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β-amyloid$_{1-42}$ (Aβ42) has been implicated in the pathogenesis of Alzheimer's disease (AD); however, the amount of this peptide in the brain does not correlate well with the presence or severity of AD. This project tested the hypothesis that individual differences exist in susceptibility to Aβ42 neurotoxicity arising from differences in the expression of α7 nicotinic acetylcholine receptors (α7 nAChRs). This hypothesis was tested in primary neuronal cultures derived from inbred mouse strains which differ in expression of α7 nAChRs. Also, the ability of nicotinic agents to modulate Aβ42 toxicity was examined. Significant strain differences in susceptibility to Aβ42 toxicity were found; however, these were not related to levels of α7 nAChRs. Additionally, strain differences were found in the ability of an α7-selective partial agonist, an α7-selective antagonist and a α4β2 nAChR-selective antagonist to protect against this toxicity. Inbred strains of mice may be useful in uncovering the pathophysiology of AD.

GENETIC MODULATION OF β-AMYLOID NEUROTOXICITY

AND PROTECTION BY NICOTINIC AGENTS

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CHAPTER I

INTRODUCTION

Summary of the Problem

The race to elucidate the pathogenesis of Alzheimer's disease (AD) has led to the emergence of multiple theories, each attributing varying levels of importance to the roles of extracellular β-amyloid$_{1-42}$ (Aβ42)-containing neuritic plaques, intraneuronal neurofibrillary tangles, and multiple other agents in the development and progression of AD. One such hypothesis put forth by Glenner, Wong, and colleagues (1984b; 1984a; 1984), the amyloid hypothesis, suggests that the aggregation of Aβ42-containing plaques are neurotoxic and play a key role in initiating a cascade of events that eventually culminates in the clinical manifestation of dementia.

This project attempted to provide a plausible explanation for the commonly reported finding at autopsy that there seems to be only a weak correlation between amyloid load in the brain and clinically-evident AD (Nagy et al., 1995; Cummings et al., 1996; Mufson et al., 1999; Giannakopoulos et al., 2003). The project was based on the hypothesis that there are individual differences in susceptibility to Aβ42-induced neurotoxicity. Differences among individuals in susceptibility to Aβ42-induced neurotoxicity may explain why two people with the same amyloid load in their brains may not both develop the disease. The project tested this hypothesis by examining Aβ42 neurotoxicity in neuronal cultures derived from three different inbred strains of mice.

Because each inbred strain has a unique genotype, a demonstration of strain differences would indicate that genotype can modulate susceptibility to Aβ42 toxicity.

Secondly, it was hypothesized that α7 nicotinic acetylcholine receptors (nAChRs), a subtype of neuronal nAChRs, are involved in modulating Aβ42 toxicity, and further, that known strain differences in the expression of α7 nAChRs (simulating potential individual differences in the human population) would predict the level of sensitivity to Aβ42-induced neuronal toxicity. The strains of mice to be utilized in these experiments, C57BL/6, C58, and DBA/2, were chosen based on published reports that they differ in α7 nAChR expression levels (Marks et al., 1989). If α7 nAChRs modulate Aβ42 toxicity, one would expect that strain differences in susceptibility to Aβ42 toxicity would be correlated with differences in α7 nAChR expression.

Data demonstrating that the α7 nAChR-selective partial agonist, (3)-2,4-dimethoxybenzylidene anabaseine (DMXB) (Meyer et al. 1997) can protect against β-amyloid$_{25\text{-}35}$ (Aβ25-35) toxicity (Kihara et al., 1997) also suggest that α7 nAChRs are involved in modulating the neurotoxic effects of amyloids. It was hypothesized that the differential expression of α7 nAChRs (in different strains) would modulate the neuroprotective effects of DMXB.

Problem/Hypotheses

The cause of most cases of AD remains unknown (i.e., most AD is idiopathic). While there is evidence to support the amyloid hypothesis, data also exist that refute this hypothesis (Hardy, 2006; Prasher et al., 1998; Younkin, 1995). Our hypothesis is one

which refines the classic amyloid cascade hypothesis. Specifically, we hypothesized that Aβ42 plays a central role in the pathogenesis of AD, but that individual differences in susceptibility to Aβ42 toxicity, partially modulated by α7 nAChRs, exist and contribute to whether an individual with a given amyloid load develops AD.

Reports that Aβ42 binds to α7 nAChRs with extremely high affinity (Wang et al., 2000a,b) support the hypothesis that these receptors may be involved in modulating the effects of Aβ42. Furthermore, agents with known activity at α7 nAChRs have been shown to be neuroprotective against various insults, including Aβ25-35 toxicity (Kihara et al., 1997). My first goal was to demonstrate that individual differences in susceptibility to Aβ42 neurotoxicity exist. My second goal was to determine whether or not genetically mediated differences in α7 nAChR expression correlate both with differences in susceptibility to Aβ42 neurotoxicity as well as the ability of DMXB to protect against this toxicity.

It was hypothesized that individual differences in susceptibility to Aβ42 toxicity exist and we attempted to demonstrate this by looking for such differences in primary cultures derived from inbred strains of mice. Further, it was hypothesized that the level of expression of α7 nAChRs in a given individual partially modulates the efficacy of Aβ42 in terms of producing neurotoxicity. If true, it was expected that a significant correlation between α7 nAChRs expression levels and the efficacy of Aβ42 in producing toxic effects would be found. Inbred strains of mice which were utilized in these experiments, C57BL/6, C58, and DBA/2, were chosen based on published reports of differences in α7 nAChR expression levels (Marks et al., 1989). Given a lack of evidence in the literature

suggesting a role for the other major nAChR subtype in brain, α4β2, in modulating the toxicity of Aβ42, it was hypothesized that α7 nAChRs, but not α4β2 nAChRs, play a role in modulating Aβ42 toxicity. It was predicted that DMXB, an α7 nAChR-selective partial agonist (Kem, 1997; Meyer et al., 1997), would protect against Aβ42 toxicity and that this protection could be blocked by methyllycaconitine (MLA), an α7 nAChR-selective antagonist (Ward et al., 1990; Yum et al., 1996), but not by dihydro-β-erythroidine (DHβE), an α4β2 nAChR-selective antagonist (Dwoskin and Crooks, 2001).

Significance of the Problem

AD affects an estimated 5.1 million Americans and its prevalence is rising, with predictions as high as 16 million Americans being afflicted with AD by the year 2050 (Alzheimer's Association, 2007). This startling statistic underscores the significant need for aggressive research in the field. Hopefully AD research will eventually culminate in the production of medications that will be effective at halting the progression of the disease or ideally allowing for the regression of the disease process along with the alleviation of its devastating symptoms. The Alzheimer's Association also asserts that given the generally advanced age of patients with symptomatic AD, a therapy capable of delaying the onset of AD symptoms for only five years could potentially reduce the number of Americans diagnosed with AD by up to 50 percent (Alzheimer's Association, 2007). Also of note is the astronomical cost to individuals, families, the insurance industry, as well as the government, associated with caring for those with AD.

A finding that the genetic makeup of an individual, possibly with regards to the expression of α7 nAChRs, affects how much damage is inflicted by the presence Aβ42 could have a tremendous impact on our understanding of AD and implications for future drug development. First, individual differences in susceptibility to Aβ42 toxicity could be one potential explanation for the apparent discrepancy often reported in human autopsy studies where there seems to be only a weak correlation between the amount of senile plaques (the core of which are primarily composed of Aβ42) present and the degree of clinically significant dementia (Nagy et al., 1995; Cummings et al., 1996; Mufson et al., 1999; Giannakopoulos et al., 2003). It logically follows that certain drugs may be effective in a certain population of AD patients, while less effective or even ineffective in other populations.

CHAPTER II

LITERATURE REVIEW

Alzheimer's Disease

AD is a neurodegenerative disorder characterized by chronic, progressive dementia. In 1906, Alois Alzheimer, a German physician, presented the case of Auguste D., a 56 year old female, who for several years prior to her death had been experiencing symptoms of memory loss, paranoid delusions, language deficits, and learning problems (Alzheimer, 1907). On autopsy, her brain revealed diffuse atrophy without any other gross abnormalities. However, when stained appropriately, Auguste D.'s brain revealed the presence of what are now identified as neuritic or senile plaques along with neurofibrillary tangles within her neurons (Cecil et al., 2004). While a patient's clinical symptomatology can be highly suggestive of a diagnosis of AD, to this day, the presence of plaques and tangles in the brain upon autopsy or biopsy remain the only definitive way to diagnose AD.

The common symptoms of AD include early memory loss, language problems (specifically word-finding difficulties), and decline in visual/spatial functions followed by apraxia, aphasia, agnosias, and behavioral issues (i.e., delusional behavior and apathy) later in the course of the disease. (Cecil et al., 2004). AD is the most common type of dementia and the sporadic form typically affects patients older than 65 years of age,

while patients with familial AD often first exhibit symptoms as young as in their 30's (Cecil et al., 2004; Blennow et al., 2006).

The Amyloid Hypothesis of Alzheimer's Disease

The brain of an AD patient is characterized by the presence of extracellular neuritic plaques and intracellular neurofibrillary tangles. AD-associated plaques contain a core of β-amyloid, primarily Aβ42, while neurofibrillary tangles are composed of paired helical filaments of hyperphosphorylated tau protein (Selkoe, 2001, 2004).

Varying lengths of β-amyloid peptides are generated by the cleavage of amyloid precursor protein (APP) by the α-, β,- and γ-secretases, and thought to be of particular importance to the development of AD is the generation of the Aβ42. While Aβ42 represents only a small portion of β-amyloid production (as compared to the more abundant β-amyloid$_{1\text{-}40}$ peptide), it is particularly prone to aggregation, accumulation in plaques, and therefore, as hypothesized by some, greater neurotoxic activity (Hardy and Selkoe, 2002; Kamboh, 2004).

The fact that accumulation of Aβ42 is a hallmark of AD and that Aβ42 has been demonstrated to be neurotoxic, itself a controversial finding, has led to the hypothesis that Aβ42 is key to the pathogenesis of AD (Hardy and Allsop, 1991). The amyloid hypothesis of AD, initially put forth over 20 years ago by Glenner, Wong, and colleagues (1984a; 1984b; 1984), states that the aggregation of Aβ42 is central to the development of AD and that the accumulation of Aβ42 in neuritic plaques initiates a cascade of events

that culminates in neuronal dysfunction and death and, subsequently, clinically evident cognitive decline.

The amyloid hypothesis was initially developed after amyloid was shown to be present in meningeal blood vessels of both patients with AD and Down's syndrome (Glenner and Wong, 1984a). The hypothesis remains one of the most promising explanations for the pathogenesis of AD (Hardy and Selkoe, 2002; Tanzi and Bertram, 2005; Hardy, 2006). Evidence supporting the amyloid hypothesis was bolstered when the APP gene was eventually cloned and localized to chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987). This provided the groundwork for some of the strongest evidence in support of the amyloid hypothesis – that several different mutations in the APP gene lead to familial AD, a form of AD inherited in an autosomally dominant fashion (Goldgaber et al., 1987; Kang et al., 1987; Goate et al., 1991). The finding that mutant form of the APP gene leads to the development of familial AD in addition to previous knowledge that Down's syndrome patients (with trisomy of chromosome 21) almost always develop an Alzheimer's-like dementia if they survive into young adulthood, support the hypothesis that the buildup of Aβ42 in the brain is the seminal event in the development of AD. Further supporting the amyloid hypothesis was the discovery that many of the mutations in the APP gene that lead to the development of familial AD were situated near the cleavage sites for the α-,β,- and γ-secretases, some of which led to the production of Aβ42 peptides which tend to aggregate (Shastry and Giblin, 1999). These mutations in the APP gene as well as in the presenilin-1 and -2 (PSEN1 and PSEN2) genes are thought to lead to increasing production of the 42 amino

acid peptide, Aβ42 (Levy-Lahad et al., 1995; Sherrington et al., 1995; Hardy and Selkoe, 2002).

Recent reports suggest that the more soluble oligomeric forms of Aβ42 may be the more relevant cause of neuronal dysfunction instead of insoluble aggregating forms (Dahlgren et al., 2002; Walsh et al., 2002b; Walsh et al., 2002a). The role of Aβ42 in the development of AD has further been refined to suggest that it can lead to activation of glia with a subsequent inflammatory response, oxidative damage, activation of cell signaling cascades, and formation of neurofibrillary tangles, all of which play a role in neuronal dysfunction/death with synaptic dysfunction and eventually lead to the clinical syndrome of chronic, progressive dementia (Hardy and Selkoe, 2002).

While much evidence exists to support the hypothesis that Aβ42 is central to the pathogenesis of AD, there continues to be debate as a result of several studies showing variable relationships between the level of clinically demonstrated dementia and the amount of Aβ42 present in the brain of AD patients versus age-matched controls on autopsy (Nagy et al., 1995; Cummings et al., 1996; Mufson et al., 1999; Giannakopoulos et al., 2003). There is clearly not a simple one to one relationship between the amyloid load in a patient's brain and the level of cognitive dysfunction present. Perhaps the lack of a one to one correlation between amyloid load and the degree of dementia is a result of Aβ42 having a differential degree of neurotoxicity in different individuals.

Inbred Mouse Strains: Tools for Studying Genetically Mediated Individual Differences

One potential way to examine individual differences in response to a compound is to utilize genetically defined strains of animals. Inbred strains of rodents or other animals (e.g., drosophila) are generated and maintained by sibling matings for 20 or more consecutive generations (Festing, 1997; Jackson Laboratories, 2006). Mice of a given inbred strain are considered genetically identical and are homozygous at practically all of their loci (Festing, 1997; Jackson Laboratories, 2006). While mice within a strain have little, if any genetic variability, considerable variability does exist among different strains. Thus different strains can be compared to assess the possibility that genetic variability may modulate a given phenotype (e.g., susceptibility to Aβ42 neurotoxicity).

While there is some variability in the levels of expression of α7 nAChRs in the brains of mice within a given strain, there is considerably more variably among various different inbred strains of mice (Marks et al., 1986; Marks et al., 1989). More specifically, significant variation in the amount of [^{125}I]α-bungarotoxin binding (α-[^{125}I]BTX), a highly selective ligand for α7 nAChRs, was demonstrated in most brain regions assayed (including cerebral cortex) among 19 inbred strains of mice tested. Among these, the C57BL/6, C58, and DBA/2J strains, the strains chosen for analysis in this project, had great variability and represent a continuum of differential α7 nAChR expression levels (Marks et al., 1989).

While receptor number was not directly measured in the current project, published data (Marks et al., 1989) shows that within the cerebral cortex of the inbred strains represented in this study, C58 mice demonstrated the highest level of α-[^{125}I]BTX

binding, followed by C57BL/6 mice, and lastly DBA/2 mice. Table 1 shows these differences in α-[^{125}I]BTX binding (using 1.1nM α-[^{125}I]BTX) as reported by Marks, et al. (1989), which indeed show considerable variation.

Table 1. α-[^{125}I]BTX Binding in Cerebral Cortical Tissue*

Strain	**Fmol/mg protein**
DBA/2	23.4 ± 1.0
C57BL/6	25.4 ± 0.9
C58	31.4 ± 0.6

***Data as reported in Marks, et al. (1989).**

Because of the differential levels of α7 nAChR expression in these strains, it was hypothesized that differences among these strains would be found in terms of susceptibility to Aβ42-induced neurotoxicity as well as the ability of nicotinic agents to produce neuroprotection against this toxicity.

Neuronal Nicotinic Receptors and Aβ42 Toxicity

Neuronal nAChRs are ligand-gated ion channels that are located in the central nervous system (as well as in non-nervous tissue) and are named for the fact that they are activated by nicotine. Neuronal nAChRs, like the nAChRs found at the neuromuscular junction, are made up of five subunits surrounding a central pore and are composed of various combinations of subunits. Those neuronal subunits identified thus far include α2-α10 and β2-β4 and are expressed in either a heterologous (e.g. α4β2) or homomeric

fashion (e.g. α7). Receptors of the α7 subtype are unique in their high affinity binding for α-bungarotoxin and relatively low affinity binding to nicotine as well as by their high permeability to calcium ions (Seguela et al., 1993; Nestler et al., 2001). These α7 receptors are also distinguished by the fact that they undergo rapid and extensive desensitization following activation by agonists.

Agents that act as agonists on α7 nAChRs are neuroprotective against various toxic insults, including ethanol (Li et al., 1999b; Li et al., 2000; Li et al., 2002; de Fiebre and de Fiebre, 2003) and Aβ42 (Kihara et al., 1997; Kihara et al.). MLA, an α7 nAChR-selective antagonist (Dwoskin and Crooks, 2001), has been reported to be able to block the neuroprotective effects of nonselective nicotinic agonists, such as nicotine (Dajas-Bailador, 2000; Kaneko, 1997; Prendergast, 2001; Tizabi et al., 2004), as well as an α7 selective nicotinic agonist, DMXB (Li et al., 2002). That agonists acting at α7 nAChRs have neuroprotective actions suggests that α7 nAChRs play a role in modulating neuronal viability.

Aβ42 has been reported to bind selectively and with very high affinity (picomolar) to α7 nAChRs (Wang et al., 2000a,b). Different labs have reported opposite effects of Aβ42 at α7 nAChRs. One lab has reported that Aβ42 binding to α7 nAChRs leads to activation (Dineley et al, 2002), while another lab has reported that this binding leads to inhibition (Liu et al., 2001; Pettit et al., 2001) of α7 nAChRs. These seemingly contradictory reports might be explained by the fact that α7 nAChRs undergo rapid and extensive desensitization, which can render activation difficult to detect. While α7 agonists activate α7 nAChRs, as would be expected from an agonist, they also can

rapidly inactivate these receptors (i.e., cause a functional antagonism (Seguela et al., 1993; de Fiebre et al., 1995; Meyer et al., 1998). However, while α7 nAChRs desensitize quickly, evidence showing that MLA can block α7 nAChR-mediated neuroprotection suggests that it is activation of these receptors that is responsible for the neuroprotective actions of α7 agents (Meyer et al., 1998; Li et al., 1999b; Li et al., 1999a; Li et al., 2000; Li et al., 2002).

Another role for α7 nAChRs is that these receptors modulate inflammation (Wang et al., 2003; Saeed et al., 2005; Wang et al., 2005). Brains of patients with AD typically show signs of chronic inflammation upon post-mortem examination as evidenced by the presence of elevated levels of activated microglia and reactive astrocytes alongside amyloid plaques, as well as increased levels of inflammatory modulators such as cytokines (Lim et al., 2000). A link between the use of anti-inflammatory medications and a reduced risk for developing AD has also been suggested in epidemiological studies (Yip et al, 2005). Also of note is the finding that α7 nAChRs have been shown to be involved in the aggregation of Aβ42 (Nagele et al., 2002).

Unlike the α7 subtype of nAChRs, the other prominent nAChR subtype, α4β2, does not appear to be involved in Aβ42 toxicity. While there may be no evidence that α4β2 nAChRs are involved in modulating Aβ42 toxicity, they are still the predominant nAChR subtype that is lost in AD (Gotti 2006). This study utilized DHβE, an α4β2 nAChR-selective antagonist, in order to examine whether α4β2 nAChRs are involved in modulating Aβ42 toxicity or the protection expected with DMXB. As DHβE was

hypothesized to not have a role in modulating this toxicity or protection, its use could be considered to be that of a negative control.

Activation Versus Desensitization of α7 nAChRs in the Modulation of Neuronal Viability

Debate remains as to whether it is agonist activation (by selective or nonselective agonists) at or subsequent desensitization/inactivation of α7 nAChRs that produces neuroprotective effects of α7 nAChR agonists. Most prior studies have utilized chronic applications of agents with activity at α7 nAChRs which produce transient stimulation quickly followed by long term desensitization (Kaneko, 1997; Li et al., 2002; Prendergast, 2001). Thus, if neuroprotection was due to activation, it would be due to a very transient event involving α7 nAChRs, as most of these receptors would be desensitized for much of the duration of chronic treatments. Nevertheless, the fact that MLA appears to block the neuroprotective effects of agonists acting at α7 nAChRs would suggest that it is activation and not the subsequent desensitization of these receptors which is responsible for the neuroprotective effects of agents like DMXB or nicotine itself.

Still, rapidly desensitizing receptors such as α7 nAChRs can potentially make it difficult to differentiate between agonist and functional antagonist effects, thus providing a logical reason for the apparent discrepant findings with Aβ42 (as discussed above). In order to further examine whether activation or antagonism/desensitization is the mechanism underlying the neuroprotective actions of α7 agents, I have employed a pharmacological approach using two α7 nAChR-selective agents, DMXB and MLA. If

MLA blocked DMXB's effects, as expected, the hypothesis that activation of α7 nAChRs is necessary for neuroprotection would be supported. However, in the unexpected event that MLA would not block the neuroprotective action, a novel hypothesis that desensitization (functional antagonism) or direct antagonism of α7 nAChRs promotes cell survival would be supported.

Given the existing literature, it was hypothesized that activation of α7 nAChRs promotes cell survival and that inhibition of these receptors would have the opposite effect. Because Aβ42 is a neurotoxic peptide, it was also hypothesized that its effects at α7 nAChRs would be one of antagonism that could be reversed by an agonist (DMXB). Further, it was hypothesized that MLA, but not DHβE would block these effects. Additionally, it was hypothesized that cultures from those strains with greater expression of α7 nAChRs would be more susceptible to Aβ42 neurotoxicity. Lastly, it was hypothesized that levels of neuroprotection afforded by DMXB would be modulated by levels of α7 nAChR expression.

CHAPTER III

METHODS

Materials

Unless stated otherwise, all cell culture media and reagents were obtained from Mediatech (Cellgro, Herndon, VA). Heat-inactivated donor horse serum was obtained from Tissue Culture Biologicals (Tulare, CA). Deoxyribonuclease I (DNAse), poly-L-ornithine, and cytosine β-D-arabinofuranoside (Ara-C) were obtained from Sigma-Aldrich (St. Louis, MO). DMXB was generously provided by Dr. Edwin Meyer of the University of Florida. MLA and DHβE were obtained from Sigma (St. Louis, MO). β-amyloid$_{1-42}$ (lyophilized) was obtained from US Peptide (Rancho Cucamonga, CA) and was resuspended in 10mM HEPES buffer followed by a 2 hour incubation at 37°C prior to initiation of each experiment. MTT was obtained from Research Organics (Cleveland, OH). All other chemicals were obtained from commercial sources and were reagent grade.

Animals

For cell culture experiments, C57BL/6, C58, and DBA/2 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were housed in the climate-controlled vivarium at the University of North Texas Health Science Center until used. Animals were maintained primarily as breeding triads in cages on a 12-hour light: 12-hour dark

cycle and were allowed free access to laboratory rodent chow and water. For the ligand binding experiments, adult Swiss-Webster mice were generously donated by Dr. Michael Forster of UNTHSC. These mice were previously utilized in a single, non-invasive study of locomotor activity carried out in the Forster lab, but were all drug-naïve (saline treated control) animals. Use of animals was approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, and all procedures abided by the guidelines set forth in the Public Health Service Policy on Humane Care and Use of Animals.

Tissue Culture Methods

Neonatal (day of birth) C57BL/6, C58, or DBA/2 mice (both male and female) were anesthetized and euthanized by hypothermia followed by decapitation. The skulls were then opened and the brains removed under aseptic conditions. The meninges were then carefully removed from the brains and the entire cerebral cortex dissected out and immersed in ice cold Hank's Balanced Salt Solution. Tissues were then crudely chopped and incubated (37°C, 20 min) in 2 ml of 0.9% sterile phosphate-buffered saline (PBS) containing trypsin (2.5%) and DNAse I (1%). Samples were centrifuged (1,500*g*, 5 min), the supernatant was removed, and tissues were resuspended in 1 ml of Dulbecco's Modification of Eagle's Medium containing L-glutamine (10 mM), $NaHCO_3$ (20 mM), sodium pyruvate (1 mM), KCl (20 mM), glucose (1%), penicillin/streptomycin (1%), fungizone (0.7%), and heat-inactivated donor horse serum (10%). The tissues were dissociated by gentle trituration using a flame-narrowed borosilicate Pasteur pipette and

an additional 2 ml of medium was added to each tube. The dissociates were then centrifuged (1,500g, 5 min), supernatants were discarded, and 1 ml of medium was added to each tube. Cells were again dissociated, and tissues were pooled and diluted to the desired concentration. Cells were plated on 96-well plates coated with poly-L-ornithine, at a plating density of 100,000 neurons/cm^2, and incubated at 37°C with 5% CO_2 in a water-jacketed incubator.

After 48 h, cells were treated with 1nM Ara-C to inhibit glial cell proliferation. Two days later, 75% of the medium was exchanged, and cells were again incubated at 37°C with 5% CO_2. Medium was completely exchanged every 3-4 days until drug treatments were initiated after a total of 14 days post-plating. Cultures were first treated with antagonists (α7-selective: MLA at 0, 1, 10, or 100 nM; α4β2-selective: DHβE at 0, 0.1, 0.3, 1, or 3 μM) by addition to the medium followed by incubation of the cultures for 30 minutes. Cultures were then treated with DMXB (0, 0.03, 0.1, 0.3., or 1 μM) also by addition to the medium followed by incubation of the cultures for 15 minutes. Finally, cells were treated with Aβ42 (0, 0.1, 0.3, 1, or 3 μM) by addition to the media followed by incubation for 48 hours. As described below, drug concentrations were chosen based on published reports of effective concentrations (ranges) and selectivity for different nAChR subtypes (Kihara et al., 1997; Kihara et al., 1999; Li et al., 1999b; Li et al., 1999a; Li et al., 2000; Li et al., 2002; de Fiebre and de Fiebre, 2003; Ferchmin, 2003; Harvey et al., 1996).

MTT Assay

After 2 days, neuronal viability was estimated by using the MTT cell proliferation assay (Mosmann, 1983). At the conclusion of each experiment, all media was removed from each well and replaced with fresh media (without phenol red) that contained MTT (0.5 mg/ml). Cells were incubated for 4 hours at 37°C with 5% CO_2. Isopropanol containing 0.08N HCl in a volume equal to the amount of media was subsequently added to each well. Plates were then sonicated for 30 seconds and placed on a vortex for an additional 1 minute to dissolve the precipitate. Plates were then read at 570 and 690 nm on a Packard Instruments SpectraCount UV/Vis plate reader. The MTT activity was expressed as the difference between these two absorbances. MTT activity, an index of mitochondrial function/dysfunction, was chosen over other methods of estimating neuronal death or viability because the experience of our laboratory has shown MTT to be a more consistent measure of Aβ42-induced toxicity than other measures of neuronal viability used in the laboratory.

Tissue Culture Experimental Design

Each experimental condition was replicated in a minimum of 7 wells over 2-3 different experiments. The majority of experimental conditions were replicated in 10-20 unique wells (with control conditions tending to be represented by a greater number of replicates.) For each experiment, tissue from all of the mouse pups of a given litter (for each separate strain) was pooled, and each sample represents a single well on a 96-well plate. Concentrations of DMXB utilized were chosen based on published data of doses

shown to have neuroprotective effects (Kihara et al., 1997; Kihara et al., 1999; Li et al., 1999b; Li et al., 1999a; Li et al., 2000; Li et al., 2002; de Fiebre and de Fiebre, 2003). Concentrations of MLA (Ferchmin, 2003) and DHβE (Harvey et al., 1996) used were chosen because they are within a range of doses known to show selectivity for α7 and α4β2 nAChRs, respectively.

Ligand Binding Studies

To assess if Aβ42 binds with high affinity to α7 nAChRs, α-[^{125}I]BTX binding studies were conducted using whole brain (less pons, medulla & cerebellum) tissue homogenates from adult male Swiss-Webster mice.

Tissue Preparation: Tissue homogenates were prepared as described by Romano and Goldstein (1980). Adult Swiss-Webster mice were sacrificed by cervical dislocation after being anesthetized with isoflorane and brains were removed and placed on an ice-cold platform. The brain tissue was then rinsed and the pons, medulla, and cerebellum were dissected and discarded. Remaining tissue was then suspended in 10 volumes of ice-cold Krebs-Ringer's HEPES (KRH) buffer (NaCl, 118 mM; KCl, 4.8 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; HEPES, 20 mM; pH adjusted to 7.5 with NaOH) and homogenized with a glass-Teflon homogenizer. Following incubation at 37°C for 5 min (to encourage hydrolysis on any endogenous acetylcholine present), the homogenate was centrifuged for 20 min at 18,000*g* at 4°C. The supernatant was then discarded and the pellet was resuspended in 20 volumes of ice-cold water. After incubation at 0°C for 60 min, the suspensions were then incubated at 37°C for 5 min and recentrifuged as described above.

The supernatant was discarded and the pellet was then resuspended in 10 volumes of KRH buffer and incubated at 37°C for 5 min followed by centrifugation as described above. The supernatant was again discarded and the pellet was resuspended in 10 volumes of KRH buffer. Samples were stored at -70° until the time of assay. On the day of the assay, the sample was thawed, resuspended in the present buffer, and centrifuged as described above. The supernatant was discarded and the pellet was then resuspended in 10 volumes of fresh KRH buffer.

α-[^{125}I]BTX Binding: The binding of α-[^{125}I]BTX was measured using a modification (de Fiebre et al., 2002) of published methods (Marks and Collins, 1982). Assays were conducted in 96-well plates at 37°C in KRH buffer containing 0.1% bovine serum albumin at an α-[^{125}I]BTX concentration of 1.04 ± 0.047 nM (100 µl ± varying concentrations of Aβ42). In separate samples in each assay, 1.6 mM nicotine was added to define non-specific binding.

An Inotech Biosystems (Rockville, MD) cell harvester was used. Both a type A/E glass fiber filter (Gelman Sci, Ann Arbor, MI) and a borosilicate microfiber filter (BMF; grade GB100R, Microfiltration Sys, Dublin, CA) were used and filtration was first through the BMF filter and then through the type A/E filter. The BMF filter was presoaked for at least 10 min in KRH buffer containing 0.25% polyethylenimine and the type A/E filter was presoaked for at least 10 min in BLOTTO (Bovine Lacto Transfer Technique Optimizer; 1.25 g/L non-fat milk (Carnation), 200 mg/L sodium azide) (Johnson et al., 1984).

After an incubation period of 180 min, 100 µl of ice-cold KRH buffer was added to each well of the microplate. This was followed by rapid vacuum filtration of the samples onto filters. Each well of the microplate was then rinsed with 200 µl of ice-cold KRH buffer which was subsequently filtered. Each sample was then washed an additional three times with 500 µl of ice-cold KRH buffer. Filters were then placed in 12x75 polypropylene tubes and radioactivity was detected by gamma counting at an efficiency of 80%. The amount of protein in each sample was estimated with the Coomassie reagent (Bradford, 1976) with bovine serum albumin as the standard. Each assay was conducted with ~150 µg of protein.

Data Analyses

MTT activity data were presented as a percentage of control activity and analyzed by two-way analysis of variance (ANOVA) to determine main and interactive effects of differing concentrations of test compounds (Aβ42, DMXB, MLA, and/or DHβE) as well as genotype (i.e., mouse strain). In addition to a large ANOVA examining all variables, separate ANOVAs were conducted to examine interactions between Aβ42 and each test compound individually. Analyses of this type were conducted both with data from the three strains combined, as well as with data from each strain analyzed separately. For those analyses for which significant effects were found, individual group differences were ascertained using the single degree of freedom F-test (Fisher's PLSD).

Binding data were expressed as a percent of control binding (i.e., binding of α-[^{125}I]BTX in the absence of Aβ42 or nicotine).

CHAPTER IV

RESULTS

Strain Differences Exist in Sensitivity to Aβ42 Toxicity

Figure 1 presents the results of a concentration-response analysis for Aβ42 applied to cerebral cortical neuron-enriched cultures derived from three inbred strains of mice (C57BL/6, C58, and DBA/2). An ANOVA showed main effects of both Aβ42 concentration and mouse strain, as well as an interactive effect between Aβ42 and strain (see figure legend for statistics). Fisher's PLSD analyses demonstrated that there were significantly fewer viable cells present in the cultures (as measured by MTT activity) at 1μM Aβ42 than at 0.3μM Aβ42 and still fewer than at 0.1μM Aβ42 *(P<0.0001)*. As for strain differences, analyses revealed lower MTT activity in C57BL/6 cultures relative to both C58 *(P=0.0003)* and DBA/2 *(P=0.0133)* cultures.

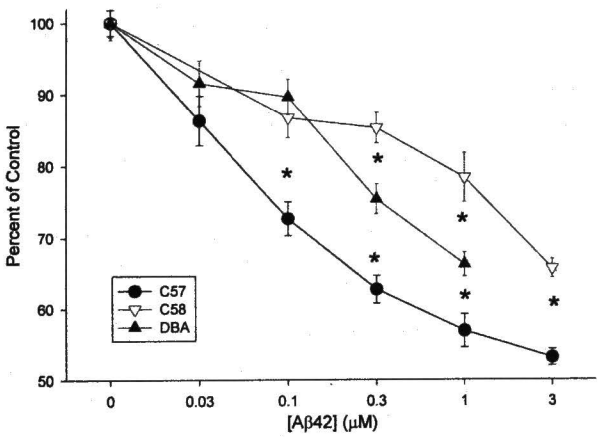

Figure 1. Neuron-enriched cultures from three inbred strains of mice differ in susceptibility to Aβ42-induced neurotoxicity. Neuronal viability was assessed by the MTT cellular proliferation assay. Data are presented as the percentage of control (0 μM Aβ42) MTT activity. Each point represents the mean±S.E.M. of a minimum of 3 cultures.
$F_{A\beta42}(3,526)=90.025$, $P<0.0001$.
$F_{strain}(2,526)=25.896$, $P<0.0001$.
$F_{A\beta42*strain}(6,526)=5.563$, $P<0.0001$.

The data shown in Figure 1 clearly demonstrate that Aβ42 is toxic to mouse cortical neurons in a concentration-dependent fashion. Furthermore, there are also obvious strain differences in terms of susceptibility to the toxicity of Aβ42. Inspection of the figure (supported by one-way analyses) allows for a simple rank-ordering of the sensitivity to Aβ42 toxicity of the strains at each concentration. Overall, the rank ordering for susceptibility to Aβ42 toxicity is C57BL/6>DBA/2>C58. At no concentration tested does this rank order coincide with the relative density of α7 nAChRs as measured by α-[^{125}I]BTX binding and reported by Marks, et al. (1989) (i.e., C58 >> C57 > DBA).

Although Aβ42 concentrations up to 3μM were examined, this was not high enough to assess differences in efficacy among the strains. These data clearly show, however, potency differences among the strains tested. A change in potency would not be expected just from differential expression of α7 nAChRs.

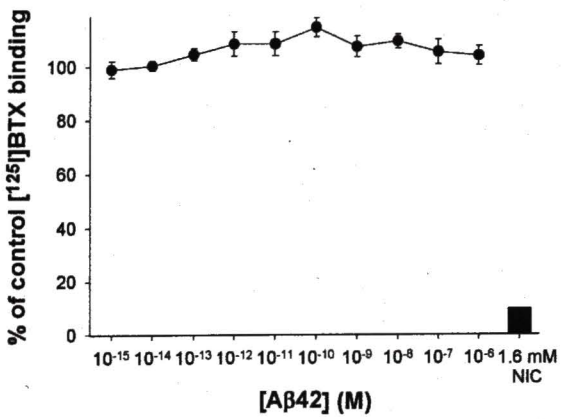

Figure 2. Aβ42 does not inhibit the binding of [^{125}I]BTX. Data are presented as a percent of control α-[^{125}I]BTX binding (i.e., in the absence of Aβ42) and each point represents the mean ± S.E.M. of four unique experiments, each done with triplicate samples.

Aβ42 Does Not Inhibit the Binding of α-[^{125}I]BTX to Mouse Brain Membranes

Separate experiments were conducted to confirm published reports that Aβ42 binds to α7 nAChRs. Specifically, the ability of Aβ42 to inhibit the binding of α-[^{125}I]BTX to mouse membranes was measured. Data

from these experiments are presented in Figure 2. In contrast to reports from other labs which utilized [^{3}H]MLA to assess potential interactions between Aβ42 and α7 nAChRs (Wang et al., 2000a), Aβ42 did not inhibit the binding of α-[^{125}I]BTX to mouse brain homogenates at concentrations even up to 1μM α-[^{125}I]BTX. The slope of this "inhibition" curve does not significantly differ from zero, demonstrating that binding did not vary with varying concentrations of Aβ42 (i.e., there was no inhibition of binding). Nicotine consistently inhibited 70-75% of total binding across all experiments (depicted by the bar at the far right of the abscissa), and thus can be considered to have served as a positive control, albeit an unplanned control. These data suggest that Aβ42 does not bind to α7 nAChRs in a competitive fashion.

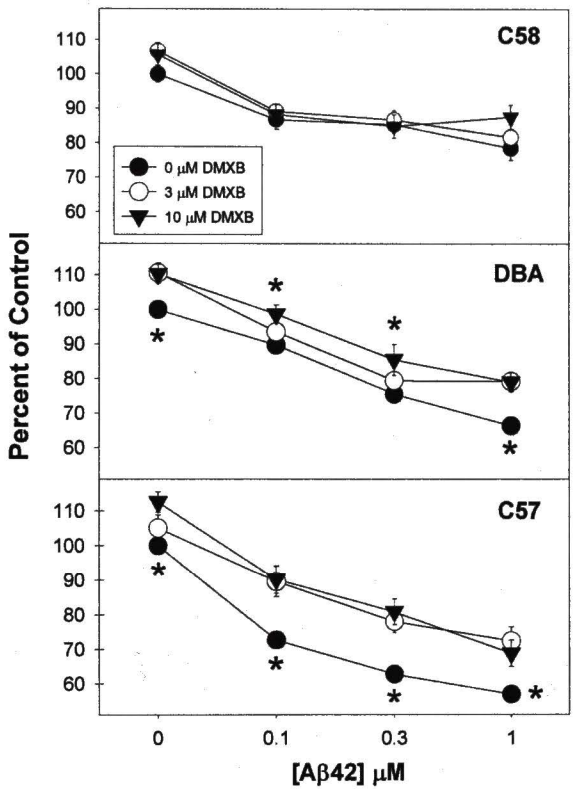

Figure 3. The ability of DMXB to protect against Aβ42-induced toxicity in cerebral cortical cultures differs among 3 inbred strains of mice. Neuronal viability was assessed by the MTT cellular proliferation assay. Data are presented as the percentage of control (0 μM DMXB) MTT activity. Each point represents the mean±S.E.M. of a minimum of separate 3 cultures.
$F_{strain}(2, 1436)=13.601, P<0.0001.$
$F_{A\beta42}(3, 1436)=283.259, P<0.0001.$
$F_{DMXB}(2,1436)=38.389, P<0.0001.$
$F_{strain*A\beta42}(6, 1436)=6.425, P<0.0001.$
$F_{strain*DMXB}(4,1436)=6.425, P<0.0001.$

Strain Differences in Protection by DMXB Against Aβ42 Toxicity

Figure 3 shows the effects of DMXB on Aβ42-induced toxicity in cerebral cortical cultures derived from the same three inbred strains of mice used in the experiments described in Figure 1. ANOVA revealed main effects of concentrations of DMXB and Aβ42, as well as a main effect of mouse strain (see figure legend for statistics). Additionally, all two-way interactive effects, except between the concentrations of DMXB and Aβ42, among these were significant, while the three-way interaction was not. Fisher's PLSD analyses demonstrated that there was significantly higher MTT activity at both 3μM and 10μM DMXB than at 0μM DMXB, which implies a protective effect. It should be noted that DMXB leads to significantly higher MTT activity of the cultures even in the absence of Aβ42 (i.e., when [Aβ42]=0) ($F_{2,612}=10.438$, $P<0.0001$). This apparent protection, both in the presence and absence of Aβ42, however, is not universal as one-way analyses revealed that in cultures from C58 mice, neither concentration of DMXB had a significant effect at any concentration of Aβ42 examined. In C57BL/6 mouse cultures, on the contrary, both concentrations of DMXB rendered cultures more viable at every concentration of Aβ42 tested. DBA cultures displayed an intermediate response with the higher concentration of DMXB providing

		C57BL/6	*C58*	*DBA/2*
DMXB	3μM	27.138%	2.754%	19.321%
	10μM	20.841%	9.659%	19.008%

Table 2. Percent of change of MTT activity from respective control values for 1μM Aβ42 data.

protection at each concentration of Aβ42, and the lower concentration of DMXB only providing protection at the highest concentration of Aβ42. These analyses suggest a rank ordering of sensitivity to DMXB protection of C57BL/6>DBA/2>C58, However, it should be noted that the differential ability of DMXB to protect against Aβ42-induced neurotoxicity may be due partially to the relatively low level of neuronal cell death, especially in C58 cultures, where the least neurotoxicity and neuroprotection is seen. These data might support the hypothesis that level of protection is at least in part determined by how much death is produced by the toxin, with the strains more sensitive to toxicity having a greater window of toxicity in which protection can occur. In a separate analysis to compare differences among the strains for protection by DMXB, a value for percent of change (of means) from each respective control was calculated (Table 2). While these values only provide a rough estimate of the efficacy of the given compound at one concentration of Aβ42 (1μM), they allow for quick comparisons among the strains. These calculations reveal that for both the 3μM and 10μM concentrations of DMXB, the greatest change in percent of MTT activity from control was seen in C57BL/6 cultures, followed by DBA/2, and lastly C58 cultures. This is in agreement with the analyses discussed in the preceding paragraph and shows that the ability of DMXB to protect is not correlated with the reported levels of α7 nAChR expression in these strains (Marks et al., 1989). However, these data suggest that the ability of DMXB to protect against Aβ42 toxicity may be influenced by the degree to which Aβ42 is toxic, as the rank ordering of strains for both susceptibility to Aβ42 toxicity and protection by DMXB is the same.

MLA May Have Protective Effects Against Aβ42 Toxicity in Some Strains

Figure 4 demonstrates the effects of MLA on Aβ42-induced toxicity in cerebral cortical cultures derived from these mice. ANOVA revealed main effects of mouse strain as well as Aβ42 concentration, and the main effect for MLA concentration approached significance. A significant interactive effect between strain and Aβ42 concentration was also found. No clear effect of MLA on neuronal viability in the presence or absence of Aβ42 was detected in Fisher's PLSD analyses including data from all three strains. That MLA alone would have no significant impact on the viability of cultures was not surprising, in that we had hypothesized that as an α7 antagonist, MLA would be able to block the protective effects of DMXB but would not have an effect by itself.

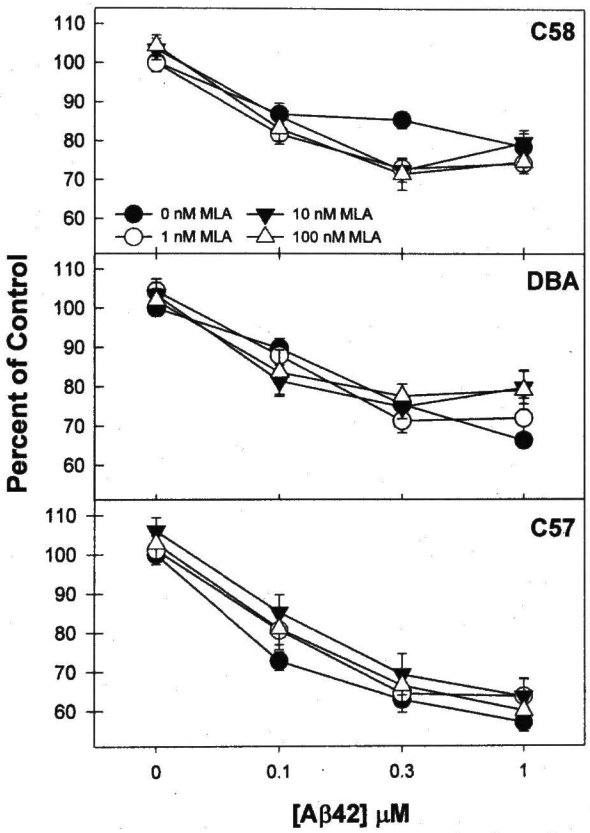

Figure 4. The effects of MLA in the presence of Aβ42 differ among cerebral cortical cultures from 3 strains of mice. Viability was assessed by the MTT proliferation assay. Data are presented as the percentage of control (0 nM MLA) MTT activity. Each point represents the mean±S.E.M. of at least 3 cultures.
$F_{strain}(2, 1138)=17.166, P<0.0001$
$F_{A\beta42}(3, 1138)=214.121, P<0.0001$
$F_{MLA}(3,1138)=2.500, P<0.0581$
$F_{strain*A\beta42}(6, 1138)=4.618, P=0.0001$

The effects of MLA were also analyzed via ANOVA in each mouse

strain separately, and similarly, no significant main effect of MLA concentration was found in any mouse strain. A more detailed analysis of these data, however, suggested that MLA may have some protective effects in some, but not all genotypes. Specifically, if data were examined at individual concentrations of Aβ42, a significant effect of MLA is seen at 1μM Aβ42 in cultures from DBA/2 mice ($F_{3,75}=4.543$, $P=0.0056$) with both the 10nM ($P=0.0019$) and 100nM ($P=0.0053$) concentrations of MLA displaying protection when compared to cultures not treated with MLA. In cultures from C57BL/6 mice, MLA may have some protective actions against 0.1μM Aβ42, especially at the 10nM concentration of MLA. At this concentration of Aβ42, a main effect of MLA concentration approached significance ($P=0.0569$) and Fisher's PLSD indicated that cultures treated with 10nM MLA were more viable than cultures not treated with MLA ($P=0.018$). In cultures from C58 mice, no concentration of MLA had a significant effect on the ability of Aβ42 to decrease neuronal viability.

The finding that MLA may be protective in two of the three strains tested was unexpected. In fact, we did not expect MLA to be neuroprotective in any strain. Later analyses both in our laboratory and the laboratories of others, however, have confirmed protective effects of MLA (Laudenbach et al., 2002; Srinivasan et al., 2003; Ferchmin et al., 2003).

DHβE May Protect Against Aβ42-Induced Toxicity in a Genotype-Dependent Fashion

Figure 5 shows the effects of DHβE on Aβ42 toxicity in cerebral cortical cultures derived from these mice. Due to missing data at some Aβ42 concentrations in DBA/2

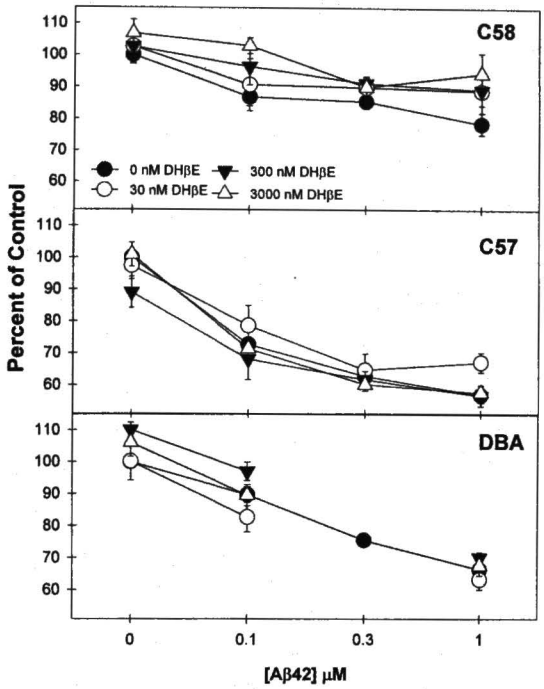

Figure 5. The effects of DHβE in the presence of Aβ42 in cerebral cortical cultures differ among 3 inbred strains of mice. Neuronal viability was assessed by the MTT cellular proliferation assay. Data are presented as the percentage of control (0 nM DHβE) MTT activity. Each point represents the mean±S.E.M. of at least separate 3 cultures.

$F_{strain}(1, 668)=156.702, P<0.0001$

$F_{A\beta42}(4, 668)=90.848, P<0.0001$

$F_{strain*A\beta42}(4, 668)=9.235, P<0.0001$

$F_{strain*DHBE}(3, 668)=3.420, P=0.017$

cultures, strain differences across multiple concentrations of Aβ42 were only assessed in C57BL/6 and C58 cultures. ANOVA revealed a main effect of mouse strain and Aβ42 concentration, but not of DHβE concentration *(P=0.0965)*. There were significant two-way interactive effects between mouse strain and both DHβE and Aβ42 concentrations. DHβE appeared to have little impact on the viability of cultures in the absence of Aβ42. Unexpectedly, analyses in C58 cultures revealed that 3000nM DHβE significantly increased MTT activity (i.e., enhanced protection) both at the 0.1 and 1.0 μM Aβ42 concentrations. Although no significant enhancements or protections against Aβ42 were seen at any concentration of Aβ42 or DHβE in C57BL/6 and DBA/2 cultures, examination of the figure provides a weak suggestion that DHβE may have toxicity- enhancing as well as protective effects, depending on the concentration examined. These data are both novel and unexpected and suggest that further studies need to be carried out in order to determine whether DHβE

may be able to enhance the neurotoxic properties of Aβ42 in cultures from some mouse strains, while protecting against toxicity in others.

Strain Differences in the Effects of Combined DMXB and MLA on Aβ42 Toxicity

Figure 6 shows the effects of combined DMXB and MLA on Aβ42-induced toxicity in cerebral cortical cultures derived from these inbred strains of mice. In contrast with my hypothesis, MLA did not appear able to block the protective effects of DMXB in cultures from any of the three mouse strains. ANOVA revealed main effects of

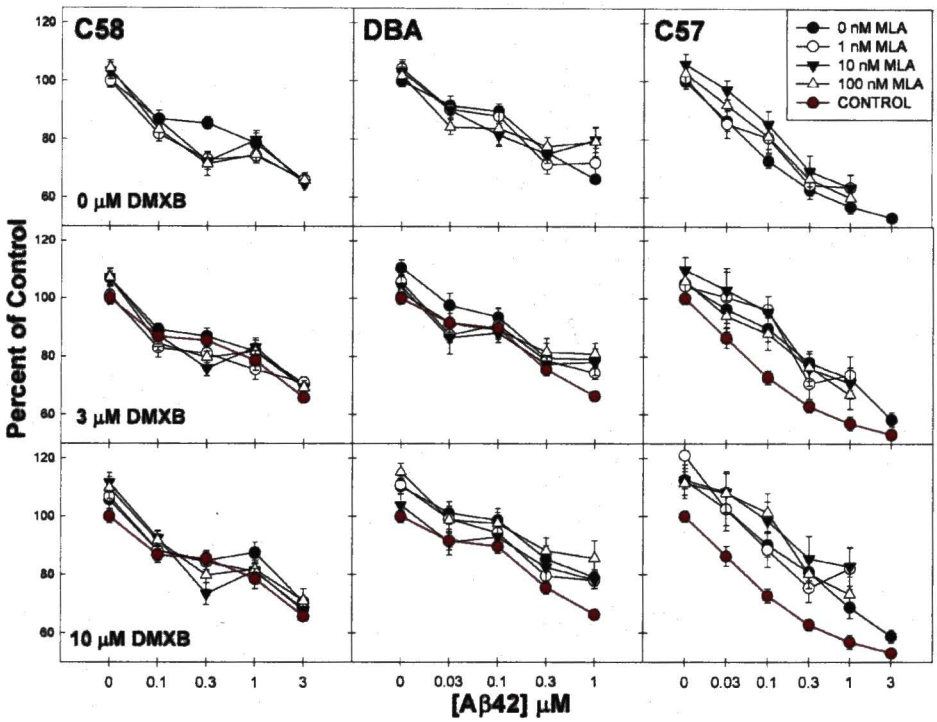

Figure 6. The effects of DMXB and MLA in the presence of Aβ42 in cerebral cortical cultures differ among 3 inbred strains of mice. Neuronal viability was assessed by the MTT cellular proliferation assay. Data are presented as the percentage of control (0 μM DMXB and 0 nM MLA) MTT activity. Each point represents the mean±S.E.M. of at least 3 cultures.

$F_{strain}(2, 2632)=6.763, P=0.0012$ $F_{A\beta42}(3. 2632)= 444.941, P<0.0001$

$F_{DMXB}(2, 2632)= 58.182, P<0.0001$ $F_{strain*A\beta42}(6, 2632)= 6.370, P<0.0001$

$F_{strain*DMXB}(4, 2632)= 5.220, P=0.0003$

concentrations of DMXB and Aβ42, as well as a main effect of mouse strain. Additionally, two-way interactive effects of mouse strain and Aβ42 concentration, as well as of mouse strain and DMXB concentration were significant. Fisher's PLSD analyses with data from all three strains examined together demonstrated that there were significantly higher MTT activities at both 3μM and 10μM DMXB than at 0μM DMXB, which is again suggestive of a protective effect. C57BL/6 cultures demonstrate a protective effect of DMXB, with a trend towards possible enhancement of this protection by MLA. Cultures derived from DBA/2 mice show less of a protective effect of DMXB (which is consistent with data previously presented in Figure 3) and less enhancement of this protective effect by MLA (although a trend toward enhancement is seen at 100nM).

Strain Differences May Exist in the Effects of Combined DXMB and DHβE on Aβ42-induced Toxicity

Figure 7 shows the effects of combined DMXB and DHβE on Aβ42-induced toxicity in cerebral cortical cultures derived from these mice. As predicted, DHβE was not able to block the protective effects of DMXB in cultures from any of the three strains of mice examined. Due to missing data at some Aβ42 concentrations in DBA/2 cultures, strain differences across multiple concentrations of Aβ42 were only assessed in C57BL/2 and C58 cultures. ANOVA revealed main effects of concentrations of DMXB, DHβE, and Aβ42, as well as a main effect of mouse strain. Additionally, two-way interactive effects of mouse strain and Aβ42 concentration, as well as of mouse strain and DHβE

concentration were significant, while none of the three-way interactions and the four-way interaction were.

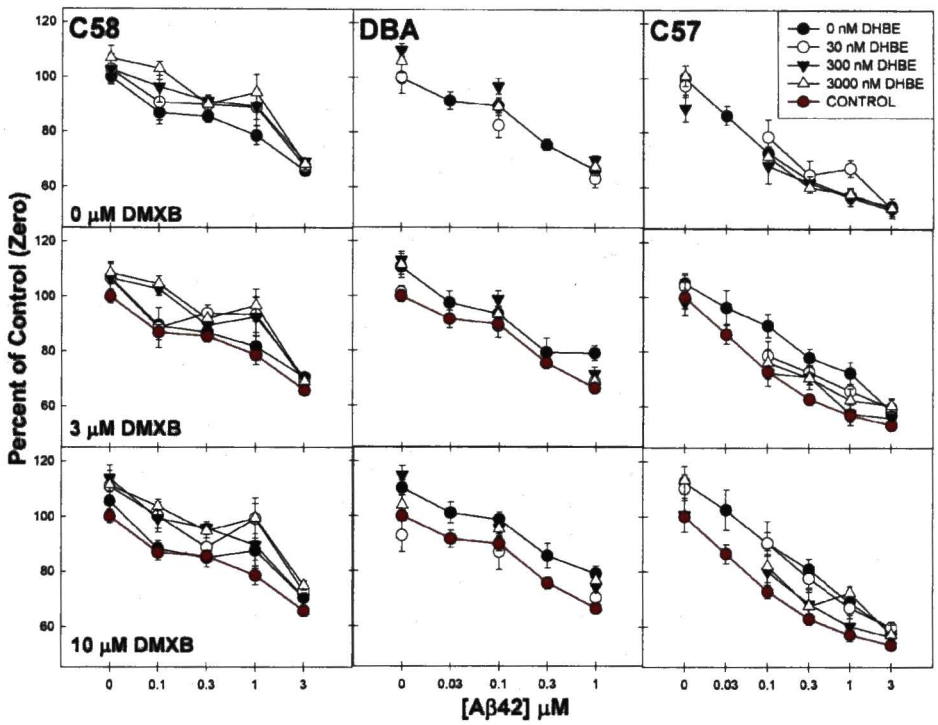

Figure 7. The effects of DMXB and DHβE in the presence of Aβ42 in cerebral cortical cultures differ among 3 inbred strains of mice. Neuronal viability was assessed by the MTT assay. Data are presented as the percentage of control (0 μM DMXB and 0 nM MLA) MTT activity. Each point represents the mean±S.E.M. of at least 3 cultures.

$F_{strain}(1, 2085)=309.813, P<0.0001$ $F_{A\beta42}(4, 2085)=274.713. P<0.0001$

$F_{DMXB}(2, 2085)=21.169, P<0.0001$ $F_{DHBE}(3, 2085)=2.643, P=0.0476$

$F_{strain*A\beta42}(4, 2085)=22.141, P<0.0001$ $F_{strain*DHBE}(3, 2085)=14.761, P<0.001$

CHAPTER V

DISCUSSION

Considerable evidence indicates that Aβ42 plays a key role in the pathogenesis of AD; however, it is clear that amyloid load is not a good predictor of disease severity or even presence or absence of AD. The principal hypothesis underlying this project, that individual differences exist in susceptibility to Aβ42 toxicity, offers an explanation for how one individual could suffer from AD while another with the same amyloid load shows no signs of dementia. The data, generated by utilizing cultures derived from inbred strains of mice, support this hypothesis. The data do not, however, support the hypothesis that these individual differences are due to differential α7 nAChR expression. Further, while the data support the hypothesis that DMXB, an α7 nAChR-selective partial agonist, would protect against this toxicity in a genotype-dependent fashion, they do not support the hypothesis that the level of protection afforded by DMXB would be correlated with levels of α7 nAChR expression. Interestingly, not only was MLA unable to block the neuroprotective effects of DMXB, but the data suggest that MLA might have a neuroprotective effect on its own. While these effects did not correlate with levels of α7 nAChR expression, they did seem to correlate with DMXB protection (i.e. those strains in which DMXB was protective, MLA was also).

The data from the experiments with α7 nAChR-selective agents also do not support nor do they refute the hypothesis that Aβ42 exerts its toxic effects through direct

action at α7 nAChRs. This is more directly refuted by the α-[^{125}I]BTX binding data which strongly suggest that Aβ42 does not bind to α7 nAChRs, at least not in a competitive fashion. Like susceptibility to Aβ42 toxicity, levels of α7 nAChR expression do not seem to correlate with levels of DMXB protection. Instead, levels of DMXB protection appear to correlate with levels of Aβ42 toxicity. Interestingly, the effects of DMXB and MLA seem to be similar in terms of strain differences (i.e. protective in C57BL/6 and DBA/2 cultures, but not effective in C58 cultures). Since both of these agents are known to be selective for α7 nAChRs, this suggests that α7 nAChRs play a role in this neuroprotection. The similar genotype dependence of MLA and DMXB protection suggest a common mechanism for the protection afforded by both agents. That both a partial agonist and an antagonist are protective suggests that inhibition or desensitization of α7 nAChRs, and not activation of these receptors, is involved in the neuroprotective actions of these agents.

In classic pharmacological theory, an antagonist simply occupies space on a receptor and prevents an agonist from exerting its physiological response. This appears at odds with our hypothesis that antagonism of α7 nAChRs (either through true antagonism or desensitization/inactivation caused by agonist activation) leads to increased cell viability. However, if one assumes that there is a normal level of stimulation of α7 nAChRs, then it is also feasible that antagonism of these receptors can reduce a normal physiologic effect caused by baseline stimulation. Therefore, even though the antagonism itself exerts no physiologic effect, its alteration of the normal level of agonist stimulation could indirectly lead to a physiologic change. In this case, we suggest that decreasing the

baseline level of stimulation of α7 nAChRs promotes cell survival. Clearly, additional studies would be required to test this hypothesis.

The binding data presented here are in disagreement with the report of Wang et al. (2000) that Aβ42 binds to α7 nAChRs with extremely high affinity. Perhaps findings are discrepant because Wang and colleagues utilized [^{3}H]MLA instead of α-[^{125}I]BTX. While it has been reported that [^{3}H]MLA binding resembles that of α-[^{125}I]BTX, it is also reported that 20-30% of [^{3}H]MLA binding cannot be displaced by α-[^{125}I]BTX (Whiteaker et al., 1999). Further, MLA has been shown to bind with high affinity to the α-conotoxin MII receptor in the brain (Mogg et al., 2002). α-[^{125}I]BTX, on the other hand, only binds to α7 nAChRs in the brain. Hence, we would argue that α-[^{125}I]BTX is a better ligand for measuring α7 nAChR expression. Also, Wang et al. (2000) reported that in order to uncover inhibition of [^{3}H]MLA binding by Aβ42, an extensive tissue preparation including multiple washes was necessary. In studies in our lab, we utilized a similar tissue preparation including multiple tissue washes and were still unable to see any inhibition of α-[^{125}I]BTX binding produced by Aβ42. Lastly, multiple preparations of Aβ42 have been utilized, which were either prepared under conditions that promote or inhibit the aggregation of the peptide. Regardless of how Aβ42 was prepared, no inhibition of α-[^{125}I]BTX binding was seen.

Additional data generated in our lab corroborate the hypothesis that Aβ42 does not exert its toxic effects through α7 nAChRs. Specifically, susceptibility to Aβ42 neurotoxicity is unaffected in cultures derived from α7 nAChR-knockout mice (de Fiebre and de Fiebre, 2004). Interestingly, while α7 nAChR null mutation did not affect

susceptibility of cultures to Aβ42-induced neurotoxicity, cultures from these mice had enhanced susceptibility to ethanol-induced toxicity in a gene dosage-related manner. These ethanol data further show that α7 nAChRs have a role in modulating neuronal viability even if Aβ42 does not produce toxicity through these receptors.

The data presented here are admittedly weak in making an argument that MLA has protective effects. In fact, MLA protection was an unexpected and novel finding. However, data generated in our lab from more extensive, but similar studies, have found a significant neuroprotective effect of MLA against Aβ42 toxicity (Martin et al., 2004). These published data were collected from cerebral cortical cultures derived from C57BL/6 mice and demonstrate that MLA alone can have neuroprotective effects against Aβ42-induced toxicity under essentially the same experimental conditions as those utilized in the studies presented here. Also, our lab has generated data demonstrating neuroprotection by MLA against ethanol-induced toxicity in rat cerebral cortical cultures (de Fiebre et al., 2002) as well as hippocampal cultures (unpublished findings). These data generated from rat cultures were also gathered in studies which attempted to use MLA to block the neuroprotective effects of DMXB, but in this case against ethanol toxicity. Both of these studies demonstrate in different animal models (both mice and rats) that MLA can exert neuroprotective effects against two different insults (ethanol and Aβ42) at concentrations where it acts selectively to block α7 nAChRs. Again, while the data in the current study show limited protection by MLA against Aβ42-induced toxicity, the apparent trend toward neuroprotection seen at some concentrations in C57BL/6 and DBA/2 cultures corroborates these other findings generated from our lab as well as

findings of MLA neuroprotection reported by other groups (Laudenbach et al., 2002; Srinivasan et al., 2003; Ferchmin et al., 2003).

The finding that MLA and DMXB share a similar genotype dependence for protective actions may be used to argue that these agents share a similar mechanism of protection. While both these agents are selective for α7 nAChRs, the data do not support the hypothesis that levels of α7 nAChR expression correlate with levels of protection produced by these agents. In fact, cultures from the strain with the highest level of α7 nAChR expression, the C58 strain, are insensitive to protection by these agents. From the data, one might instead conclude that the efficacy of an agent in producing protection is related to the level of toxicity produced by Aβ42.

As expected, DHβE was not able to block the neuroprotective actions of DMXB. Unexpectedly, our data provided weak evidence that DHβE might be able to protect neurons from C58 mice from Aβ42-induced neurotoxicity. If proven true in further studies, this novel finding would argue against the conclusion that the efficacy of an agent in producing protection is related to the level of toxicity produced by Aβ42 (i.e., the level of toxicity produced by Aβ42 in a given strain does not necessarily dictate how well another agent can protect against it).

Some aspects of the cell signaling pathways thought to be involved in α7 nAChR-mediated neuroprotection have been well characterized. α7 nAChR-mediated protection is a calcium dependent process (Dajas-Bailador et al., 2000) and likely involves the PI-3K/Akt pathways through the activation of Janus kinase 2 (JAK2) (Kihara et al., 1997; Shaw et al., 2002). However, further investigation into which

signaling pathways are responsible for α7 nAChR-mediated neuroprotection needs to be done in order to better answer the question as to whether it is activation or inactivation/desensitization of α7 nAChRs which is responsible for neuroprotection through these receptors.

It is important to appreciate that the cause of AD is likely multifactorial. The finding that different inbred strains of mice differ in their susceptibility to the toxic effects of Aβ42 demonstrates that genetic factors can modulate the neurotoxic properties of this peptide. It follows that individual humans likely differ in their own genetically-mediated susceptibility to Aβ42 toxicity. This could explain differential expression of AD in individuals possessing similar amyloid loads. While genetic factors may predispose some individuals towards developing AD, environmental factors more than likely also play a role. Many recent studies have emphasized the critical role that oxidative damage plays in the development of AD. In fact, Aβ42 has been shown to directly cause oxidative damage (Calabrese et al., 2006; Yatin et al., 1999a,b). It is therefore probable that environmental factors, such as level of exercise and type of diet, could alter the probability of a genetically-susceptible individual developing AD.

Limitations of the Current Study

There have been reports of problems associated with the MTT assay when utilized to assess neuronal viability in the presence of Aβ42 (Hertel et al., 1996; Wogulis et al., 2005). Specifically, it has been reported that Aβ42 can interact with MTT formazan crystal such that the crystals produced in the presence of Aβ42 assume a dagger-like

shape which pierces cell membranes and kills cells. However, unlike studies that have identified these potentially problematic interactions between the MTT formazan crystal formation and Aβ42, the culture media in our studies was replaced with fresh DMEM with 20% horse serum (not containing any Aβ42) directly prior to carrying out the MTT assay. This should have greatly reduced or even completely removed any direct interaction between the Aβ42 peptide and MTT that could have occurred. Furthermore, it is highly unlikely that an Aβ42-MTT interaction would differ in cultures derived from different inbred strains of mouse. The fact that strain differences were found in concentration-dependent Aβ42-induced decreases in MTT activity argues against the data presented here arising from an artifact of Aβ42-MTT interactions.

It should be noted that our experiments were conducted in cortical cultures. While hippocampus could be considered a better choice for studying α7 nAChR regulation of cellular events, because both total expression of α7 nAChRs and differences among strains in α7 nAChR expression are greater, we chose to use cortical tissue due to the relative abundance of cortical tissue that one can obtain from a neonatal mouse pup. This allowed us to carry out for more extensive experiments with a greater number of replicates in the time available. Nevertheless, different results may have been obtained had these studies been carried out in hippocampal tissues.

It also should be noted that differences in glial content of cultures across the strains used in these experiments can not be ruled out. While each culture was treated with an anti-mitotic agent and preliminary examination revealed that glial content in our cultures was limited to ~10-20% of all cells present, rigorous analyses of glial content in

each strain were not conducted. It is possible that glial content among the strains differed both due to possible experiment to experiment variability as well as due to possible strain differences in the efficacy of the mitotic inhibitor in blocking glial cell proliferation.

Because an anti-mitotic agent was added, all of our cell culture experiments were done in cultures with a reduced glial concentration. The binding reported by Marks et al. (1989), the basis for our assumption of strain differences in α7 nAChR expression in our cultures, was carried out in tissue containing glia. It has been demonstrated that glia express α7 nAChRs (Gahring et al., 2004; Suzuki et al., 2006; Teaktong et al., 2003; Xiu et al., 2006; Yu et al., 2005). Hence, it is possible that strain differences in α7 nAChR expression reported by Marks et al. (1989) could be due to differential expression in glial cells, but not in neurons. If this were the case, strain differences in Aβ42 which correlated with α7 nAChR expression would not be expected. That α7 nAChRs are present in glial cells, however, is of questionable importance in affecting the conclusions that we have discussed thus far because binding data, obtained from tissue containing glia, suggest that Aβ42 does not directly interact with α7 nAChRs. Nevertheless, glial expression of α7 nAChRs may affect Aβ42 toxicity in a fashion not readily apparent from our studies.

The studies reported here were undertaken to generate preliminary data for later grant submission. Specifically, these experiments were conducted in hopes of supporting the hypothesis that Aβ42 exerted some of its toxic effects through α7 nAChRs. When the data did not support this hypothesis, this line of research was not continued in our lab and therefore, further elucidation of the role of glia in the effects seen was not attempted.

Future Directions and Concluding Remarks

Despite the fact that Aβ42 may not exert its toxic effects through α7 nAChRs, this study does emphasize the utility of inbred strains of mouse when attempting to determine the pathophysiological mechanism underlying diseases like AD. Just as different inbred strains of mice likely differ in their response to multiple toxic insults as well as to pharmaceuticals used to treat different diseases, human individuals manifest diseases in unique fashions and respond to treatments in distinct ways. Not only could the use of inbred strains of mice be used to explain differential expression of diseases, they could also be utilized in the development of highly specific pharmacotherapies. It is for these reasons, that further characterization of more inbred strains of mice could be useful for future study of human diseases, especially those that show marked differences in phenotypic manifestations.

While our strongest finding is that strain differences exist in susceptibility to Aβ42 toxicity, our other data are admittedly weaker, but thought provoking nonetheless. Clearly, future studies need to be carried out in order to confirm and elucidate the mechanisms behind the neuroprotective effects seen with nicotinic agents. The finding of strain differences in susceptibility to Aβ42 toxicity, however, offers novel tools for identifying the biological substrates for these strain differences and possibly developing novel therapies targeted toward these substrates for treating AD in humans.

CHAPTER VI

APPENDIX

Martin SE, de Fiebre NC, de Fiebre CM (2004) The α7 nicotinic acetylcholine receptor-selective antagonist methyllycaconitine, partially protects against β-amyloid$_{1-42}$ toxicity in primary neuron-enriched cultures. Brain Res 1022:254-256.

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Short communication

The α7 nicotinic acetylcholine receptor-selective antagonist, methyllycaconitine, partially protects against β-amyloid$_{1-42}$ toxicity in primary neuron-enriched cultures

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Abstract

Studies have suggested that the neuroprotective actions of α7 nicotinic agonists arise from activation of receptors and not from the extensive desensitization which rapidly follows activation. Here, we report that the α7-selective nicotinic antagonist, methyllycaconitine (MLA), protects against β-amyloid-induced neurotoxicity; whereas the α4β2-selective antagonist, dihydro-β-erythroidine, does not. These findings suggest that neuroprotective actions of α7-acting agents arise from receptor inhibition/desensitization and that α7 antagonists may be useful neuroprotective agents.

Theme: Disorders of the nervous system
Topic: Neurotoxicity

Keywords: Methyllycaconitine; β-Amyloid; Neuroprotection; Nicotinic receptor; Dihydro-β-erythroidine; MTT

Numerous studies have identified α7 nicotinic acetylcholine receptors (nAChRs) as targets for neuroprotective drug development [8]. The α7-selective partial agonist (3)-2,4-dimethoxybenzylidene anabaseine (DMXB) [12] as well as the α7-selective agonist, choline (and choline analogs), protect against a number of neural insults [3,6,10,18]. Most studies which have examined neuroprotective actions of these agents have utilized protocols whereby receptors are exposed to agonists for extended periods. Because α7 nAChRs undergo rapid and extensive desensitization [16], the predominant effect of prolonged agonist treatment on α7 nAChR function is one of antagonism due to receptor desensitization/inactivation.

Although α7 nAChRs undergo extensive desensitization, many studies suggest that agonists and partial agonists acting at α7 nAChRs produce their protective actions through receptor activation. That activation of α7 nAChRs is necessary for neuroprotective actions is supported by studies with agents which differ in their agonist/antagonist properties at α7 nAChRs [13]. Also, studies have reported that the α7-selective antagonist, methyllycaconitine (MLA) [20], blocks the protective actions of α7-selective and nonselective nicotinic agonists [6,7,15]. Preliminary data from our laboratory, however, has suggested that MLA itself has neuroprotective actions [2] leading to the study reported here.

The ability of MLA to protect against the toxicity produced by the Alzheimer's disease-related peptide, β-amyloid$_{1-42}$ (Aβ), was examined in primary neuron-enriched cultures using slight modifications to published methods [3]. Cultures were established from neonatal (day of birth) C57BL/6J mouse cerebral cortices and were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing heat-inactivated horse serum (20%). Cultures were exposed to β-D-arabinofuranoside (1 nM) 2–

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4 days post-plating to inhibit glial cell proliferation. After 2 weeks, cultures were exposed to Aβ (30 nM to 1 μM), MLA (1–100 nM), dihydro-β-erythroidine (DHβE, 30–300 nM), an antagonist selective for α4β2 nAChRs, and/or vehicle (media) control. Following 2 days exposure to drugs or vehicle, the viability of each culture was assessed with the MTT assay, which estimates cell viability by estimating mitochondrial activity [14].

MLA attenuated Aβ-induced neurotoxicity (Fig. 1a). An ANOVA examining for main and interactive effects of test compound concentrations revealed significant concentration dependencies for both Aβ ($F_{3,252}$=*59.309*, p<*0.0001*) and MLA ($F_{3,252}$=*5.333*, p=*0.0014*). Fisher's post hoc analyses revealed that each of the three concentrations of MLA tested decreased the toxicity produced by Aβ. In contrast, DHβE did not significantly affect Aβ-induced neurotoxicity (Fig. 1b).

The experiments reported here were initiated following attempts to use relatively high (nonselective) concentrations of MLA to block DMXB-induced protection against ethanol neurotoxicity in rat cortical cultures [2]. Then, we were surprised to see apparent protection by MLA itself. The results presented here corroborate and expand upon these earlier results. The data demonstrate that MLA is neuroprotective at concentrations where it possesses selectivity

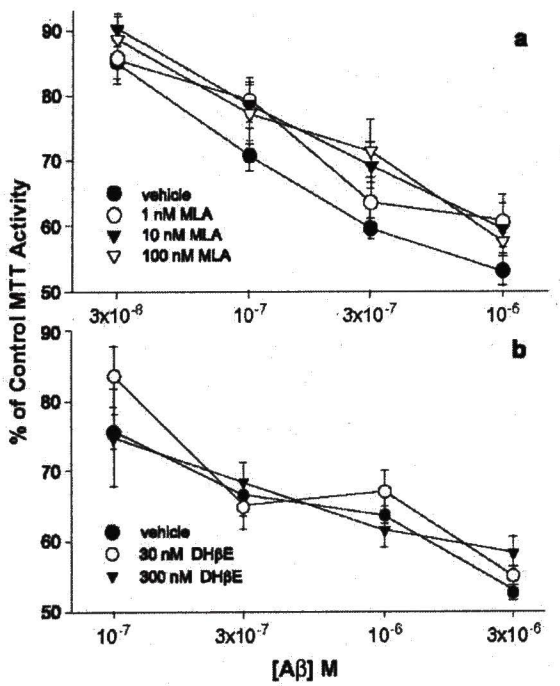

Fig. 1. Aβ-induced neurotoxicity in cerebral cortical cultures derived from C57BL/6J mice. After establishment in culture for 2 weeks, cells were exposed to constant Aβ for 2 days prior to assay for viability with the MTT assay. Data demonstrate (a) a protection against Aβ-induced toxicity by MLA and (b) a lack of protection against this toxicity by DHβE. Data are presented as the percent of control activity and each point represents the mean±S.E.M. of 11–15 separate cultures.

for α7 nAChRs. Corroborating findings with ethanol, near-maximal neuroprotection is seen at the lowest concentration tested (1 nM). Follow-up studies will need to be conducted to define the lower limits of concentrations of MLA that retain protective effects. However, data with DHβE suggest that inhibition of α4β2 nAChRs does not contribute to the neuroprotective actions of MLA at non-α7-selective concentrations. Together with the previous results, these data represent a demonstration of neuroprotective actions of MLA in neuronal cultures from two different species (mouse and rat) and with protection against two different toxicants (Aβ and ethanol).

That MLA has neuroprotective actions brings into question whether it was agonist activity or the desensitizing/inactivating activity of agonists and partial agonists at α7 nAChRs which produced neuroprotective effects. Jonnala and Buccafusco [5] have suggested that upregulation of α7 nAChRs, due to desensitization/inactivation, is responsible for neuroprotective effects of nicotine. Although these investigators did not find neuroprotective effects of MLA by itself, in contrast to our findings, they found that a 96-h pretreatment with a non-α7-selective concentration of MLA (100 μM), which produces maximum upregulation of α7 nAChRs, enhanced the neuroprotective effects of nicotine. In this report, nicotine exposure (10 μM) for 24 h was protective; whereas 10 min nicotine exposure was not. This suggests that long-term α7 nAChR inactivation by nicotine was required.

In light of the findings presented here coupled with other recent reports of protective actions of MLA [4,11,19], it is not readily apparent why these and other investigators have seen blockade of agonist-induced neuroprotection by MLA [6,7,15]. Since each study utilized different models of neurotoxicity in terms of type of insult, duration of treatment, and cell type, it may not be surprising that different results were obtained. Supportive of our findings, however, Laudenbach et al. [11] and Srinivasan et al. [19] have reported neuroprotection by MLA in vivo, and Ferchmin et al. [4] have reported MLA protection in brain slices.

Aspects of the cell signaling pathways involved in α7 nAChR-mediated neuroprotection have been elucidated. Not surprisingly, α7-mediated protection is calcium dependent [1] and may involve the PI-3K/Akt pathways via the activation of Janus kinase 2 (JAK2) [9,17]. These findings, however, have not been conclusively linked to the activation of α7 nAChRs as opposed to the inactivation or desensitization of these receptors. Further study into the signaling mechanisms involved in producing α7 nAChR-mediated neuroprotection, especially with regard to the kinetics of signaling following (1) agonist activation (in the presence or absence of an antagonist), (2) agonist-induced desensitization, or (3) antagonist-induced receptor blockade may lead to an explanation for the seemingly incongruent results obtained by different laboratories. Nevertheless, the findings reported here that MLA has neuroprotective actions against

Aβ-induced neurotoxicity clearly demonstrate that α7 nAChRs are involved in modulating neuronal viability and suggest that α7-selective antagonists might be useful therapeutics in treating neurodegenerative disorders such as Alzheimer's disease.

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