ABSTRACT

Title of Document: DEVELOPMENT OF ALZHEIMER'S-LIKE PATHOLOGY IN NON-HUMAN PRIMATES WITH REDUCED LEVELS OF NOREPINEPHRINE

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Synthesis of the norepinephrine distributed to sub-cortical and cortical brain regions occurs in the locus coeruleus. Impaired function results in reduced availability of norepinephrine. Locus coeruleus degeneration is a well-documented feature of Alzheimer's disease; however, the role of catecholaminergic dysfunction remains unclear. Deregulation of this system may accelerate the development and progression of Alzheimer's disease, particularly in patients without familial gene mutations. Currently no animal model exists for idiopathic Alzheimer's disease, which accounts for the majority of human cases. To ascertain the role of the noradrenergic system on the development of amyloid pathology and amyloid-β synthesis pathway, female non-human primates received injection of 40 mg/kg of the neurotoxin DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) or vehicle and subsequent injections three and six months later (10 mg/kg DSP-4). At nine months, brain samples were processed for catecholamines. Distribution of amyloid identified by 6E10 and

localization of dopamine β-hydroxylase to visualize locus coeruleus neurons was examined using immunostaining in tissue sections. Brain levels of amyloid precursor protein, soluble amyloid-β peptides (1-40 and 1-42) along with β-site APP cleaving enzyme-1 were also measured. Results showed norepinephrine depletion in the locus coeruleus following DSP4 injection. Reduction of dopamine β-hydroxylase was detected in aged rhesus monkeys after DSP4. Distribution of amyloid identified by 6E10 was exacerbated in squirrel monkeys following DSP4 and elevated in aged rhesus monkeys after DSP4; additionally DSP4 increased the amyloid-β42 to amyloid-β40 ratio in aged rhesus monkeys. Species specific alterations in amyloid precursor protein and β-site amyloid precursor protein cleaving enzyme-1 were observed and rhesus monkeys were more sensitive to effects of DSP4. These data provide evidence for a potential mechanism important in Alzheimer's disease pathology development and indicate that decreased norepinephrine contributed to an increase in soluble amyloid isoforms and increased accumulation in neocortex in non-human primates. Altered amyloid precursor protein processing contributes to increased amyloid pathology in the absence of chronic neuroinflammation. Non-human primates are an ideal candidate for an animal model because amyloid pathology and neurodegenerative disease characteristics occur naturally later in life, similar to humans.

DEVELOPMENT OF ALZHEIMER'S-LIKE PATHOLOGY IN NON-HUMAN PRIMATES WITH REDUCED LEVELS OF NOREPINEPHRINE

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Dedication

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Central Nervous System

Introduction

The interdisciplinary branch of Neuroscience aims to further the study of the nervous system, developing experiments to unravel the mystery of the brain and diseases that impact its function are at the forefront of modern science. The nervous system is the most complex system in the body and neurological diseases represent a large and increasing burden; through careful study scientists have made great strides in comprehending modes of cellular communication along with mechanisms for regulating brain activity and behavior. Neurons receiving a threshold stimulus from neighboring cells transmit an action potential down the axon. Since they do not physically contact one another neurons communicate through an extracellular space called the synapse. Electrochemical signaling results in propagation of an electrical signal and conversion to chemical release into the synaptic cleft. These neurotransmitters, functioning in both inhibitory and excitatory capacities, act on receptors and second messenger systems; this mechanism dramatically amplifies the signal. Levels of neurotransmitters, the receptors, and their action upon signaling pathways require balance to maintain proper function. Many neurological and psychiatric diseases result from imbalances in neurotransmitters or inappropriate signaling. Early studies regarding neurons and interactions between neuronal systems by Donald Hebb and later Eric Kandel modernized the study of Neuroscience. Yet many questions still exist and understanding this information processing and the

downstream consequences of aberrant signaling remains one of the major challenges within the field of neuroscience.

Parts of the central nervous system include the brain and spinal cord, with the brain being further divided into brainstem (mesencephalon and rhombencephalon), cerebellum (metencephalon) and cerebrum (telencephalon and diencephalon). Voluntary actions such as movement, sensory integration, learning and memory are all controlled by activity in the central nervous system. Specific regions are involved in regulation of central nervous system activity, though tasks are often interdependent on multiple areas. The largest and most developed region of the brain, the cerebrum, is divided into equal hemispheres and further classified into four major lobes: frontal, parietal, temporal, and occipital cortex. Brain tissue can be grossly sub-divided into white and grey matter regions, with neuronal cell bodies contained mainly within the grey matter neocortex. Ramon y Cajal clearly identified the neuron as the most fundamental unit in the brain, using the recently developed Golgi staining method to label individual cerebral cortex cells and presenting his work in 1888 (De Carlos and Borrell 2007).

Neurons function as the core of the central nervous system to integrate signals, pass on pertinent information and produce outcomes. Synthesis of specific proteins and receptors determine the neuronal physiology and action; two major excitatory neurotransmitters include acetylcholine and norepinephrine, while γ-aminobutyric acid is the main inhibitory neurotransmitter in the brain. These chemicals are released into the extracellular space and bind to receptors on the post-synaptic neuron to propagate the signal. The axons and dendrites extending from

neuronal cell bodies and resulting synaptic connections are essential for cellular communication and activity regulation. Neuroglia, such as microglia and astrocytes, populate the white matter and are an integral support system in the central nervous system. Microglia are widely distributed throughout the brain and derived from the immune system; astrocytes are derived from neural stem cells and integrate neurons to the central nervous system blood supply. Microglia monitor their environment and act as resident macrophages in the brain, rapidly altering their morphology and proliferating after trauma in order to phagocytize cellular debris (Kettenmann, Hanisch et al. 2011; Wake and Fields 2011). Working together with the blood-brain barrier this response is vital to protecting the central nervous system from invading pathogens and maintaining homeostasis in the brain after trauma. Astrocytes also provide metabolic and structural support to synapses and the blood-brain barrier; regulating the chemical environment of the synapse by removing excess ions and neurotransmitters (Sofroniew and Vinters 2010).

Structure and Function

The mammalian cortex has a laminated appearance which contains distributions of neuronal sub-types. Neurons vary in morphology, both size and shape, and multipolar neurons are the most common in vertebrate systems. Functionally organized into vertical columns, six distinct layers extend from the surface of the brain through the gray matter. Their appearance varies depending on their association area with associative fiber tracts connecting regions within a hemisphere. Commissural fiber tracts to the opposite hemisphere traverse the corpus callosum in order to reach their target. In order to increase size and efficiently

maximize grey matter area, the cerebral hemispheres contain multiple involutions called sulci which surround gyri to give human and other large mammal brains their characteristic appearance. In higher order primates and humans, it is believed that increased development of neocortex in conjunction with intricate brain networks enable more complex social interactions, emotions, and memory.

Importantly for neuroanatomical characterization, while there is individual variation in smaller sulcal patterning, the large sulci provide consistency for nomenclature and identification of neuroanatomical landmarks across subjects. The medial longitudinal fissure divides the cerebral hemispheres and the central sulcus divides the frontal lobe from the parietal lobe. The horizontal lateral sulcus (also referred to as the Sylvian fissure or lateral cerebral fissure) divides the temporal lobe from the dorsal lobes, while the parieto-occipital sulcus divides the parietal and occipital lobes (Figure 1). Specific delineations are made within each lobe depending on the sulci/gyri patterning and cytoarchitectural observations first defined by neuroanatomist K. Brodmann in 1909. Utilizing the Nissl staining method he recorded the laminar organization of the neocortex. Brodmann defined up to 52 regions in mammalian species, using the same number to label homologous regions across species to provide a means of comparison (Zilles and Amunts 2010). While Brodmann is probably the most well-known and his contribution undeniable to the study of neuroanatomy, his work has been debated and revised over the last century. Numerous groups in the 1990's used more precise staining methods, including antibodies to neurofilament proteins and calcium binding proteins, to better isolate borders of specific architectonic regions in non-human primates (Carmichael and

Price 1994; Hof, Nimchinsky et al. 1995). The use of Brodmann's numbering system became well established when it was used in conjunction with stereotaxic atlases for labeling specific structure locations in space; the additional introduction of functional brain imaging s have allowed for correlations between Brodmann areas and cortical function.

Figure 1.1: Human Brain Anatomy. Lateral (A) and medial surface (B) of a human cerebral hemisphere and gyri patterning for lobe identification. Adapted from Gray's Anatomy.

Many early insights into cortical function came from medical observations of disease and injury. Impaired function in cortical regions negatively impacts sensing, perceiving and interacting with the surrounding environment. Confirmed at autopsy, in some of these medical cases damage to specific regions of the cortex provided neurologists with evidence linking their behavioral phenotype to brain damage. Similar lesions located during autopsy along two separate patients' frontal gyri led Dr. Broca to link his observed phenotype of difficultly producing speech to a narrow region roughly corresponding to Brodmann's area 44 and 45. Broca's patients comprehended language and followed spoken directions, however could not speak. Dr. Wernke observed a different phenotype, an inability to comprehend spoken or

written words, and it was associated with damage to the superior temporal gyrus. Two well-known and classic examples further illustrate the importance of more complex cortical connectivity and function: Phineas Gage and an amnesiac case-study patient referred to as "H.M" (Henry Molaison).

Mr. Gage survived a railway accident in which his left frontal lobe was severely damaged and second-hand accounts note significant behavioral changes after the traumatic injury. He died in 1860, however his skull was recently imaged to further examine the extent of damage and the researchers reconsidered alterations of brain networks based on our current understanding of cortical connectivity (Van Horn, Irimia et al. 2012). Recent animal and human studies have correlated damage to frontal cortices to impairments in executive function, decision making, and memory (Moore, Schettler et al. 2009; Kesner and Churchwell 2011; Stuss 2011). Even more important to the study of cortical and sub-cortical function came from the patient H.M., who underwent a bilateral medial temporal lobectomy in an attempt to prevent persistent epileptic seizures in the early 1950's. Post-op he suffered profound memory impairment, specifically anterograde amnesia or inability to convert short term working memory into new long-lasting memories (Scoville and Milner 1957; Squire 2009). H.M's inability to retain information and to form new memories coupled with his relatively unimpaired memory for events that occurred before his lobectomy imply that the medial temporal lobe region is necessary to form new memories but old consolidated memories are not stored in the same location. Dr. Milner's findings regarding H.M. forced reevaluation regarding memory function and the neural structures regulating it; additionally her work set the foundation for

discriminating between types of memory and demonstrated the potential of multiple memory systems.

Portions of cortex can also be broadly subdivided based on their function into sensory (auditory, visual and somatosensory) and motor (pre- and primary), while cortical regions involved in complex relationships (e.g. learning, emotion and attention) are termed associative and tend to have more inter-cortical connections. Associative regions within the frontal lobe mediate executive function actions such as working memory, attention, decision making and task-oriented behaviors (Ofen, Kao et al. 2007; Axmacher, Schmitz et al. 2008). Behavioral tasks such as the Wisconsin Card Sorting Task (Jodzio and Biechowska ; Nyhus and Barcelo 2009) and the Stroop Interference Test (Hyafil, Summerfield et al. 2009) illustrate intact executive function in humans. Further evidence supporting frontal lobe mediation in executive functions comes from electrophysiology recordings in non-human primates showing activation of neurons within a specific context (Mansouri, Matsumoto et al. 2006; Zaksas and Pasternak 2006). The temporal lobe contains auditory cortex (BA 41 and 42) and processes sound, additionally the medial portion encapsulates the hippocampus and functions in declarative memory processing (Gour, Ranjeva et al. 2011). The hippocampus is integral to formation of new memories regarding experience, detection of novel stimuli and spatial navigation (O'Keefe and Nadel 1978; Driscoll and Sutherland 2005).

Recent groundbreaking studies coupled with enhanced imaging technology and the ability to detect brain activity in normal individuals illustrates several relevant principles (Fakhri, Sikaroodi et al. 2012). Specific regions in the brain are

responsible for specific functions. Memory involves multiple systems and is not compartmentalized to one region. Injury and/or disease impact and cause specific impairments to sub-sets of function depending on location, e.g. short vs. long-term memory (Levy and Goldman-Rakic 1999; Marklund, Fransson et al. 2007).

Brain Barriers and Fluids

The brain depends on delivery of molecules, such as oxygen and glucose, from the peripheral blood supply; however transportation across the blood-brain barrier must be tightly regulated to maintain homeostasis. Underneath the meninges a thin collection of glial processes connect to the basal lamina of the cerebral cortex to form the glial limitans and divides the central nervous system from the rest of the body. Larger blood vessels penetrating the cerebrum pass through the subarachnoid space and are covered by a thin meningeal layer, the pia mater, which results in a perivascular space and serves as a drainage route for brain interstitial fluid. Astrocytes also support and provide signals to capillary endothelial cells to form tight junctions, which are essential for maintenance of the blood-brain barrier (Abbott, Ronnback et al. 2006). These junctions along brain vasculature create the seal necessary for optimal operation of the blood-brain barrier, and along with the glia limitans and the perivascular space regulate fluid flow in the central nervous system. Together these components separate blood from the interstitial space, carefully policing the movement of molecules from the periphery and serving to protect the brain and spinal cord from foreign substances.

Produced in the choroid plexus from arterial blood, the cerebrospinal fluid fills the ventricles, the subarachnoid space and central canal of the spinal column.

Cerebrospinal fluid is continually made and reabsorbed into the blood and in addition to providing mechanical support it serves an important role in facilitating cerebral blood flow, movement of nutrients, and removal of waste through excretion to venous blood. While brain interstitial fluid contacts neurons and glial cells directly, cerebrospinal fluid remains separate but mixes with brain interstitial fluid and thus contributes to the local environment surrounding brain tissues. Sampling the cerebrospinal fluid provides scientists with a closer view of the brain environment and examining the composition of cerebrospinal fluid provides useful information regarding health and neurological diseases. Certain stimuli and chemical substances are capable of opening up the blood-brain barrier and excessive release of pro-inflammatory cytokines can disrupt the blood-brain barrier during a neuro-immune response (Coisne and Engelhardt 2011).

Alzheimer's disease (AD)

Introduction

Exceptionally complex interactions between many levels and systems, both inside and outside the body, impact an organism's lifespan and contribute to a differential aging phenotype. Alzheimer's disease (AD) is an age-related progressive neurological form of dementia. First described as presenile degeneration by Dr. Alois Alzheiemer in 1906, major characteristics of AD include behavioral and cognitive problems such as degeneration of memory and changes in personality that affect the ability of a person to carry out daily activities. AD patients exhibit profound memory loss which progresses with disease severity, delusions and significant emotional

changes and eventual failure to communicate with friends and family. According to the Alzheimer's Association one in 10 people over the age of 65 and close to half over the age of 85 have AD. The ADAMS report (Plassman, Langa et al. 2007) indicates in America alone, nearly 2.5 million dementia patients over the age 71 have Alzheimer's disease. Annual cost for care for patients with AD exceeds billions of dollars and excessive stress takes its toll on caregivers.

Researchers cannot yet pinpoint specific causes of the disease, because the etiology of AD is still poorly understood. Currently a multitude of factors including sex, race, socioeconomic status, and education are assessed in order to determine an overall risk for the disease. Professionals agree (ADEAR 2007) that the development of AD is the result of a complicated course of both genetic and non-genetic factors, with increasing age being the biggest determining aspect. Even with advancing scientific tools and technologies, a medical diagnosis of AD is usually based on criteria outlined in the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, (APA 2000) and neuropsychological evaluation using the Mini-Mental State Examination (MMSE). The patient must exhibit impaired memory along with at least one of the following conditions: problems with executive function, aphasia, apraxia or agnosia. According to the criteria, these cognitive impairments occur gradually and continue over time interfering with occupational functioning and social interaction. According to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ARDA) rating scale, established in 1984, a definite AD diagnosis relies on post-mortem pathological analysis of brain tissue (Dubois,

Feldman et al. 2007). This analysis utilizes Braak staging of I – VI; stage I denotes the earliest stage of pathology located around the entorhinal cortex and stage VI represents spreading of pathology throughout the neocortex into the occipital lobe (Braak and Braak 1991; Braak, Thal et al. 2011). However, recently the National Institute on Aging (NIA) and Alzheimer's Association proposed new guidelines for a medical diagnosis of AD and incorporates criteria for stages of disease progression along with utilization of biomarker tests (Jack, Albert et al. 2011)

An estimated 5% of AD cases are early onset (Alzheimer's Association 2008); affecting people aged 30-65. Early onset AD seems to follow a rigorous pattern of inheritance and is often referred to as familial AD. Cases have been linked to mutations of the amyloid precursor protein (APP) gene on chromosome 21, mutations that impact functioning of γ-secretase in the presenilin 1 (PSEN-1) gene located on chromosome 15 or a similar gene called presenilin 2 (PSEN-2) on chromosome 1 (McClearn and Vogler 2001; ADEAR 2007). Remaining cases of AD are late onset and idiopathic, with no clear pattern in onset or severity of disease pathology. A widely recognized genetic risk factor in late onset AD is a particular form of the apolipoprotein E (ApoE) gene on chromosome 19. Inheritance of one or two copies of the ε4 allele increases risk for idiopathic AD (McClearn and Vogler 2001; Serretti, Olgiati et al. 2007). However, presence of this allele does not predict whether or not a person will develop AD. While the mechanisms are not clearly understood, different ApoE isoforms preferentially bind Aβ peptides and that affinity may contribute to the higher risk of ε4 carriers in developing AD due to reduced clearance of amyloid from the brain (Petrlova, Hong et al. 2010). Interestingly, a study done in

southern Italy reports a protective effect of the ApoE ε2 allele in familial AD and idiopathic AD due to more efficient action of the isoform (Panza, Solfrizzi et al. 2000; Rebeck, Kindy et al. 2002), while others suggest that ApoE ε2 improves cognition in the oldest old but does not reduce accumulation of Alzheimer's pathology (Berlau, Corrada et al. 2009). Other contributing factors linked to idiopathic AD include cardiovascular disease, type II diabetes, oxidative damage and inflammation, and traumatic brain injury (Weller, Yow et al. 2002; Mahley, Weisgraber et al. 2009; Johnson, Stewart et al. 2010; Bosco, Fava et al. 2011)

Irrespective of type, the major pathological characteristics of AD are very similar and include abnormal protein aggregation, neuronal loss, brain shrinkage, and cognitive decline. More specifically the development and deposition of amyloid-β plaques in brain parenchyma and cerebral vessels (angiopathy) along with neurofibrillary tangles containing aggregates of hyperphosphorylated tau occurs with advancing age. Several studies have documented and reviewed neuronal losses in Alzheimer's relevant regions including basal forebrain, hippocampus, cortex and locus coeruleus (Zarow, Lyness et al. 2003; Chetelat, Villemagne et al. 2010; Brunnstrom, Friberg et al. 2011; Schliebs and Arendt 2011). Additional magnetic resonance imaging (MRI) studies reveal alterations in brain volume and have identified other key locations such as cingulate cortex (Jones, Barnes et al. 2006; Amanzio, Torta et al. 2011). Aberrant microglial activation and astrocyte pathology correspond to elevated pro-inflammatory processes and changes in permeability of the blood-brain-barrier (Li, Zhao et al. 2011; Crehan, Hardy et al. 2012). Cell culture studies examining ApoEε4 function also demonstrate impaired tight junction and

reduced integrity of the blood-brain barrier (Nishitsuji, Hosono et al. 2011). These combinations of factors directly impact brain regions important for memory processes and lead to the varied symptoms experienced by AD patients.

Alzheimer's pathology

Biochemical alterations lead to cortical atrophy and apoptosis leading to enlargement of brain ventricles and cortical thinning. Coupled with neuronal loss in the basal forebrain and hippocampus (Zarow, Vinters et al. 2005), overall shrinkage of brain tissue impacts memory recall and formation of new memories. Plaques containing Aβ form due to irregular amyloid precursor protein processing (Hardy and Allsop 1991; Selkoe 2000; Tseng, Kitazawa et al. 2004), accumulation ultimately results in degeneration of axons and dendrites. Dysfunctions in microtubule associated protein tau lead to neurofibrillary tangles inside the cell body (LaFerla and Oddo 2005; Gendron and Petrucelli 2009), impairing neuronal stabilization and contributing to neuronal loss.

In the central nervous system, acetylcholine functions as the major neurotransmitter of the basal forebrain cholinergic system. Acetylcholine enhances hippocampal and cortical processing; contributing to arousal, maintaining attention, learning and preserving memory (Hasselmo and Giocomo 2006; Roberts and Thiele 2008; Klinkenberg, Sambeth et al. 2011). Early studies of AD reported significant decreases in synthesis and release of acetylcholine, which was linked to neuronal loss in the basal forebrain and dementia (Whitehouse, Price et al. 1982; Coyle, Price et al. 1983). Researchers proposed that the cognitive decline in AD was due to cholinergic deficits and that dementia might be reversed by elevating acetylcholine; these studies

formed the basis for one of the oldest AD hypotheses. This hypothesis focused attention on the basal forebrain and proposed the loss of acetylcholine in the central nervous system as the central pathological feature for development of AD. Deterioration of memory and cognitive abilities occurs due to degeneration of cerebral presynaptic cholinergic neurons and resulting imbalance in acetylcholine metabolism (Smith and Swash 1978; Bartus, Dean et al. 1982). Reversible inhibition of acetylcholine causes memory impairment in animal models and application of an acetylcholinesterase inhibitor reverses cognitive deficits (Arendt, Schugens et al. 1990; Van Dam, Abramowski et al. 2005). Based on these sorts of studies, acetylcholinesterase inhibitors were developed for pharmacological interventions and the majority of Food and Drug Administration approved drugs in use to treat AD target the acetylcholine system. These drugs inhibit the breakdown of acetylcholine, leading to an increase in the brain and making it available for re-release at the synapse. Support for this hypothesis declined due to an overall lack of effectiveness in AD patients to sufficiently restore acetylcholine, nor do these drugs significantly reduce amyloid burden or improve cognitive status long term.

A hallmark feature of AD is the deposition of amyloid-β plaques first developing in areas of the brain relating to memory. It is a protein fragment cleaved from a larger complex called amyloid precursor protein. This protein contains enzymatic cleavage sites for α-, β-and γ-secretase (Figure 1.2) resulting in two major cleavage pathways. Cleavage of amyloid precursor protein and activity in the presenilin family (PS1/PS2) influences γ-secretase and leads to the generation of amyloid peptides. While not clearly understood, some evidence supports normal

function of amyloid precursor protein in neuronal development and regulation of synaptic plasticity (Priller, Bauer et al. 2006) and in normal healthy neurons α-secretase cleavage predominates to form non-pathogenic peptides. The pathogenic pathway characterized by cleavage of amyloid precursor protein by β-secretase instead of α-secretase results in generation of distinct pathogenic amyloid-β peptide isoforms (Hardy and Selkoe 2002). In AD there is a shift in processing to slightly longer protein 42 amino acids in length, forming amyloid beta-42. Amyloid-β42 aggregates with other amyloid fragments to form deposits (Lorenzo, Yuan et al. 2000; Selkoe 2000). Compared to the Amyloid-β40 isoform, Amyloid-β42 aggregates more readily and is more cytotoxic (Chen and Glabe 2006; Jan, Gokce et al. 2008; Hedskog, Petersen et al. 2010; Kuperstein, Broersen et al. 2010). Another factor in progression of AD pathology is the ratio of amyloid-β42:amyloid-β40 (Lewczuk, Esselmann et al. 2004; Wiltfang, Esselmann et al. 2007). Conversion from monomeric amyloid peptides into oligomeric amyloid deposits contribute to neuronal loss resulting in memory deterioration and observable cognitive deficits. Under normal functioning circumstances amyloid-β is catabolized and cleared by endogenous mechanisms; however, in AD it accumulates extracellularly and intraneuronally (Oddo, Caccamo et al. 2006). Recently it has also been reported that soluble Aβ fractions, including monomers and oligomers, correlate better to cognitive impairment (Tomic, Pensalfini et al. 2009) and soluble fractions impair synaptic plasticity in experimental animal models (Shankar, Li et al. 2008).

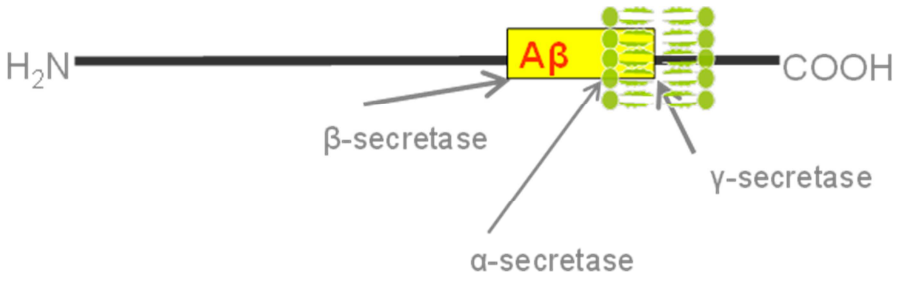

Figure 1.2: Amyloid Precursor Protein. Liberation of amyloid-β peptide fragment via sequential secretase cleavage of the full length protein.

The discovery that amyloid was the main component of these deposits led to the belief that the progressive buildup of Aβ in the brain caused AD. Proponents of the amyloid hypothesis (Hardy and Allsop 1991; Hardy and Higgins 1992; Coria, Rubio et al. 1994) believe amyloid precursor protein processing and the formation of plaques to be the key events in development of AD. Changes in amyloid-β metabolism then lead to the downstream changes: (1) increased formation of pathogenic amyloid isoforms (2) microglia and astrocyte activation (3) neuronal loss (4) senile plaque and tangle pathology. Many support this hypothesis because amyloid-β accumulates and forms aggregated deposits prior to other definable symptoms of AD. While APP/PS1 mutations in familial AD could account for the initial generation of amyloid-β this hypothesis does not adequately explain the trigger in idiopathic AD; in humans plaques also increase with aging and a relatively high amyloid burden present in only mild cases of cognitive impairment or even in cognitively normal subjects suggest that other mechanisms should be considered.

The last major feature of AD relates to the cytoskeleton protein tau and tangle formation. Tau plays an important role in cellular trafficking, organizing and stabilizing the cell by binding to microtubules; however in an aberrant

hyperphosphorylated form it cannot bind microtubules. Instead tau aggregates into twisted protein and accumulates in nerve cell bodies, forming tangles. It is generally thought that neurofibrillary tangles disrupt axonal transport eventually leading to neuronal death. Advocates for a tau hypothesis of AD (Goedert, Spillantini et al. 1992; Strittmatter, Weisgraber et al. 1994; Schmitz, Rutten et al. 2004) assign the major causative role to neurofibrillary tangles; compared to the amyloid cascade hypothesis where the influence of tau occurs downstream of amyloid aggregation. Disrupted axonal transport impairs the ability of neurons to properly export proteins to distal regions or receive trophic factors at terminals compromising survivability of neurons with accumulating NFTs (Trojanowski and Lee 2005) and thus contributing to the pathology of AD independently of amyloid. Other support for this angle comes from studies indicating that amyloid load does not correlate with cognitive decline or neuronal loss (Schmitz, Rutten et al. 2004). However, on their own, tau mutations do not lead to significant production of amyloid pathology.

Modeling AD

Experimental animal models provide precise control over experimental manipulations allowing researchers to minimize extraneous noise in data collection. Shorter lifespans are also appealing, allowing for easier repetition and larger sample sizes for studies. Essential tools in translating our understanding into medical advances in the study of disease include integrative approaches using animal models. Animal models of AD include transgenic mice expressing selected atypical human genes resulting in production of AD pathology and pharmacological manipulations

targeting specific neurotransmitter systems thought to be involved in the development of AD.

Transgenic animal models only relate to a small proportion of AD cases due to gene mutations. While similar challenges to human studies exist, experiments with non-human primates can be carefully controlled and monitored throughout their time course. Humans and non-human primates share significant physiological aspects and they develop amyloid pathology naturally with age. Currently no animal exists for late onset AD, this idiopathic form accounts for approximately 95% of reported cases.

Alzheimer's disease greatly impacts the lives of patients and caregivers. Complicated patient history and compliance issues are two important factors impacting a researchers control over the experiment along with scope of inference in studies with human subjects; experiments require extremely large sample sizes in order to draw meaningful conclusions from the data. Additionally, in studies with humans, obtaining optimally prepared tissue samples can be difficult and time consuming given their extended lifespans.

Neuroinflammation

Introduction

An inflammatory response is an early immune reaction to trauma or invading pathogens; in the central nervous system microglia act as the resident immune system and mediate neuroinflammatory processes (Streit, Mrak et al. 2004). Activation of microglia and increased cytokine production characterize immune responses in the brain. Under normal circumstances microglia exhibit a resting (ramified) phenotype, characterized by a uniform compact cell body and long extending processes. When

microglia sense a change in the environment, they respond by a shift in phenotype and become activated. These activated microglia migrate and proliferate at the site of trauma. They communicate with astrocytes and release effector molecules to deal with the insult; released cytokines may be beneficial or detrimental, often depending on the period of time microglia remain activated. Therefore, in terms of pathology, it is important to distinguish between acute and chronic neuroinflammation.

Cytokines

Cytokines are central to immune responses in the brain and regulate inflammatory pathways. Different families of cytokines are involved in pro- or anti-inflammatory mechanisms and released by glial cells. Interleukin-1β, interleukin -6, and tumor necrosis factor- α are all expressed during pro-inflammatory actions, while interleukin -10 family cytokines are considered anti-inflammatory. During an inflammatory response, microglia and astrocytes release cytokines in response to trauma. The production of these different families of cytokines regulates both the strength and length of an immune response (Tuppo and Arias 2005).

Relationship to AD

Activated microglia are thought to play a role in development of age-related inflammatory diseases and have also been shown to increase during aging in non-human primates (Sheffield and Berman 1998). Alzheimer's disease has been associated with chronic microglia activation and morphological changes leading to increased secretion of pro-inflammatory factors and recruitment of astrocytes which

may further accentuate the neurotoxic response. In rodents, these alterations impact the integrity of surrounding neurons often exacerbating disease conditions.

Oxidative stress and neuroinflammation play critical roles in the pathogenesis of AD due to interactions between astrocytes, microglia and AD pathology within the inflammatory response. Further study of free radicals has shown that they promote amyloid aggregation. This interaction of β-amyloid and reactive oxygen species (ROS) may enhance damage to brain tissue resulting in the increasing neuronal damage seen in AD. Dead and damaged neurons promote immune responses triggering activation of microglia which respond at the injured locations to scavenge debris and remove it. However, when microglia remain in their activated state for a sustained period of time they also release harmful pro-inflammatory cytokines which promote chronic neuroinflammation and contribute to neuronal death. The amyloid-β protein sustains microglia activation and leads to increased secretion of cytokines that impact neuronal viability (Barger and Harmon 1997; Barger and Basile 2001; Sondag, Dhawan et al. 2009).

Several cytokines are relevant to AD and have been measured in areas surrounding amyloid-β plaques, notably the interleukins and tumor necrosis factor-α. A cycle of activation, initiated microglial release of interleukin-1 and production of interleukin-6 in in astrocytes, enhances further activation and production of cytokines. In this way interleukin-6 promotes neuroinflammation and astrogliosis. High levels of tumor necrosis factor-α released from microglia contribute to neurotoxic activation and indicate ongoing inflammation. Cell culture experiments show dramatic increases in cytokine mRNA after exposure to amyloid.

Catecholamine System

Locus Coeruleus

In the periphery, norepinephrine is synthesized and released from chromaffin granules in the adrenal gland into the blood stream where it acts as a hormone via the endocrine system. Results of increased norepinephrine from sympathetic neurons in the periphery are increased cardiac muscle contractions and enhanced blood flow to muscles during the flight-or-flight responses. The locus coeruleus is a small midbrain structure contained in the pons and is responsible for norepinephrine release in the central nervous system (Figure 1.3). The locus coeruleus contains a dense cluster of neuronal cell bodies that synthesize norepinephrine and fibers project to regions of the brain that utilize norepinephrine as a neurotransmitter, e.g the hippocampus and prefrontal cortex (Thompson 2000; Sharma, Xu et al. 2010).

Locus coeruleus neuronal firing patterns change dramatically when an animal is awake versus sleeping, suggesting that this system contributes to wakefulness (Devilbiss, Page et al. 2006) and altering electrical stimulation patterns in the locus coeruleus impacts norepinephrine release in projection regions (Florin-Lechner, Druhan et al. 1996). Furthermore, these neurons display burst firing patterns in response to behavioral sensory stimuli and activation of the locus coeruleus is associated with detecting salience, an important mechanism for focusing attention which facilitates learning (Aston-Jones, Rajkowski et al. 1999; Bouret and Sara 2004). A functional role for the locus coeruleus in decision making and memory processing has been proposed (Aston-Jones, Rajkowski et al. 2000; Rajkowski, Majczynski et al. 2004) and several studies with non-human primates have illustrated

activation of locus coeruleus neurons during behavioral tasks which require a change in attention or an alteration in the reinforcement paradigm during a testing session.

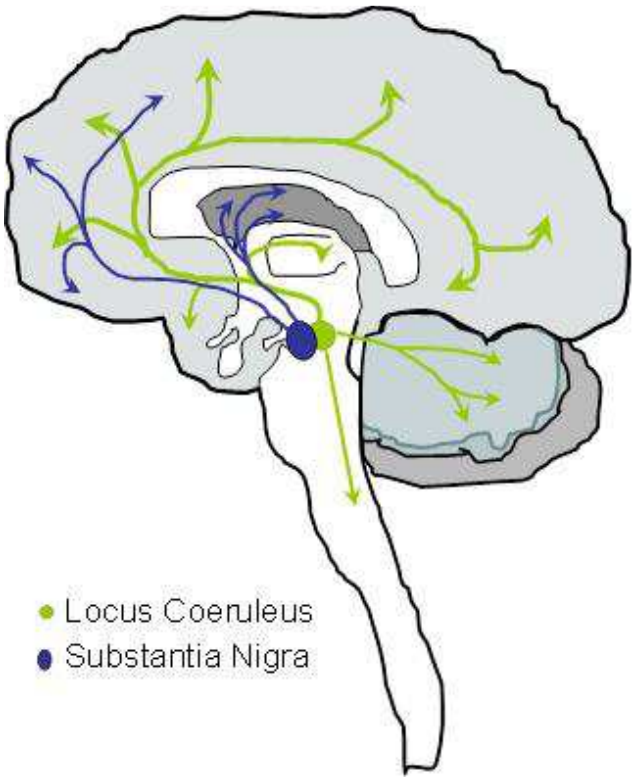

Figure 1.3: Location and projections from Locus Coeruleus. Brain schematic illustrates projections of dopamine from the Substantia Nigra (blue arrows) and norepinephrine (green arrows) from the Locus Coeruleus. Mid-sagittal view.

Synthesis, Degradation and Regulation

Dopamine and norepinephrine are prevalent neurotransmitters in both the central and peripheral nervous system. The initial building block for these catecholamines, tyrosine, is an amino acid acquired from protein food sources and can be synthesized in the body from phenylalanine. Tyrosine is converted into L-dopa by the enzyme tyrosine hydroxylase, which is in turn converted into dopamine by dopa-decarboxylase. After transport into a synaptic vesicle, dopamine β-

hydroxylase changes dopamine into norepinephrine (Figure 1.4). Tyrosine hydroxylase is present in all catecholamine-producing neurons and therefore considered to be a general marker of catecholamine neurons. Dopamine β-hydroxylase, which is present within vesicles of cells producing norepinephrine, specifically labels noradrenergic neurons. Due to its small densely packed morphology and more isolated location within the midbrain, both enzymes have been used to visualize locus coeruleus cells using immunoreactivity staining.

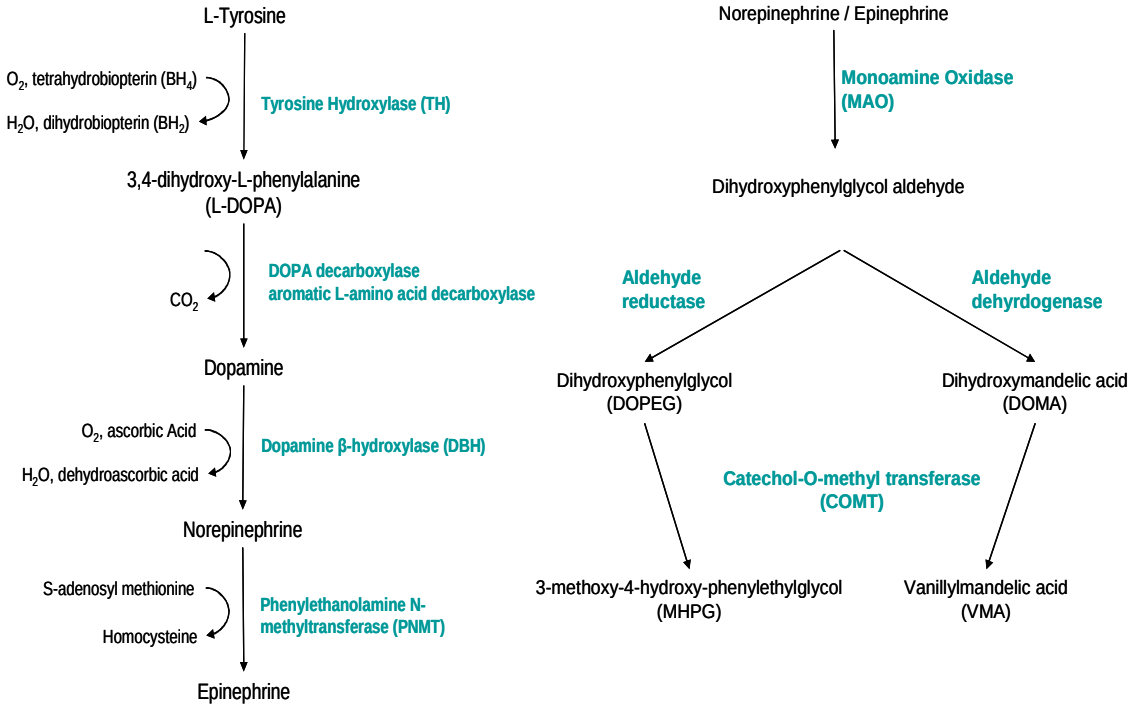

Figure 1.4: Synthesis pathway for dopamine and norepinephrine. Synthesis occurs via DOPA decarboxylase and dopamine-β-hydroxylase (A). Monoamine Oxidase and Catechol-O-methyl transferase are the main steps in norepinephrine degradation, resulting in formation of two amine metabolites: 3-methoxy-4-hydroxy-phenylethylglycol and vanillylmandelic acid (B).

Noradrenergic receptors on post-synaptic terminals in locus coeruleus projection regions contain two major types of adrenergic receptors, generally categorized as α-receptors and β-receptors. Activation of post-synaptic α1- and β-

receptors typically leads to excitation; while α2-receptors on post-synaptic membranes lead to inhibition and α2-receptors on presynaptic membranes provide autoregulation inhibiting locus coeruleus activity via negative feedback. Downstream transduction of α1-receptor activation proceeds through Phospholipase-C resulting in elevated Ca+2; whereas, stimulation of α2-receptors inhibits adenylate cyclase and reduced excitability decreases neurotransmitter release. Stimulation of β-receptors impacts protein kinase A phosphorylation cascades and enhances long term potentiation. Obvious complexities arise as regional differences in norepinephrine action occur due to varying receptor distributions and norepinephrine concentration.

After signaling, norepinephrine degradation occurs through monoamine oxidase or reuptake occurs through two main transporters: Norepinephrine transporter from the synaptic cleft and vesicular monoamine transporter inside the terminal. In addition to synthesis, autoregulation and reuptake impact the availability of norepinephrine. The most common metabolite of norepinephrine, 3-methoxy-4-hydroxyphenylglycol, can be measured in cerebrospinal fluid and used as a general marker for norepinephrine turnover.

Pathology

Deficits in other neurotransmitter systems have also been documented in aging and neurodegenerative disease, although the clinical significance of the locus coeruleus-norepinephrine system in AD is not well understood. Early research describes decreases of norepinephrine in the hindbrain with age along with increase in monoamine oxidase-B, the enzyme responsible for degrading norepinephrine (Robinson, Nies et al. 1972). Locus coeruleus neuronal loss in AD has been

described (Wilcock, Esiri et al. 1988) and others have documented losses of norepinephrine in locus coeruleus projection areas along with alterations in amine metabolites (Adolfsson, Gottfries et al. 1979).

After trauma, plasticity related changes such as increased tyrosine hydroxylase mRNA expression, dendritic sprouting, and altered receptor expression in hippocampus has been associated with reduction of locus coeruleus neurons. The amount of tyrosine hydroxylase in a neuron is important because it is the rate limiting step for the synthesis of both dopamine and norepinephrine. Even with a loss of norepinephrine neurons, reports of increased activity in surviving neurons (Szot, White et al. 2006) indicate that the brain may be able to compensate for some amount of time.

Two types of axon terminals releasing norepinephrine have been described, a conventional terminal with classic norepinephrine neurotransmitter release to activate adrenergic receptors and release of norepinephrine from extra-synaptic varicosities to diffuse into the microenvironment to act on glial cells and other neurons (Marien, Colpaert et al. 2004). Norepinephrine suppresses inflammatory gene expression (Feinstein, Heneka et al. 2002) and reduces expression of inflammatory proteins (Frohman, Vayuvegula et al. 1988); significant decreases in norepinephrine could then contribute to elevated cytokine production from activated microglia and apoptosis signaling molecules leading to further neuroinflammation and neuronal loss.

N-(2-bromobenzyl)-N-(2-chloroethyl) ethylamine hydrochloride (DSP-4)

Prior to discovery and use of neurotoxins, scientists studied nerve function and resultant physiological perturbations following physical damage to nerves and/or transection of axons. However, this type of approach is not as practical in the brain because nerve bundles are difficult to isolate from one another or the target brain regions may not be easily accessible. Furthermore, mechanical or electrolytic lesions are nonspecific and often result in damage outside the target. Neurotoxins can be administered to produce site specific lesions of neurotransmitter systems in the brain. The discovery of various toxins and their effects has allowed for extensive study of morphology, biochemistry and physiology in specific brain systems. This approach provides a more precise experimental method to study the function of particular neurotransmitters and to model certain disease pathologies.

The noradrenergic neurotoxin DSP-4, originally described by Ross & colleagues in 1973, crosses the blood brain barrier and is converted to an aziridinium ion to exert neurotoxic effects in the locus coeruleus leading to depletion of norepinephrine. Ross and Renyl's original experiments demonstrated that DSP-4 blocked norepinephrine uptake in brain slices and further that the effect they reported was specific to norepinephrine, suggesting that DSP4 interacted with a norepinephrine-specific transport mechanism into the neuron. Using their slice culture model, they described a long lasting impairment of norepinephrine uptake up to 4 weeks after a single dose of 100 mg/kg and doses of 25mg/kg and 50 mg/kg produced similar effects 24 hours after injection (Ross and Renyl 1976).

The precise mechanism by which DSP-4 exerts cytotoxic denervation is not well understood, however effects are duplicated by the highly reactive aziridinium ion form. The neurotoxin must be prepared and injected quickly prior to this spontaneous reaction because the ionic form does not cross the blood brain barrier (Zieher and Jaim-Etcheverry 1980). The negative effects of DSP4 can be mitigated by pre-treatment with desipramine, a norepinephrine transport blocker, suggesting that the transporter is required for the neurotoxic effects of DSP4.

Experimentally induced locus coeruleus lesions in AD-transgenic models produce elevated amyloid and induce inflammation (Kalinin, Gavrilyuk et al. 2007). Reported locus coeruleus degeneration in cases of mild cognitive impairment, often considered a pre-AD stage, suggests that locus coeruleus dysfunction occurs early on prior to more significant amyloid deposition (Grudzien, Shaw et al. 2007).

Studies with non-human primates

Amyloid

Long-lived animal models with naturally developing amyloid are necessary in order to better understand effects of deposition and aggregation of amyloid on brain function. The common squirrel monkey (*Saimiri sciureus*), a small new world primate, shows more vascular amyloid deposition of amyloid (Walker, Masters et al. 1990; Elfenbein, Rosen et al. 2007); while more parenchymal deposition occurs in senile plaques of the rhesus macaque (*Macaca mulatta*), a larger old world monkey (Wei, Walker et al. 1996; Shah, Lal et al. 2010). Both amyloid precursor protein processing pathways occur in non-human primates (Podlisny, Tolan et al. 1991) and deposition increases in an age-dependent manner. Similar to humans, parenchymal

deposits present as both diffuse and compact morphologies, containing both Aβ40 and Aβ42 (Kanemaru, Iwatsubo et al. 1996; Hartig, Goldhammer et al. 2010). However, the amount and isoform distribution varies depending on species, and similar to humans large variation is noted among individuals of similar age.

Rationale: Locus Coeruleus, Norepinephrine and AD

Degeneration in the locus coeruleus is a documented feature of AD; however its role remains unclear (Bondareff, Mountjoy et al. 1987; Burke, Chung et al. 1988; Marien, Colpaert et al. 2004). The DSP-4 model will provide a means to further investigate the role of norepinephrine depletion prior to onset of AD pathology using two species of non-human primates. This approach is ideal because while mice have a much shorter lifespan and are easily manipulated genetically, they do not develop AD pathology in an idiopathic manner. Non-human primates develop amyloid pathology naturally (Walker, Masters et al. 1990; Podlisny, Tolan et al. 1991) and the rhesus monkey genome is 93% similar to humans (Gibbs, Rogers et al. 2007). Currently no animal model exists for idiopathic AD, which accounts for the vast majority of all human cases. Development of idiopathic model AD would provide a new valuable model for exploring novel therapeutics and interventions designed to slow down pathogenesis, ultimately lessening costly health consequences.

Hypothesis and predictions

Age is the largest risk factor for development of AD pathology and degeneration in locus coeruleus has been documented in cases of AD. Norepinephrine deficiency promotes neurodegeneration, contributing to onset and progression of AD. Therefore

amyloid pathology and neuroinflammation will increase with age. Pharmacological reduction of norepinephrine with DSP4 will be permissive for pathology development resulting in onset and increased accumulation of Alzheimer's pathology, chronic neuroinflammation and elevated AD relevant biomarkers.

Chapter 2: Increased Amyloid pathology in non-human primates following DSP-4 injection resulting in impaired locus coeruleus function

Introduction

Alzheimer's disease (AD) affects 5.4 million Americans and the World Health Organization estimates 18 million people worldwide; without successful intervention the number of AD patients could easily triple by 2050, considering longer life expectancies and the aging baby boomer population (Alzheimer's-Association 2012). This progressive neurological disease impairs cognitive and executive functions, including severe impairments of memory and attention. Cognitive and sensory neural systems seem preferentially impacted by AD with amyloid plaque accumulation, hyperphosphorylated tau formation, neuroinflammation, and neuronal loss. Biochemical alterations and deposition of pathology begin well before behavioral symptoms and the onset of dementia.

One widely supported hypothesis of AD highlights amyloid as the central player in disease progression, with focused attention on generation and deposition of amyloid-β with subsequent plaque formation (Hardy and Allsop 1991; Hardy and Selkoe 2002). More recently, the primary hypothesis has shifted to focus on the importance of soluble amyloid isoforms and their contribution to AD pathology and cognitive decline (Selkoe 2008). Formation of amyloid-β occurs via a proteolytic cleavage pathway in which amyloid precursor protein forms isoforms based on the site specific cleavage by β- and γ-secretase. These peptides also undergo post-translational modifications which impact the solubility, aggregation and degradation of the various isoforms (Atwood, Martins et al. 2002). Parenchymal deposits isolated

from clinical AD cases primarily contain aggregates of Aβ-40 and Aβ-42, while vascular deposits are made up of mainly Aβ-40. Soluble monomers of these peptides form larger oligomers, which have been shown to interfere with synaptic function and have the potential to disrupt neurotransmission including long term potentiation (Walsh, Klyubin et al. 2002; Selkoe 2008).

The locus coeruleus contains the highest density of noradrenergic system cell bodies that synthesize and distribute norepinephrine to wide-ranging brain regions, including neocortex and hippocampus. Locus coeruleus degeneration and norepinephrine loss are also well documented features of AD (Mann and Yates 1981; Mann, Yates et al. 1982; German, Manaye et al. 1992) along with other types of dementia (Zarow, Lyness et al. 2003; Brunnstrom, Friberg et al. 2011) and aging (Manaye, McIntire et al. 1995); although other reports suggest similar neuronal counts in adult and non-demented aged cases (Mouton, Pakkenberg et al. 1994; Ohm, Busch et al. 1997). Changes in norepinephrine transporter and adrenergic receptor subtypes are associated with locus coeruleus impairment; both contribute to reduced norepinephrine in projection regions and likely contribute to bio-psychiatric symptoms observed in AD patients (Tejani-Butt, Yang et al. 1993; Matthews, Chen et al. 2002). Demonstration of neuronal loss in the locus coeruleus actually exceeding cholinergic loss increased attention on the involvement of the locus coeruleus-norepinephrine dysfunction in developing AD (Zarow, Lyness et al. 2003).

Several studies utilizing various transgenic AD mouse models expressing APP mutations further supported the hypothesis of norepinephrine involvement in AD associated neurodegeneration. These studies demonstrated that treatment with the

noradrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) increased Aβ pathology (Heneka, Ramanathan et al. 2006; Kalinin, Gavrilyuk et al. 2007; Jardanhazi-Kurutz, Kummer et al. 2010). These early studies also reported increased astrocyte and microglia expression, induction of inflammatory pathways, and cognitive deficits following targeted locus coeruleus degeneration in AD models (Heneka, Galea et al. 2002). These findings suggested that locus coeruleus impairment and norepinephrine depletion was permissive for neuroinflammation. Further research confirmed suppression of both proinflammatory gene expression and cytokine production in microglia by norepinephrine; whereby induced degeneration by DSP4 increased secretion of these markers from microglia cells and impaired beneficial actions including phagocytosis (Heneka, Nadrigny et al. 2010).

Relating specific genetic mutations in APP to development of early onset AD provides important insights into disease progression. Studies with transgenic models continue to further understanding of aberrant disease pathways and are important for unraveling the complexities of AD; however their significance in fully comprehending idiopathic AD processes may not be adequate. Many studies using AD transgenic murine models have examined the deposition of cerebral amyloid in parenchymal and vascular tissue. However, this differs in non-human primates in which age related increases occur in aggregated amyloid similarly to humans; although deposition patterns vary between species (Uno and Walker 1993; Gearing, Tigges et al. 1996; Bons, Rieger et al. 2006). Uno and colleagues (1996) examined brains from 81 rhesus macaques (16-39 yrs) and 25/41 (21%) animals between the ages of 26 and 31 showed plaques, while only 5/41 (12%) showed cerebral amyloid

angiopathy. In comparison, cortical cerebral amyloid angiopathy deposition was considerably higher than amyloid-β plaque formation in squirrel monkeys (Elfenbein, Rosen et al. 2007). Non-human primates develop amyloid pathology naturally (Walker, Masters et al. 1990; Podlisny, Tolan et al. 1991). The appearance of plaques and development of cerebral amyloid angiopathy is similar to observations from AD patients (Selkoe, Bell et al. 1987). APP_{695} is predominantly expressed in neuronal cells and homology between the human sequence of amyloid precursor protein and both cynomolgus monkey and squirrel monkey is nearly identical (Selkoe 1991; Levy, Amorim et al. 1995).

Few studies have investigated locus coeruleus-norepinephrine systems and their role in amyloid deposition using an animal model with natural age-related accumulation. Thus non-human primates provide an opportunity to examine idiopathic development of early stage Alzheimer's-like pathology following depletion of norepinephrine. The aim of this study was to determine if DSP4 could induce and increase cerebral amyloid pathology in two species of non-human primates which develop amyloid deposits naturally.

Materials and Methods

Subjects

Pilot study: 3 female adult common squirrel monkeys (*Saimiri sciureus,* aged 15 years*)* and 3 female adult rhesus monkeys (*Macacca mulatta,* aged 15-16 yrs*)* were studied in an initial short-term study (Table 1). Long term study: 8 adult (11 years old) and 5 aged (19-20 years old) squirrel monkeys were studied. Additionally, 14 adult (14-17 years old) and 7 aged (19-25 years old) rhesus monkeys were studied (Table 1). All monkeys were female.

Animals were obtained from approved sources and maintained at the National Institute on Aging/NIH primate facility prior to the study. Animals were housed in standard non-human primate caging and kept on 12:12hr light cycle, had *ad libitum* access to water and were fed standard NIH diet twice daily approximating *ad libitum* levels. Animals were observed daily by trained observers, including checks on food consumption and well-being. Routine health monitoring, TB tests and blood collections were done quarterly.

Animal husbandry and all experimental procedures in the study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were conducted under an approved protocol by the NIA Institutional Animal Care and Use Committee (IACUC). In addition, an IACUC protocol was also approved at the University of Maryland for all experiments utilizing non-human primate tissue and fluid samples.

Table 2.1: Animal Distribution and Treatment Groups. All animals were housed at the National Institute on Aging (NIA) primate facility.

Species	Age	Group	Study	Treatment	n
S. sciureus	15	Adult	30 day	Con	1
	15	Adult		DSP-4	2
	11	Adult	9 month	Con	4
	20	Aged		Con	2
	11	Adult		DSP-4	4*
	19-20	Aged		DSP-4	3
M. mulatta	16	Adult	30 day	Con	1
	15	Adult		DSP-4	2
	14-17	Adult	9 month	Con	5
	19-25	Aged		Con	3
	14-17	Adult		DSP-4	6
	19-25	Aged		DSP-4	4*

*One adult squirrel and one aged rhesus animal died before the end of study

Experimental Design

Previous work with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) in several other species consistently reported norepinephrine depletion with a dose of 50 mg/kg (Heneka, Ramanathan et al. 2006; Waterman and Harding 2008). To confirm the utility and dosage of the DSP4 neurotoxin in non-human primates, 2 control animals received vehicle and 4 adults received a single injection of DSP4 *(*50 mg/kg); all animals were necropsied 30 days post injection.

In the long term study (Figure 2.1), 4 adult and 3 aged squirrel monkeys received three injections of DSP-4 spaced 3 months apart (n=7), along with a control group receiving saline vehicle (n=6, Table 2.1). To minimize transient peripheral side effects the dose used in the long term study was lowered to 40 mg/kg for the initial dose and 10 mg/kg for subsequent dosing. Six adult and four aged rhesus monkeys received three injections of DSP4 spaced 3 months apart (n=10), along with

a control group receiving saline vehicle (n=8, Table 1). To prevent incidental serotonergic depletion each animal received an intraperitoneal (i.p.) injection of Zimelidine (Sigma: 10 mg/kg). The initial DSP-4 dose (Sigma: 40 mg/kg, i.p.) was administered 45 minutes later. Subsequent injections of Zimelidine followed by DSP-4 (10 mg/kg, i.p.) or vehicle were given at the 3 and 6 month time points. At each time point (baseline, 3, 6 and 9 months), before neurotoxin injection, blood was collected for serum analysis. In the rhesus monkeys baseline behavioral testing was done prior to injections and then 1 month following each subsequent injection. Three months after the final injections animals were necropsied.

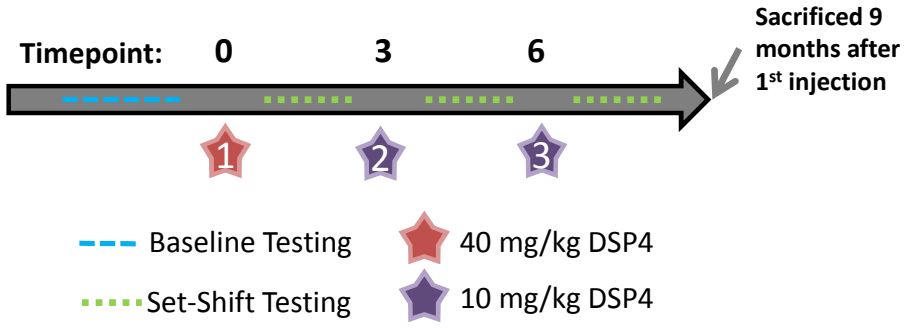

Figure 2.1: Long-term Study Design Squirrel and Rhesus monkeys were injected with DSP4 3 times over 9 months. Behavioral testing (baseline and set-shift) was conducted with the rhesus monkeys 1 month following injections.

Tissue Collection

Animals were restrained with ketamine and both blood and cerebrospinal fluid were collected. Animals were deeply anesthetized using B-euthanasia-D (80 mg/kg, IV) and then perfused transcardially with cold 0.9% saline. Brain tissue was prepared for biochemical and immunohistochemical assays. Each brain was divided along the medial longitudinal fissure and blocked in 1 cm increments using an adult monkey coronal brain matrix with 2mm slots. The right half was immediately frozen in

isopentane and stored at -80°C for biochemical analyses; while the other hemisphere was immersion-fixed in 4% paraformaldehyde for 48 hrs, placed through a series of graded sucrose solutions until blocks sank in 30% sucrose and then frozen at -80°C.

Enzyme Linked Immuno-Sorbet Assays (ELISAs)

Tissue punches from specific brain regions were taken from fresh frozen tissue blocks for analysis of catecholamines and amyloid levels. Punches for catecholamine measurements were homogenized (Fisher Scientific PowerGen125) in 0.1N HCL, centrifuged (15k G) for 15 minutes at 4°C, and the supernatant was collected for ELISA. Norepinephrine and dopamine were measured in prefrontal cortex (PF-C), caudate striatum (CS), hippocampus (HC), cingulate (CING-C), temporal cortex (TEM-C) and locus coeruleus (LC) using an ELISA specific to each catecholamine (Rocky Mountain Diagnostics, Inc. Colorado Springs CO) and run according to the manufacturer's instructions. All samples were run in duplicate. Absorbance values were determined using a microplate reader (BioRad 480) with a 450nm filter. The average optical density values for each region were interpolated on 4-PL standard curves to determine catecholamine concentrations. Catecholamine levels were standardized by sample protein levels measured using the BCA method (Pierce-Thermo Scientific, Rockford IL).

Additional punches from prefrontal and temporal cortex were homogenized in 5 volumes of homogenization buffer (50mM Tris-HCl, 150mM NaCl, with protease inhibitor cocktail) and then centrifuged at 100k g for 60 minutes at 4°C to generate a soluble fraction supernatant. Amyloid-β40 and amyloid-β42 were measured in each region by ELISA (Invitrogen, Camarillo CA) according to the manufacturer's

instructions. Samples were run in duplicate. Absorbance values were determined using a microplate reader and a 540nm filter. The average optical density values for each region were interpolated on 4-PL standard curves to determine catecholamine concentrations. Levels were standardized for each sample according to protein content.

Immunohistochemistry (IHC)

Fixed blocks were sectioned (50µm) on a freezing stage sliding microtome and placed into cryopreservation buffer for storage at -20°C until immuno-histo-chemical (IHC) staining. Serial sections were collected from blocks throughout the brain to examine frontal cortex, hippocampus, parietal, entorhinal and temporal cortex. Regions of interest were identified based on landmarks from a rhesus monkey stereotaxic atlas (Paxinos and Watson, 2009). Subsets were stained with the following antibodies: Anti-Aβ 6E10 (1:1000, Covance, Emeryville, CA), Anti-DBH (1:1000 Millipore, Temecula CA) and Anti-Tau HT7 (1:500 Thermo Scientific, Rockford, IL).

Stained sections were mounted onto PLUS slides and allowed to air dry for 3 days. Slides were counter stained with cresyl violet and/or congo red, dehydrated, and then cover-slipped using permanent mounting medium. Slides dried for 1 week and were analyzed for amyloid subtypes in neocortex and norepinephrine containing cell bodies located in the locus coeruleus.

Images were quantified using ImageJ (NIH) to analyze specific staining patterns for each antibody. Thresholding was adjusted to isolate positively stained regions from background to determine % area stained within the region of interest

(Tynan, Naicker et al.). The locus coeruleus region was imaged using a 4x objective; the %area was calculated and averaged on 2-3 serial sections per animal spaced 100μm apart in the rhesus monkey and 50μm in the squirrel monkey. Squirrel monkey 6E10-IR was determined on 2 serial sections spaced 500μm apart from each animal; within each section three non-overlapping adjacent 10x images from each region of interest and %area stained was calculated. Due to the rarity of 6E10-IR in rhesus monkeys under the age of 25 (Uno, Alsum et al. 1996) each observation of amyloid was counted using a 10x objective on two serial sections from frontal, entorhinal/temporal, and parietal regions. Within each region instances of amyloid were summed for a regional count and then total neocortical load was determined by summing the counts from each region for each treatment group.

Behavioral Measurements

The rhesus monkeys used in this study were behaviorally naïve and first trained to respond/use a novel touchscreen apparatus. The apparatus consisted of a touch sensitive computer screen and food pellet dispenser which attached to the monkey's home cage during the testing session and was controlled by a computer. This pre-training (acclimation) task required the monkey to touch a single stimulus which appeared in a random location on the screen to receive the reward during each trial. Rewards consisted of fruit flavored pellets. Acclimation was continued until the monkey responded to 20 consecutive trials in one testing session.

Animals that responded and reached criterion passed on to a simplified two-choice conceptual set shifting task (CSST) to assess executive function and the ability to shift their response strategy to receive the food reward. Animals were tested at 4

time points throughout the study (Figure 2.1): Baseline testing prior to the first injection of DSP4, 1 month after the high dose injection, after the second dose, and prior to necropsy. The CSST task is divided into discrimination (OD) and reversal (RV) phases, monkeys responded by touching one of two objects presented on the screen: a green star or red star. The location of both objects on the touchscreen varied with each trial and the maximum number of trials within a testing session was 60. During baseline OD, monkeys were trained to select the green star and the criterion for passing into the RV phase was 90% positive selections over 3 consecutive sessions. Correct selection resulted in delivery of a fruit pellet. After reaching criterion the animal switched to the RV phase and was rewarded for selecting the red triangle, the phase was continued until the individual reached a criterion of 90% positive selections over 3 consecutive days. During each subsequent round of testing, beginning 30 days after injection of DSP4, the OD and RV stimuli were reversed (Table 2.2). Total trials per session and errors were recorded during each round of testing.

Table 2.2: Behavioral Testing Paradigm. Alternating stimulus choice for each round of object discrimination (OD) and reversal (RV) testing.

Round	Phase	OD (S+)	RV (S+)
B	Baseline	Green Star	Red Triangle
1	DSP4; 40 mg/kg	Red Triangle	Green Star
2	DSP4; 10 mg/kg	Green Star	Red Triangle
3	DSP4; 10 mg/kg	Red Triangle	Green Star

Statistics

Data were analyzed by one-way ANOVA and directional contrasts were used to determine significant differences. The specific a priori hypotheses tested in this study were the following: (h1) DSP4 will exert deleterious effects in adult animals

compared to age matched animals injected with vehicle (e.g. adult animals injected with DSP4 will have significantly depleted norepinephrine); (h2) aged animals injected with DSP4 will exhibit increased pathology compared to age-matched controls; (h3) aged animals would show significantly more pathology than adults. The number of planned comparisons for each dependent measure was restricted (k−1) to test the three above hypotheses in order to minimize family wise type I error. Behavioral data were analyzed by repeated measures MANOVA.

Results

DSP4 impairment of the catecholamine system

Female adult and aged monkeys were treated with DSP4 3 times over 9 months. Levels of norepinephrine and dopamine were measured in locus coeruleus (Figure 2.2) along with cortical and subcortical regions (Figure 2.3, Figure 2.4) to assess alterations in tissue content in the treatment groups. In squirrel monkeys DSP4 reduced norepinephrine in the locus coeruleus of adults ($p=0.011$). Aged squirrel monkeys injected with DSP4 did not differ from aged controls ($p=0.242$). Although there is a trend for age-related decline of norepinephrine, the decrease was not statistically significant ($p=0.069$, Figure 2.2A). In rhesus monkeys, DSP4 depleted norepinephrine in the locus coeruleus of adults ($p= 0.008$). Age alone did not reduce norepinephrine, adult and aged rhesus controls had similar levels of norepinephrine ($p=0.373$). In aged rhesus DSP4 decreased norepinephrine ($p=0.037$) in the locus coeruleus (Figure 2.2B).

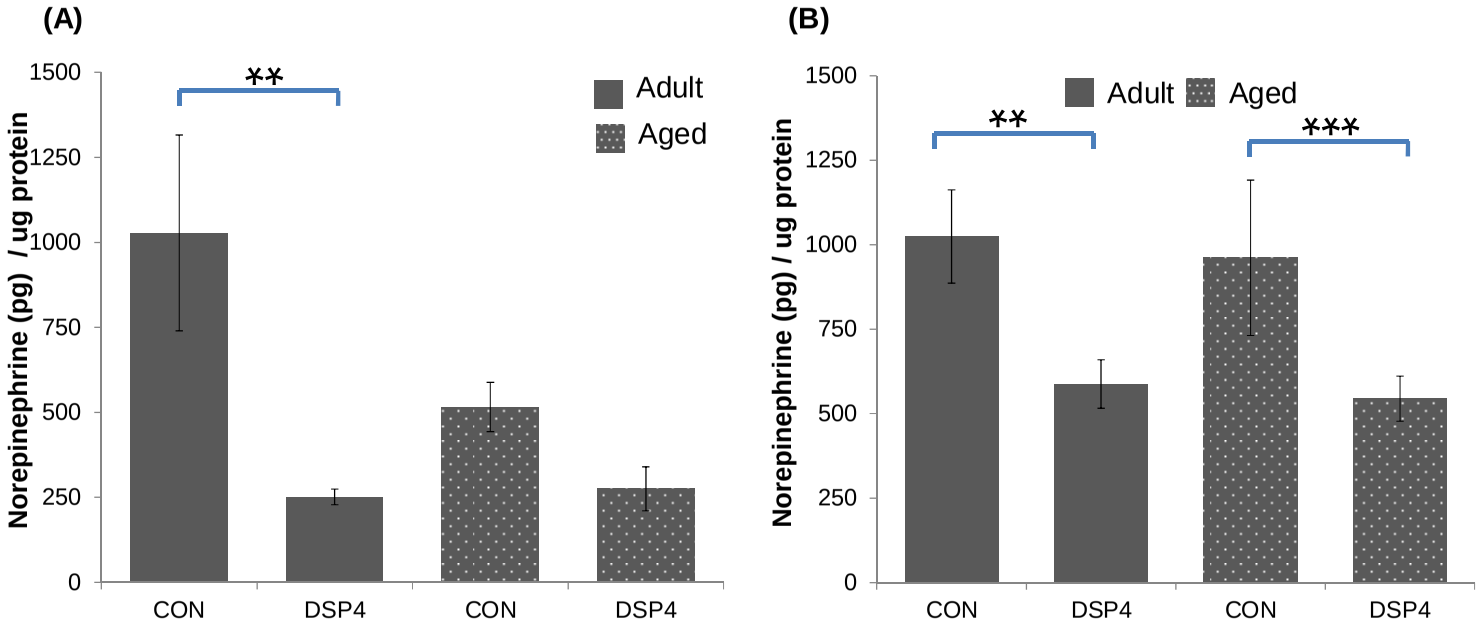

Figure 2.2: Norepinephrine in Locus Coeruleus. Adult and aged squirrel monkeys (A) and rhesus monkeys (B) received 3 injections of DSP4 or vehicle (CON) over 9 months. Norepinephrine decreased following DSP4 injection in adult squirrel and rhesus monkeys, significant depletion was also detected in aged rhesus monkeys after DSP4. Norepinephrine tissue content measured in brain punches collected after necropsy by ELISA and normalized to sample protein levels. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

Mean norepinephrine values in cortical projection areas were slightly lower following DSP4 (Figure 2.3). In adult squirrel monkeys (A) DSP4 reduced norepinephrine in the prefrontal cortex ($p=0.041$); whereas norepinephrine in rhesus monkey (B) temporal cortex was lower in aged controls compared to adults ($p=0.046$). Mean norepinephrine content in remaining regions was not decreased when compared to controls in rhesus and squirrel monkeys ($p > 0.10$) following DSP4; with similar norepinephrine concentrations in remaining projection regions for adult and aged animals ($p > 0.10$).

Subcortical projection (hippocampus) and non-projection (striatum) regions were evaluated for changes in norepinephrine content (Figure 2.4). In the hippocampus variable results were noted across individuals; decreases were not detected in squirrel (A, $p > 0.10$) or rhesus females (B, $p > 0.10$) following DSP4 or with increased age. In striatum, norepinephrine was elevated in adult squirrel monkeys after DSP4 (Figure 2.3A, $p=0.010$). No age-related alteration or change in aged animals following DSP4 ($p > 0.10$) or any rhesus monkey groups ($p > 0.10$) was detected.

In squirrel monkeys DSP4 reduced mean dopamine in the striatum of adults (Table 2.3, $p=0.02$). Remaining comparison between aged squirrel monkeys injected with DSP4 was not different nor was an age-related change detected in striatum ($p > 0.10$). Dopamine was not significantly altered in prefrontal cortex, hippocampus, or the locus coeruleus of squirrel monkeys ($p > 0.10$) and no group differences were detected in rhesus monkeys ($p > 0.10$).

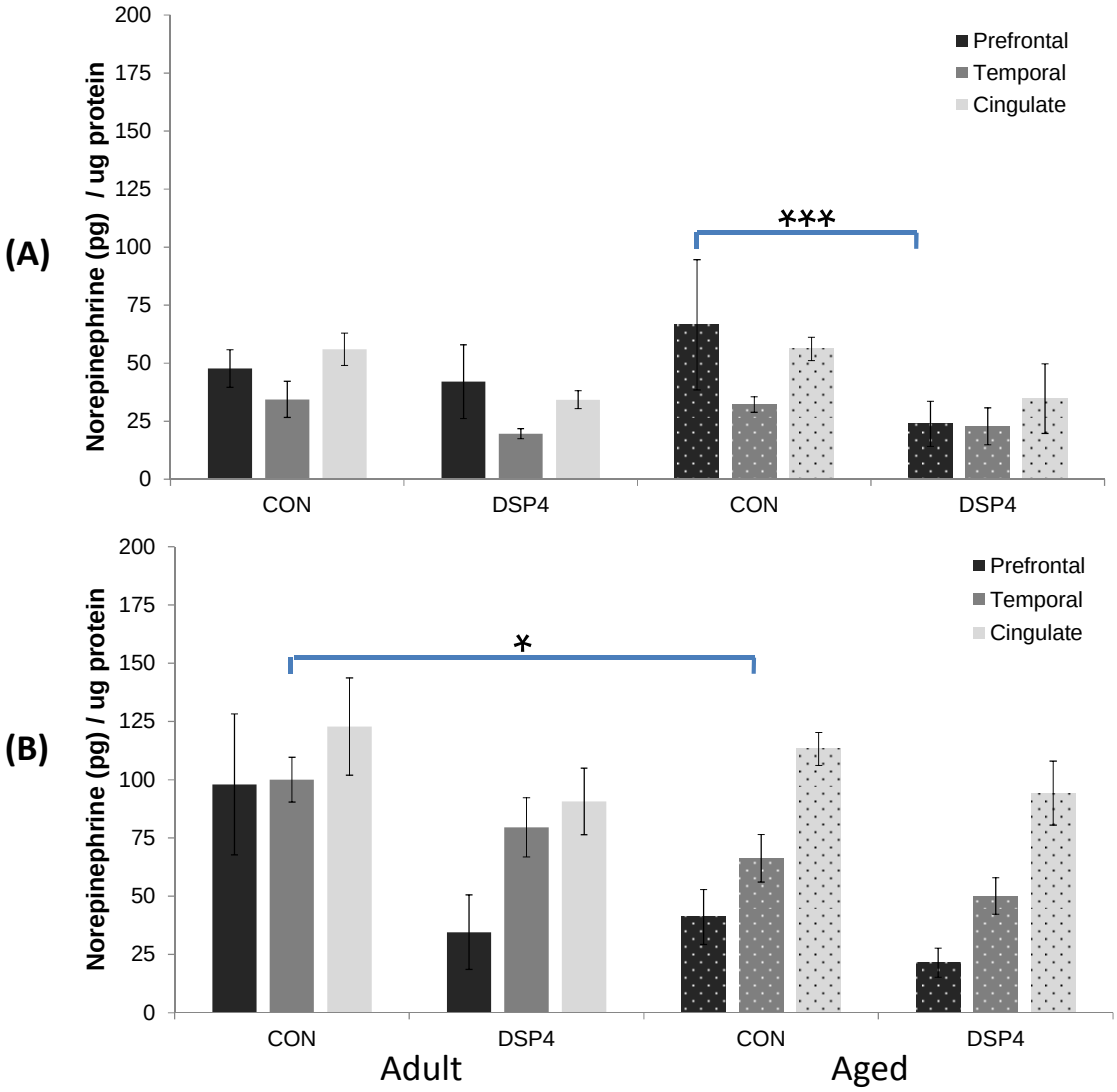

Figure 2.3: Norepinephrine in cortical projection regions. Adult and aged squirrel monkeys (A) and rhesus monkeys (B) received 3 injections of DSP4 or vehicle (CON) over 9 months. Prefrontal cortex was most sensitive to DSP4 and an age-related decrease was detected in temporal cortex of rhesus monkeys. Norepinephrine tissue content measured in brain punches collected after necropsy by ELISA and normalized to sample protein levels. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

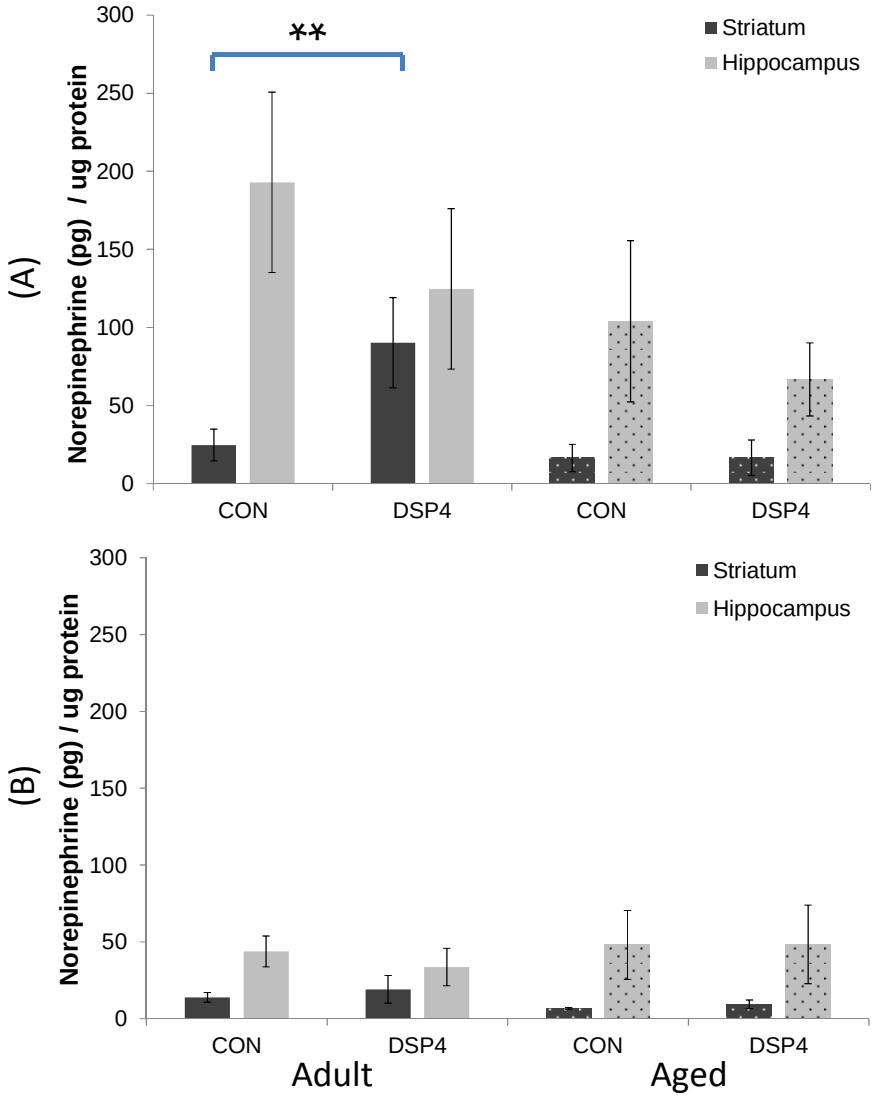

Figure 2.4: Norepinephrine in subcortical projection and non-projection region. Adult and aged squirrel monkeys (A) and rhesus monkeys (B) received 3 injections of DSP4 or vehicle over 9 months. Variable response was detected in hippocampus of each species while an increase of norepinephrine was detected in the striatum of adult squirrel monkeys. Norepinephrine tissue content measured in brain punches collected after necropsy by ELISA and normalized to sample protein levels. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

Table 2.3: Dopamine in locus coeruleus, projection and non-projection regions. Adult and aged squirrel monkeys (A) and rhesus monkeys (B) received 3 injections of DSP4 or vehicle (CON) over 9 months. Dopamine level was highest in striatum and a decrease was detected in adult squirrel monkeys after DSP4 injection. Dopamine tissue content was measured in brain punches collected after necropsy by ELISA and normalized to sample protein levels. DSP4 = 40 mg/kg, CON = saline vehicle, * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

(A) Squirrel Monkey					(B) Rhesus Monkey				
Dopamine (DA) pg/ ug protein					Dopamine (DA) pg/ ug protein				
Prefrontal	**Mean**		**SEM**	**% depletion**	**Prefrontal**	**Mean**		**SEM**	**% depletion**
Adult Con	9.51	±	4.38		Adult Con	23.12	±	3.22	
Adult DSP4	19.05	±	13.01	-100.44	Adult DSP4	24.54	±	5.87	-6.14
Aged Con	17.96	±	2.43	-88.94	Aged Con	18.68	±	6.10	19.20
Aged DSP4	12.44	±	5.10	30.71	Aged DSP4	15.42	±	2.88	17.45
Striatum					**Striatum**				
Adult Con	632.05	±	134.94		Adult Con	780.94	±	159.42	
****Adult DSP4**	**31.85**	**±**	**14.30**	**94.96**	Adult DSP4	738.11	±	148.59	5.48
Aged Con	413.01	±	113.16	34.66	Aged Con	753.60	±	162.82	3.50
Aged DSP4	447.38	±	121.19	-8.32	Aged DSP4	741.00	±	173.77	1.67
Hippocampus					**Hippocampus**				
Adult Con	13.03	±	3.99		Adult Con	10.42	±	4.22	
Adult DSP4	5.71	±	0.56	56.20	Adult DSP4	9.97	±	2.68	4.32
Aged Con	5.49	±	0.81	57.89	Aged Con	11.62	±	3.28	-11.52
Aged DSP4	15.17	±	9.21	-176.40	Aged DSP4	8.27	±	4.25	28.83
Locus Coeruleus					**Locus Coeruleus**				
Adult Con	16.20	±	8.96		Adult Con	21.06	±	8.10	
Adult DSP4	69.95	±	54.19	-331.84	Adult DSP4	55.50	±	28.22	-163.53
Aged Con	7.50	±	2.40	53.67	Aged Con	15.88	±	7.64	24.60
Aged DSP4	96.77	±	90.87	-1189.69	Aged DSP4	61.76	±	20.56	-288.92

Serial brain sections were collected and analyzed for dopamine β-hydroxylase immunoreactive neurons (Figure 2.5) approximately midway along the rostro-caudal distance of the locus coeruleus where the cell density is high and significant cell loss has been documented in AD patients (Marien, Colpaert et al. 2004). DSP4 reduced % area stained in aged rhesus monkeys (D, $p=0.040$); however there was no reduction due to DSP4 in adult groups ($p > 0.10$) or a significant reduction due to age alone ($p > 0.10$).

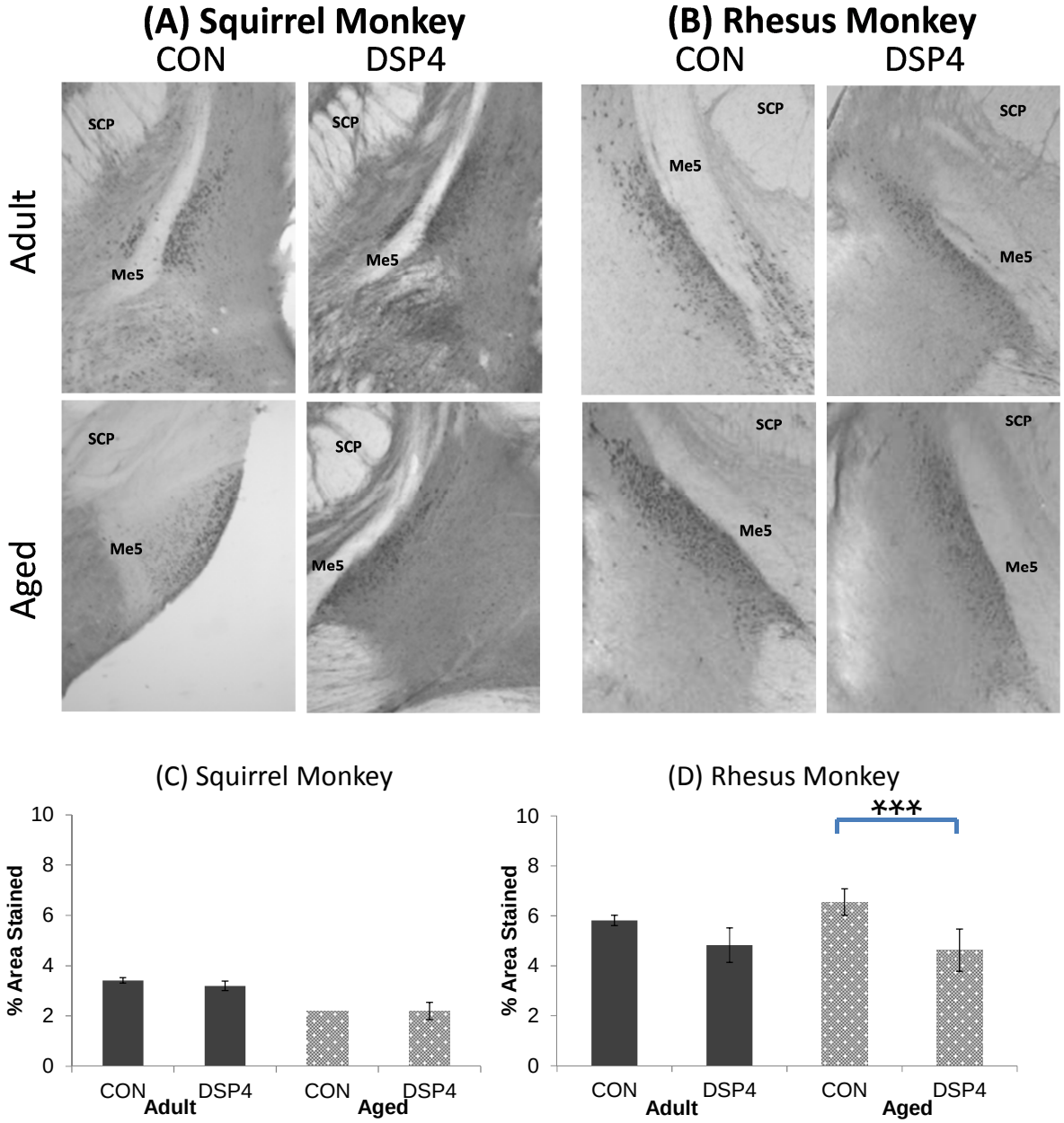

Figure 2.5: Norepinephrine neurons in the locus coeruleus Photomicrograph through the locus coeruleus identifying dopamine β-hydroxylase neurons from the squirrel monkey (A) and rhesus monkey (B). Semi-quantitative determination of %area stained in serial sections through locus coeruleus of squirrel monkey (C,D). No difference in the squirrel monkey (C) and reduction in aged rhesus monkeys (D). DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

DSP4 influence on Alzheimer's-like pathology

Serial brain sections were collected and analyzed for extracellular deposits of amyloid in frontal and temporal cortex along with hippocampus using 6E10 immunostaining and congo red histological staining in adult and aged non-human primates. An increase in % area stained for 6E10-immunostaining was detected due to aging in squirrel monkeys (Figure 2.6B, $p=0.012$); a trend for further increase due to DSP4 was noted however the effect was not statistically significant ($p=0.059$) for area stained. Diffuse and compact morphology was observed in squirrel monkeys with pathology, with more pathology in deep layers of cortex and then extending into superficial layers in individuals with higher amounts of staining. No pathology was observed in hippocampus or other subcortical regions.

Amyloid staining identified by 6E10-IR was infrequent and isolated in rhesus monkeys; furthermore it was present only in the aged groups. Total amyloid counts were significantly elevated with age ($p=0.0001$) and following DSP4 ($p=0.0001$, Figure 2.7B). Diffuse and compact staining was observed in neocortex and preferentially localized to deep layers in aged rhesus monkeys. Subcortical areas were devoid of amyloid and all animals were congo red negative (Table 2.4) and vascular deposition nearly non-existent across individuals.

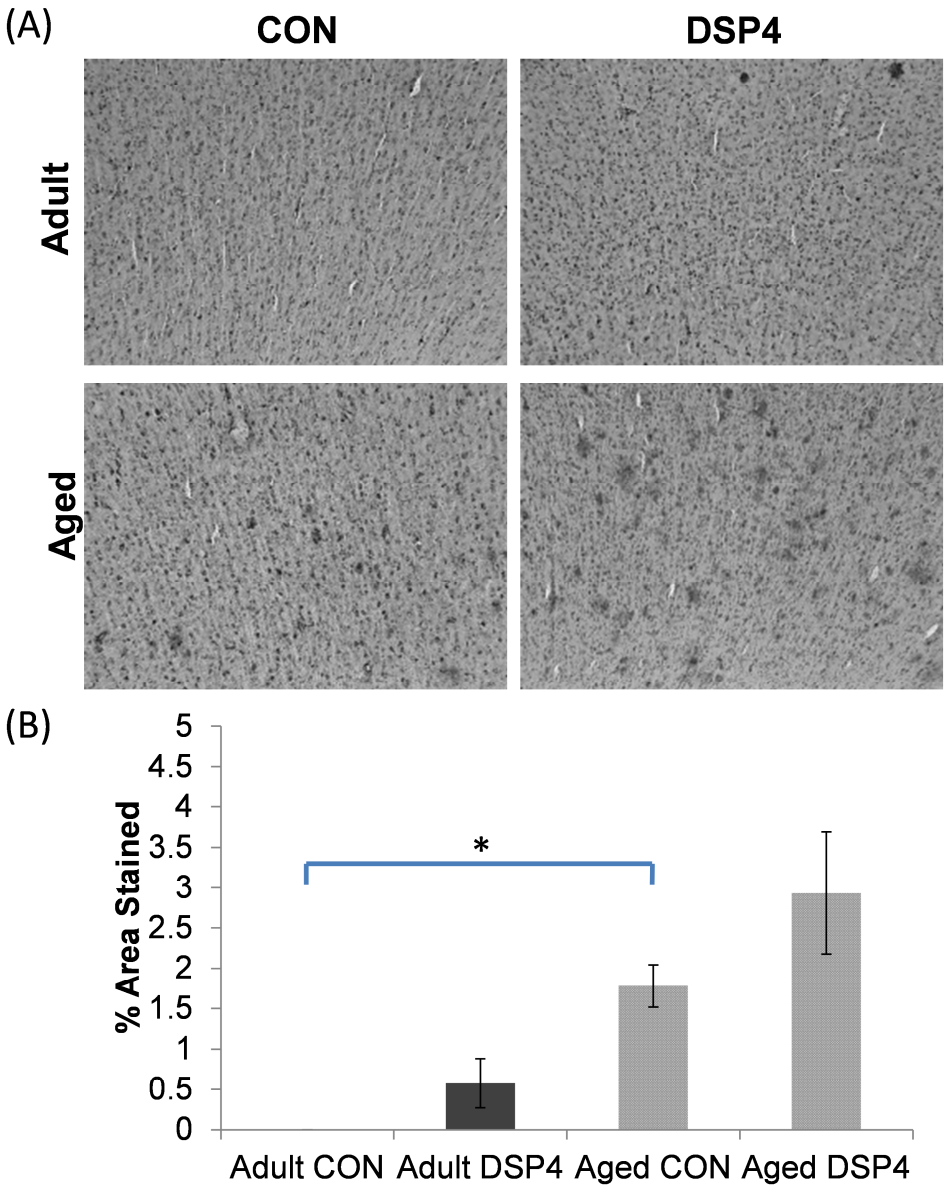

Figure 2.6: Diffuse and compact amyloid pathology in squirrel monkeys. Photomicrographs of 6E10-immunostaining in representative areas of frontal cortex from squirrel monkey groups (A) and an age-related increase in % area (B). DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

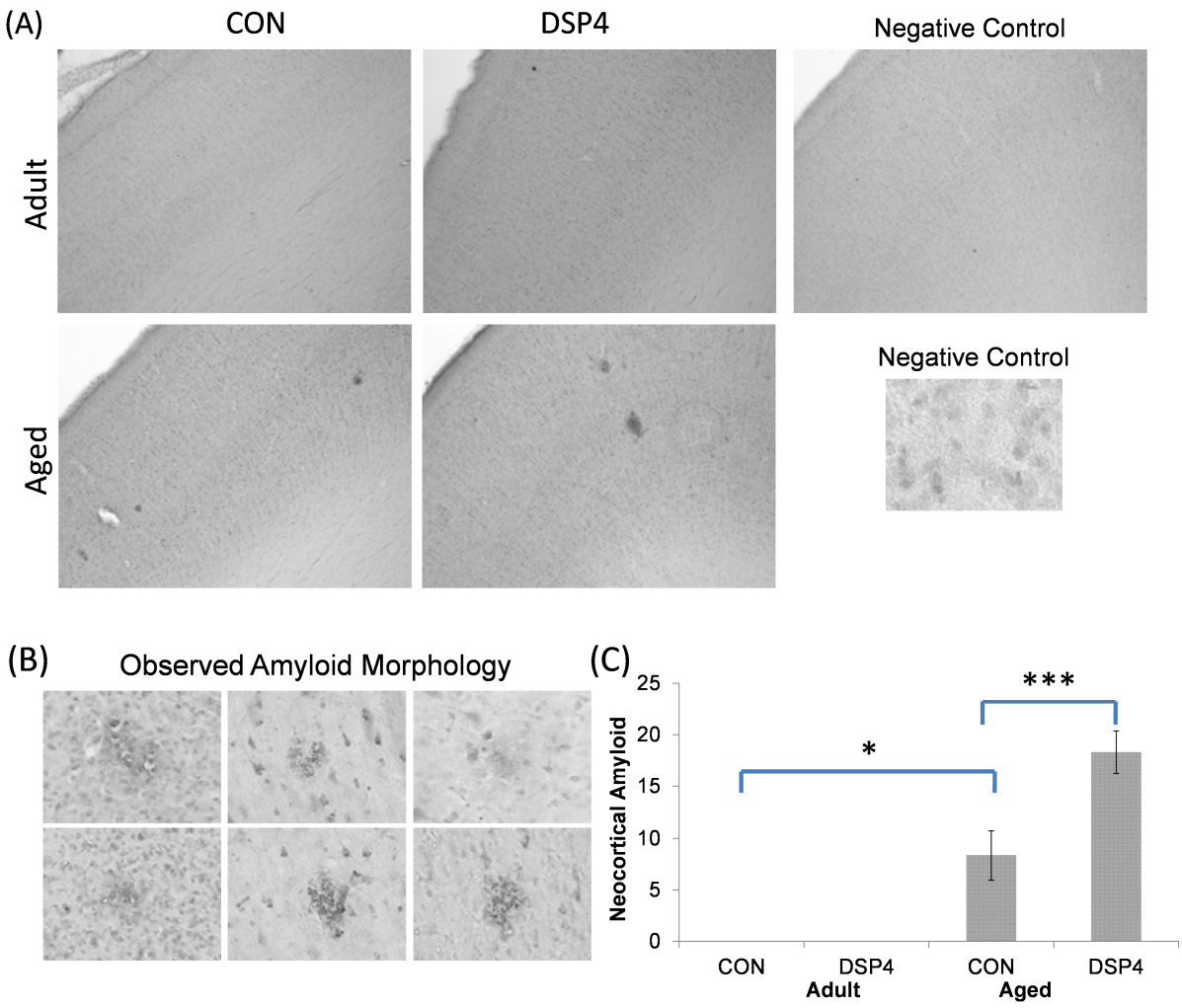

Figure 2.7: Observed amyloid pathology in rhesus monkeys. Photomicrographs of 6E10-IR in representative areas of frontal cortex of the rhesus monkey (A), observed amyloid morphology (B) and % area stained for each treatment group (C). DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

Table 2.3: Norepinephrine depletion modulates Aβ deposition in rhesus monkeys. Regional distribution of amyloid deposition varied by individual. Instances of neocortical amyloid in aged animals was counted on serial sections and increased following DSP4 in cortical LC projection regions. Extracellular 6E10-immunoreactivty from each region was summed to determine the total neocortex score.

Amyloid Distribution										
ID		*Brain Region*								
		Frontal		Entorhinal / Temporal		Parietal		Hippocampus		Neocortex
Group	Trt	6E10	CR	6E10	CR	6E10	CR	6E10	CR	Total
Adult	Con	No pathology observed.								0
Adult	Con									0
Adult	Con									0
Adult	Con									0
Adult	Con									0
Adult	DSP4									0
Adult	DSP4									0
Adult	DSP4									0
Adult	DSP4									0
Adult	DSP4									0
Adult	DSP4									0
Aged	Con	1	0	3	0	3	0	0	0	7
Aged	Con	0	0	2	0	3	0	0	0	5
Aged	Con	10	0	0	0	3	0	0	0	13
Aged	DSP4	7	0	8	0	7	0	0	0	22
Aged	DSP4	8	0	2	0	8	0	0	0	18
Aged	DSP4	2	0	11	0	2	0	0	0	15

Tissue punches from prefrontal and temporal cortex were prepared for soluble amyloid-β40 and amyloid-β42 ELISAs to determine the ratio of isoforms in each cortical region. The ratio of soluble Aβ42:Aβ40 increased in aged squirrel and rhesus monkeys after DSP4 animals (Figure 9). The response was highly variable in PF-C ($p > 0.10$) and significantly elevated in TEM-C ($p = 0.003$) from squirrel monkeys (Figure 9A). In aged rhesus monkeys, the ratio was significantly elevated in both prefrontal ($p = 0.025$) and temporal cortex ($p = 0.028$). In adult squirrel and rhesus monkeys the ratio was not elevated after DSP4 ($p > 0.10$). A trend for an age-related increase was noted in squirrel monkeys ($p = 0.054$), however no difference between adult and aged rhesus monkeys was measured ($p > 0.10$).

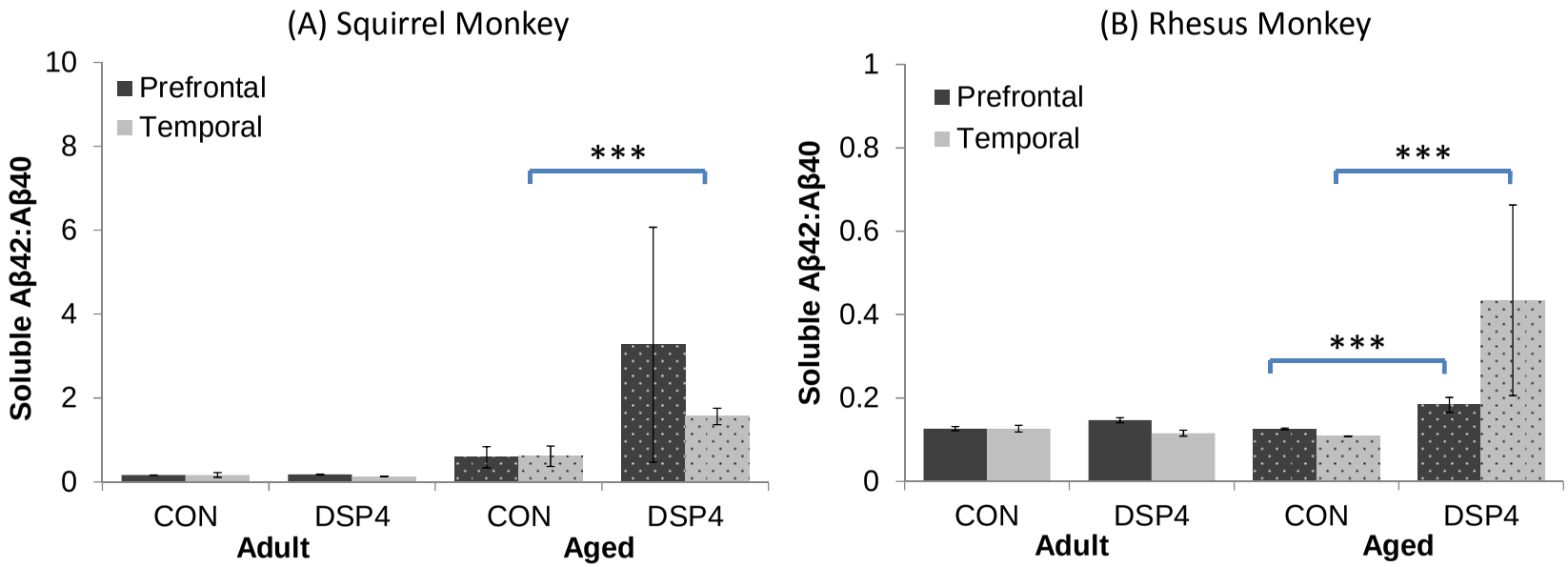

Figure 2.8: Ratio of soluble amyloid isoforms. Shift toward more pathological ratio of soluble amyloid isoforms in squirrel (A) and rhesus monkey (B) cortex. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$)

Conceptual Set Shift Testing

Analysis of behavioral testing data using repeated measures revealed no significant interaction between testing round and group for OD-errors ($p > 0.10$) and RV-errors ($p > 0.10$); although a significant effect of time for OD-errors ($p=0.003$) but not RV-errors ($p > 0.10$) was detected.

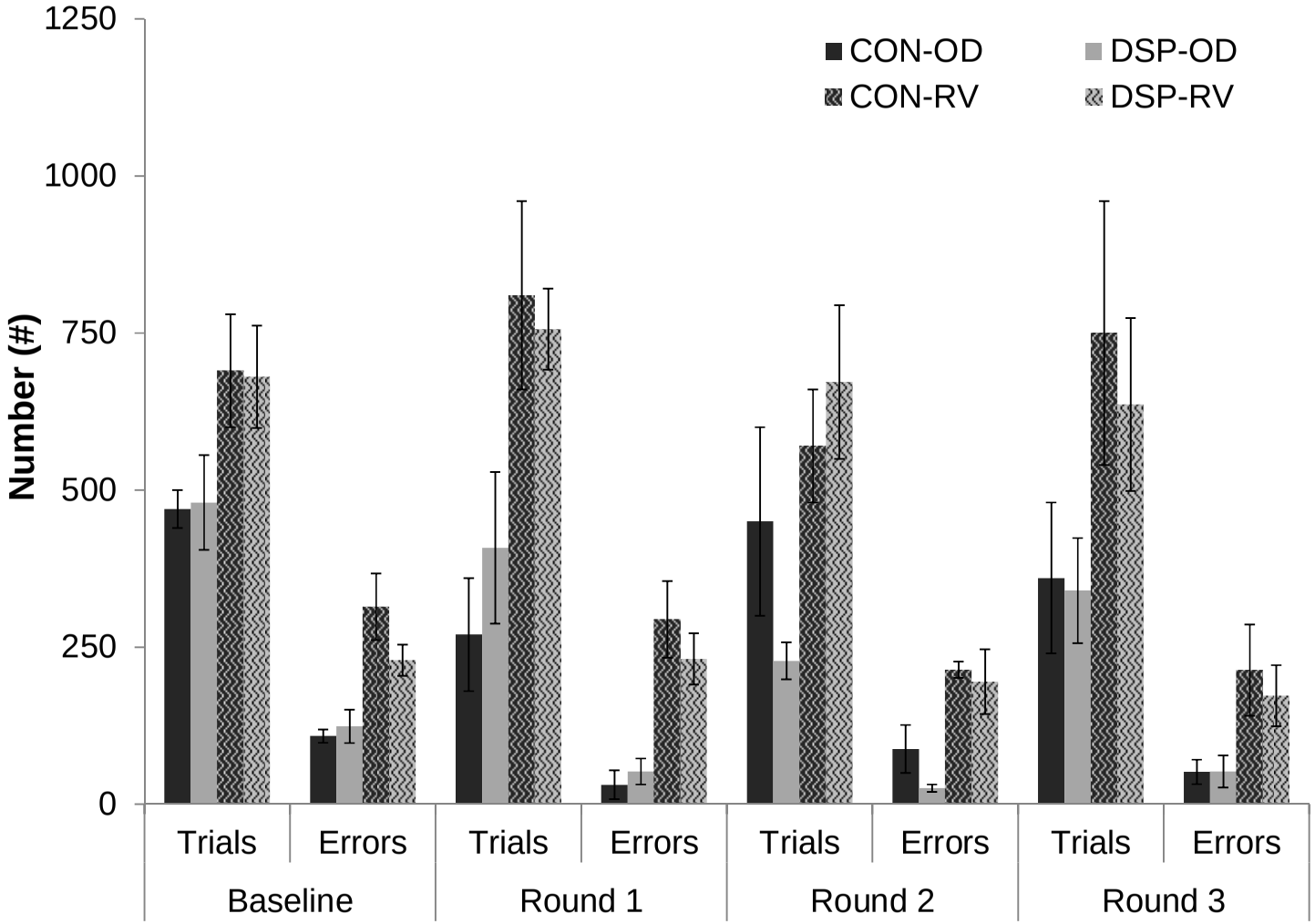

Figure 2.9: Object Discrimination and Reversal Testing in Rhesus Monkeys. A simplified conceptual set shifting task was utilized to test cognitive flexibility in rhesus monkeys one month following DSP4 injections. Total # of trials and # of errors during each testing phase were recorded.

Discussion

In the current study, increased age and long-term norepinephrine depletion in the locus coeruleus with DSP4 increased amyloid-β pathology in cortical regions of non-human primates. Nearly twice as much amyloid was observed in depleted aged animals of both species. While previous studies with transgenic AD-animals and post-mortem examination of AD-human brains show significant loss of norepinephrine neurons in the locus coeruleus (Zarow, Lyness et al. 2003; O'Neil, Mouton et al. 2007) a reduction with age was not observed in either non-human primate species examined in this study.

Conflicting reports on the degree and duration of norepinephrine depletion due to DSP4 are interwoven with dosing paradigm. Differences between studies include the amount injected, number of injections provided and timeframe. Early experiments also demonstrated that sensitivity often differed with strain and species (Jaim-Etcheverry and Zieher 1980; Jonsson, Hallman et al. 1981; Grzanna, Berger et al. 1989; Fornai, Bassi et al. 1996). In contrast to previous experiments with DSP4 in AD transgenic mice which most often utilized multiple high dose injections; the paradigm used in the current study did not produce a permanent or long-lasting lesion in adult monkeys although the long-term reduction of norepinephrine suggests that locus coeruleus function was impaired by DSP4. These results also support the primary action of DSP4 was not restricted to locus coeruleus terminals and that direct influence of DSP4 also occurred at locus coeruleus cell bodies through action at norepinephrine transporters (Ordway, Stockmeier et al. 1997; Sanders, Happe et al. 2005). Equivalent levels of norepinephrine in cortical projection regions coupled

with reduced norepinephrine content in the locus coeruleus support the notion that increased activity could compensate for a subset of impaired neurons (Szot, White et al. 2006); although locus coeruleus dysfunction still permits increased amyloid deposition. A novel finding in this study, specific to the adult squirrel monkeys, was elevated norepinephrine and decreased DA in striatum following DSP4. This result was indicative of further adaptive changes within the monoaminergic system; however the mechanism or benefit remains unclear. Non-coeruleun noradrenergic projections may contribute to the increase, although the corresponding decrease of DA within the local environment suggests a role for increased dopamine β-hydroxylase activity.

Species comparison of dopamine β-hydroxylase immunoreactivity indicated that the rhesus monkey locus coeruleus was approximately twice the size of the squirrel monkey locus coeruleus. This was not surprising given the difference in brain size and trends noted in other primate species regarding neuronal number in the locus coeruleus (Sharma, Xu et al. 2010). Interpretation of squirrel monkey dopamine β-hydroxylase immunoreactivity was hampered by low sample size and for this endpoint only 1 aged control was measured due to improper hemi-section during tissue processing. However, the individual measured had lower localization of dopamine β-hydroxylase when compared to the adult group and considering the age-related loss of norepinephrine in aged squirrel monkeys it appeared feasible that neuronal loss might contribute to the reduction. In the rhesus monkey, slight reduction of dopamine β-hydroxylase detected at necropsy in adults following DSP4 indicated a capacity for recovery. While not the focus of the early experiments,

moderate recovery within weeks to months following single injections of DSP4 has been reported in young rodents (Jonsson, Hallman et al. 1981; Fornai, Bassi et al. 1996); Szot et al. (2010) report restored norepinephrine tissue content in locus coeruleus and projection regions 3 months following a single injection of DSP4 in Spraque-Dawley rats. The long-term reduction of dopamine β-hydroxylase immunoreactivity and decreased norepinephrine after DSP4 in aged rhesus monkeys demonstrated that they may be more sensitive to long-term effects of DSP4 and thus less able to recover after exposure. In that regard the results discussed here are striking, considering the parameters of DSP4 dosing and 3 month period between injections. The long term decrease of norepinephrine in the locus coeruleus after DSP4 in both adult and aged groups contributed to increased amyloid in adult and aged squirrel monkeys along with aged rhesus monkeys.

Similar to humans, an age-related increase of amyloid occurred in both squirrel and rhesus monkey brain. Consistent with other reports of cerebral amyloid in non-human primates, diffuse and compact extracellular amyloid pathology in squirrel monkeys was observed in frontal and temporal cortex; rhesus monkey pathology was infrequent and isolated in cortex. In both species more amyloid was measured following DSP4 injection (Figure 2.6 and Figure 2.7) and these results demonstrated that norepinephrine depletion contributed to the deposition and accumulation of amyloid in the neocortex of aged rhesus monkeys. The earlier onset of pathology in adult squirrel monkeys suggests locus coeruleus impairment may also accelerate pathology. Locus coeruleus degeneration has been reported in cases of mild cognitive impairment and may serve as a pathological trigger in early stages of

AD (Grudzien, Shaw et al. 2007). A more pronounced effect of DSP4 on amyloid occurred in squirrel monkeys due to dense reciprocal fiber connections between the FC and locus coeruleus; compared to other regions the transient loss of norepinephrine exacerbated FC more extensively and squirrel monkeys were more sensitive due to their relatively older age compared to rhesus monkeys. Loss of norepinephrine also altered the ratio of soluble amyloid in cortical regions from aged animals, soluble monomers represent an earlier stage of pathology and results suggest DSP4 also influences the generation and aggregation of amyloid isoforms. Importantly, elevated levels of these monomers and oligomers correlate better to impaired cognition in AD patients and in animal models soluble oligomers impair synaptic function.

Executive functions decline in AD and the locus coeruleus regulates attention and decision making processes involved in executive function tasks mediated by prefrontal cortex (Chang, Jacobson et al. 2009). Studies with non-human primates have illustrated activation locus coeruleus neurons during behavioral tasks which require a change in attention or an alteration in the reinforcement paradigm during a testing session (Rajkowski, Majczynski et al. 2004). Analysis of behavioral data did not reveal significant differences in object discrimination or reversal endpoints. While animals improved over time, total number of trials and errors for each testing phase were not different between treatment groups. Taking into account depletion data from the pilot study where levels of norepinephrine were affected to a greater degree at 30 days suggested increased activity in the locus coeruleus could be compensating during behavioral testing and allowing DSP4 animals to perform

adequately. Over the long term the DSP4 injection schedule may have been insufficient to cause long lasting cortical differences in norepinephrine, equivalent performance on this behavioral task is less surprising during round 2 and 3 given the lower dose of DSP4 used in the study and lack of permanent lesion. Additional factors such as anxiety and motivation also contribute to individual performance.

The goal of this study was to determine if DSP4 could induce and increase cerebral amyloid pathology in two species of non-human primates which develop amyloid deposits naturally. The results from both squirrel and rhesus monkeys support the experiments suggesting that locus coeruleus impairment plays a role in the development of AD. The data discussed here highlight the importance for considering dosing paradigm used to induce degeneration, in future studies it would be beneficial to use low dose chronic infusions of DSP4 to better mimic a gradual decline similar to what was done in Kalinin, Gavrilyuk et al. (2007) study with a transgenic mouse model or consider monthly injections to ensure a long-lasting lesion. Compensatory actions and recovery mechanisms are interesting mediators to overall dysfunction but require further experiments to elucidate their contribution in non-human primates.

Chapter 3: Altered amyloid precursor protein processing in non-human primates with impaired locus coeruleus function

Introduction

Degeneration in the locus coeruleus is a major pathological feature of Alzheimer's disease (AD); studies have demonstrated neuronal loss and dysfunction occur early in progression of pathogenesis (Mann, Lincoln et al. 1980; Bondareff, Mountjoy et al. 1982; Grudzien, Shaw et al. 2007). In efforts to explain the cognitive decline and pathological changes occurring with AD, the cholinergic system received much consideration with observations of neurochemical deficits in choline acetyltransferase and neuronal loss in basal forebrain resulting in presynaptic decline in acetylcholine contributing to memory problems (Bartus, Dean et al. 1982; Whitehouse, Price et al. 1982). The cholinergic hypothesis gained traction within the AD field, while concurrent observations regarding the locus coeruleus were generally overlooked. Interest in the locus coeruleus and the contribution of locus coeruleus projections to AD gained attention when Zarow and colleagues (2003) reported more cell loss in the locus coeruleus compared to basal forebrain of AD patients. Subsequent studies utilizing animal models provided further evidence for interactions between amyloid pathology and locus coeruleus impairment (Heneka, Ramanathan et al. 2006; Jardanhazi-Kurutz, Kummer et al. 2010; Lockrow, Boger et al. 2011).

Distribution of norepinephrine is regulated by the locus coeruleus, which is the main site of central nervous system synthesis. Cortical and subcortical regions receive noradrenergic input and utilize norepinephrine in a variety of receptor-mediated functions, including more classical neurotransmitter actions modulating

attentional behavior and extrasynaptic mediation of inflammatory responses from neurons and neuroglia (Feinstein, Heneka et al. 2002; Marien, Colpaert et al. 2004; Sara 2009). In the central nervous system, norepinephrine suppresses inflammatory gene expression and reduces inflammatory responses by suppression of cytokine production (Galea, Heneka et al. 2003; Madrigal, Feinstein et al. 2005; Heneka, Nadrigny et al. 2010) thus acting in a neuroprotective capacity. In addition to deposition of amyloid, AD transgenic mouse models with experimentally depleted norepinephrine exhibited increased neuroinflammatory markers including sustained microglia and astrocyte activation along with elevated pro-inflammatory cytokine release (Heneka, Ramanathan et al. 2006; Pugh, Vidgeon-Hart et al. 2007; Jardanhazi-Kurutz, Kummer et al. 2010).

The mechanisms responsible for increased AD pathology due to locus coeruleus degeneration are not clearly understood or established. Generation of amyloid-β occurs through processing of amyloid precursor protein and sequential cleavage by β- and γ- secretase (Hardy and Selkoe 2002). Levels of amyloid-β are regulated by differential activity in production, clearance and degradation pathways (Wang, Wang et al. 2010). Post-processing clearance of amyloid isoforms occurs through blood-brain barrier dynamics and multiple degradation mechanisms, including the protease neprilysin and microglial phagocytosis. Thus, an imbalance due to increased amyloid-β synthesis along with decreased clearance contributes to aggregation of amyloid and the pathological progression of AD (Miners, Baig et al. 2008). In AD transgenic mice, reduced neprilysin and elevated amyloid deposition was reported in animals after a DSP4 induced reduction in norepinephrine (Kalinin,

Gavrilyuk et al. 2007). Further, addition of norepinephrine in microglia culture effectively enhanced uptake and degradation of Aβ mediated through adrenergic receptor signaling (Kong, Ruan et al. ; Heneka, Nadrigny et al. 2010).

Locus coeruleus impairment and norepinephrine depletion could contribute to amyloid pathology from uncontrolled inflammation due to reduced anti-inflammatory regulation or directly alter amyloid precursor protein processing. Furthermore, because neuroglia far outnumber neurons in the central nervous system, resulting dysfunction in astrocytes and microglia could contribute significantly to development of pathology. The aim of the current study was to examine the role of norepinephrine depletion on elevated amyloid deposition in non-human primates. Non-human primates develop amyloid pathology naturally which was exacerbated by reduced norepinephrine in the locus coeruleus due to systemic injection of DSP4 (Chapter 2). Therefore this model provides the opportunity to examine pathways responsible for the elevation of pathology using non-transgenic animals, specifically the contribution of neuroinflammation and pathogenic changes in APP processing in conjunction with central depletion of norepinephrine were examined in two species of non-human primates.

Materials and Methods

Subjects

Pilot study: 3 female adult common squirrel monkeys (*Saimiri sciureus*, aged 15 years*)* and 3 female adult rhesus monkeys (*Macacca mulatta*, aged 15-16 yrs*)* were studied in an initial short-term study (Table 1). Long term study: 8 adult (11 years old) and 5 aged (19-20 years old) squirrel monkeys were studied. Additionally, 14 adult (14-17 years old) and 7 aged (19-25 years old) rhesus monkeys were studied (Table 1). All monkeys were female.

Animals were obtained from approved sources and maintained at the National Institute on Aging/NIH primate facility prior to the study. Animals were housed in standard non-human primate caging and kept on 12:12hr light cycle, had *ad libitum* access to water and were fed standard NIH diet twice daily approximating *ad libitum* levels. Animals were observed daily by trained observers, including checks on food consumption and well-being. Routine health monitoring, TB tests and blood collections were done quarterly.

Animal husbandry and all experimental procedures in the study complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were conducted under an approved protocol by the NIA Institutional Animal Care and Use Committee (IACUC). In addition, an IACUC protocol was also approved at the University of Maryland for all experiments utilizing non-human primate tissue and fluid samples.

Table 3.1: Animal Distribution and Treatment Groups. All animals were housed at the National Institute on Aging (NIA) primate facility.

Species	Age	Group	Study	Treatment	n
S. sciureus	15	Adult	30 day	Con	1
	15	Adult		DSP-4	2
	11	Adult	9 month	Con	4
	20	Aged		Con	2
	11	Adult		DSP-4	4*
	19-20	Aged		DSP-4	3
M. mulatta	16	Adult	30 day	Con	1
	15	Adult		DSP-4	2
	14-17	Adult	9 month	Con	5
	19-25	Aged		Con	3
	14-17	Adult		DSP-4	6
	19-25	Aged		DSP-4	4*

*One adult squirrel and one aged rhesus animal died before the end of study

Experimental Design

Previous work with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) in several other species consistently reported norepinephrine depletion with a dose of 50 mg/kg (Heneka, Ramanathan et al. 2006; Waterman and Harding 2008). A short-term pilot experiment was conducted to confirm the utility and dosage of the neurotoxin in non-human primates. Two adults served as controls and received an injection of saline vehicle; while 4 adults received an injection of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; 50 mg/kg). All animals were necropsied 30 days post injection (Table 3.1).

In the long-term study (Figure 3.1), 10 adult and 7 aged animals received 3 injections DSP-4 spaced 3 months apart (n=17); controls received the saline vehicle (n=14; Table 3.1). To minimize transient peripheral side effects the dose used in the long term study was lowered to 40 mg/kg for the initial dose and 10 mg/kg for

subsequent dosing. To prevent incidental serotonergic depletion each animal first received an intraperitoneal (i.p.) injection of Zimelidine (Sigma: 10 mg/kg). The initial DSP-4 dose (Sigma: 40 mg/kg, i.p.) was administered 45 minutes later. Subsequent injections of Zimelidine followed by DSP-4 (10 mg/kg, i.p.) or vehicle were administered at the 3 and 6 month time points. Blood was collected prior to each injection series. Three months after the final injections animals were necropsied

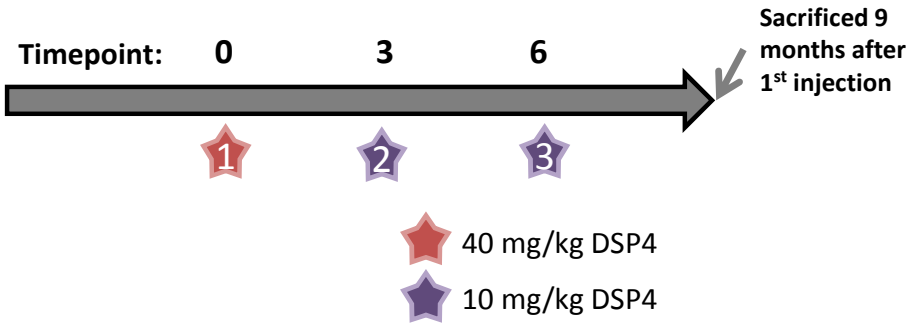

Figure 3.1: Long Term Study Design. Squirrel and Rhesus monkeys were injected 3 times with indicated dose of DSP4 over 9 months.

Tissue Collection

Animals were restrained with ketamine and both blood and cerebrospinal fluid were collected. Animals were deeply anesthetized using B-euthanasia-D (80 mg/kg, IV) and then perfused transcardially with cold 0.9% saline. Brain tissue was prepared for biochemical and immunohistochemical assays. Each brain was divided along the medial longitudinal fissure and blocked in 1 cm increments using an adult monkey coronal brain matrix with 2mm slots. The right half was immediately frozen in isopentane and stored at -80°C for biochemical analyses; while the other hemisphere

was immersion-fixed in 4% paraformaldehyde for 48 hrs, placed through a series of graded sucrose solutions until blocks sank in 30% sucrose and then frozen at -80°C.

Immunohistochemistry (IHC)

Fixed blocks were sectioned (50µm) on a freezing stage sliding microtome and placed into cryopreservation buffer for storage at -20°C until immuno-histo-chemical (IHC) staining. Serial sections were collected from blocks throughout the brain to examine frontal cortex, hippocampus, parietal, entorhinal and temporal cortex. Regions of interest were delineated based on landmarks from a rhesus monkey stereotaxic atlas (Paxinos and Watson, 2009). Subsets were stained with the following antibodies: Anti-GFAP Ab-4 (1:500, Thermo Scientific, Freemont CA), Anti-IBA1 (1:3000, Wako Chemicals, Richmond VA), Anti-MHCII HLA-DR Clone LN-3 (1:1000, Pierce, Rockford IL), Anti-CD10 (1:1000, Acris, San Diego CA)

Stained sections were mounted onto PLUS slides and allowed to air dry for 3 days. Slides were counter stained with 2% cresyl violet and/or 5% congo red solutions, dehydrated, and then cover-slipped using permanent mounting medium. Slides dried for 1 week to assess microglia and astrocyte morphology along with neprilysin distribution. Images were analyzed using ImageJ (NIH) to analyze specific staining patterns for each antibody. Thresholding was adjusted to isolate positively stained regions from background to determine % area stained. In cresyl violet co-stained tissue size criteria were added to exclude cresyl violet stained nuclei and to ensure % area reflected only staining for each primary antibody. Three non-overlapping adjacent 10x images from each region of interest were calculated on 2

serial sections spaced 500μm apart from each animal and averaged to obtain a semi-quantitative measure of % area stained.

Enzyme Linked Immuno Sorbet Assays (ELISAs)

Serum, cerebrospinal fluid and cerebral cortex brain homogenate samples were prepared and analyzed for inflammatory markers analysis. Brain punches were collected from fresh-frozen tissue blocks and homogenized (Fisher Scientific PowerGen125) in 0.1N HCL, centrifuged (15k G) for 15 minutes at 4°C, and the supernatant was collected for subsequent ELISA procedures. Monkey interleukin -6, rhesus monkey tumor necrosis factor-α, and human interleukin-1β (Invitrogen, Camarillo, CA) were assayed according to the manufacturer's instructions. All samples were run in duplicate. Absorbance values were measured by microplate reader (BioRad 480) with a 450nm filter. The average optical density values for each region were interpolated on 4-PL standard curves to determine levels of pro-inflammatory cytokines. Levels in brain homogenate were standardized by sample protein levels measured using the BCA method (Pierce-Thermo Scientific, Rockford IL).

Luminescence Assays

Baseline, 3 month, and end of study serum samples were analyzed for Caspase 3/7 and Caspase 9 activity according to the manufacturer's standard protocol (Promega, Madison, WI). Briefly, a 25 ul sample was mixed gently for 30s with 25 ul Caspase-Glo 3/7 reagent in white-walled 96-well plates and incubated for 2h at room temperature in the dark. Lysis buffer with the caspase reagent served as a blank

and stripped serum served as a negative control. All samples were run in duplicate. Luminescence was measured using a Biotek Synergy HT plate reader and values were expressed as relative intensity units (RIU). Relative intensity values were normalized to individual baseline values and then examined for group differences.

Western Blot Analyses

Sample lysates from prefrontal and temporal cortex were prepared using M-PER (Pierce, Rockford IL) to analyze levels of amyloid precursor protein (APP) and β-site APP cleaving enzyme-1. An equal amount of lysate protein was loaded onto 10% polyacrylamide gels containing SDS. SDS-PAGE was run and transferred to PVDF membranes. The membranes blocked for 1 h in nonfat milk; then incubated overnight with primary antibodies against APP-CT (Calbiochem, Billerica MA), BACE-1 (3D5) and β-actin (Sigma, St Louis MO). Membranes were then incubated with secondary antibody for 1 h. Bands were visualized and normalized to β-actin.

Statistics

Data were analyzed by one-way ANOVA and directional contrasts were used to determine significant group differences. The specific *a priori* comparisons used were (1) adult animals injected with DSP4 vs. adult CON (2) aged animals injected with DSP4 vs aged CON and (3) aged animals vs. adults to test whether development of amyloid pathology in animals with impaired LC function occurs due to increased neuroinflammation, apoptosis, and altered APP processing. The number of planned comparisons for each dependent measure was restricted (k−1) to test the above hypotheses in order to minimize family wise type I error.

Results

Evaluation of Neuroinflammation

Markers of inflammation were examined in serum, cerebrospinal fluid, and brain homogenate. Level of interleukin-6 was elevated in aged squirrel monkeys after DSP4 ($p=0.0484$), however remaining comparisons were not increased in either species ($p > 0.10$). Tumor necrosis factor-α level in adult compared to aged squirrel monkeys was increased ($p=0.0015$); while remaining comparisons were not elevated ($p > 0.10$) at the 9-month time point in brain homogenate; serum collected at necropsy and cerebrospinal fluid were below assay detection. In all samples, results from interleukin-1β assays were below detection (b.d).

Table 3.2: Inflammatory Cytokines. Low levels of interleukin -6 and tumor necrosis factor-α were measured in adult and aged non-human primate brain homogenate and normalized to sample protein levels; cytokines were below detection in cerebrospinal fluid and serum collected at necropsy. Interleukin-1β was below assay detection in all samples. DSP4 = 40 mg/kg, CON = saline vehicle, * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$). Samples below assay detection are noted by b.d.

Interleukin-6								Tumor Necrosis Factor-α								Interleukin-1β							
Squirrel Monkey				Rhesus Monkey				Squirrel Monkey				Rhesus Monkey				Squirrel Monkey				Rhesus Monkey			
CSF	**Mean**		**SEM**	**CSF**	**Mean**		**SEM**	**CSF**	**Mean**		**SEM**	**CSF**	**Mean**		**SEM**	**CSF**	**Mean**		**SEM**	**CSF**	**Mean**		**SEM**
Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--
Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--
Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--
Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--
Serum				**Serum**				**Serum**				**Serum**				**Serum**				**Serum**			
Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--	Adult Con	b.d	±	--
Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	--
Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--	Aged Con	b.d	±	--
Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	--
Brain	**Mean**		**SEM**	**Brain**	**Mean**		**SEM**	**Brain**	**Mean**		**SEM**	**Brain**	**Mean**		**SEM**	**Brain**				**Brain**			
Adult Con	12.58	±	1.54	Adult Con	4.93	±	0.88	Adult Con	2.27	±	0.17	Adult Con	3.12	±	0.37	Adult Con	b.d	±	--	Adult Con	b.d	±	
Adult DSP4	15.64	±	1.53	Adult DSP4	6.78	±	0.75	Adult DSP4	2.87	±	0.24	Adult DSP4	2.24	±	0.79	Adult DSP4	b.d	±	--	Adult DSP4	b.d	±	
Aged Con	11.50	±	0.55	Aged Con	6.04	±	0.99	*Aged Con	4.96	±	0.89	Aged Con	2.84	±	0.30	Aged Con	b.d	±	--	Aged Con	b.d	±	
**Aged DSP4	18.85	±	4.15	Aged DSP4	6.50	±	0.47	Aged DSP4	4.65	±	0.58	Aged DSP4	2.46	±	0.23	Aged DSP4	b.d	±	--	Aged DSP4	b.d	±	

Microglia were assessed using IBA1-IR and identification of cell hypertrophy to distinguish resting and activated microglia (Streit, 2009). Microglia cells were widely distributed throughout the brain (Figure 3.2A) and an activated morphology was rarely observed (Figure 3.2B). The % area stained was determined for each group and no differences were detected in either species ($p > 0.10$, Figure 3C).

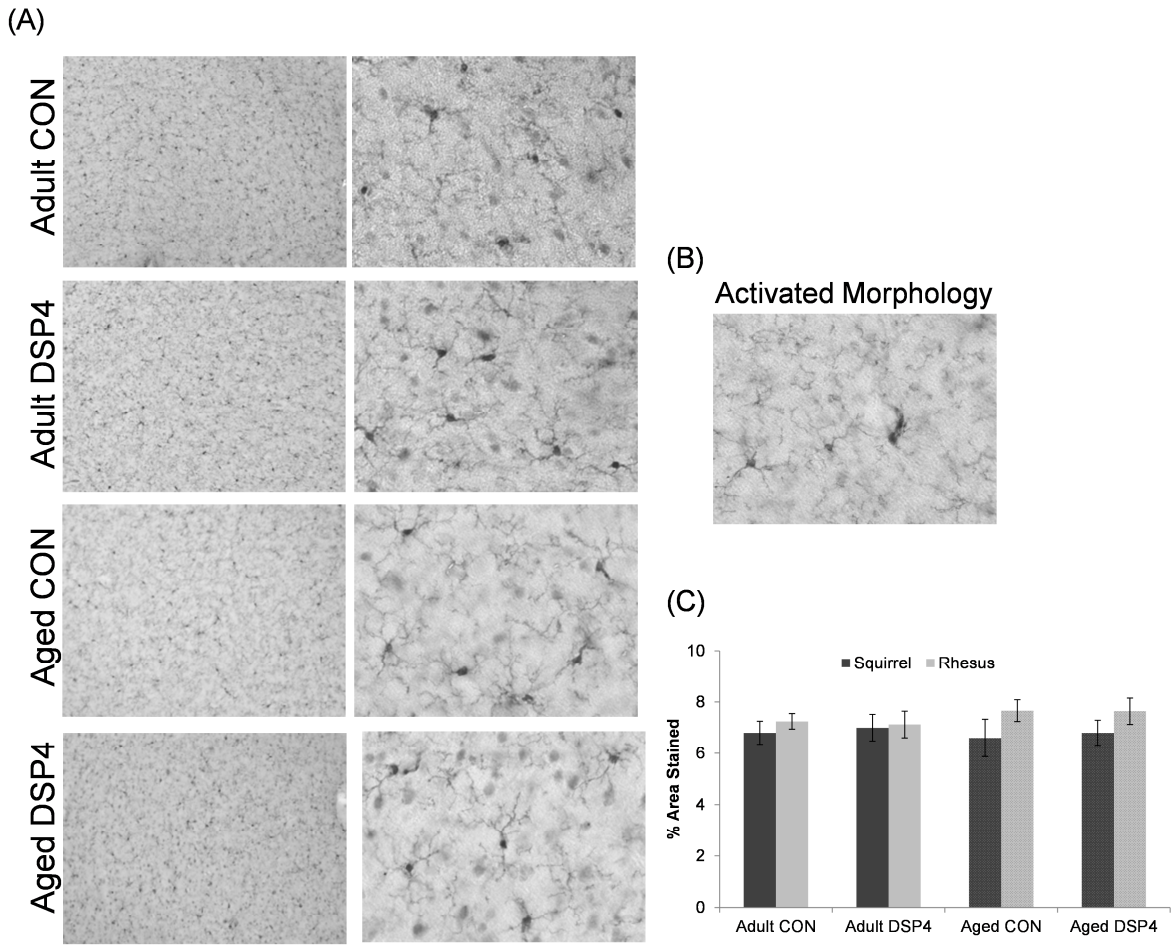

Figure 3.2: Microglia distribution and morphology. Representative photomicrographs of non-human primate frontal cortex Iba1-immunoreactivity (A) Example of activated morphology, rarely observed (B) and % area stained in non-human primates (C) DSP4 = 40 mg/kg, CON = saline vehicle, * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$).

Astrocyte distribution and morphology were examined using GFAP-IR in grey matter and white matter regions (Figure 3.3). GFAP-IR in grey matter was very low and when present was associated with vasculature, GFAP was constitutively expressed in white matter and cell body morphology showed no sign of hypertrophy. In each region, GFAP-IR % area stained did not increase ($p > 0.10$).

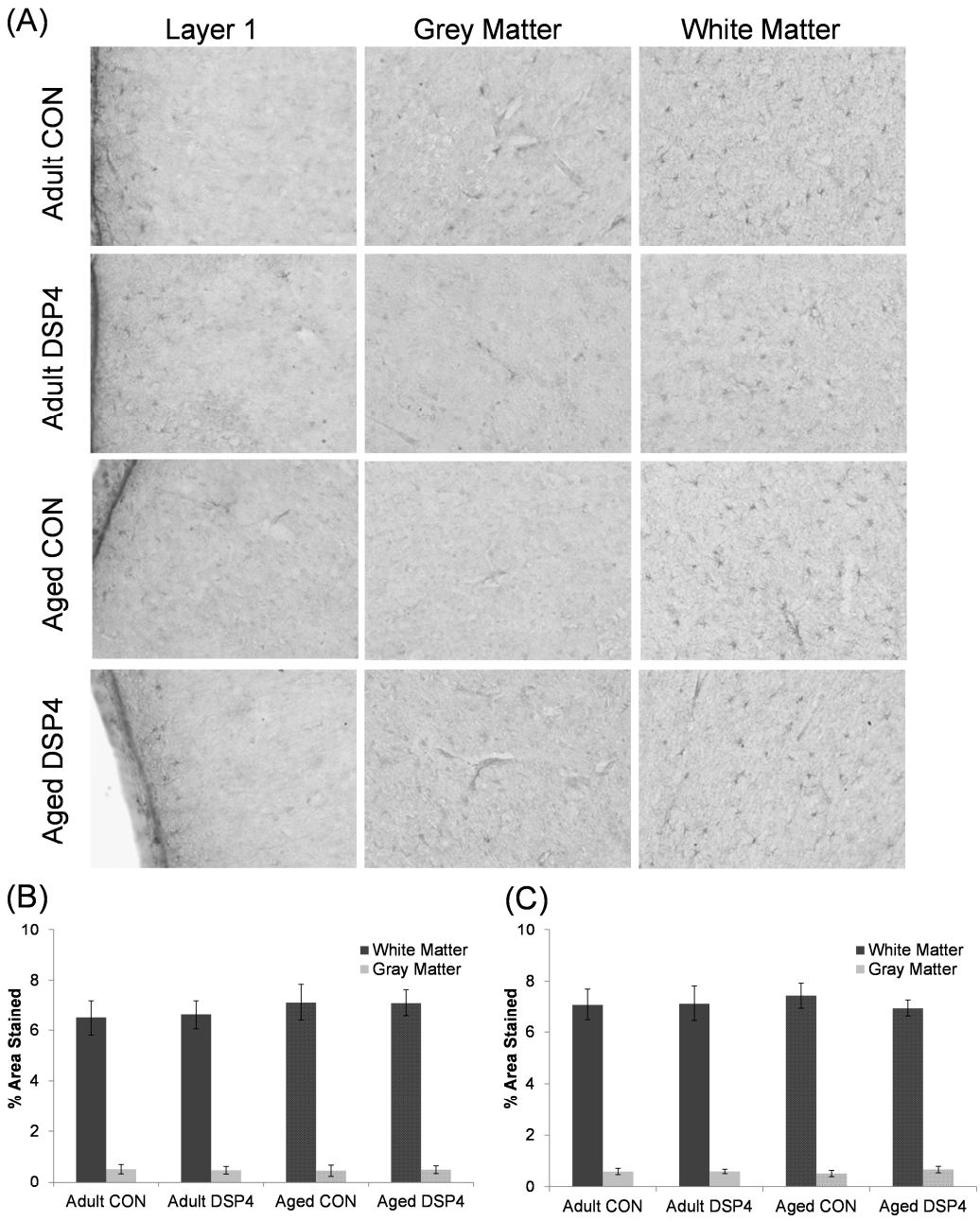

Figure 3.3: Astrocyte distribution and morphology. Representative photomicrographs in non-human primate frontal cortex (grey) and white matter GFAP-IR (A) %area stained in white (dark bars) and grey (light bars) matter neocortex in rhesus (B) and squirrel (C) monkeys DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$).

Analysis of cell-death related pathways

In order to examine intrinsic and extrinsic apoptosis pathways as potential biomarkers, caspase 3/7 and caspase 9 were measured in serum collected at baseline, 3-month, and necropsy. 3-month and necropsy measurements were normalized to baseline and analysis revealed an increase of caspase 9 in adult rhesus monkeys following DSP4 ($p = 0.015$, Figure 3.4B). Remaining comparisons of caspase 3/7 and caspase 9 activities were not significant ($p > 0.10$) for squirrel or rhesus monkeys.

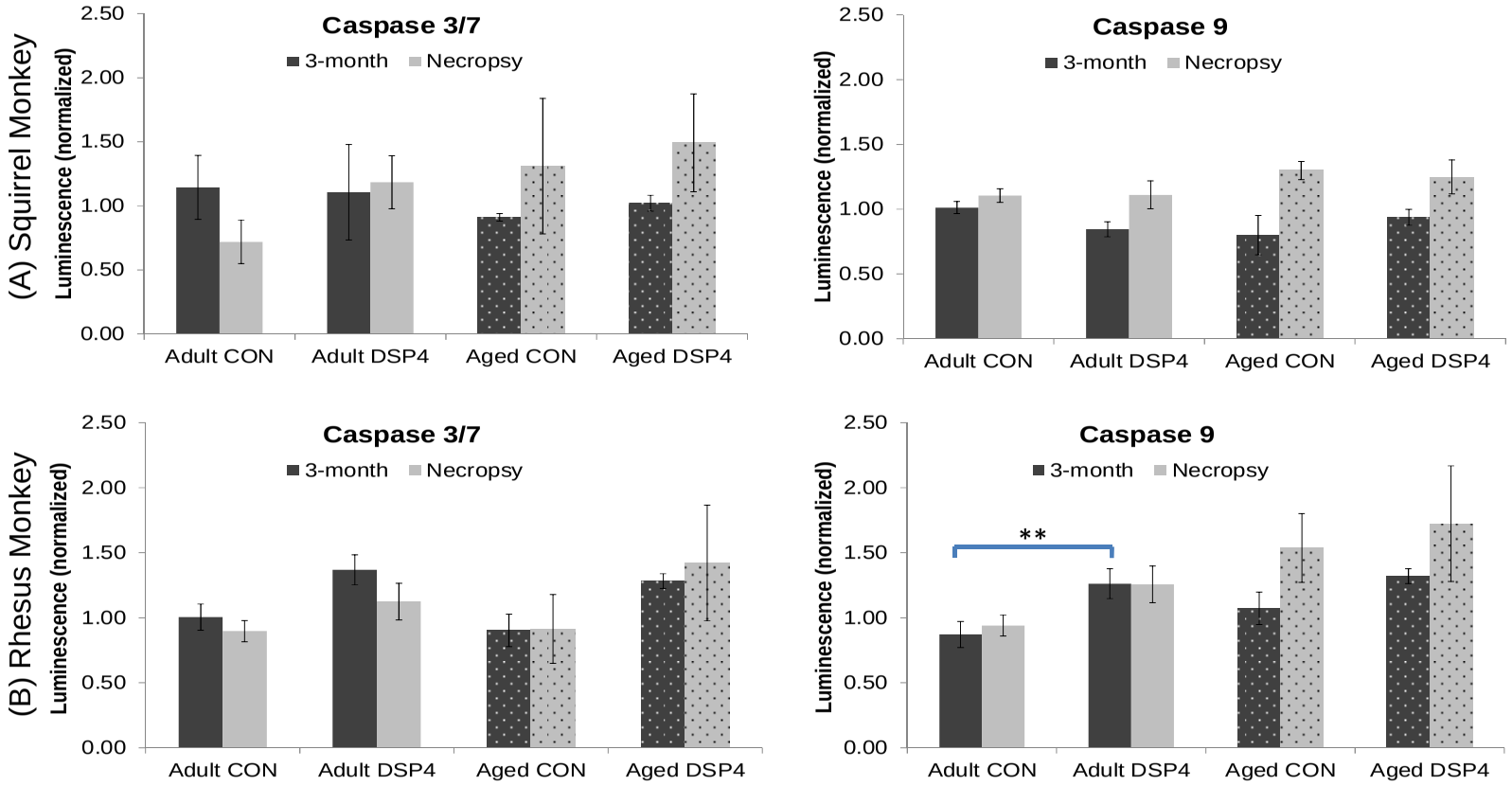

Figure 3.4: Caspase Activation. Caspase 3/7 (left) and caspase 9 (right) activities were measured by luminescence in 3-month (dark bars) and necropsy (light bars) serum samples collected from squirrel (A) and rhesus (B) monkeys. Relative luminescence was normalized to individual baseline levels. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$).

Examination of APP processing

In order to assess production and cleavage of amyloid precursor protein (APP) after DSP4, levels of intracellular APP and β-site APP cleaving enzyme-1 were determined in frontal and temporal cortex. In squirrel monkeys APP increased in frontal cortex ($p = 0.032$) with aging; β-site APP cleaving enzyme-1 also increased with age in frontal ($p = 0.038$) and temporal cortex ($p = 0.016$). However, DSP4 did not further exacerbate these age-related changes. In rhesus monkeys there was an age-related increase of APP in temporal cortex ($p = 0.0004$) and DSP4 treatment increased APP in adults ($p = 0.00006$). Additional increases of β-site APP cleaving enzyme-1 following DSP4 were detected in frontal cortex of aged rhesus monkeys ($p = 0.007$) and temporal cortex in adult rhesus monkeys ($p = 0.0009$).

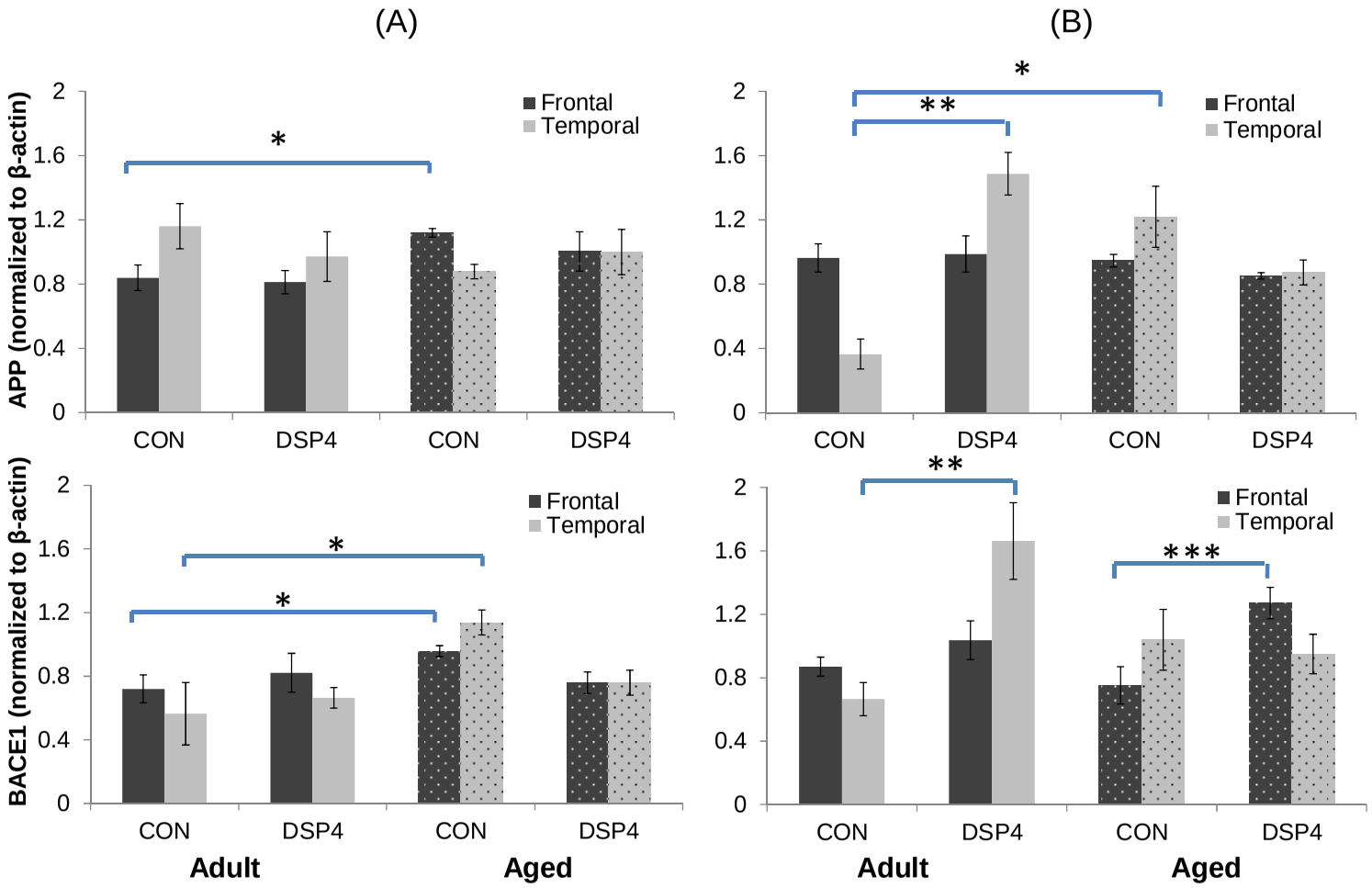

Figure 3.5: APP processing. Total amyloid precursor protein (APP) and β-site APP cleaving enzyme-1 (BACE-1) were measured in frontal and temporal cortex samples in squirrel (A) and rhesus (B) monkeys and normalized to β-actin. DSP4 = 40 mg/kg, CON = saline vehicle, Bar graphs display Mean ± SEM. * adult vs. aged control ($p < 0.05$), ** adult control vs. adult DSP4 ($p<0.05$), *** aged control vs. aged DSP4 ($p < 0.05$).

To examine amyloid precursor protein degradation, the protease neprilysin (CD10) was examined in non-human primate frontal cortex, striatum, hippocampus and temporal cortex. Neprilysin immunoreactivity was observed and widely distributed in the rhesus monkey striatum (Figure 6A), but was not present in other brain regions. Analysis of % area stained showed equivalent staining in all groups (Figure 6B, $p > 0.10$). No specific binding was detected in the squirrel monkey.

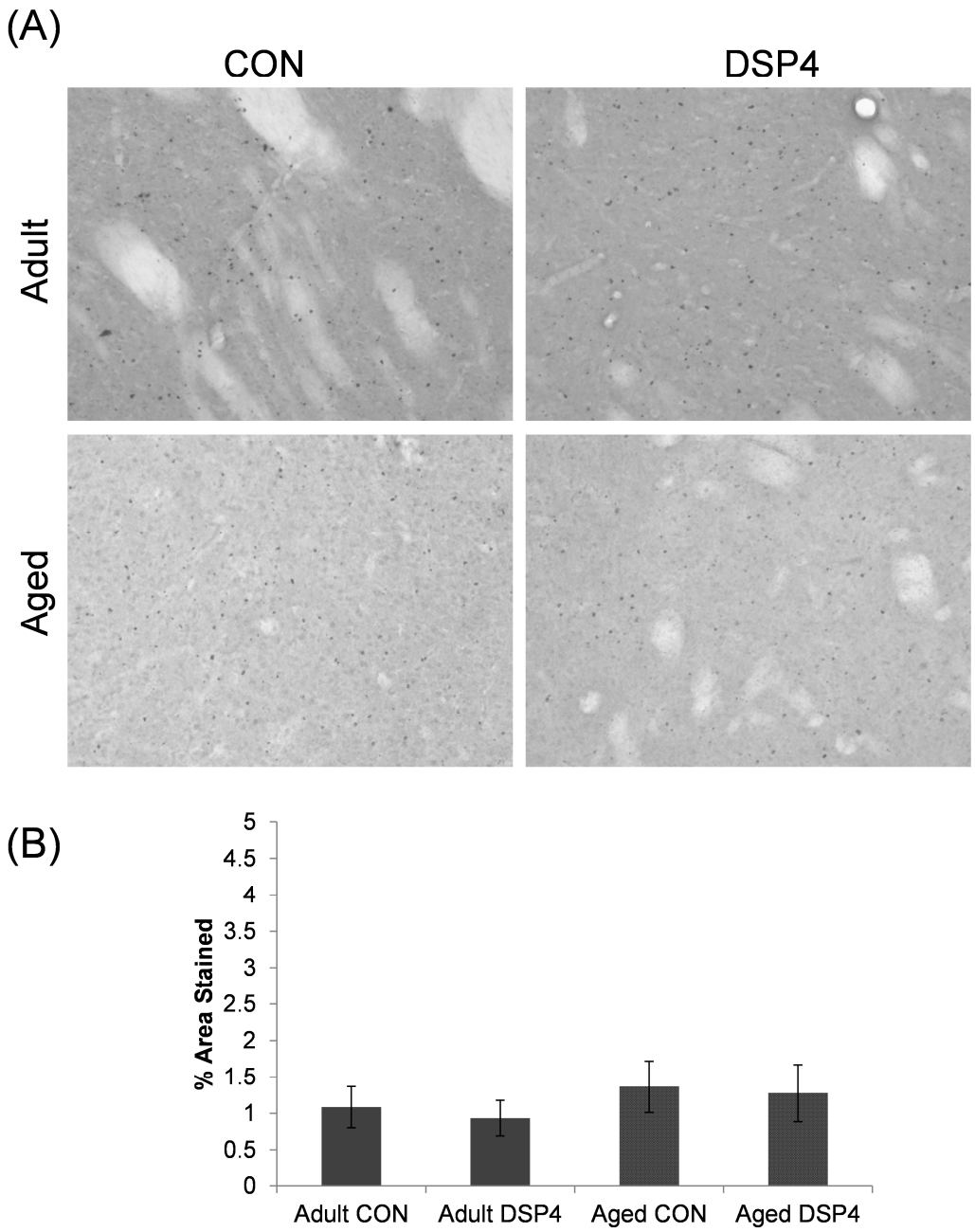

Figure 6: Amyloid-β degradation. Representative photomicrographs depicting neprilysin immunoreactivity in striatum of rhesus monkeys (A) and % area stained (B)

Discussion

These experiments were focused on revealing potential mechanisms and pathways impacted by DSP4 treatment in adult and aged female non-human primates, contributing to the elevation of amyloid pathology in non-human primates reported previously (Chapter 2). We chose markers for serum and cerebrospinal fluid for neuroinflammation, apoptosis and amyloid precursor protein processing in AD relevant brain regions to elucidate the mechanisms influencing amyloid accumulation in animals with impaired locus coeruleus function and significant long term reduction of norepinephrine. Here we demonstrated that norepinephrine depletion increased levels of β-site APP cleaving enzyme-1, the secretase enzyme responsible for pathogenic formation of amyloid-β, and also influenced amyloid precursor protein in the absence of chronic neuroinflammation.

Microglia cells are located throughout the central nervous system and are critical transducers of neuroimmune responses to inflammation. Activated microglia are essential for phagocytosis of damaged cells and debris. Microglia respond quickly to central nervous system injury and are important for clearance of amyloid with chronic activation occurring during disease (Streit, Mrak et al. 2004). After injury microglia increase in cell number, change morphological appearance, and migrate to site of trauma during activation. These microglia regain their resting state morphology after the return of homeostasis while sustained microglial activation results in increased and aberrant production of pro-inflammatory cytokines which contribute to further damage to surrounding tissue. Numerous AD-transgenic mouse studies demonstrated robust microglia and astrocyte activation following DSP4 with

increased secretion of proinflammatory cytokines from microglia (Heneka, Ramanathan et al. 2006; Heneka, Nadrigny et al. 2010). In our study the morphology of Ibal1 immunoreactive stained microglia across age and treatment showed no evidence for activation necessary for release of pro-inflammatory cytokines and the widely distributed uniform appearance of Iba1 positive microglia was typical of a resting phenotype without proliferation. These results indicated that chronic neuroinflammation did not occur with our DSP4 dosing paradigm in non-human primates. Further our experimental design allowed for sufficient time between injections to restore a resting state within the microglia population.

Astrocytes make extensive contacts with blood vessels and play a neurosupportive role in conjunction with the Blood-Brain-Barrier; aiding homeostasis through interactions with neurons. The GFAP protein was isolated from a pathological disease state and is consequently very sensitive to detection of reactive astrogliosis but does not label all non-reactive astrocytes (Sofroniew and Vinters 2010). In white matter GFAP can also be used as a general indicator for blood-brain barrier integrity (Liedtke, Edelmann et al. 1996). Similar staining across groups demonstrated normal blood-brain barrier morphology and provided evidence for proper myelination of axons, which are densely populated within white matter brain regions. The lack of proliferation or increase in cell hypertrophy in grey matter regions supported the conclusion that the dosing paradigm did not induce chronic neuroinflammation and astrocyte function was maintained at the conclusion of this study. Further, the presence of increased amyloid (Chapter 2) without coexisting activation of microglia or astrocytes suggested that the observed amyloid deposits

alone were not sufficient to cause a sustained inflammatory response. Relatively low levels of oligomeric and fibrillar species of amyloid-β compared to higher levels of diffuse amyloid-β deposition might contribute to the lack of activation due to amyloid alone (Stalder, Phinney et al. 1999; White, Manelli et al. 2005; Sondag, Dhawan et al. 2009).

Cytokines are central to both pro and anti-inflammatory pathways in the brain and are a key regulator of neuro-immune responses including modulation of inflammation. After insult to the central nervous system and during subsequent inflammatory response, microglia and astrocytes release cytokines into the local environment. Acute release of inflammatory cytokines is essential for stimulating appropriate reparative processes and acts in a generally beneficial manner; however under pathological conditions damage increases and persistent activation is considered an important component of neurological diseases including AD (Dello Russo, Boullerne et al. 2004). Levels of pro-inflammatory cytokines are generally very low or even undetectable in normal brain, however they become rapidly induced following injury or sustained release during disease (Allan, Tyrrell et al. 2005). Notably interleukin-1β, tumor necrosis factor-α and interleukin-6 are localized close in close proximity to amyloid plaques in AD (Cacquevel, Lebeurrier et al. 2004); and amyloid deposits perpetuate the release of more interleukin-1β, driving the cycle of aberrant cytokine expression (Forloni, Demicheli et al. 1992). Inflammatory cytokines measured in this study were below detection in cerebrospinal fluid and sera from both species; low levels and small increases of interleukin-6 were detectable in brain homogenate of aged squirrel monkeys after DSP4. These cytokines have been

postulated as potential biomarkers for AD; however, conflicting results from clinical studies examining serum and cerebrospinal fluid bring their utility as biomarkers into question (Mrak and Griffin 2005; Schuitemaker, Dik et al. 2009). These data, along with IBA1 and GFAP immunoreactivity, supported the conclusion that neuroinflammation was not persistent or heightened by the end of the long term study. Transient increases of neuroinflammation cannot be ruled out and based on the literature likely do contribute to the longterm effects on amyloid deposition in a short or intermediate manner not picked up in this study. Data from our short term pilot study (appendix) demonstrated more depletion compared to the longterm study and the elevated inflammatory measures in brain homogenate provided some initial evidence to support this conclusion.

Extrinsic signals, such as binding at the TNFα receptor, and intrinsic mitochondrial pathways contribute to neuronal cell death via caspase-3 (Cas3) activation. Cas3 is a key enzyme in apoptosis cell-signaling cascades and also acts on a caspase cleavage site on the cytoplasmic domain of amyloid precursor protein to form the C31 fragment. This fragment further contributes to apoptosis in AD by complexing with amyloid precursor protein to enhance signals related to neurotoxicity (Bertrand, Brouillet et al. 2001; Park, Shaked et al. 2009). Intrinsic activation may alter mitochondrial membrane permeability, thereby releasing cytochrome c, which then interacts with caspase-9 upstream of cas3. Observations of cas3/7 and cas9 activities from both species showed trends for increased activity in rhesus monkeys at the 3-month and necropsy time points following injection with DSP4. Analyses also revealed a small but significant increase of cas9 detected in

serum from adult rhesus monkeys 3 months after the high dose of DSP4, indicating transient increase of mitochondrial membrane permeability or possible activation by C31 (Lu, Soriano et al. 2003); however, a change in the periphery might also contribute to an elevation of caspase 9 in serum and further investigation would be required to determine brain specific effects. Given the lack of cytokine activation and probable recovery between injection time points, the rhesus monkeys may be more sensitive to long term changes in caspase activity. The transient nature of the effects after the DSP4 treatment paradigm implies a more continuous exposure or repeated high doses through the experiment may have been more effective in sustained elevations of these biomarkers and more effective in chronic elevations reported in AD.

The accumulation of various amyloid isoforms and plaque formation occurs through an imbalance between amyloid-β production and clearance. Pro-inflammatory cytokines have been shown to modulate pathogenic amyloid precursor protein processing, increasing generation of amyloid precursor protein itself and elevated β-secretase cleavage (Moore and O'Banion 2002; Bourne, Ferrari et al. 2007; Carrero, Gonzalo et al. 2012); while neuroglia and proteases actively degrade amyloid (Hersh and Rodgers 2008; Lee and Landreth 2010; Pihlaja, Koistinaho et al. 2011). Age related changes in amyloid precursor protein and β-site APP cleaving enzyme-1 were detected in the squirrel monkey; however DSP4 did not increase either endpoint in frontal or temporal cortex. Elevated amyloid deposition was reported in these animals (Chapter 2) and the age-related increase can be partially explained by the increases in APP processing. The further significant elevation of

amyloid deposition after DSP4 in squirrel monkeys cannot be explained by long-term changes in amyloid precursor protein processing. However, transient increases in inflammation or reduced degradation would explain the increase. DSP4 also increased amyloid deposition in neocortex of rhesus monkeys and the sustained elevations in APP and β-site APP cleaving enzyme-1 detected following injection with DSP4 suggest altered amyloid precursor protein processing contributed to increased deposition. The Aβ degrading enzyme neprilysin was detected in the striatum of rhesus monkeys; however IR was not significantly decreased after DSP4. Thus the elevation in APP and increased pathogenic cleavage by β-site APP cleaving enzyme-1 both contribute to the elevation of amyloid deposition in rhesus monkeys, rather than a decreased degradation of amyloid-β by neprilysin.

In summary, DSP4 treatment effectively accelerated the onset and incidence of AD-like pathology in non-human primates due to altered APP processing. This provides support for the contribution of impaired noradrenergic systems in progression of AD pathology and elucidated some of the pathways involved in amyloid accumulation following long term depletion of norepinephrine in the locus coeruleus. Specifically, our findings reveal that the long term reduction of norepinephrine contributed to increased amyloid pathology due to altered APP processing and not degradation via neprilysin. Furthermore, these long lasting modifications were maintained in the absence of chronic neuroinflammation.

Chapter 4: Concluding Remarks

Overall Discussion

Understanding the neurobiological basis for the accumulation of pathology in AD remains elusive. Studies focused on charactering the neuropathology in transgenic AD-mouse models but often focused on degeneration in basal forebrain, hippocampus, and temporal cortex. Despite the challenges of small sample sizes, heterogeneous sample populations and individual variation our findings provide valuable insights into the role of the noradrenergic system as a key component in AD associated neurodegenerative processes. Moreover, the data presented in Chapters 2 and 3 builds the groundwork to demonstrate an influence of locus coeruleus-norepinephrine changes in non-human primates that develop amyloid pathology in an idiopathic manner.

It is remarkable to observe significant long-term changes in AD pathology based on impairment of a single small population of neurons. The observation of a significant change from permuting one neurotransmitter system is significant because it illustrates the importance of the norepinephrine system in the upregulation and accumulation of amyloid pathology. At the same time, our experiments also demonstrated resilience in norepinephrine and associated other neural systems in regard to mediating a neurotoxic insult and minimizing chronic neuroinflammation. The potential for recovery of norepinephrine containing neurons projecting to AD sensitive regions is further evidence for a capacity for compensation by multiple brain systems. This capacity also highlights the importance of redundancy in neural systems and more importantly for plasticity in the event of disease states. These

types of mechanisms allow organisms to potentially cope with loss of neurons and resulting dysfunction up to a point, until multiple networks fail resulting in significant disease progression.

The brain uses a series of checks and balances. Within this complex tapestry, norepinephrine provides wide-ranging modulation throughout the cortex. Based on our data, the loss of norepinephrine neural systems in key areas of the brain appears to be permissive for the accumulation of amyloid pathology, thereby contributing to the development of AD. In the short-term experiment, widespread norepinephrine depletion in the locus coeruleus, cortical and subcortical regions resulted in evidence of inflammatory responses at 30 days following a single injection of DSP4. Conversely, the treatments and measurements taken 3 months after the final dosing in the long-term study provides evidence to support the restoration of norepinephrine in cortical regions may occur at the expense of developing AD pathology. Considering the long-term reduction of norepinephrine in the locus coeruleus, the brain may compensate for loss of norepinephrine in the case of limited physiological functions. However, the norepinephrine compensation did not appear to be adequate to modulate processes that contribute more directly to increased Aβ production or decreased clearance.

Contributions from biology-related fields and medicine have dramatically enhanced our understanding of the brain and central nervous system. Neuroscience and molecular biology techniques are ever evolving and have made incredible technological advances in the last few decades helping scientists to further their study of neurons and key players regulating function. Idiopathic AD is a multi-faceted and

likely caused by a complex interaction of environmental and genetic factions leading to eventual dysfunction. There is no single risk factor or cerebral alteration that guarantees a shift to an Alzheimer's phenotype of progressive and severe cognitive decline.

So why are humans so susceptible to Alzheimer's disease? One answer is that we live too long; specifically we outlive our reproductive lifespan. This may also be a contributing factor for higher incidence of AD in women than men; perhaps due in part of the menopausal related loss of gonadal steroids. Genetic lineages for human and ape separated about 7 million years ago with significant differences in maximum lifespan between the species; maximum lifespan for the rhesus monkey is 40, while the oldest living female human died at the age of 122. AD pathology occurs late in life, well beyond reproductive senescence but at least a decade before the average mortality in the United States. In contrast, the majority of non-human primates do not survive long after reproduction stops and the prevalence of Alzheimer's-like pathology is lower compared to humans. Perhaps the evolutionary forces that selected for our larger brain size and increased longevity also contributed to vulnerability in our neural systems that lead to neurodegenerative diseases. Given the increased incidence of neurodegenerative diseases with aging, one wonders why humans want to live so long without a guarantee of well-being. Alternatively, the more we understand about these diseases, the closer we come to prevention or at least effective intervention.

There is no denying that the human body suffers wear and tear throughout the lifespan; however there are examples of healthy human aging extending well beyond

the average (Cevenini, Invidia et al. 2008; Willcox, Willcox et al. 2009; Guralnik and Kritchevsky 2010). Overall, our goal will be to minimize the impact of many age-related diseases of aging or perhaps even avoid persistent disease states altogether. Pathological pathways and mechanisms of age-related diseases have been carefully studied and categorized by gerontologists over the years (Birren and Schaie 2006; Masoro and Austad 2006). I hope that by contributing and expanding this knowledge will help to extend and improve the quality of life of our elders, and our own lives as we age.

Future Directions

The novel finding of increased norepinephrine in the striatum deserves further consideration and follow-up to determine the mechanism and utility. While we did not observe motor deficits, the concurrent decrease of dopamine in striatum would potentially be harmful to an individual if maintained long term. Deficits of dopamine in striatum are observed in cases of Parkinson's disease, and this region of the brain is essential for controlling balance, movement and walking.

Clearance of amyloid isoforms across the blood-brain barrier mediated by low-density lipoprotein receptor and receptor for advanced glycation end products also warrants further investigation. Due to the higher incidence of cerebral amyloid angiopathy, the squirrel monkey would serve as a better model as this pathology was not observed in our rhesus monkey cohorts. It is worthwhile to consider the relative ages between the squirrel and rhesus monkeys used in the current study; in order to make more relevant comparisons between aged groups the inclusion of an older

rhesus monkey group would be beneficial toward assessment of age-related changes and examine the potential for reduced plasticity in aged animals.

Appendices

Table A.1: Greater than 60% reduction in prefrontal cortex, hippocampus, cingulate (Retrosplenial) cortex and locus coeruleus 30 days following a single injection of DSP4

Brain Region	**% NE depletion**	
	Rhesus	***Squirrel***
Hippocampus	74.72	68.49
Pre-Frontal Cortex	72.24	78.11
Retrosplenial Cortex	91.52	97.37
Hypothalamus	32.17	3.24
Cerebellum	30.73	50.39
Locus Coeruleus	61.66	97.29

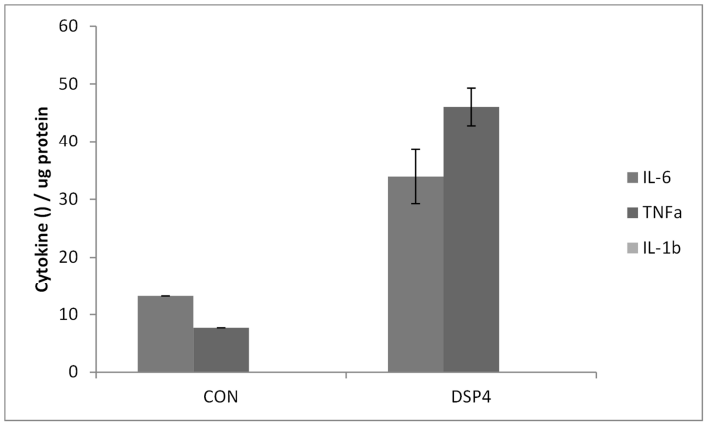

Figure A1. Increased pro-inflammatory cytokines in squirrel monkey brain. 30 days following injection with DSP4 IL-6 and TNFα cytokines were elevated. IL-1β was not detected.

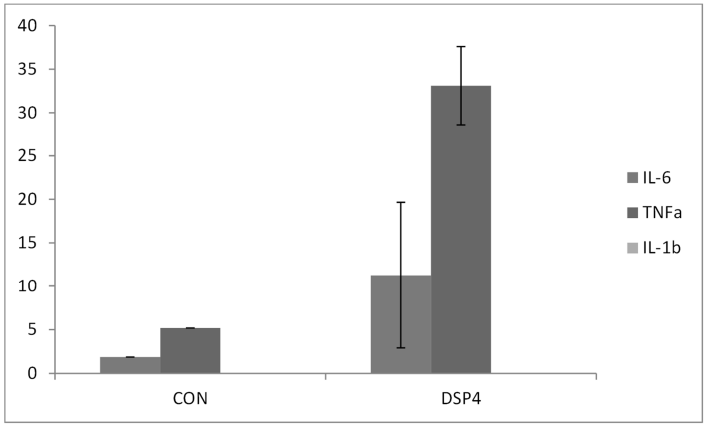

Figure A2. Increased pro-inflammatory cytokines in rhesus monkey brain. 30 days following IL-6 and TNFα cytokines were elevated in animals that received DSP4. IL-1β was not detected.

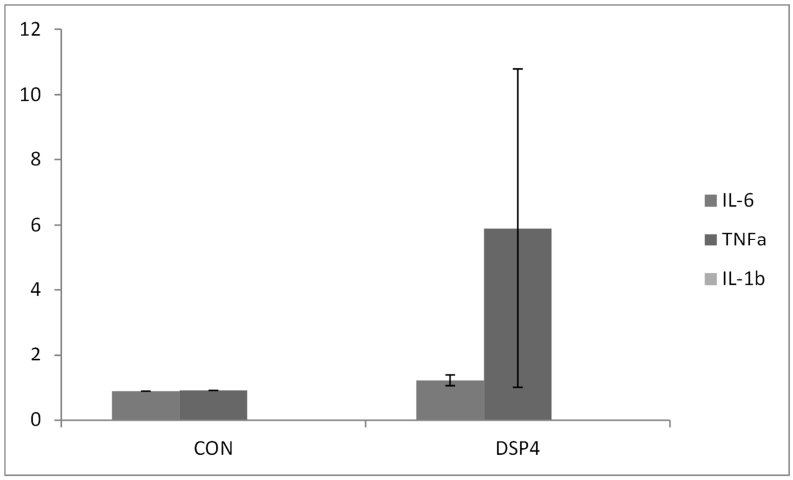

Figure A3. Increased pro-inflammatory cytokines in rhesus monkey cerebrospinal fluid. Low levels of pro-inflammatory cytokines detected in cerebrospinal fluid indicating TNFα may be a more sensitive measure. IL-1β was not detected.

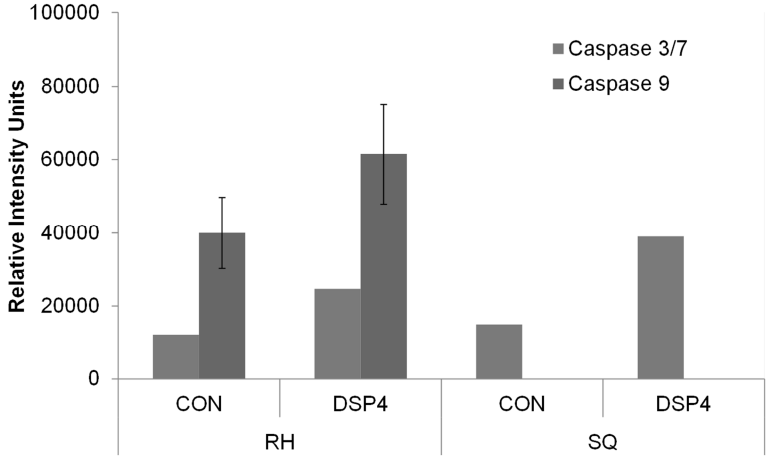

Figure A4. Increased apoptosis in non-human primates. Measurement of Caspase 3/7 and Caspase 9 showed increases in both species following DSP4 at 30 days.

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