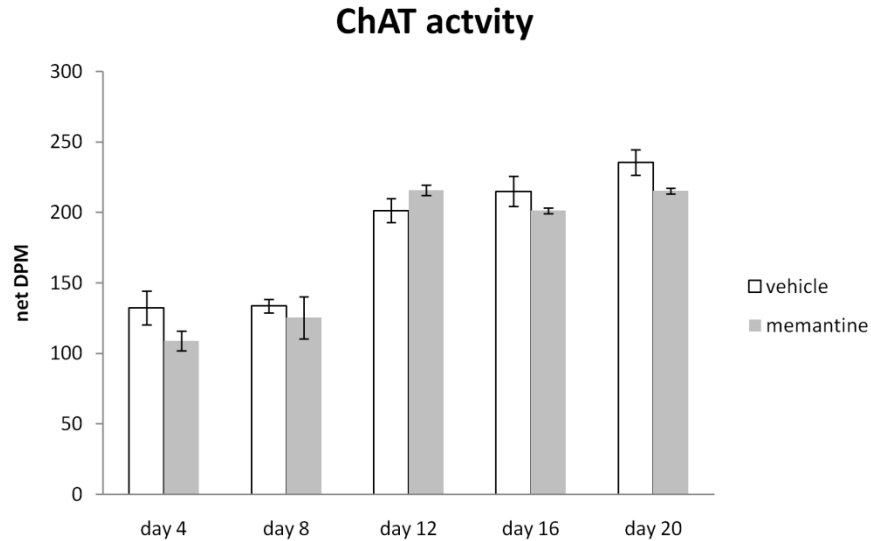
interesting that levels of this protein did not follow SNAP-25 closely, with NR1 protein persisting in vehicle-treated cells until at least day 16 after SNAP-25 had become undetectable. This suggests that either the post-synaptic portion of the synapse is more stable in these cultures, or NMDA receptors are maintained in the cell bodies of neurons with degenerating neurites, or they are sequestered in storage vesicles after synapses have degenerated.

Effect of memantine on HACU and ChAT activities

To determine the effect of memantine treatment on cholinergic activity in these cultures, high-affinity choline uptake (HACU) was measured (figure 38). It was expected that memantine treatment would increase HACU in parallel with the synaptic terminal markers SNAP-25 and synaptophysin because this is where HACU is generally thought to occur. In several similar experiments, HACU activity was observed, however memantine treatment did not consistently produce any observable effect. It was recently observed that astrocytes can exhibit hemicholinium-3 sensitive choline uptake (Inazu et al., 2005), which could explain the consistent observation of HACU in all lots of cells examined with this assay that did not correlate with synaptic markers.

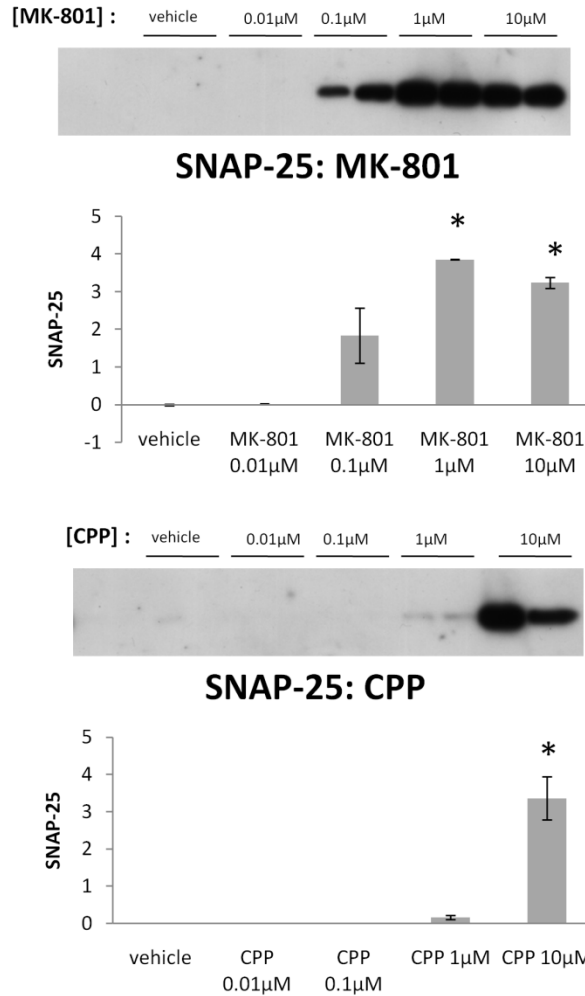
Choline acetyltransferase (ChAT) was measured in primary cortical cultures incubated with memantine or vehicle starting at day 4 (figure 39). Choline acetyltransferase is a marker specific to cholinergic cells, and differences in ChAT activity would be expected if memantine preserved cholinergic terminals. Similar to the HACU

Figure 39: ChAT, vehicle vs. memantine



Lysate samples from vehicle and memantine treated cells were assayed for ChAT. Activity was low, with only 0.9% of the [³H]acetyl-CoA converted into acetylcholine. ChAT activity was also not related to memantine treatment or any synaptic protein measured in these cells. Strong ChAT activity in vehicle treated cells at day 20 relative to other time points suggests a non-neuronal source of this activity. In similar assays, it has been observed that acetylcarnitine can be produced, and this is one possible source of activity in the absence of synaptic acetylcholine synthesis.

Figure 40: SNAP-25 Western blot, vehicle vs. MK-801 vs. CPP



The effects of MK-801 and CPP on SNAP-25 levels were compared using a broad concentration range of both drugs. MK-801 was more potent, reaching a maximum effect at about 1 μ M, while no significant increase in SNAP-25 was observed with less than 10 μ M CPP. These two drugs differ in their mechanism of action, with MK-801 blocking the ion channel, and CPP antagonizing ligand binding. Since drugs of varying mechanism can both increase SNAP-25 levels, this effect is not specific channel blocking drugs and NMDA inhibition by any mechanism is likely to produce similar effects.

data, ChAT activity was observed to increase with time, but did not correlate with either SNAP-25 or synaptophysin. It was expected that ChAT activity would decline as synaptic markers declined starting around day 16 in vehicle treated cultures, however no such decline was observed. This suggests that ChAT activity in these samples is not due to synaptic enzyme activity. This assay does not distinguish between production of acetylcholine and acetylcarnitine, and the low level of ^3H incorporation into the measured product could represent acetylcarnitine synthesized with a small amount of indigenous substrate.

Effect of MK-801 and CPP on SNAP-25

To further evaluate the specificity of the effect of memantine on synaptic markers for the NMDA receptor, two other NMDA receptor inhibiting drugs were tested in this culture system (figure 40). MK-801 is a channel blocking drug with a similar mode of action to memantine, and CPP differs from memantine in that it inhibits glutamate binding to its receptor site. Cells were treated for 48 hours starting at day 14, during the time when neurites and synaptic markers are predicted to be in decline based on previous experiments. Both drugs produced increases in SNAP-25 levels in a concentration-dependent fashion. Both drugs produced an increase in SNAP-25 levels, suggesting that the inhibition of the NMDA receptor is responsible for the observed effects. An approximately 10-fold higher concentration of CPP was required for observable effects as compared to MK-801, which is consistent with the relatively higher potency of MK-801.

Effect of memantine on APP secretion

To determine the effect of memantine on APP processing, the time-dependent pattern of APP secretion was first determined (figure 12). Total secreted APP levels increased until day 12, but were then reduced at day 16, simultaneous with synaptic marker loss. Secreted APP appeared as a characteristic pair of bands of approximately 100kDa and 130kDa that both increased from day 4 to day 12. At later time points, intensity of the lower APP band declined while the top band became the predominant form at day 20. These changes that occur as the relative cell populations in the culture plate shift from neuron-rich to glia-rich suggest that the higher molecular weight form of secreted APP is primarily of glial origin, while the lighter form is primarily neuronal in origin.

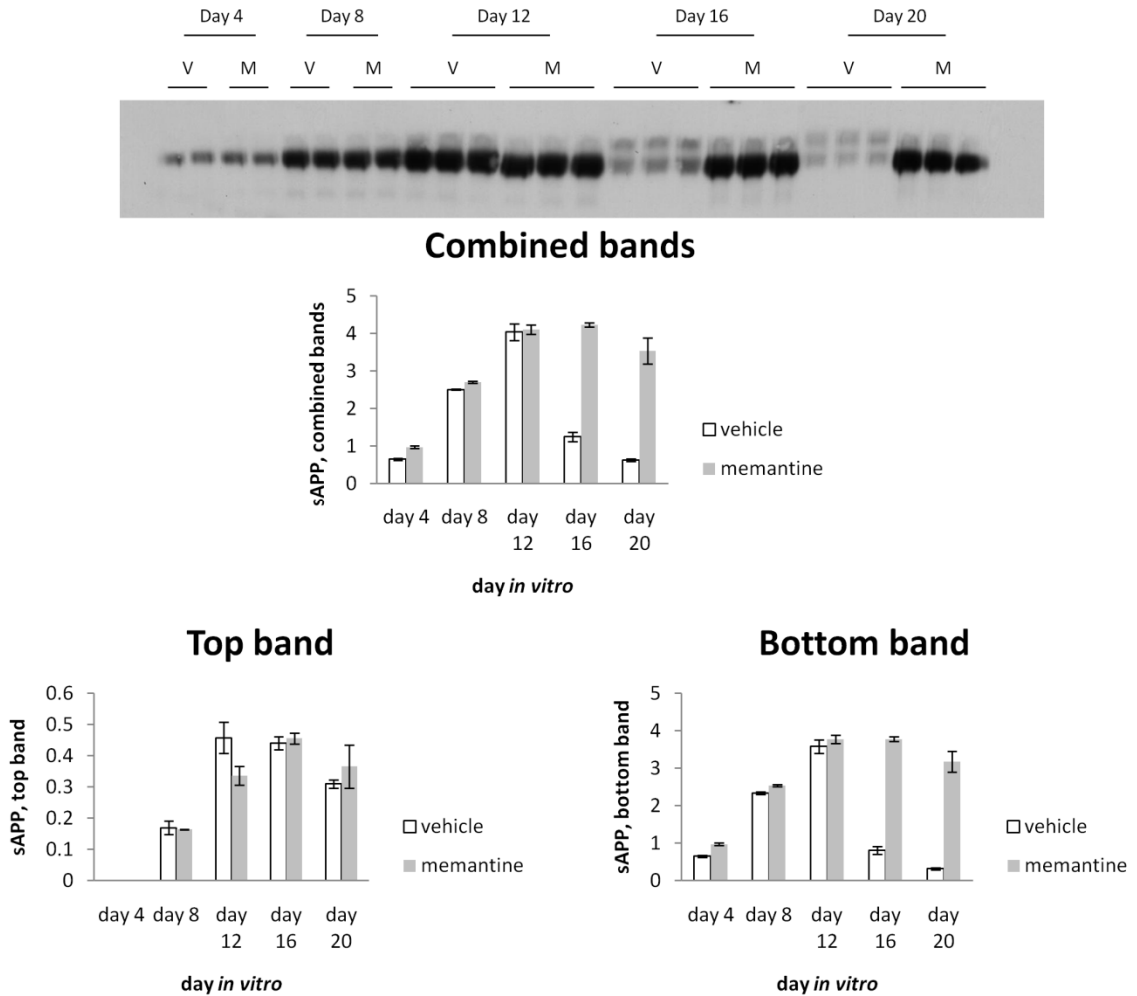
If the lower molecular weight form is of neuronal origin, this band would be expected to be preserved or enhanced by memantine treatment at the later time points. Total secreted APP was maintained at peak day 12 levels by 20 μ M memantine treatment, while it declined dramatically at day 16 and day 20 in vehicle treated cells (figure 41). When analyzed separately, the two bands are clearly distinct in their response to treatment. The higher molecular weight band is relatively unresponsive to memantine treatment, however the bottom band diverges dramatically between treated and untreated cells. Memantine treatment maintains the bottom band, which represents the majority of sAPP, throughout the remaining duration of the time course at near peak levels. Intensity of the top band follows the pattern of GFAP levels more

closely than other cellular proteins measured, further suggesting that high molecular weight secreted APP is mostly of glial origin.

Effect of memantine on A β ₁₋₄₀ and A β ₁₋₄₂ secretion

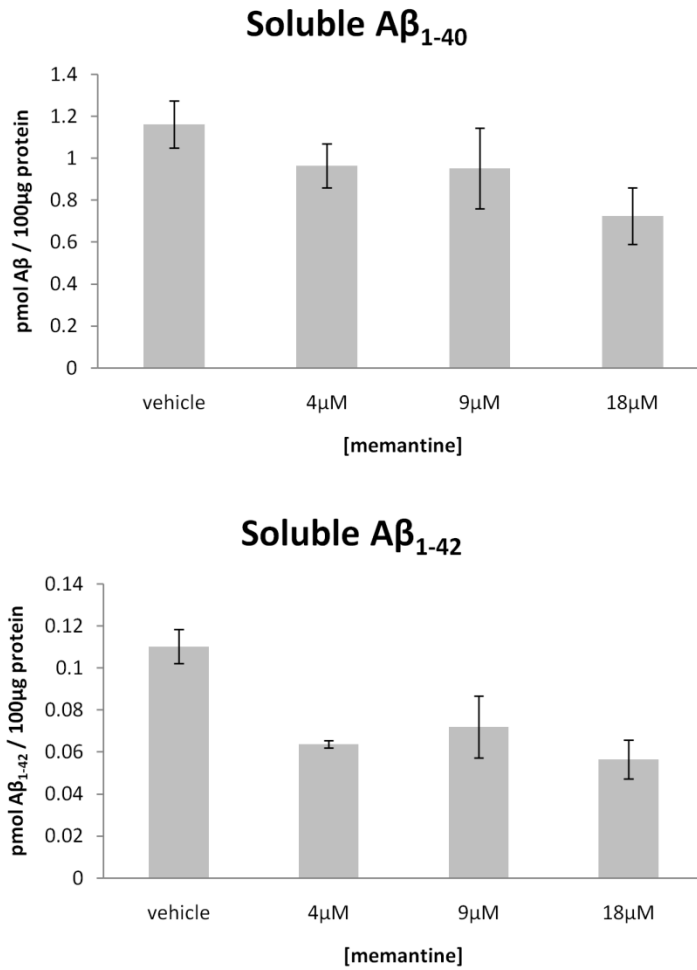
A β ₁₋₄₀ and A β ₁₋₄₂ was measured in primary neuronal cultures treated with memantine for 48 hours starting at day 7 *in vitro* (figure 42). No significant change was observed in A β ₁₋₄₀, however statistically significant decreases in A β ₁₋₄₂ were observed in 4 μ M and 18 μ M treatments, but not in the 9 μ M treatment, perhaps due to greater variability in this group. A response in A β levels to the 4 μ M memantine treatment suggests that this may be an effect distinct from effects observed on synaptic proteins and APP, as no other effects were observed this early in culture or below 10 μ M. Several attempts were made to quantify A β at later time points when other effects could be observed, however A β levels were consistently below detection threshold, either due to variability in A β secretion between batches of neurons, or lot-to-lot variations in the ELISA kits used for detection.

Figure 41: sAPP Western blot, vehicle vs. memantine



Total sAPP was measured in vehicle and memantine treated conditioned medium samples. Two distinct bands were observed, possibly related to different splice variants of APP, the top band corresponding to APP₇₅₁ and APP₇₇₀, the bottom band corresponding to APP₆₉₅. When quantified individually, the top band does not respond to memantine treatment, while the bottom band is maintained in treated samples at later time points while this band declines in untreated samples. APP₆₉₅ is the predominant form produced in neurons, so these data are consistent with neuronal preservation.

Figure 42: A β ELISA quantification, vehicle vs. memantine



Secreted A β_{1-40} and A β_{1-42} were measured by ELISA in cultures treated from day 14 to day 16 with the indicated concentrations of memantine. A β_{1-40} was reduced only at the highest concentration tested, while A β_{1-42} was reduced by both 4 μ M and 18 μ M memantine. These data suggest both a reduction in total A β production, and modulation of the γ -secretase complex, favoring production of A β_{1-40} over A β_{1-42} .

Discussion

Of particular interest in this present work is the study of Alzheimer's disease and the possible effects NMDA receptor inhibition on neuronal viability, synaptic marker levels, and APP processing. The timing and levels of the proteins of interest to this work were incompletely characterized previously, so it was necessary first to track the development of these markers over time. It was found that primary cultures maintained under the conditions used show a relatively predictable increase and subsequent decline in neuronal and synaptic markers, and an increase in glial activation that coincides with synapse loss. Furthermore, memantine treatment of these cultures appears to preserve neuronal and synaptic markers after untreated neurons are lost. And finally, it was demonstrated that memantine treatment affects APP processing such that the high level of APP secretion in these cells is maintained in a pattern similar to that of synaptic markers, suggesting different cell type origins of the several forms of secreted APP observed. Taken together, this work implicates the NMDA receptor in presynaptic terminal loss and changes in APP processing, both of which are thought to be causative agents in the clinical manifestations of AD. Furthermore, this work supports the modulation of NMDA receptor activity as a valid strategy for the treatment of AD.

The steady increase in glutamate in the medium over time is of great interest with respect to these primary cultures as a neurodegenerative model. Glutamate toxicity has been implicated as a possible mechanism in several neurodegenerative disorders. Glutamate is not provided to the cells in the medium, thus they must convert

glutamine in the medium to glutamate to subserve protein synthesis and synaptic transmission functions. Extracellular glutamate levels are typically highly regulated in neuronal tissue, and excessive extrasynaptic NMDA receptor activation is potentially highly toxic to neurons, relative to synaptic receptor activation (Leveille et al., 2008). It has been observed that impaired uptake by glia under some conditions, such as hypoxia or glial activation can lead to toxic levels of extracellular glutamate (Fuller et al., 2009; Jabaudon et al., 2000). Additionally, neuroinflammatory signaling such as that mediated by nitric oxide, can potentially stimulate glutamate release from cultured astrocytes (Brown and Bal-Price, 2003). Based on the increases in GFAP levels observed both by ICC and Western blot, it appears that glial activation occurs spontaneously in these cultures, and thus it seems likely that glial-mediated damage, including glutamate release and other possible activities of astrocytes, such as cytokine release (Hua et al., 2002), is the cause of neurodegeneration in these cultures. Glial-mediated neuronal damage is very relevant to AD and several other neurodegenerative diseases, which supports the use of this tissue culture model in the study of neuroprotective mechanisms. Given the still somewhat ambiguous cause of the neurodegeneration in this model, it should not be assumed that this model is specific to AD, but rather it should be considered as a more general model of synapse and neuron degeneration. Thus, the protective effects observed may have relevance to other diseases.

Neuron viability was similar between memantine treated and untreated cultures up until day 12 *in vitro*, after which point several measures including CTG, LDH assays, and morphological measurements indicate that the two groups diverged dramatically.

Memantine increased viability in the cultures and the ICC data suggest this is a neuron-specific effect. ICC shows enhanced neuronal morphology and greater numbers of neurons in treated cultures relative to vehicle treated cells, while glial cells appear unaffected. However, it is unexpected that while glia appear to proliferate, or at least expand in size, that the CTG data still indicate a steady decline in untreated cells. This suggests that at earlier time points, the bulk of the metabolic activity (i.e., ATP) measured by the CTG assay is neuronal in origin, and that the neuronal portion of the cells in these cultures are responsible for the majority of the metabolic activity in these cultures. It was observed using both Western blot and ICC techniques that in the absence of NMDA inhibitors, synaptic markers declined rapidly between day 12 and day 16, coincident with the sharp rise in GFAP. These data suggest that neuron decline is brought on by activity of the glial cells in this mixed culture, which could have important correlates with the state of glial-neuronal interactions in brains with AD or other neurodegenerative disease and perhaps traumatic brain injury.

It is interesting that the timing of synaptic terminal marker (e.g., SNAP-25) loss precedes post-synaptic marker (NR1) and neuronal marker (NSE) loss. This could suggest a mechanism of neuronal loss wherein neurodegeneration occurs through a mechanism initiated at the synaptic terminal rather than at the cell body. This would suggest that endpoints of experiments aimed at preserving neuronal function in AD and some other disorders should primarily focus on the synapse as the initiation point of toxicity and degeneration. Recent data suggest that memantine protects neurons by preferential inhibition of extrasynaptic NMDA receptors (Xia et al., 2010). This suggests alternative

mechanisms to direct postsynaptic to presynaptic signaling (e.g., NO mediated) in presynaptic terminal loss, wherein NMDA receptor mediated signaling outside the synapse may down-regulate the production of proteins involved in presynaptic vesicle release, such as SNAP-25 and synaptophysin.

Variations in the pattern of APP secretion suggest that part of the total secreted APP in these cultures is produced by glia, and the rest is produced by neurons, and it is possible to distinguish APP of these two origins by molecular weight. In western blots of secreted APP from vehicle and memantine treated cultures, the lower molecular weight forms of APP co-varied with synaptic markers, suggesting that this form is secreted by neurons, and perhaps more specifically, at the synapse. Memantine treatment preserved the high levels of low molecular weight APP secretion. Alternatively, the higher molecular weight form appears to follow the pattern of GFAP, suggesting a glial origin that is not affected by memantine treatment. It is likely that the two forms of APP detected by western blot are splice variants, with a Kunitz protease inhibitor domain included in the larger form. Thus, it is possible that the relative levels of secretion of these two forms could have important implications based on inclusion or exclusion of this domain.

Furthermore, it was observed at an early time point in these cultures that memantine does not alter $A\beta_{1-40}$ secretion, but does reduce secretion of the more toxic and amyloidogenic $A\beta_{1-42}$. It is likely that $A\beta$ has some role in normal neuronal processes, such as regulating neurotransmitter release (Abramov et al., 2009), however this has been overshadowed by the clearly detrimental role it plays in AD. The results

presented here suggest that modulating the relative levels of these two forms of A β via action on the NMDA receptor may be possible. This could be a promising mechanism to explore in more detail, as reducing the highly toxic A β_{1-42} while leaving the less toxic A β_{1-40} to perform its normal function could produce greater beneficial effects than indiscriminately removing all forms of A β .

With respect to guiding therapeutic interventions in AD, the disparity between SNAP-25 and NSE levels over time is particularly interesting. It appears that in response to glia-mediated toxicity, presynaptic terminals are lost first while at least some of the neuronal structure and post-synaptic markers persist for some time later. This suggests a potentially important therapeutic window during which synaptic contacts are lost, perhaps resulting in the early cognitive symptoms of AD, but restoration of these contacts could still be achieved and perhaps cognitive function could be improved to some extent. It is not expected that this type of toxicity and degeneration will affect every neuron simultaneously *in vivo* as passive observations indicated that it does not occur in this simpler *in vitro* system. Therefore early intervention followed by a maintained protective therapy may be required in order to restore then preserve function over the long term.

Overall summary and future directions

Summary

Research in neurodegeneration and many other areas is greatly aided by the use of well characterized tissue culture models. In the present work, a model of primary fetal neurons was characterized with respect to synaptic protein marker levels, glial activation, neuronal morphology, and other parameters relevant to AD in greater detail than was previously available. These data provide evidence that this primary tissue culture system provides a model wherein neurons degenerate as a downstream effect of glial activation, which is an important part of several neurodegenerative conditions, including AD and traumatic brain injury (Li et al., 2009; Simpson et al., 2010). Time-dependent synaptic terminal protein marker loss that coincides with glial activation suggests that this is a useful model of glia-mediated neuronal damage, which may provide a valuable research tool for AD and other neurodegenerative diseases.

It was shown that presynaptic markers that are lost in AD may be preserved by inhibition of the postsynaptic NMDA receptor. While protection of neurons against glutamate-mediated excitotoxicity with glutamate receptor antagonists is an established phenomenon, the effect on presynaptic terminals was not previously characterized in this type of neurodegeneration model. In this model, it was shown that NMDA receptor inhibition can protect neurons in the presence of activated glial cells and increased glutamate concentrations that otherwise lead to synapse pruning and then neuron death. With respect to AD specifically, it was also shown that memantine treatment preserves at later time points the band pattern of secreted APP observed when

neuronal protein expression was at its peak. Similarly, APP in media samples of treated cells at later time points was found to more closely resemble the levels and molecular weight band pattern observed during the peak of neuronal protein expression.

Processing of APP may have also been altered by this treatment. A β_{1-42} levels were decreased by memantine treatment but A β_{1-40} levels were unaffected, suggesting a γ -secretase modulating effect.

The utility of this model in studies of gene regulation was also demonstrated. It was shown that the activities of the *APP*, *BACE1*, and *MAPT* gene promoters vary with neuronal differentiation and degeneration. These changes may represent opportunities for therapeutic interventions in AD. To further understand the regulation of these gene promoters, activities of deletion series of *BACE1* and *MAPT* promoters was evaluated, and some subregions that represent transcriptional “hot spots” were identified, and these should be further evaluated as therapeutic targets. Regulation of these promoters by A β and p25, both of which are dysregulated in AD, was also examined. It was found that p25 overexpression up-regulates *MAPT* promoter activity. A β treatment was found to reduce *BACE1* promoter activity, suggesting a possible negative feedback mechanism. However, the negative control, reverse-sequence A β peptides were also observed to inhibit *MAPT* and *BACE1* promoter activities, but not *APP* promoter activity, suggesting a novel interaction with an as-yet unidentified target in these cells.

It is concluded that this tissue culture system models neurodegeneration with a mechanism that involves glial activation, which is known to be involved in the pathogenesis of AD and other disorders. Furthermore, this model is useful for the study

of gene expression at the level of the promoter which, as our understanding of gene regulation expands, may become a fruitful place to search for the next generation of drug targets. Finally, the utility of this model in evaluating the mechanisms of therapeutic agents was demonstrated. It was determined that memantine acts through preservation of synapses in degenerating neurons, which may largely explain any clinical improvements experienced by patients using this drug. In all, these data suggest mechanisms for therapeutic agents currently in use, propose a potentially fruitful search for new therapeutic targets within the promoter regions of genes known to be dysregulated in AD, and provide a useful tool to evaluate potential drug targets and therapeutic agents in the future.

Future Directions

While these experiments have provided important information regarding the nature of this tissue culture system and the mechanisms by which NMDA inhibition may counteract the effects of glial activation, several important questions remain unanswered. Of particular relevance to AD, somewhat dramatic differences between the APP band patterns observed in western blots of memantine treated and vehicle treated cultures were observed. Perhaps most importantly, the lowest molecular weight secreted form appears to follow closely the time- and treatment-dependent pattern of synaptic markers. At present it is unclear what combinations of post-transcriptional and post-translational modification produce these apparent differences in the molecular weight of APP, however the differences observed between cultures at different time points and treatment-based differences suggest that APP may be involved in the glial activation modeled in these cultures, and in the protective effects of NMDA receptor inhibition. A reasonable hypothesis is that the differences between the sAPP bands visualized by Western blot correspond to the different splice variants of APP, although this has not been tested in these cultures. A full understanding of all the various secreted and cell-associated forms of APP observed by Western blots from these cultures may be a large undertaking, given that there are at least three major splice variants and several other forms of post-translational modifications (e.g., glycosylation, phosphorylation, sulfonation) that could modify the apparent molecular weight of the APP protein. However, a more complete understanding of the APP variants observed in

these cultures may have important implications for the various functions of APP and the observed effect of memantine on the various forms of sAPP.

The NMDA receptor antagonists memantine, CPP and MK-801 were demonstrated to have apparently protective effects on synapses, however it is presently unclear what effect these drugs have on receptor activity, or if there is any baseline NMDA receptor activity in these cultures. To further characterize the tissue culture model, it would be useful to know at what point in time these cells begin to show NMDA receptor activation. It has been observed in similar cultures that cortical cells become responsive to NMDA treatment between day 9 and day 11 *in vitro* (Mizuta et al., 1998), however, it is less clear at what point NMDA receptor activation occurs through endogenous glutamate release. It is important to determine this behavior of cells cultured under the described conditions, as this could inform the interpretation of the drug effects described here. For example, by comparing glutamate concentrations measured by HPLC to glial and synaptic markers, it appears that the primary source of glutamate in these cultures is glial. However, micromolar concentrations of glutamate were observed in the medium at all time points, even before GFAP is present in detectable quantities. The glutamate concentrations observed should be sufficient to activate NMDA receptors, however, there is not an observable response in viability or in any protein level measured to NMDA receptor inhibition until day 16 *in vitro* when toxicity develops. It would be useful to know if there is a change in baseline NMDA receptor activity or subcellular distribution between day 12 and day 16 *in vitro*. Additionally, only the NR1 subunit of the NMDA receptor was measured in these

cultures, while changes in NR2 receptor isoform expression can influence receptor activity. For example, inclusion of the NR2A or NR2B subunits in the heteromeric NMDA receptor complex can influence neuron survival and death, depending on the specific configuration of the receptor (Liu et al., 2007). Changes in the specific subunits included in the NMDA receptors of these cells over time could at least partially explain the sudden neuronal cell loss that occurs between day 12 and day 16. An alternate strategy would be to repeat some of the experiments detailed herein using organotypic hippocampal slices. Although more time and resource-intensive, this would have the added benefits of some intact hippocampal circuitry involving mature neurons with active receptors (Di Simoni and Yu, 2006). This model is currently under development and optimization for this purpose.

Promoter regions of genes can be quite large and often do not have clearly defined boundaries, although the data presented here provide a rough idea of what relatively constrained (~0.5 kilobase) areas of the *BACE1* and *MAPT* promoter regions may positively regulate transcription most strongly. Down-regulating promoter activity of either of these genes may represent workable goals for slowing AD progression. Upstream gene promoter regions typically bind numerous proteins or protein complexes (i.e., transcription factors) involved in transcriptional activity of that gene. Informatic tools that can predict binding sites for these proteins are constantly becoming more effective and reliable, such that transcription factors that act on these gene promoters can be predicted *in silico* and tested in the laboratory. Thus, it seems a potentially fruitful avenue of research to pursue in this tissue culture model, to identify

and test potential transcription factors or the pathways that activate them in order to determine which become active during neurodegeneration, and to pursue them as therapeutic targets.

Another very interesting line of research has recently suggested that A β itself can act as a transcription factor in such a way as to regulate its own production (Lahiri and Maloney, 2010). It was previously reported that intracellularly produced A β can enter the nucleus and induce transcription of the TP53 gene during oxidative stress, which was suggested to be a mechanism of neuron death in AD (Ohyaqi et al., 2005). We have attempted to extend our knowledge in this area, showing that extracellular A β can be transported to the nucleus under oxidative stress conditions, and that this nuclear localization affects both the *APP* and the *BACE1* gene promoters such that A β acts in a positive feedback loop to stimulate its own production under certain conditions (Bailey et al., *Gene* 2011, in press). Furthermore, extensive investigations have been carried out to determine the DNA binding sequence specific to A β (Maloney et al., *Gene* 2011, in press). Regulation of the A β -producing genes by A β itself presents the possibility of several new drug targets that could be used to produce more effective treatments for AD, for example, little is known about the A β death inducing protein (Lakshmana et al., 2005), which may be an important transporter of A β in AD, and therefore a potential drug target. It can be expected that continued investigation of A β transport and its interaction with these gene promoters will provide important clues about the pathogenesis of AD and reveal novel drug targets.

The data presented here demonstrate that in a tissue culture model of glial activation induced neuronal toxicity, NMDA receptor inhibition can produce a strong synapse- and neuron-preserving effect. This effect appears to be specific for the NMDA receptor, although the exact mechanism of this effect is still not completely understood. Further investigation of the possible mechanisms behind the effects observed here may suggest more specific drug targets and guide the development of future therapeutic agents for the treatment of AD, traumatic brain injury, or other glial-mediated disorders. For example, it was recently demonstrated that the synaptogenic effects of ketamine, another NMDA receptor antagonist that was used experimentally to treat depression like symptoms, is dependent on the mammalian target of rapamycin (mTOR) pathway (Li et al., 2010). It is possible that the mTOR pathway is also involved in the effects of memantine described here. If mTOR is indeed involved in the mechanism of memantine's effects, the numerous components of the mTOR pathway could be considered as therapeutic targets, which could lead to the development of more specific therapeutic agents to treat AD. Given the similarities between this tissue culture model, with respect to glial activation, synaptic marker loss, and neurodegeneration, this model provides a workable starting point for these investigations.

Reference List

- Abramov E, Dolev I, Fogel H, Ciccotosto GD, Ruff E, Slutsky I. 2009. Amyloid-beta as a positive endogenous regulator of release probability at hippocampal synapses. *Nat. Neurosci.* 12:1567-1576.
- Akasofu S, Kimura M, Kosasa T, Sawada K, Ogura H. 2008. Study of neuroprotection of donepezil, a therapy for Alzheimer's disease. *Chem. Biol. Interact.* 175:222-226.
- Akasofu S, Kosasa T, Kimura M, Kubota A. 2003. Protective effect of donepezil in a primary culture of rat cortical neurons exposed to oxygen-glucose deprivation. *Eur. J. Pharmacol.* 472:57-63.
- Albin RL, Greenamyre JT. 1992. Alternative excitotoxic hypotheses. *Neurology* 42:733-738.
- Alley GM, Bailey JA, Chen D, Ray B, Puli LK, Tanila H, Banerjee PK, Lahiri DK. 2010. Memantine lowers amyloid-beta peptide levels in neuronal cultures and in APP/PS1 transgenic mice. *J. Neurosci. Res.* 88:143-154.
- Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR. 1995. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". *Clin. Anat.* 8:429-431.
- Apparsundaram S, Ferguson SM, George AL, Jr., Blakely RD. 2000. Molecular cloning of a human, hemicholinium-3-sensitive choline transporter. *Biochem. Biophys. Res. Commun.* 276:862-867.
- Armstrong RA. 1994. Differences in beta-amyloid (beta/A4) deposition in human patients with Down's syndrome and sporadic Alzheimer's disease. *Neurosci. Lett.* 169:133-136.
- Bailey JA, Ge YW, Lahiri DK. 2007. Sensitive detection of reporter gene assay results. *Cell Notes* 17:13-15.
- Bailey JA, Lahiri DK. 2010. A novel effect of rivastigmine on pre-synaptic proteins and neuronal viability in a neurodegeneration model of fetal rat primary cortical cultures and its implication in Alzheimer's disease. *J. Neurochem.* 112:843-853.
- Bailey JA, Maloney BJ, Ge YW, Lahiri DK. 2009. Internalization of the amyloid beta (A β) peptide may result in binding to the regulatory region of the APP gene promoter. *Alzheimers. Dement.* 5:P315-P316.
- Bailey J.A., Ray, B., Greig N.H., Lahiri D.K. Rivastigmine lowers A β and increases sAPP α levels, which parallel elevated synaptic markers and metabolic activity in degenerating primary rat neurons. *PLoS ONE. In press, 2011.*
- Bains M, Heidenreich KA. 2009. Live-cell imaging of autophagy induction and autophagosome-lysosome fusion in primary cultured neurons. *Methods Enzymol.* 453:145-158.
- Barber RC. 2010. Biomarkers for early detection of Alzheimer disease. *J. Am. Osteopath. Assoc.* 110:S10-S15.
- Bartus RT, Dean RL, III, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. *Science* 217:408-414.

- Becher A, Drenckhahn A, Pahner I, Margittai M, Jahn R, Hnert-Hilger G. 1999. The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation. *J. Neurosci.* 19:1922-1931.
- Becker RE, Greig NH. 2008. Alzheimer's disease drug development in 2008 and beyond: problems and opportunities. *Curr. Alzheimer Res.* 5:346-357.
- Biscaro B, Lindvall O, Hock C, Ekdahl CT, Nitsch RM. 2009. Abeta immunotherapy protects morphology and survival of adult-born neurons in doubly transgenic APP/PS1 mice. *J. Neurosci.* 29:14108-14119.
- Braak H, Braak E. 1997a. Diagnostic criteria for neuropathologic assessment of Alzheimer's disease. *Neurobiol. Aging* 18:S85-S88.
- Braak H, Braak E. 1997b. Staging of Alzheimer-related cortical destruction. *Int. Psychogeriatr.* 9 Suppl 1:257-261.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 72:248-254.
- Brewer GJ. 1995. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. *J. Neurosci. Res.* 42:674-683.
- Brewer GJ, Boehler MD, Jones TT, Wheeler BC. 2008. Neurobasal/B27 increases neuron synapse densities and network spike rates on multielectrode arrays. *J. Neurosci. Methods* 170:181-187.
- Brewer GJ, Torricelli JR, Evege EK, Price PJ. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* 35:567-576.
- Brown GC, Bal-Price A. 2003. Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol. Neurobiol.* 27:325-355.
- Buell SJ, Coleman PD. 1979. Dendritic growth in the aged human brain and failure of growth in senile dementia. *Science* 206:854-856.
- Camins A, Verdaguer E, Folch J, Canudas AM, Pallas M. 2006. The role of CDK5/P25 formation/inhibition in neurodegeneration. *Drug News Perspect.* 19:453-460.
- Cao X, Sudhof TC. 2001. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* 293:115-120.
- Casas C, Sergeant N, Itier JM, Blanchard V, Wirths O, van der KN, Vingtdoux V, van de SE, Ret G, Canton T, Drobecq H, Clark A, Bonici B, Delacourte A, Benavides J, Schmitz C, Tremp G, Bayer TA, Benoit P, Pradier L. 2004. Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated Abeta42 accumulation in a novel Alzheimer transgenic model. *Am. J. Pathol.* 165:1289-1300.
- Caumont AS, Octave JN, Hermans E. 2006. Amantadine and memantine induce the expression of the glial cell line-derived neurotrophic factor in C6 glioma cells. *Neurosci. Lett.* 394:196-201.
- Chen HS, Pellegrini JW, Aggarwal SK, Lei SZ, Warach S, Jensen FE, Lipton SA. 1992. Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. *J. Neurosci.* 12:4427-4436.

- Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res.* 21:2895-2900.
- Colonnese MT, Zhao JP, Constantine-Paton M. 2005. NMDA receptor currents suppress synapse formation on sprouting axons in vivo. *J. Neurosci.* 25:1291-1303.
- Czech MP, Aouadi M, Tesz GJ. 2011. RNAi-based therapeutic strategies for metabolic disease. *Nat. Rev. Endocrinol.*
- Davies P, Maloney AJ. 1976. Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* 2:1403.
- DeKosky ST, Scheff SW. 1990. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann. Neurol.* 27:457-464.
- DeKosky ST, Scheff SW, Styren SD. 1996. Structural correlates of cognition in dementia: quantification and assessment of synapse change. *Neurodegeneration.* 5:417-421.
- Di Simoni A, Yu LM. 2006. Preparation of organotypic hippocampal slice cultures: interface method. *Nat. Protoc.* 1:1439-1445.
- Dickey CA, Ash P, Klosak N, Lee WC, Petrucelli L, Hutton M, Eckman CB. 2006. Pharmacologic reductions of total tau levels; implications for the role of microtubule dynamics in regulating tau expression. *Mol. Neurodegener.* 1:6.
- Domercq M, Brambilla L, Pilati E, Marchaland J, Volterra A, Bezzi P. 2006. P2Y1 receptor-evoked glutamate exocytosis from astrocytes: control by tumor necrosis factor-alpha and prostaglandins. *J. Biol. Chem.* 281:30684-30696.
- Evans NA, Facci L, Owen DE, Soden PE, Burbidge SA, Prinjha RK, Richardson JC, Skaper SD. 2008. Abeta(1-42) reduces synapse number and inhibits neurite outgrowth in primary cortical and hippocampal neurons: a quantitative analysis. *J. Neurosci. Methods* 175:96-103.
- Floden AM, Li S, Combs CK. 2005. Beta-amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor alpha and NMDA receptors. *J. Neurosci.* 25:2566-2575.
- Fu AK, Fu WY, Ng AK, Chien WW, Ng YP, Wang JH, Ip NY. 2004. Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity. *Proc. Natl. Acad. Sci. U. S. A* 101:6728-6733.
- Fuller S, Munch G, Steele M. 2009. Activated astrocytes: a therapeutic target in Alzheimer's disease? *Expert. Rev. Neurother.* 9:1585-1594.
- Ge YW, Maloney B, Sambamurti K, Lahiri DK. 2004. Functional characterization of the 5' flanking region of the BACE gene: identification of a 91 bp fragment involved in basal level of BACE promoter expression. *FASEB J.* 18:1037-1039.
- GERZON K, KRUMKALNS EV, BRINDLE RL, MARSHALL FJ, ROOT MA. 1963. THE ADAMANTYL GROUP IN MEDICINAL AGENTS. I. HYPOGLYCEMIC N-ARYLSULFONYL-N'-ADAMANTYLUREAS. *J. Med. Chem.* 6:760-763.
- Ghosh AK, Kumaragurubaran N, Hong L, Koelsh G, Tang J. 2008. Memapsin 2 (beta-secretase) inhibitors: drug development. *Curr. Alzheimer Res.* 5:121-131.

- Glenner GG, Wong CW. 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* 120:885-890.
- Gomez de BE, Perez M, Gomez RP, de CJ, Martin-Maestro P, Moran A, Dawson HN, Vitek MP, Lucas JJ, Hernandez F, Avila J. 2010. Tau-knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits. *Neurobiol. Dis.* 37:622-629.
- Gong X, Tang X, Wiedmann M, Wang X, Peng J, Zheng D, Blair LA, Marshall J, Mao Z. 2003. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. *Neuron* 38:33-46.
- Goodman DR, Adatsi FK, Harbison RD. 1984. Evidence for the extreme overestimation of choline acetyltransferase in human sperm, human seminal plasma and rat heart: a case of mistaking carnitine acetyltransferase for choline acetyltransferase. *Chem. Biol. Interact.* 49:39-53.
- Gotz M, Williams BP, Bolz J, Price J. 1995. The specification of neuronal fate: a common precursor for neurotransmitter subtypes in the rat cerebral cortex in vitro. *Eur. J. Neurosci.* 7:889-898.
- Goux E, Leveille F, Nicole O, Melon C, Had-Aissouni L, Buisson A. 2009. Reverse glial glutamate uptake triggers neuronal cell death through extrasynaptic NMDA receptor activation. *Mol. Cell Neurosci.* 40:463-473.
- Grabrucker A, Vaida B, Bockmann J, Boeckers TM. 2009. Synaptogenesis of hippocampal neurons in primary cell culture. *Cell Tissue Res.* 338:333-341.
- Greenamyre JT. 1986. The role of glutamate in neurotransmission and in neurologic disease. *Arch. Neurol.* 43:1058-1063.
- Greig NH, Lahiri DK, Sambamurti K. 2002. Butyrylcholinesterase: an important new target in Alzheimer's disease therapy. *Int. Psychogeriatr.* 14 Suppl 1:77-91.
- Han RZ, Hu JJ, Weng YC, Li DF, Huang Y. 2009. NMDA receptor antagonist MK-801 reduces neuronal damage and preserves learning and memory in a rat model of traumatic brain injury. *Neurosci. Bull.* 25:367-375.
- Hardingham GE, Bading H. 2010. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat. Rev. Neurosci.* 11:682-696.
- Hartlage-Rubsamen M, Zeitschel U, Apelt J, Gartner U, Franke H, Stahl T, Gunther A, Schliebs R, Penkowa M, Bigl V, Rossner S. 2003. Astrocytic expression of the Alzheimer's disease beta-secretase (BACE1) is stimulus-dependent. *Glia* 41:169-179.
- Hartman RE, Shah A, Fagan AM, Schwetye KE, Parsadonian M, Schulman RN, Finn MB, Holtzman DM. 2006. Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. *Neurobiol. Dis.* 24:506-515.
- Hauss-Wegrzyniak B, Lynch MA, Vraniak PD, Wenk GL. 2002. Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. *Exp. Neurol.* 176:336-341.

- Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, Van LF. 2005. Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. *J. Neuroinflammation*. 2:22.
- Henley DB, May PC, Dean RA, Siemers ER. 2009. Development of semagacestat (LY450139), a functional gamma-secretase inhibitor, for the treatment of Alzheimer's disease. *Expert. Opin. Pharmacother*. 10:1657-1664.
- Hesselink MB, De Boer BG, Breimer DD, Danysz W. 1999. Brain penetration and in vivo recovery of NMDA receptor antagonists amantadine and memantine: a quantitative microdialysis study. *Pharm. Res*. 16:637-642.
- Hills ID, Vacca JP. 2007. Progress toward a practical BACE-1 inhibitor. *Curr. Opin. Drug Discov. Devel*. 10:383-391.
- Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G. 2002. Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann. Neurol*. 51:783-786.
- Honda M, Tsuruta R, Kaneko T, Kasaoka S, Yagi T, Todani M, Fujita M, Izumi T, Maekawa T. 2010. Serum glial fibrillary acidic protein is a highly specific biomarker for traumatic brain injury in humans compared with S-100B and neuron-specific enolase. *J. Trauma* 69:104-109.
- Hua LL, Kim MO, Brosnan CF, Lee SC. 2002. Modulation of astrocyte inducible nitric oxide synthase and cytokine expression by interferon beta is associated with induction and inhibition of interferon gamma-activated sequence binding activity. *J. Neurochem*. 83:1120-1128.
- Huang HC, Jiang ZF. 2009. Accumulated amyloid-beta peptide and hyperphosphorylated tau protein: relationship and links in Alzheimer's disease. *J. Alzheimers. Dis*. 16:15-27.
- Huettner JE, Bean BP. 1988. Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. U. S. A* 85:1307-1311.
- Inazu M, Takeda H, Matsumiya T. 2005. Molecular and functional characterization of an Na⁺-independent choline transporter in rat astrocytes. *J. Neurochem*. 94:1427-1437.
- Iqbal K, Alonso AC, Chen S, Chohan MO, El-Akkad E, Gong CX, Khatoon S, Li B, Liu F, Rahman A, Tanimukai H, Grundke-Iqbal I. 2005. Tau pathology in Alzheimer disease and other tauopathies. *Biochim. Biophys. Acta* 1739:198-210.
- Iqbal K, Liu F, Gong CX, Alonso AC, Grundke-Iqbal I. 2009. Mechanisms of tau-induced neurodegeneration. *Acta Neuropathol*. 118:53-69.
- Jaubaudon D, Scanziani M, Gahwiler BH, Gerber U. 2000. Acute decrease in net glutamate uptake during energy deprivation. *Proc. Natl. Acad. Sci. U. S. A* 97:5610-5615.
- Jantas D, Lason W. 2009. Anti-apoptotic effect of memantine against staurosporine- and low-potassium-induced cell death in cerebellar granule cells: a development-dependent effect. *Pharmacol. Rep*. 61:827-937.

- Joseph JA, Denisova NA, Arendash G, Gordon M, Diamond D, Shukitt-Hale B, Morgan D. 2003. Blueberry supplementation enhances signaling and prevents behavioral deficits in an Alzheimer disease model. *Nutr. Neurosci.* 6:153-162.
- Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Muller-Hill B. 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325:733-736.
- Kangas L, Gronroos M, Nieminen AL. 1984. Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. *Med. Biol.* 62:338-343.
- Kawahara M, Ohtsuka I, Yokoyama S, Kato-Negishi M, Sadakane Y. 2011. Membrane Incorporation, Channel Formation, and Disruption of Calcium Homeostasis by Alzheimer's beta-Amyloid Protein. *Int. J. Alzheimers. Dis.* 2011:304583.
- Kolodny JM, Leonard JL, Larsen PR, Silva JE. 1985. Studies of nuclear 3,5,3'-triiodothyronine binding in primary cultures of rat brain. *Endocrinology* 117:1848-1857.
- Kornhuber J, Bormann J, Retz W, Hubers M, Riederer P. 1989. Memantine displaces [3H]MK-801 at therapeutic concentrations in postmortem human frontal cortex. *Eur. J. Pharmacol.* 166:589-590.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- LaFerla FM, Tinkle BT, Bieberich CJ, Haudenschild CC, Jay G. 1995. The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. *Nat. Genet.* 9:21-30.
- Lahiri DK. 1994. Reversibility of the effect of tacrine on the secretion of the beta-amyloid precursor protein in cultured cells. *Neurosci. Lett.* 181:149-152.
- Lahiri DK, Chen D, Maloney B, Holloway HW, Yu QS, Utsuki T, Giordano T, Sambamurti K, Greig NH. 2007. The experimental Alzheimer's disease drug posiphen [(+)-phenserine] lowers amyloid-beta peptide levels in cell culture and mice. *J. Pharmacol. Exp. Ther.* 320:386-396.
- Lahiri DK, Farlow MR, Hintz N, Utsuki T, Greig NH. 2000. Cholinesterase inhibitors, beta-amyloid precursor protein and amyloid beta-peptides in Alzheimer's disease. *Acta Neurol. Scand. Suppl* 176:60-67.
- Lahiri DK, Farlow MR, Nurnberger JI, Jr., Greig NH. 1997. Effects of cholinesterase inhibitors on the secretion of beta-amyloid precursor protein in cell cultures. *Ann. N. Y. Acad. Sci.* 826:416-421.
- Lahiri DK, Farlow MR, Sambamurti K. 1998. The secretion of amyloid beta-peptides is inhibited in the tacrine-treated human neuroblastoma cells. *Brain Res. Mol. Brain Res.* 62:131-140.
- Lahiri DK, Farlow MR, Sambamurti K, Greig NH, Giacobini E, Schneider LS. 2003. A critical analysis of new molecular targets and strategies for drug developments in Alzheimer's disease. *Curr. Drug Targets.* 4:97-112.
- Lahiri DK, Ge YW, Maloney B. 2005a. Characterization of the APP proximal promoter and 5'-untranslated regions: identification of cell type-specific domains and implications in APP gene expression and Alzheimer's disease. *FASEB J.* 19:653-655.

- Lahiri DK, Ge YW, Maloney B, Wavrant-De VF, Hardy J. 2005b. Characterization of two APP gene promoter polymorphisms that appear to influence risk of late-onset Alzheimer's disease. *Neurobiol. Aging* 26:1329-1341.
- Lahiri DK, Ge YW, Rogers JT, Sambamurti K, Greig NH, Maloney B. 2006. Taking down the unindicted co-conspirators of amyloid beta-peptide-mediated neuronal death: shared gene regulation of BACE1 and APP genes interacting with CREB, Fe65 and YY1 transcription factors. *Curr. Alzheimer Res.* 3:475-483.
- Lahiri DK, Maloney B. 2010. Beyond the signaling effect role of amyloid-ss42 on the processing of APP, and its clinical implications. *Exp. Neurol.* 225:51-54.
- Lahiri DK, Maloney B, Bailey JA, Ge YW. 2009a. Role of Alzheimer's amyloid-beta peptide as a putative transcription factor. *Peptide Science* 92:354-355.
- Lahiri DK, Maloney B, Zawia NH. 2009b. The LEARN model: an epigenetic explanation for idiopathic neurobiological diseases. *Mol. Psychiatry* 14:992-1003.
- Lahiri DK, Robakis NK. 1991. The promoter activity of the gene encoding Alzheimer beta-amyloid precursor protein (APP) is regulated by two blocks of upstream sequences. *Brain Res. Mol. Brain Res.* 9:253-257.
- Lahiri DK, Sambamurti K, Bennett DA. 2004. Apolipoprotein gene and its interaction with the environmentally driven risk factors: molecular, genetic and epidemiological studies of Alzheimer's disease. *Neurobiol. Aging* 25:651-660.
- Lakshmana MK, Araki W, Tabira T. 2005. Amyloid beta peptide binds a novel death-inducing protein, AB-DIP. *FASEB J.* 19:1362-1364.
- Lanz TA, Carter DB, Merchant KM. 2003. Dendritic spine loss in the hippocampus of young PDAPP and Tg2576 mice and its prevention by the ApoE2 genotype. *Neurobiol. Dis.* 13:246-253.
- LaPointe NE, Morfini G, Pigino G, Gaisina IN, Kozikowski AP, Binder LI, Brady ST. 2009. The amino terminus of tau inhibits kinesin-dependent axonal transport: implications for filament toxicity. *J. Neurosci. Res.* 87:440-451.
- Lee LJ, Iwasato T, Itohara S, Erzurumlu RS. 2005. Exuberant thalamocortical axon arborization in cortex-specific NMDAR1 knockout mice. *J. Comp Neurol.* 485:280-292.
- Legrand C, Bour JM, Jacob C, Capiaumont J, Martial A, Marc A, Wudtke M, Kretzmer G, Demangel C, Duval D, . 1992. Lactate dehydrogenase (LDH) activity of the cultured eukaryotic cells as marker of the number of dead cells in the medium [corrected]. *J. Biotechnol.* 25:231-243.
- Lehmann J, Schneider J, McPherson S, Murphy DE, Bernard P, Tsai C, Bennett DA, Pastor G, Steel DJ, Boehm C, . 1987. CPP, a selective N-methyl-D-aspartate (NMDA)-type receptor antagonist: characterization in vitro and in vivo. *J. Pharmacol. Exp. Ther.* 240:737-746.
- Leskovjan AC, Lanzirotti A, Miller LM. 2009. Amyloid plaques in PSAPP mice bind less metal than plaques in human Alzheimer's disease. *Neuroimage.* 47:1215-1220.
- Leuba G, Savioz A, Vernay A, Carnal B, Kraftsik R, Tardif E, Riederer I, Riederer BM. 2008. Differential changes in synaptic proteins in the Alzheimer frontal cortex with marked increase in PSD-95 postsynaptic protein. *J. Alzheimers. Dis.* 15:139-151.

- Leussis MP, Lawson K, Stone K, Andersen SL. 2008. The enduring effects of an adolescent social stressor on synaptic density, part II: Poststress reversal of synaptic loss in the cortex by adinazolam and MK-801. *Synapse* 62:185-192.
- Leveille F, El GF, Gouix E, Lecocq M, Lobner D, Nicole O, Buisson A. 2008. Neuronal viability is controlled by a functional relation between synaptic and extrasynaptic NMDA receptors. *FASEB J.*
- Li DR, Ishikawa T, Zhao D, Michiue T, Quan L, Zhu BL, Maeda H. 2009. Histopathological changes of the hippocampus neurons in brain injury. *Histol. Histopathol.* 24:1113-1120.
- Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS. 2010. mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329:959-964.
- Lindwall G, Cole RD. 1984. Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J. Biol. Chem.* 259:5301-5305.
- Liu J, Chang L, Roselli F, Almeida OF, Gao X, Wang X, Yew DT, Wu Y. 2010. Amyloid-beta Induces Caspase-Dependent Loss of PSD-95 and Synaptophysin Through NMDA Receptors. *J. Alzheimers. Dis.*
- Liu Y, Wong TP, Aarts M, Rooyackers A, Liu L, Lai TW, Wu DC, Lu J, Tymianski M, Craig AM, Wang YT. 2007. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J. Neurosci.* 27:2846-2857.
- Liu Y, Yoo MJ, Savonenko A, Stirling W, Price DL, Borchelt DR, Mamounas L, Lyons WE, Blue ME, Lee MK. 2008. Amyloid pathology is associated with progressive monoaminergic neurodegeneration in a transgenic mouse model of Alzheimer's disease. *J. Neurosci.* 28:13805-13814.
- Long JM, Lahiri DK. 2011. MicroRNA-101 downregulates Alzheimer's amyloid-beta precursor protein levels in human cell cultures and is differentially expressed. *Biochem. Biophys. Res. Commun.* 404:889-895.
- Lopes JP, Oliveira CR, Agostinho P. 2010. Neurodegeneration in an Abeta-induced model of Alzheimer's disease: the role of Cdk5. *Aging Cell* 9:64-77.
- Lu CW, Lin TY, Wang SJ. 2010. Memantine depresses glutamate release through inhibition of voltage-dependent Ca²⁺ entry and protein kinase C in rat cerebral cortex nerve terminals: an NMDA receptor-independent mechanism. *Neurochem. Int.* 57:168-176.
- Ma YY, Cepeda C, Cui CL. 2009. The role of striatal NMDA receptors in drug addiction. *Int. Rev. Neurobiol.* 89:131-146.
- Marlow L, Canet RM, Haugabook SJ, Hardy JA, Lahiri DK, Sambamurti K. 2003. APH1, PEN2, and Nicastrin increase Abeta levels and gamma-secretase activity. *Biochem. Biophys. Res. Commun.* 305:502-509.
- Masliah E, Mallory M, Alford M, DeTeresa R, Hansen LA, McKeel DW, Jr., Morris JC. 2001. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology* 56:127-129.
- Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. U. S. A* 82:4245-4249.

- Matsui T, Ingelsson M, Fukumoto H, Ramasamy K, Kowa H, Frosch MP, Irizarry MC, Hyman BT. 2007. Expression of APP pathway mRNAs and proteins in Alzheimer's disease. *Brain Res.* 1161:116-123.
- Matsuoka Y, Picciano M, Malester B, LaFrancois J, Zehr C, Daeschner JM, Olschowka JA, Fonseca MI, O'Banion MK, Tenner AJ, Lemere CA, Duff K. 2001. Inflammatory responses to amyloidosis in a transgenic mouse model of Alzheimer's disease. *Am. J. Pathol.* 158:1345-1354.
- McConlogue L, Buttini M, Anderson JP, Brigham EF, Chen KS, Freedman SB, Games D, Johnson-Wood K, Lee M, Zeller M, Liu W, Motter R, Sinha S. 2007. Partial reduction of BACE1 has dramatic effects on Alzheimer plaque and synaptic pathology in APP Transgenic Mice. *J. Biol. Chem.* 282:26326-26334.
- McDonald CR, McEvoy LK, Gharapetian L, Fennema-Notestine C, Hagler DJ, Jr., Holland D, Koyama A, Brewer JB, Dale AM. 2009. Regional rates of neocortical atrophy from normal aging to early Alzheimer disease. *Neurology* 73:457-465.
- Medvedev NI, Popov VI, Dallerac G, Davies HA, Laroche S, Kraev IV, Rodriguez Arellano JJ, Doyere V, Stewart MG. 2010. Alterations in synaptic curvature in the dentate gyrus following induction of long-term potentiation, long-term depression, and treatment with the N-methyl-d-aspartate receptor antagonist CPP. *Neuroscience.*
- Miklossy J. 2008. Chronic inflammation and amyloidogenesis in Alzheimer's disease -- role of Spirochetes. *J. Alzheimers. Dis.* 13:381-391.
- Mizuta I, Katayama M, Watanabe M, Mishina M, Ishii K. 1998. Developmental expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary cultures of murine cerebral cortical neurons. *Cell Mol. Life Sci.* 54:721-725.
- Monaco EA, III. 2004. Recent evidence regarding a role for Cdk5 dysregulation in Alzheimer's disease. *Curr. Alzheimer Res.* 1:33-38.
- Moriguchi S, Zhao X, Marszalec W, Yeh JZ, Narahashi T. 2005. Modulation of N-methyl-D-aspartate receptors by donepezil in rat cortical neurons. *J. Pharmacol. Exp. Ther.* 315:125-135.
- Morsch R, Simon W, Coleman PD. 1999. Neurons may live for decades with neurofibrillary tangles. *J. Neuropathol. Exp. Neurol.* 58:188-197.
- Mrak RE, Griffin WS. 2005. Glia and their cytokines in progression of neurodegeneration. *Neurobiol. Aging* 26:349-354.
- Mucke L, Masliah E, Johnson WB, Ruppe MD, Alford M, Rockenstein EM, Forss-Petter S, Pietropaolo M, Mallory M, Abraham CR. 1994. Synaptotrophic effects of human amyloid beta protein precursors in the cortex of transgenic mice. *Brain Res.* 666:151-167.
- Myers AJ, Kaleem M, Marlowe L, Pittman AM, Lees AJ, Fung HC, Duckworth J, Leung D, Gibson A, Morris CM, de SR, Hardy J. 2005. The H1c haplotype at the MAPT locus is associated with Alzheimer's disease. *Hum. Mol. Genet.* 14:2399-2404.
- Niewiadomska G, Baksalerska-Pazera M, Riedel G. 2009. The septo-hippocampal system, learning and recovery of function. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33:791-805.

- Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. 2001. Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 60:759-767.
- Ohyagi Y, Asahara H, Chui DH, Tsuruta Y, Sakae N, Miyoshi K, Yamada T, Kikuchi H, Taniwaki T, Murai H, Ikezoe K, Furuya H, Kawarabayashi T, Shoji M, Checler F, Iwaki T, Makifuchi T, Takeda K, Kira J, Tabira T. 2005. Intracellular Abeta42 activates p53 promoter: a pathway to neurodegeneration in Alzheimer's disease. *FASEB J.* 19:255-257.
- Ono K, Hasegawa K, Naiki H, Yamada M. 2004. Curcumin has potent anti-amyloidogenic effects for Alzheimer's beta-amyloid fibrils in vitro. *J. Neurosci. Res.* 75:742-750.
- Papadia S, Soriano FX, Leveille F, Martel MA, Dakin KA, Hansen HH, Kaindl A, Sifringer M, Fowler J, Stefovaska V, McKenzie G, Craigon M, Corriveau R, Ghazal P, Horsburgh K, Yankner BA, Wyllie DJ, Ikonomidou C, Hardingham GE. 2008. Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. *Nat. Neurosci.* 11:476-487.
- Parsons CG, Danysz W, Quack G. 1999. Memantine is a clinically well tolerated N-methyl-D-aspartate (NMDA) receptor antagonist--a review of preclinical data. *Neuropharmacology* 38:735-767.
- Parsons CG, Quack G, Bresink I, Baran L, Przegalinski E, Kostowski W, Krzascik P, Hartmann S, Danysz W. 1995. Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists in vitro with anticonvulsive and motor impairment activity in vivo. *Neuropharmacology* 34:1239-1258.
- Parsons CG, Stoffler A, Danysz W. 2007. Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system--too little activation is bad, too much is even worse. *Neuropharmacology* 53:699-723.
- Patrick GN, Zukerberg L, Nikolic M, de La MS, Dikkes P, Tsai LH. 1999. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* 402:615-622.
- Phillips LL, Lyeth BG, Hamm RJ, Reeves TM, Povlishock JT. 1998. Glutamate antagonism during secondary deafferentation enhances cognition and axo-dendritic integrity after traumatic brain injury. *Hippocampus* 8:390-401.
- Puntel RL, Nogueira CW, Rocha JB. 2005. N-methyl-D-aspartate receptors are involved in the quinolinic acid, but not in the malonate pro-oxidative activity in vitro. *Neurochem. Res.* 30:417-424.
- Rammes G, Danysz W, Parsons CG. 2008. Pharmacodynamics of memantine: an update. *Curr. Neuropharmacol.* 6:55-78.
- Ray B, Bisht S, Maitra A, Maitra A, Lahiri DK. 2011a. Neuroprotective and neurorescue effects of a novel polymeric nanoparticle formulation of curcumin (NanoCurc) in the neuronal cell culture and animal model: implications for Alzheimer's disease. *J. Alzheimers. Dis.* 23:61-77.

- Ray B, Chauhan NB, Lahiri DK. 2011b. Oxidative insults to neurons and synapse are prevented by aged garlic extract and S-allyl-l-cysteine treatment in the neuronal culture and APP-Tg mouse model. *J. Neurochem.* 117:388-402.
- Ray B, Simon JR, Lahiri DK. 2009. Determination of high-affinity choline uptake (HACU) and choline acetyltransferase (ChAT) activity in the same population of cultured cells. *Brain Res.* 1297:160-168.
- Roberson ED, Scarce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L. 2007. Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. *Science* 316:750-754.
- Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT. 1998. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Donepezil Study Group. *Neurology* 50:136-145.
- Ronn LC, Ralets I, Hartz BP, Bech M, Berezin A, Berezin V, Moller A, Bock E. 2000. A simple procedure for quantification of neurite outgrowth based on stereological principles. *J. Neurosci. Methods* 100:25-32.
- Ryu JK, McLarnon JG. 2009. A leaky blood-brain barrier, fibrinogen infiltration and microglial reactivity in inflamed Alzheimer's disease brain. *J. Cell Mol. Med.* 13:2911-2925.
- Salinovich O, Montelaro RC. 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* 156:341-347.
- Salmina AB. 2009. Neuron-glia interactions as therapeutic targets in neurodegeneration. *J. Alzheimers. Dis.* 16:485-502.
- Salminen A, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. 2009. Inflammation in Alzheimer's disease: amyloid-beta oligomers trigger innate immunity defence via pattern recognition receptors. *Prog. Neurobiol.* 87:181-194.
- Sambamurti K, Greig NH, Utsuki T, Barnwell EL, Sharma E, Mazell C, Bhat NR, Kindy MS, Lahiri DK, Pappolla MA. 2011. Targets for AD treatment: conflicting messages from gamma-secretase inhibitors. *J. Neurochem.* 117:359-374.
- Sambamurti K, Kinsey R, Maloney B, Ge YW, Lahiri DK. 2004. Gene structure and organization of the human beta-secretase (BACE) promoter. *FASEB J.* 18:1034-1036.
- Sandbrink R, Masters CL, Beyreuther K. 1994. Beta A4-amyloid protein precursor mRNA isoforms without exon 15 are ubiquitously expressed in rat tissues including brain, but not in neurons. *J. Biol. Chem.* 269:1510-1517.
- Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ. 2007. Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. *Neurology* 68:1501-1508.
- Schipke CG, Ohlemeyer C, Matyash M, Nolte C, Kettenmann H, Kirchhoff F. 2001. Astrocytes of the mouse neocortex express functional N-methyl-D-aspartate receptors. *FASEB J.* 15:1270-1272.
- Schneider LS, Lahiri DK. 2009. The perils of Alzheimer's drug development. *Curr. Alzheimer Res.* 6:77-78.

- Schneppenheim R, Rautenberg P. 1987. A luminescence Western blot with enhanced sensitivity for antibodies to human immunodeficiency virus. *Eur. J. Clin. Microbiol.* 6:49-51.
- Schnitzler AC, Lopez-Coviella I, Blusztajn JK. 2008. Purification and culture of nerve growth factor receptor (p75)-expressing basal forebrain cholinergic neurons. *Nat. Protoc.* 3:34-40.
- Shimohama S, Kamiya S, Taniguchi T, Akagawa K, Kimura J. 1997. Differential involvement of synaptic vesicle and presynaptic plasma membrane proteins in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 236:239-242.
- Simon JR, Kuhar MG. 1975. Impulse-flow regulation of high affinity choline uptake in brain cholinergic nerve terminals. *Nature* 255:162-163.
- Simpson JE, Ince PG, Lace G, Forster G, Shaw PJ, Matthews F, Savva G, Brayne C, Wharton SB. 2010. Astrocyte phenotype in relation to Alzheimer-type pathology in the ageing brain. *Neurobiol. Aging* 31:578-590.
- Sinhorin VD, Roehrs C, Pasin JS, Belle NA, Rubin MA, Mello CF. 2005. Succinate causes oxidative damage through N-methyl-D-aspartate-mediated mechanisms. *Brain Res.* 1051:66-71.
- Stein TD, Johnson JA. 2002. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. *J. Neurosci.* 22:7380-7388.
- Steinerman JR, Irizarry M, Scarmeas N, Raju S, Brandt J, Albert M, Blacker D, Hyman B, Stern Y. 2008. Distinct pools of beta-amyloid in Alzheimer disease-affected brain: a clinicopathologic study. *Arch. Neurol.* 65:906-912.
- Stockley JH, Ravid R, O'Neill C. 2006. Altered beta-secretase enzyme kinetics and levels of both BACE1 and BACE2 in the Alzheimer's disease brain. *FEBS Lett.* 580:6550-6560.
- Sun W, Jia J. 2009. The +347 C promoter allele up-regulates MAPT expression and is associated with Alzheimer's disease among the Chinese Han. *Neurosci. Lett.* 450:340-343.
- Sun X, He G, Qing H, Zhou W, Dobie F, Cai F, Staufenbiel M, Huang LE, Song W. 2006. Hypoxia facilitates Alzheimer's disease pathogenesis by up-regulating BACE1 gene expression. *Proc. Natl. Acad. Sci. U. S. A* 103:18727-18732.
- Takada-Takatori Y, Kume T, Sugimoto M, Katsuki H, Sugimoto H, Akaike A. 2006. Acetylcholinesterase inhibitors used in treatment of Alzheimer's disease prevent glutamate neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol 3-kinase cascade. *Neuropharmacology* 51:474-486.
- Terry RD. 2000. Where in the brain does Alzheimer's disease begin? *Ann. Neurol.* 47:421.
- Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* 30:572-580.

- Theuns J, Brouwers N, Engelborghs S, Sleegers K, Bogaerts V, Corsmit E, De PT, van Duijn CM, De Deyn PP, Van BC. 2006. Promoter mutations that increase amyloid precursor-protein expression are associated with Alzheimer disease. *Am. J. Hum. Genet.* 78:936-946.
- Thies W, Bleiler L. 2011. 2011 Alzheimer's disease facts and figures. *Alzheimers. Dement.* 7:208-244.
- Thornton E, Vink R, Blumbergs PC, Van Den HC. 2006. Soluble amyloid precursor protein alpha reduces neuronal injury and improves functional outcome following diffuse traumatic brain injury in rats. *Brain Res.* 1094:38-46.
- Tilleux S, Hermans E. 2007. Neuroinflammation and regulation of glial glutamate uptake in neurological disorders. *J. Neurosci. Res.* 85:2059-2070.
- Touchon J, Bergman H, Bullock R, Rapatz G, Nagel J, Lane R. 2006. Response to rivastigmine or donepezil in Alzheimer's patients with symptoms suggestive of concomitant Lewy body pathology. *Curr. Med. Res. Opin.* 22:49-59.
- Ullrich C, Pirchl M, Humpel C. 2010. Hypercholesterolemia in rats impairs the cholinergic system and leads to memory deficits. *Mol. Cell Neurosci.*
- Van Nostrand WE, Wagner SL, Suzuki M, Choi BH, Farrow JS, Geddes JW, Cotman CW, Cunningham DD. 1989. Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor. *Nature* 341:546-549.
- Volbracht C, van BJ, Zhu C, Blomgren K, Leist M. 2006. Neuroprotective properties of memantine in different in vitro and in vivo models of excitotoxicity. *Eur. J. Neurosci.* 23:2611-2622.
- von Koch CS, Zheng H, Chen H, Trumbauer M, Thinakaran G, van der Ploeg LH, Price DL, Sisodia SS. 1997. Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. *Neurobiol. Aging* 18:661-669.
- Wati H, Kawarabayashi T, Matsubara E, Kasai A, Hirasawa T, Kubota T, Harigaya Y, Shoji M, Maeda S. 2009. Transthyretin accelerates vascular Abeta deposition in a mouse model of Alzheimer's disease. *Brain Pathol.* 19:48-57.
- Wen Y, Yu WH, Maloney B, Bailey J, Ma J, Marie I, Maurin T, Wang L, Figueroa H, Herman M, Krishnamurthy P, Liu L, Planel E, Lau LF, Lahiri DK, Duff K. 2008. Transcriptional regulation of beta-secretase by p25/cdk5 leads to enhanced amyloidogenic processing. *Neuron* 57:680-690.
- Wiesenhofer B, Humpel C. 2000. Lipid-mediated gene transfer into primary neurons using FuGene: comparison to C6 glioma cells and primary glia. *Exp. Neurol.* 164:38-44.
- Wu J, Basha MR, Brock B, Cox DP, Cardozo-Pelaez F, McPherson CA, Harry J, Rice DC, Maloney B, Chen D, Lahiri DK, Zawia NH. 2008. Alzheimer's disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD. *J. Neurosci.* 28:3-9.
- Wyss-Coray T, Loike JD, Brionne TC, Lu E, Anankov R, Yan F, Silverstein SC, Husemann J. 2003. Adult mouse astrocytes degrade amyloid-beta in vitro and in situ. *Nat. Med.* 9:453-457.

- Xia P, Chen HS, Zhang D, Lipton SA. 2010. Memantine preferentially blocks extrasynaptic over synaptic NMDA receptor currents in hippocampal autapses. *J. Neurosci.* 30:11246-11250.
- Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kaye R, Glabe CG, Frautsch SA, Cole GM. 2005. Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J. Biol. Chem.* 280:5892-5901.
- Yang H, Cong R, Na L, Ju G, You SW. 2010. Long-term primary culture of highly-pure rat embryonic hippocampal neurons of low-density. *Neurochem. Res.* 35:1333-1342.
- Yin YI, Bassit B, Zhu L, Yang X, Wang C, Li YM. 2007. γ -Secretase Substrate Concentration Modulates the A β 42/A β 40 Ratio: IMPLICATIONS FOR ALZHEIMER DISEASE. *J. Biol. Chem.* 282:23639-23644.
- Zaja-Milatovic S, Gupta RC, Aschner M, Milatovic D. 2009. Protection of DFP-induced oxidative damage and neurodegeneration by antioxidants and NMDA receptor antagonist. *Toxicol. Appl. Pharmacol.* 240:124-131.
- Zheng H, Jiang M, Trumbauer ME, Hopkins R, Sirinathsinghji DJ, Stevens KA, Conner MW, Slunt HH, Sisodia SS, Chen HY, van der Ploeg LH. 1996. Mice deficient for the amyloid precursor protein gene. *Ann. N. Y. Acad. Sci.* 777:421-426.

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Publications:

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Bailey J.A., Ray, B., Greig N.H., Lahiri D.K. Rivastigmine lowers $A\beta$ and increases sAPP α levels, which parallel elevated synaptic markers and metabolic activity in degenerating primary rat neurons. *PLoS ONE. In press, 2011.*

Bondy S.C., Li H., Zhou J., Wu M., Bailey J.A., Lahiri D.K. Melatonin Alters Age-Related Changes in Transcription Factors and Kinase Activation. *Neurochemical Research. 35(12):2035-42, 2010.*

Li Y., Duffy K.B., Ottinger M.A., Ray B., Bailey J.A., Holloway H.W., Tweedie D., Perry T., Mattson M.P., Kapogiannis D., Sambamurti K., Lahiri D.K., Greig N.H. GLP-1 receptor stimulation reduces amyloid-beta peptide accumulation and cytotoxicity in cellular and animal models of Alzheimer's disease. *Journal of Alzheimers Disease. 19(4):1205-19, 2010.*

Bailey, J.A., Lahiri, D.K. A novel effect of rivastigmine on presynaptic proteins and neuronal viability in a neurodegeneration model of fetal rat primary cortical cultures and its implication in Alzheimer's disease. *Journal of Neurochemistry. 112(4):843-53, 2010.*

Alley G.M., Bailey J.A., Chen D., Ray B., Puli L.K., Tanila H., Banerjee P.K., and Lahiri D.K. Memantine Lowers Amyloid-beta Peptide Levels in Neuronal Cultures and in APP/PS1 Transgenic Mice. *Journal of Neuroscience Research*. *Journal of Neuroscience Research*. 88(1):143-54, 2010.

Ray B., Bailey J.A., Sarkar S., Lahiri D.K. Molecular and immunocytochemical characterization of primary neuronal cultures from adult rat brain: Differential expression of neuronal and glial protein markers. *Journal of Neuroscience Methods*. 184(2):294-302, 2009.

Wen Y., Yu W.H., Maloney B., Bailey J., Ma J., Marié I., Maurin T., Wang L., Figueroa H., Herman M., Krishnamurthy P., Liu L., Planel E., Lau L.F., Lahiri D.K., Duff K. Transcriptional regulation of beta-secretase by p25/cdk5 leads to enhanced amyloidogenic processing. *Neuron*. 57(5):680-90, 2008.

Bailey J.A., Ge Y-W., Lahiri D.K. Sensitive Detection of Reporter Gene Assay Results. *Cell Notes*. 17:13-15, 2007.

Tweedie D., Brossi A., Chen D., Ge Y.-W., Bailey J., Yu Q.S., Kamal M.A., Sambamurti K., Lahiri D.K., Greig N.H. Neurine, an acetylcholine autolysis product, elevates secreted amyloid-beta protein precursor and amyloid-beta peptide levels, and lowers neuronal cell viability in culture: A role in Alzheimer's disease? *Journal of Alzheimer's Disease*. 10(1):9-16, 2006.

Bailey J.A., Lahiri D.K. Neuronal differentiation is accompanied by increased levels of SNAP-25 protein in fetal rat primary cortical neurons- Implications in neuronal plasticity and Alzheimer's disease. *Annals of the New York Academy of Science*. 1086:54-65, 2006.

Posters and Presentations:

Bailey, J.A., Ray B., Banerjee P.K., Greig N.H., Lahiri D.K. Neuroprotection mechanisms of Alzheimer's disease drugs memantine and rivastigmine involving the BDNF and mTOR pathways. Society for Neuroscience 41st Annual Meeting, November 12-16, 2011, Washington, DC.

Lahiri D.K., Ray B., Bailey, J.A., Long, J. Early-life environment and epigenetics in Alzheimer's disease, a new paradigm represented by the LEARN model. Society of Biological Psychiatry 66th Annual Convention and Scientific Program, May 12-14, 2011, San Francisco, CA.

Lahiri D.K., Ray B., Chauhan N.B., Bailey J.B., Long J.M. a Novel Therapeutic Agent for Alzheimer's Disease (AD) Based on Studies from Neuronal Cultures and APP-transgenic Model for AD. 4th International Conference on Drug Discovery and Therapy, February 7-10, 2011, Dubai, UAE.

Lahiri D.K., Bailey J.A., Ray B., Banerjee P.K. Rapamycin treatment reverses memantine mediated increased synaptic signaling proteins in degenerating primary neurons: Implications in Alzheimer's disease. Alzheimer's Association International Conference on Alzheimer's Disease, July 16-21, 2011, Honolulu, HI.

Lahiri D.K., Bailey J.A., Ray B., Tanila H., Banerjee P.K., Long J. The effect of memantine and rivastigmine on synaptic markers and amyloid processing in *in vivo* and *in vitro* models of Alzheimer's disease. Alzheimer's Association International Conference on Alzheimer's Disease, July 10-15, 2010, Honolulu, HI.

Bailey J.A., Ray B., Banerjee P.K., Lahiri D.K. Novel effects of memantine on APP and synaptic proteins in different cell lines are mediated by intracellular targets. Society for Neuroscience 40th annual meeting, November 13-17, 2010, San Diego, CA.

Ray B., Bailey J.A., Long J.M., Pathak K., Sambamurti K., Rogers J.T., Greig N.H., Maitra D., Lahiri D.K. Historical journey of the application and use of curcumin from culinary purposes to therapeutics in neurodegenerative disorders. Society for Neuroscience 40th annual meeting, November 13-17, 2010, San Diego, CA.

Ray B., Bailey J.A., Greig N.H., Lahiri D.K. Rivastigmine promotes alpha-secretase pathway of β -amyloid precursor protein (APP) processing by up-regulating ADAM complex and its implication in Alzheimer's disease (AD). Society for Neuroscience 40th annual meeting, November 13-17, San Diego, 2010, CA.

Bailey J.A., Lahiri D.K. Nuclear localization of the amyloid-beta peptide in response to oxidative stress in neuronal cells. Society for Neuroscience 39th annual meeting, October 17-21, 2009, Chicago, IL.

Ray B., Bailey J.A., Greig N.H., Lahiri D.K. Role of rivastigmine in the processing of amyloid beta precursor and synaptic protein: implication in Alzheimer's disease. Society for Neuroscience 39th annual meeting, October 17-21, 2009, Chicago, IL.

Simon J.R., Ray B., Bailey J.A., Sambamurti K., Greig N.H., Tweedie D., Lahiri D.K. Role of rivastigmine in the processing of amyloid beta precursor and synaptic protein: implication in Alzheimer's disease. Society for Neuroscience 39th annual meeting, October 17-21, 2009, Chicago, IL.

Lahiri D.K., Ray B., Bailey, J., Sambamurti K., Greig N., Chauhan N. Neuroprotective role of novel natural products: Implications in aging and Alzheimer's disease. International Society for Neurochemistry/Asian Pacific Society for Neurochemistry 22nd Biennial Meeting, Busan, Korea, August 23-28, 2009.

Bailey J.A., Maloney, B., Ge, Y-W., Lahiri D.K. Internalization of the amyloid beta-peptide (A β) may result in binding to the regulatory region of the APP gene promoter. International Conference on Alzheimer's Disease. July 11-16, 2009, Vienna, Austria.

Bailey J.A., Lahiri D.K. Nuclear localization of the amyloid β -peptide during oxidative stress in neuronal cells. 21st American Peptide Symposium. June 7-12, 2009, Bloomington, IN.

Lahiri, D.K., Maloney B., Bailey, J.A., Ge, Y-W. Role of Alzheimer's amyloid-beta peptide as a putative transcription factor. 21st American Peptide Symposium. June 7-12, 2009, Bloomington, IN.

Lahiri, D.K., Bailey, J.A., Maloney, B., Greig, N., Duff, K., Ray, B. Regulation of BACE1 and Tau genes: Inhibitors of p25 and STAT sites as unique therapeutic targets for Alzheimer's disease. International Conference on Alzheimer's Disease and Parkinson's Disease. March 11-15, 2009, Prague, Czech Republic.

Bailey J.A., Ray B., Lahiri D.K. Inhibition of the NMDA receptor preserves synaptic marker expression and synaptic function in primary cortical cell cultures. Society for Neuroscience 38th annual meeting. November 15-19, 2008, Washington, D.C.

Bailey J.A., Lahiri D.K. Rivastigmine Decreases Amyloid- β Precursor Protein (app) And Increases Synaptic Markers in Primary Neuronal Cultures. International Conference on Alzheimer's Disease. July 26-31, 2008, Chicago, IL.

Bailey J.A., Lahiri D.K. NMDA receptor channel blockade preserves synaptic markers in primary cortical cell cultures. Society for Neuroscience 37th annual meeting. November 3-7, 2007, San Diego, CA.

Bailey J.A., Lahiri D.K., Evidence for a role of amyloid-beta precursor protein (APP) in neurite outgrowth and synaptogenesis. Society for Neuroscience 36th annual meeting. October 14-18, 2006, Atlanta, GA.

Kubek M.J., Yard M., Bailey J.A., Veronesi, M.C., Lahiri, D.K., Thyrotropin-releasing hormone (TRH) is protective against DHPG plus NMDA glutamate agonist-induced toxicity in cultured fetal hippocampal neurons. Society for Neuroscience 36th annual meeting. October 14-18, 2006, Atlanta, GA.

Bailey J.A., Lahiri D.K., Role of Amyloid Precursor Protein (APP) and Synaptic Proteins During Synaptogenesis. 10th International Conference on Alzheimer's Disease and Related Disorders. July 14-20, 2006, Madrid, Spain.

Bailey J.A., Lahiri D.K., Altered processing of the Alzheimer amyloid- β precursor protein during retinoic acid-induced differentiation of human SK-N-SH neuroblastoma cells. Society for Neuroscience 35th annual meeting. November 12-16, 2005, Washington, DC.

Tweedie D., Lahiri D.K., Chen D.M., Ge Y.W., Bailey J., Brossi, A., Soncrant, T.T., Greig, N.H. Actions of Neurine, a product of acetylcholine (ACh) autolysis product, on neuronal cell viability, APP and the amyloid- β peptide in cell culture. Society for Neuroscience 35th annual meeting. November 12-16, 2005, Washington, DC.

Lahiri D.K., Bailey J., Chen D.M., Farlow M.A., Banerjee P.K. Memantine decreases the levels of Alzheimer β -amyloid proteins in rat primary cortical neurons and human neuroblastoma cells. Society of Biological Psychiatry, 60th annual meeting. May 19-21, 2005, Atlanta, GA.

Bailey J.A., Lahiri D.K. Retinoic acid differentiation of SK-N-SH human neuroblastoma cells enhances APP secretion and NMDA receptor expression. Society for Neuroscience 34th annual meeting. October 23-27, 2004, San Diego, CA.

Lahiri D.K., Bailey J., Alley G., Chen D., Sambamurti K., Greig, N. Effects of diverse cholinesterase inhibitors and memantine on the amyloid pathway and its implications in Alzheimer's disease. 9th International Conference on Alzheimer's Disease and Related Disorders. July 17-22, 2004, Philadelphia, PA.

Campbell M.C., Wylie S.A., Bailey J.A., and Stout J.C. Specificity of the relationship between motor symptoms and cognitive functioning in Parkinson's disease. Mental and Behavioral Dysfunction in Movement Disorders International Symposium, October 10-13, 2001, Montreal, Canada. Movement Disorders, 16(1), S24.