Efficacy of GFAP as a biomarker for Alexander disease and estrogenic regulation of GFAP

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EFFICACY OF GFAP AS A BIOMARKER FOR ALEXANDER DISEASE AND ESTROGENIC REGULATION OF GFAP

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ABSTRACT

Alexander disease (AxD) is a rare and fatal neurodegenerative disorder caused by mutations in glial fibrillary acidic protein (GFAP), which leads to GFAP accumulation above an unknown toxic threshold. There is no cure for AxD, but reduction of GFAP may alleviate AxD patient symptoms. In anticipation of future drug therapies, we explored the potential of GFAP as a biomarker for analysis of drug efficacy in mouse models of AxD and AxD patients. In chapter 2, we investigated two independent measures of GFAP expression in AxD mouse models: a genetic reporter of promoter activity and quantification of GFAP protein directly in a manner that could also be employed in human studies. Using a reporter mouse line which expresses firefly luciferase under the control of the murine *Gfap* promoter, we found that luciferase activity reflected the regional CNS variability of *Gfap* mRNA in *Gfap*$^{+/+}$ mice, and increased in a mouse model of AxD. We also quantified GFAP protein in CSF from three different AxD mouse models and found GFAP levels increased in all models. In chapter 3, we analyzed GFAP protein in CSF and plasma from AxD patients, finding GFAP was significantly elevated in both CSF and plasma of AxD patients compared with controls. The results from Chapters 2 and 3 suggest we have good biomarkers in mouse models of AxD (*Gfap* promoter activity and GFAP in CSF) and AxD patients (GFAP in both CSF and plasma). In chapter 4, we

analyzed the potential GFAP regulatory effects of estradiol (E2). A previous drug screen revealed E2 decreased *Gfap* promoter activity by 26% (Cho et al. 2010). In a mouse model of AxD, we found minimal changes in GFAP expression resulting from either surgical or pharmacological manipulation of estradiol levels. In addition, no fluctuation in GFAP expression was evident during the natural variations in endogenous estrogens occurring during the estrous cycle. These studies suggest caution in the implementation of estrogen-based treatments for AxD. Overall, the conclusions of the work described in this report support GFAP as a biomarker for AxD that can be utilized in future clinical trials to determine drug efficacy.

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Abbreviations

16α-LE2 – 16α-lactone-estradiol

ACM – astrocyte conditioned media

AD – Alzheimer's disease

ALS – amyotrophic lateral sclerosis

AP – activator protein

AxD – Alexander disease

BLD – biological limit of detection

bp – base pair

BSA – bovine albumin serum

CNMC – Children's National Medical Center

CNS – central nervous system

COV – coefficient of variation

CRE – cAMP response element

CSF – cerebrospinal fluid

DBD – DNA binding domain

DE – diestrus

DES – disethylstibestrol

DPN – diarylpropiolnitrile

E – estrus

E2 – estrogen

EAE – experimental autoimmune encephalomyelitis

ECL – entorhinal cortex lesion

ELISA - enzyme-linked immuno sorbent assay

ER – estrogen receptor

ERE – estrogen response element

ERK – extracellular signal-related kinase

fLuc – firefly luciferase

Gdx – gonadectomy

GATA – globin transcription factor

GFAP – glial fibrillary acidic protein

GPR30 – G-protein coupled receptor 30

GSH – glutathione

HPG – hypothalamic-pituitary-gonadal

HPTE – 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane

HSP – heat shock protein

IF – intermediate filament

IgG – immunoglobulin G

INFγ – interferon gamma

IRB – Institutional Review Board

JNK – c-Jun N-terminal kinase

kDa – kilodalton

L – liter

LBD – ligand binding domain

LIQ – liquiritigenin

LLD – lower limit of detection

LP – lumbar puncture

LPS – lipopolysaccharide

MAPK – mitogen-activated protein kinase

MCAO – middle cerebral artery occlusion

mGluR – metabotropic glutamate receptors

μL – microliter

mL – milliliter

ME – metestrus

MLK – mixed linage kinases

MRI – magnetic resonance imaging

MS – multiple sclerosis

mTOR – mammalian target of rapamycin

NF – nuclear factor

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

ng – nanogram

NTD – amino-terminal domain

OGD – oxygen-glucose deprivation

OVX – ovariectomy

PBS – phosphate buffered saline

PE – proestrus

PI – protease inhibitors

PL – placebo

PPT – propyl-pyrazole-triol

RF – Rosenthal fiber

R,R-THC – R,R-tetrahydrochrysene

RSV – respiratory syncytial virus

RT – room temperature

SCI – spinal cord injury

SD – standard deviation

SOCS3 – suppressor of cytokine signal 3

SOD – superoxide dismutase

SP – specificity protein

STAT3 – signal transducer and activator of transcription 3

TBI – traumatic brain injury

TGFβ – transforming growth factor beta

TNFα – tumor necrosis factor alpha

TX – Triton-X-100

Tw20 – Tween 20

Chapter 1:

Introduction

Alexander disease (AxD) is an untreatable, rare, and fatal disorder caused by abnormalities in brain cells known as astrocytes. In astrocytes mutations in the main cytoskeletal structure alters astrocyte function which results in a variety of symptoms in AxD patients through unknown mechanisms. In the next several sections, we will explore AxD and determine potential biomarkers in mice and humans as well as elucidate the role of estradiol as a potential treatment in an effort to better understand AxD. First, I will give a brief overview of the central nervous system (CNS) and the role of astrocytes during normal function. Then, I will explain what is known about AxD including mouse models and potential drug treatments. Finally, I will discuss potential biomarkers of AxD and their use in determining the efficacy of potential drug therapies such as estradiol.

Central Nervous System and Astrocytes

The main two cell types present in the brain are neurons and glia. Neurons are involved in processing and transmitting information through electrical and chemical signals, while glia are traditionally thought to support neuronal function. There are several different types of glial cells present in the brain, each with complex processes or projections extending from a cell body. Glia are important regulators of nerve signal propagation, synaptic transmission, and recovery from neural injury among other functions. The three main types of glial cells are oligodendrocytes, microglia, and astrocytes.

Oligodendrocytes surround neuronal axon projections with myelin, an insulating material, to reduce ion leakage and increase synaptic transmission velocity. Microglia are a type of immune cell which defends the CNS from infection. Astrocytes have a diverse function throughout the brain. Astrocytes maintain the blood-brain barrier (Brightman and Reese 1969; Bush et al. 1999), regulate blood flow (Lovatt et al. 2007), maintain neuronal circuits through regulation of the synaptic environment and neurotransmitter concentrations (Rothstein et al.

1996; Araque et al. 1998; Oliet et al. 2001; Hirrlinger et al. 2004; Regan et al. 2007), and maintain sleep through release of adenosine (Benington et al. 1995; Thakkar et al. 2003; Thakkar et al. 2003; Halassa et al. 2009).

Astrocytes also display regional variation in astrocyte cell sub-type as well as in astrocyte-neuron interactions, receptor expression, second messenger responses, and protein concentration in various brain regions (Hansson 1990; Regan et al. 2007; Yeh et al. 2009; Fitting et al. 2010), which is referred to as astrocyte heterogeneity. In the late nineteenth century, astrocytes were divided into two main subtypes (Andriezen 1893; Sofroniew and Vinters 2010). Protoplasmic astrocytes are found in gray matter and have thick, highly branched processes. Fibrous astrocytes, located in white matter, have long, thin processes with few branches (Andriezen 1893). Since then, several other astroglial cells have been identified including Muller glia in the retina, Bergman glia in the cerebellum, tanycytes in the ventricles, as well as others (Sofroniew and Vinters 2010). The reason astrocytes vary regionally is unclear, though gene expression patterns vary regionally in astrocytes when comparing cortex, cerebellum, and brain stem both *in vitro* and *in vivo* (Yeh et al. 2009; Fitting et al. 2010).

Astrocytes also play an essential role after neurological insult due to injury, disease, or infection by becoming reactive. Reactive astrocytosis, or astrogliosis, is characterized by hypertrophy, or increased cell size, as well as upregulation and reorganization of glial filaments, such as glial fibrillary acidic protein (GFAP), vimentin, and nestin (Andriezen 1893; Eliasson et al. 1999; Wilhelmsson et al. 2004; Middeldorp and Hol 2011). Reactive astrocytes form a glial scar at the injury location which may reduce or exacerbate damage to neuronal tissue depending on the damage type, severity, and time point observed (Pekny et al. 1999; Bal-Price and Brown 2001; Wilhelmsson et al. 2004; Voskuhl et al. 2009). Astrocytes protect neurons through regulation of antioxidant levels, blood brain barrier, cell infiltration, and neurogenesis by releasing neurotrophic factors after injury (Friedman et al. 1996; Bush et al. 1999; Song et al.

2002; Shih et al. 2003; Zhao et al. 2003; Faulkner et al. 2004; Pekny and Nilsson 2005; Barkho et al. 2006; Okada et al. 2006; Lovatt et al. 2007; Li et al. 2008; Voskuhl et al. 2009). Neuronal damage is exacerbated by astrocytes after injury through expression of anti-regeneration chemicals and enzymes such as nitric oxide synthase, nuclear factor kappa B (NFκB), and suppressor of cytokine signal 3 (SOCS3) (Bal-Price and Brown 2001; Heneka et al. 2001; Heneka et al. 2005; Okada et al. 2006; Brambilla et al. 2009; Brambilla et al. 2009; Sofroniew 2009).

Astrocyte response to injury also varies in different brain regions. When comparing oxygen-glucose deprivation (OGD), peroxide, and excitotoxic insults to primary astrocyte cultures, cortical, hippocampal and striatal astrocytes responses differed to each insult (Xu et al. 2001). Cortical astrocytes were more sensitive to oxidative stress caused by peroxide exposure compared with hippocampal and striatal astrocytes, while striatal astrocytes showed more injury with OGD. Further analysis of several antioxidant parameters in the primary astrocyte cultures revealed differential protein expression with higher levels of glutathione (GSH) in striatal astrocytes, intermediate levels in cortical astrocytes, and the lowest levels of GSH in hippocampal astrocytes. Superoxide dismutase (SOD), another antioxidant protein, was highest in striatal astrocytes with the lowest concentrations of SOD found in cortical astrocytes. The apoptosis regulator protein, bcl-X_L had higher concentrations in striatal astrocytes with the lowest concentration in hippocampal astrocytes (Xu et al. 2001). These results indicate injury and astrocyte location influence protein expression in astrocytes, indicating astrocytes display regional heterogeneity both during normal function as well as response to injury.

Astrocyte Intermediate Filaments

In astrocytes, the main intermediate filaments are GFAP and vimentin which are type III intermediate filaments (IFs) (Fuchs and Weber 1994). Vimentin is the most widely expressed IF

while GFAP is mainly found in astrocytes (Mamber et al. 2012). IFs are unique in the lack of structural polarity as well as the mode and location of assembly and turnover (Coulombe and Wong 2004). All IFs are composed of a central α helical domain known as a rod with non-helical head and tail domains (Pauling and Corey 1953; Geisler and Weber 1982; Marchuk et al. 1984; Albers and Fuchs 1987; Wong and Cleveland 1990). The head and tail domains can vary in length between different IFs, but the rod domain is conserved. Three short non-helical linkers segment the rod domain, with the helical domains between known as 1A, 1B, 2A, and 2B (McLachlan 1978; Conway and Parry 1990; Lu and Lane 1990). IFs can self-assemble *in vitro* with no other auxillary protein necessary for assembly. Amino acid substitutions within the 1A or 2B rod domains can disrupt IF assembly (Hatzfeld and Weber 1991; Letai et al. 1992).

GFAP is the main intermediate filament within astrocytes and is also the hallmark of reactive astrogliosis (Fuchs and Weber 1994; Pekny et al. 1999; Pekny and Pekna 2004; Pekny and Nilsson 2005; Middeldorp and Hol 2011). The human *GFAP* gene is located on chromosome 17 at position 21 and spans 9869 nucleotides. There are several different GFAP isoforms including GFAPα, GFAPβ, and GFAPδ. GFAPα, the major isoform of GFAP, is encoded by 9 exons, contains 432 amino acids, has a molecular mass of 50 kilodaltons (kDa), and is the isoform hitherto referred to as GFAP (Brenner et al. 1990; Inagaki et al. 1994; Middeldorp and Hol 2011). Sequence analysis of GFAP reveals high sequence similarity between humans and mice (Brenner et al. 1990).

In mice and humans, the *Gfap* promoter is known to have several transcriptional regulators important for *Gfap* expression in astrocytes and upregulation of GFAP in response to injury (Brenner 1994; Brenner et al. 1994; Johnson et al. 1995; Lee et al. 2006; Lee et al. 2008). Specific analysis of a 2.2 kb *GFAP* promoter revealed a TATA-like sequence required for transcription located 29 bp upstream of the transcription start site as well as several positive and negative regulators which upregulate or inhibit transcription respectively (Sarid 1991; Sarkar

and Cowan 1991; Brenner 1994; Kaneko et al. 1994), such as globin transcription factor (GATA), interferon gamma (IFNγ) and NFκB which can negatively regulate GFAP (Brenner 1994; Lee et al. 2008) and activator protein (AP), signal transducer and activator of transcription 3 (STAT3), and cAMP response elements (CRE) which can positively regulate GFAP (Besnard et al. 1991; Brenner 1994; Laping et al. 1994; Takizawa et al. 2001; Lee et al. 2008).

GFAP expression is detectable early in development and is highly variable depending upon the brain region observed (Laping et al. 1994; Martin and O'Callaghan 1995; Mamber et al. 2012). *Gfap* mRNA and protein in mice and humans is highest in the spinal cord and brain stem followed in descending order by hippocampus, olfactory bulb, and cerebellum (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007). Vimentin is normally expressed early in development, with continued expression in fibroblasts, ependymal cells, astrocytes, and glial precursors in adult cells (Schnitzer et al. 1981; Mamber et al. 2012). A third cytoskeletal protein found in astrocytes is nestin. Nestin is expressed in several different types of dividing cells during development, but expression typically does not continue into adulthood (Yachnis et al. 1993; Loo et al. 1994; Von Visger et al. 1994; Mamber et al. 2012). During astrocyte activation, all three intermediate filaments are upregulated, though only vimentin and GFAP appear to have a critical function for astrocyte reactivity (Wilhelmsson et al. 2004).

GFAP ablation in astrocytes limits astrocyte hypertrophy and wound healing though IFs are still present (Eliasson et al. 1999; Pekny et al. 1999; Wilhelmsson et al. 2004; Macauley et al. 2011). Analysis reveals IFs in astrocytes with no GFAP are composed of vimentin and nestin. When vimentin is removed, GFAP is the only protein detectable in the IFs and the IFs are compactly bundled (Eliasson et al. 1999). In both cases, response to injury decreases, healing is prolonged, and limited astrocyte hypertrophy, determined by astrocyte process length, is present (Eliasson et al. 1999; Wilhelmsson et al. 2004; Macauley et al. 2011). Removal of

both GFAP and vimentin reveals no IF formation and limited reactive astrocytosis after injury (Eliasson et al. 1999; Pekny et al. 1999; Wilhelmsson et al. 2004). Overall, this indicated that after an injury vimentin and GFAP were both important for astrocyte hypertrophy and healing function.

Alexander Disease

Alexander disease (AxD) is a rare, fatal neurodegenerative disease first described in 1949 by W. Stewart Alexander (Alexander 1949). Several neurodegenerative diseases occur when proteins begin to abnormally accumulate. Many neurodegenerative diseases impair protein control and degradation pathways such as proteasomes or autophagosomes, resulting in intracellular inclusions or extracellular aggregates (Rubinsztein 2006; Takalo et al. 2013). These aggregates may induce toxicity both in the affected cell and surrounding cells, resulting in loss of structure and function in neural cells (Rubinsztein 2006; Takalo et al. 2013). Several common neurodegenerative diseases include Alzheimer's, Parkinson's, and Huntington's diseases. Some of the current therapies focus on decreasing toxicity and protein accumulation, and increasing degradation pathways (Rubinsztein 2006; Takalo et al. 2013).

AxD was first described in a 15 month old male with an enlarged head, or megalencephaly, developmental deficits, and seizures (Alexander 1949). The individual had no family history of disease and after death, inspection of the brain revealed white matter abnormalities and homogeneous bodies around blood vessels and subpial zones, areas directly below the pia mater or membrane enveloping the brain and spinal cord. Astrocytes were hypertrophied and were closely associated with the bodies (Alexander 1949). In 1964, the disease was first designated Alexander disease and defined by characteristic granular, eosinophilic deposits in perivascular tissue as well as along the pial surface (Friede 1964).

There are three different forms of AxD distinguishable by age of onset (Brenner et al. 2009). Infantile AxD, the most common form, is characterized by white matter degeneration, especially in the frontal portion of the brain (Fig. 1.1A), megalencephaly, seizures, and developmental deficits with onset between 0 and 2 years of age. Death occurs approximately 4 years after presentation with most deaths due to respiratory complications. Juvenile AxD occurs between 2 and 12 years of age and usually presents with bulbar and pseudobulbar difficulties such as difficulties in speech, swallowing and coordination. Megalencephaly is less consistent in the juvenile form of AxD and these individuals typically have later and less rapid deterioration with death normally 8 years after onset. The adult form of AxD occurs after the age of 12, has variable presentation, and is usually the least severe form of AxD. Adult AxD presents with hindbrain symptoms such as ataxia and spasticity with death ranging from 2 to 20 years after onset (Brenner et al. 2009). Recently, a re-classification based on symptoms has been performed (Prust et al. 2011). The new classification has only two different forms, type 1 and type 2, of AxD. To prevent confusion, this paper will only refer to the classic infantile, juvenile, and adult forms of AxD.

One characteristic common to all AxD forms is the presence of the cytoplasmic inclusions known as Rosenthal fibers (RFs) (Herndon et al. 1970; Goldman and Corbin 1988; Tomokane et al. 1991; Wippold et al. 2006). RFs were first noted in 1898 as inclusions closely associated with gliosis (Wippold et al. 2006). RFs are beaded, elongated or corkscrew shaped and are found within the cytoplasm of astrocytes. They are eosinophilic, electron dense structures that stain positive for GFAP (Fig. 1.1B-C) (Herndon et al. 1970; Li et al. 2005; Wippold et al. 2006). Analysis of RFs in AxD reveals not only GFAP immunoreactivity but positive staining for heat shock proteins such as heat shock protein 27 (hsp27) (Head et al. 1993), and alpha-B-crystallin (Iwaki et al. 1992), as well as activated c-Jun N-terminal kinase (JNK), a regulator of apoptosis, and components of the 20S proteasome (Tang et al. 2006).

Finding proteins involved in stress resistance such as heat shock protein and apoptotic regulators may indicate sequestration of the protein into RFs which attenuates stress resistance (Head et al. 1994; Iwaki et al. 1994; Perng et al. 1999). RFs have been noted in multiple sclerosis (MS) (Herndon et al. 1970; Nishie et al. 2004), AxD (Herndon et al. 1970; Goldman and Corbin 1988), brain tumors (Yano et al. 2011; Sugita et al. 2013), and after nickel-induced lesions (Kress et al. 1981), but the diffuse distribution of RFs in the brain of AxD patients distinguishes it from other diseases.

In 2001, five magnetic resonance imaging (MRI) criteria were defined for AxD diagnosis based on typical MRI findings from infantile AxD patients (van der Knaap et al. 2001). Of the five criteria described (1 – white matter abnormalities primarily in the frontal lobe; 2 – enhanced signal on T1-weighting and decreased signal on T2-weight in periventricular rim; 3 – physical and signal abnormalities present in the basal ganglia and thalami; 4 – similar abnormalities in brain stem; 5 – contrast enhancement) only four are necessary for AxD diagnosis (van der Knaap et al. 2001). These characteristics are also present in other white matter disorders, though none of the other white matter disorders meet all the criteria set for AxD diagnosis. However, these characteristics may not recognize atypical cases, especially the adult forms of AxD where some individuals may have only 1 or 2 of the MRI criteria (van der Knaap et al. 2001; Probst et al. 2003; Brenner et al. 2009).

Until 2001, the cause of AxD was unknown though astrocytes and GFAP were hypothesized to be involved (Brenner et al. 2009). It was known that GFAP ablation did not produce symptoms similar to AxD (Gomi et al. 1995; Pekny et al. 1995; Eliasson et al. 1999; Pekny et al. 1999; Brenner et al. 2009). In 1998, Messing, et al (1998) overexpressed wild-type human GFAP in mice and found astrocyte hypertrophy, upregulation of small heat shock proteins, and increased fatality before postnatal day 14. Examination of the brain revealed structures within astrocytes that had similar appearance and characteristics to RFs. These

observations led to GFAP characterization in AxD patients and the discovery of mutations within GFAP (Brenner et al. 2001), providing another type of diagnostic test for AxD.

Mutations in GFAP

Mutations in GFAP are found in greater than 90% of the known AxD cases and usually occur spontaneously in the germ line or during the first stages of development (Li et al. 2006). The individuals without GFAP coding region mutations may have alterations in regulatory genes such as proteins known to modulate IFs which are present in RFs such as αB-crystallin, hsp27, and plectin (Iwaki et al. 1989; Perng et al. 1999; Tian et al. 2006), or mutations occurring later in embryo development which are not present in all cells and are thus undetectable by typical sequencing techniques (Li et al. 2006). Mutations in GFAP are heterozygous and lead to filament disorganization *in vitro* (Hsiao et al. 2005). Most are simple missense mutations caused by changes in a single amino acid (Fig. 1.2, all known mutations found at http://www.waisman.wisc.edu/alexander-disease/mutation-table.pdf). The few familial forms of AxD have no recessive inheritance and present with variable phenotypes (Messing et al. 2012). While the mutations can occur throughout the protein, the highest frequency of mutations associated with AxD occur at the arginine amino acids located at position 79 (R79) and 239 (R239) (36%) (Prust et al. 2011). Interestingly, the same mutation can lead to different forms of AxD, suggesting other alterations such as changes in genetic modifiers which regulate GFAP may be involved (Brenner et al. 2009) .

Mutations in GFAP lead to a gain-of-function phenotype, which is contrary to results in other intermediate filament diseases with homologous mutations (Li et al. 2002). A gain-of-function phenotype was first suggested when GFAP null mice presented no phenotype, were overtly healthy and lived normal life spans (Eliasson et al. 1999; Pekny et al. 1999) while over-expression of GFAP led to increased mortality and presentation of RFs (Messing et al. 1998).

Analysis of the mutations in AxD also shows that none of the known GFAP mutations led to inactivation of the protein such as null, nonsense, or frameshift mutations (Brenner et al. 2009) . Together, this evidence suggests the mutations which are present in AxD lead to a gain-of-function phenotype for GFAP.

Feedback Loops

GFAP mutations may create a positive feedback loop which helps induce GFAP upregulation above an unknown toxic threshold. There are several instances of AxD presentation in patients occurring after injury (Meins et al. 2002; Namekawa et al. 2002) or infection (Herndon et al. 1970; Shiroma et al. 2001; Kyllerman et al. 2005), suggesting an initial reactive gliosis event. While not all AxD patients require an initial stress event, it does suggest that GFAP mutations may create a positive feedback loop with stress response pathways which induces further upregulation of GFAP protein. Some of the stress response pathways involved may be phosphorylation and activation of the JNK pathway which may further impair the proteasome system leading to progressive accumulation of GFAP (Tang et al. 2006; Brenner et al. 2009). Feedback loops may also be exacerbated by the slow turnover rate of GFAP (Chiu and Goldman 1984; Morrison et al. 1985; DeArmond et al. 1986). GFAP and vimentin have a half-life of approximately 8 days in rat primary cultures (Chiu and Goldman 1984; Morrison et al. 1985). In mice, GFAP has a half-life of 9 weeks in the spinal cord (DeArmond et al. 1986) while GFAP in the brain has a half-life of 28 days (Price et al. 2010). Prolonged protein life may also play an important role in GFAP accumulation and feedback loops in AxD.

Mouse Models of AxD

Several mouse models have been created for the study of AxD. The first model, *GFAP*^{Tg}, over-expresses human wild-type GFAP which induces several characteristics similar to human AxD such as upregulation of GFAP, hypertrophy of astrocytes, increased instance of

death compared to wild-type littermate controls, and upregulation of stress pathways (Messing et al. 1998; Hagemann et al. 2005). *GFAP*^{Tg} mice also have RFs and a significant reduction in body weight (Messing et al. 1998). In addition to increased stress response pathway genes, immune response genes are upregulated, including tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), and Mac1, indicating activation of microglia. In *GFAP*^{Tg} mice, after the upregulation in GFAP, microglia appear ramified indicating activation, suggesting a microglial response to the disease itself (Hagemann et al. 2005).

Other mouse models contain knock-in point mutations in GFAP. *Gfap*$^{R236H/+}$ and *Gfap*$^{R76H/+}$ mice contain mutations homologous to common AxD patient mutations (R239H and R79H respectively), and both mutations are present in infantile AxD patients with severe phenotypes. In mice, these mutations present with different severities and GFAP concentrations. Compared with wild-type littermate controls, *Gfap*$^{R236H/+}$ mice have an approximate 4-fold increase in both GFAP protein and mRNA concentrations, a significant reduction in body weight, and an increased susceptibility to seizures (Hagemann et al. 2006). The *Gfap*$^{R76H/+}$ mice have an approximate 1.5-fold increase in GFAP protein and mRNA, with only males showing a significant reduction in body weight (Hagemann et al. 2006). Both *Gfap*$^{R236H/+}$ and *Gfap*$^{R76H/+}$ mice contain RFs especially in the hippocampus, olfactory bulb, corpus callosum, and pial surfaces, but RFs are less prevalent in *Gfap*$^{R76H/+}$ mice. Neither show any overt phenotypes other than the reduced body weight nor do they have unusual mortality or myelination (Hagemann et al. 2006). All three of the mouse models described here provides a spectrum of disease severities for studying AxD in mice.

Genetic background is also important and influences, reproduction, behavior, response to injury, and gene and protein expression including GFAP. Due to the high use of C57BL/6J (B6) and FVB/N mice by our lab, we will focus on known differences between these two strains. Reproductive performance is varied across different mouse strains with FVB/N mice having

larger litter sizes and better maternal instincts and fertility compared with B6 mice (Mouse Phenome Project, http://phenome.jax.org/). Comparison of behavior reveals FVB/N mice are less anxious compared with B6 (Kim et al. 2002). Injury and disease response reveals FVB/N mice are more sensitive to diabetes and insulin resistance (Haluzik et al. 2004) as well as kainic acid-induced seizures (Santos and Schauwecker 2003; Schauwecker 2010).

Comparison of gene expression profiles between different genetic background strains as well as different brain regions reveals differences in genes involved long-term potentiation, spatial learning, structural function, transcriptional regulation, as well as others (Fernandes et al. 2004; Hovatta et al. 2007). Specifically, in hippocampi from both B6 and FVB/N, *Gfap* mRNA expression was significantly higher in FVB/N mice (Fernandes et al. 2004). Bai et al. (in press) compared *Gfap* promoter activity through analysis of green fluorescent protein which was under the control of a human *GFAP* promoter in both B6 and FVB/N mice and found FVB/N mice had elevated transgene expression compared to B6 mice. In mouse models of AxD, FVB/N mice have increased GFAP protein concentration as well as a higher reduction in body weight compared with B6 mice (unpublished observation, T.L. Hagemann; P.L. Jany; C. Lapash Daniels). Overall, these comparisons reveal genetic backgrounds play a role in the levels of endogenous expression of *Gfap* with FVB/N mice displaying the highest level of expression. The difference in *Gfap* expression between B6 and FVB/N may be related to other differences noted in these mice, such as reproductive performance, anxiety, and susceptibility to kainic acid-induced seizures. These results suggest careful consideration and analysis of genetic background during experiments.

Discovery of Potential Drug Treatments for AxD

The only treatments currently available for AxD focus on alleviating symptoms through suppression of seizures, treatment of pulmonary function, providing nutritional supplementation

as well as others (Messing et al. 2010). Two individuals have received treatment with either thyrotropin releasing hormone or ceftriaxone which have resulted in improvement of symptoms, but no change in MRI (Messing et al. 2010). Finding a reliable treatment for AxD which may help alleviate symptoms is highly desirable. Since GFAP appears to play a pivotal role not only in the cause of AxD but also in the regulation of other critical pathways (see feedback loop above), focus has been on drugs which down-regulate GFAP as potential treatments. In a drug screen of FDA-approved drugs, Cho et al. (2010) found several potential drug candidates which decreased both *GFAP* promoter activity and protein concentrations *in vitro*. Studies are currently underway with several of these potential drug candidates to determine their effects on GFAP concentrations *in vivo* in various AxD mouse models.

Drug targets for AxD treatment may also involve indirect regulation of GFAP. A small heat shock protein, αB-crystallin is upregulated both in patients and mouse models of AxD and is also found within RFs (Iwaki et al. 1989; Head et al. 1993; Hagemann et al. 2005; Tian et al. 2006). αB-crystallin attenuates stress response and its sequestration into RFs may lead to increased detrimental stress responses and apoptosis (Head et al. 1994; Iwaki et al. 1994; Mehlen et al. 1995; Koyama and Goldman 1999; Perng et al. 1999). GFAP assembly is regulated by αB-crystallin (Koyama and Goldman 1999; Perng et al. 1999) and over-expression of αB-crystallin results in fewer GFAP aggregates and decreases stress response and mortality in mouse models of AxD (Hagemann et al. 2009). Other drug targets may be revealed as we continue to explore the role of GFAP and other proteins in AxD.

GFAP as a Potential Biomarker of AxD

For testing of drug treatments in both mouse models of AxD and patients, an easily measurable biomarker is necessary. Since the current focus is on drugs which downregulate GFAP either directly or indirectly, measurement of GFAP may provide a reliable biomarker.

Monitoring changes in both GFAP protein and mRNA at various times during treatment will determine if and when a drug is decreasing GFAP. Monitoring both protein and mRNA requires a method for longitudinal studies which may be useful in mouse models of AxD and which might translate into understanding biomarker changes in human AxD patients.

Reporter Mice

A reporter mouse expressing the firefly luciferase gene under the control of a *Gfap* promoter allows indirect analysis of *Gfap* promoter activity in mice and may be useful for studying long-term effects of a drug on *Gfap* promoter activity in the same mouse. Cho et al. (2009) previously created a reporter mouse line expressing firefly luciferase under the control of a 2.2 kb human *GFAP* promoter with an internal control of renilla luciferase controlled by the *GAPDH* promoter, a housekeeping gene (dual luciferase mice). The 2.2 kb human *GFAP* promoter contains most of the regulatory elements required for *GFAP* expression in astrocytes and shows increased *GFAP* gene activity after injury (Brenner 1994; Brenner et al. 1994; Lee et al. 2006; Lee et al. 2008; Cho et al. 2009). Firefly luciferase activity in the dual luciferase mice was highest in the brain with low but detectable levels in the heart. Analysis revealed firefly luciferase immunoreactivity colocalized to GFAP-positive cells in the hippocampus, and luciferase activity preceded protein increases due to seizures (Cho et al. 2009). Interestingly, when mated to $Gfap^{R236H/+}$ mice, luciferase activity was significantly elevated compared with wild-type mice at 3 weeks of age, but not at 6 weeks of age (Cho et al. 2009). In comparison, *Gfap* mRNA in $Gfap^{R236H/+}$ mice was elevated through 8 weeks of age (Hagemann et al. 2006), suggesting some regulatory elements may be missing from the 2.2 kb *GFAP* promoter.

Another reporter mouse line created by Caliper©, *Gfap*-luc, expresses firefly luciferase under the control of a 12 kb mouse *Gfap* promoter (Zhu et al. 2004). These mice have been used in several different injury and disease models to monitor astrogliosis. Initial experiments

subjected *Gfap*-luc mice to kainic acid injections which increased luciferase activity in the brain 40-fold during live *in vivo* imaging. Comparison of mRNA concentrations showed *Gfap* and luciferase mRNA concentrations were positively correlated (Zhu et al. 2004). Subsequent experiments demonstrated upregulated luciferase activity after bacterial infection (Kadurugamuwa et al. 2005), in experimental autoimmune encephalomyelitis (EAE) models of MS (Luo et al. 2008), in prion disease (Tamguney et al. 2009), and in amyotrophic lateral sclerosis (ALS) mouse models (Keller et al. 2009). *Gfap*-luc mice have also been used to study *Gfap* promoter activity after middle cerebral artery occlusion (MCAO) ischemic injury (Cordeau et al. 2008) and in mouse models of Alzheimer's disease (AD) (Watts et al. 2011). In all of these injury and disease models, luciferase activity increases significantly compared with controls and is comparable to mRNA and/or protein levels of GFAP, suggesting *Gfap*-luc reporter mice may provide another method for monitoring *Gfap* promoter activity in mouse models of AxD.

GFAP in Cerebrospinal Fluid

We may also utilize measurement of GFAP concentrations in cerebrospinal fluid (CSF) for monitoring drug treatment effects, a method which easily adapts to human studies. CSF is a clear bodily fluid produced within the choroid plexus which flows throughout the ventricles and surrounds the brain and spinal column (Dandy 1919; Heisey et al. 1962; Milhorat et al. 1971). CSF maintains an external environment for neurons and glia and removes brain metabolites, some of which may be harmful to the brain (Pratico et al. 2004; Iliff et al. 2012). CSF also provides a mechanical cushion to protect the brain from movement and injury. In humans, CSF volume is estimated to be 150 mL with most of the volume contained within the subarachnoid space and major cisterns, and is made at a rate of 0.4 mL per minute (Rubin et al. 1966; Atack et al. 1998; Silverberg et al. 2001). CSF is cleared through arachnoid and nasal lymphatic

vessels where CSF and its molecular components, such as protein, glucose, and metabolites, are absorbed into venous blood (Sweet and Locksley 1953; Hochwald and Wallenstein 1967; Kida et al. 1993; Papaiconomou et al. 2002; Johanson et al. 2008). CSF composition is altered during disease states, allowing detection of distortions in brain metabolism, blood brain barrier transport and permeability, and possible biomarkers for CNS diseases (Lamers et al. 1995; Lamers et al. 2003; Johanson et al. 2008; Czeiter et al. 2012).

In humans, GFAP is detectable in control individuals and elevated after neuropathological conditions such as injury and diseases. Specifically, GFAP is elevated in CSF during MS (Norgren et al. 2004; Axelsson et al. 2011), Parkinson's disease (Constantinescu et al. 2009), AD (Jesse et al. 2009), stroke (Aurell et al. 1991), traumatic brain injury (Zoltewicz et al. 2012), and spinal cord injury (Kwon et al. 2010). In a study by Kyllerman et al. (2005), GFAP was measured in the CSF of three individuals suffering from AxD. GFAP concentrations were significantly higher in all three patients compared with controls, but a study composed of more patients is required to determine whether GFAP is increased in the CSF of all AxD patients and elucidate the range of GFAP concentrations in CSF.

For monitoring drug efficacy in future experiments, we need to establish a correlation between GFAP concentration in CSF and brain. Measuring GFAP concentration in mouse CSF and brain is a feasible way to determine this correlation. The mouse brain contains 40 µL of CSF which is created at a rate of 0.33 µL per minute (Rudick et al. 1982; Oshio et al. 2005; Johanson et al. 2008). Methods of CSF collection in mice are established (DeMattos et al. 2002), though few researchers have utilized these methods. A comparison of mouse CSF and astrocyte-conditioned media (ACM) revealed approximately 2/3 of the detectable proteins were common to both, suggesting astrocytic contribution to mouse CSF (Lafon-Cazal et al. 2003) though GFAP has never been specifically measured in mouse CSF. While it is well established that GFAP is detectable in CSF, it is unclear how GFAP gets into CSF, though possible

mechanisms include astrocytic death, GFAP degradation, and/or GFAP secretion (discussed in more detail in Chapter 5 on page 167).

GFAP in Blood

Measurement of GFAP in blood may also be used for monitoring drug effects on GFAP during treatments. As mentioned previously, CSF is cleared into the venous blood system, therefore GFAP may also be detectable in blood. In humans, GFAP is detectable in the blood of some controls depending on the sensitivity of the assay and elevated in neuropathological conditions such as stroke (Foerch et al. 2012), traumatic brain injury (Nylen et al. 2006), and in Parkinson's disease (Su et al. 2012). Currently, GFAP measurements in blood and CSF are used to determine severity of diseases or long-term consequences of head injury and disease. In most cases, a comparison of values between the different studies suggests GFAP concentrations are lower in blood compared with CSF with a range of 70 ng/L (Vissers et al. 2006; Honda et al. 2010; Foerch et al. 2012) to 400 ng/L (Vos et al. 2004; Wunderlich et al. 2006) in blood compared with a range of 70 ng/L (Rosengren et al. 1992; Gurnett et al. 2003) to 1300 ng/L (Rosengren et al. 1994; Jesse et al. 2009) in CSF. A comparison of GFAP in blood and CSF from the same individual has only been done in a few studies. Notturno et al. (2008) found little difference when comparing blood and CSF GFAP concentrations from the same individual with axonal Guillain-Barre syndrome, a peripheral nerve disease, while Kwon et al. (2010) found GFAP concentrations in CSF were 1000-fold higher compared to serum after spinal cord injury.

GFAP concentration in CSF and blood may also change with age. In both mouse and rat brains, GFAP concentrations have previously been shown to increase with age (Goss et al. 1991; O'Callaghan and Miller 1991), though our lab has found GFAP concentrations only increase up to four weeks of age and then remain unchanged through six months of age

(current study and unpublished observations, J.X. Connor, P.L. Jany). Comparison of GFAP concentration in CSF or blood and age revealed significant linear positive correlation in some studies (Rosengren et al. 1992; Rosengren et al. 1994; Axelsson et al. 2011) while others found no correlation (Ahlsen et al. 1993; Herrmann et al. 2000; Fukuyama et al. 2001; Petzold et al. 2004). Protein concentrations in CSF are influenced by severity and extent of damage, regional variability of protein concentrations, and degradation by proteinases locally or in CSF (Lamers et al. 1995). With increased age, CSF volume may increase due to increased brain atrophy or decreased turnover (Takeda and Matsuzawa 1984; Gideon et al. 1994; Matsumae et al. 1996; Wahlund et al. 1996; Lamers et al. 2003; Nagra and Johnston 2007) which together results in increased protein concentrations. CSF clearance may also alter protein concentrations. Removal of the water channel aquaporin-4 from astrocytes reduced CSF clearance thus increasing protein concentrations (Iliff et al. 2012), suggesting careful comparison of age, disease effects on CSF clearance, and protein concentrations in CSF and/or blood should be considered during biomarker analysis.

Estradiol Effects on GFAP

Potential drug treatments for AxD aim to reduce GFAP protein concentration in the brain below an unknown toxic threshold which may help to alleviate AxD patient symptoms. From the FDA-approved drug screen described previously, several candidate drug therapies were uncovered. One of these drugs was 17β-estradiol (E2) (Cho et al. 2010). E2 is the predominant sex hormone in females and is derived from cholesterol. E2 is produced in the gonads of males and females but can also be produced in fat cells and in the brain (Lara et al. 1990; Fluker et al. 1991; Akwa et al. 1992; Christian and Moenter 2010; Giatti et al. 2012). In AxD, there was some early evidence for sex difference and thus potential E2 regulation of GFAP (Messing et al. 2001; Li et al. 2005), but in a later study comparing a larger group, no sex

differences were found (Prust et al. 2011). Therefore, it is unlikely endogenous E2 plays a role in AxD, but this does not exclude the possibility of treating AxD with E2.

E2 is neuroprotective in rodents after stroke (Lu et al. 2002; Suzuki et al. 2007; Raval et al. 2011) and mouse models of neurological diseases such as Parkinson's disease (Baraka et al. 2011), AD (Cizas et al. 2011), and MS (Bebo et al. 2001; Morales et al. 2006; Offner and Polanczyk 2006; Lelu et al. 2010; MacKenzie-Graham et al. 2012). E2 may also regulate GFAP. Previous studies found two estrogen response elements in the *Gfap* promoter which regulate GFAP expression (Stone et al. 1998; Rozovsky et al. 2002). After injury in primary astrocyte cultures, E2 decreases gliosis and GFAP concentrations (Rozovsky et al. 2002). Mutations within either of the estrogen response elements (EREs) reduce E2 efficacy on gliosis and GFAP (Fig. 1.3A) (Rozovsky et al. 2002). After injury or in neurological disease, E2 may decrease GFAP concentrations in rodents (Rozovsky et al. 2002; Martinez and de Lacalle 2007; Cordeau et al. 2008; Barreto et al. 2009), though GFAP may also increase with E2 treatment (Ritz and Hausmann 2008). Gonadectomy, or removal of gonads, may also alter GFAP concentrations (Day et al. 1993; Garcia-Segura et al. 1994; Martinez and de Lacalle 2007). Different studies indicate that the regulation of GFAP expression by E2 may vary with injury model, E2 dose, treatment timing, gonad contributions, brain region observed, and animal model (Table 1.1).

The Estrus Cycle

Several studies have also looked at GFAP concentration changes during the estrus cycle both with and without injury. In females, E2 regulates the estrus cycle along with several other hormones (Allen 1922; Lara et al. 1990; Fluker et al. 1991; Marcondes et al. 2002; Goldman et al. 2007; Christian and Moenter 2010). E2 regulates hormones such as follicle-stimulating hormone, luteinizing hormone, and gonadotropin-releasing hormone by controlling

the feedback loops to the hypothalamic-pituitary-gonadal (HPG) axis, an important pathway in the estrus cycle (Allen 1922; Christian and Moenter 2010). The estrus cycle is characterized by four different stages: proestrus (PE), estrus (E), metestrus (ME), and diestrus (DE). E2 concentrations rise significantly during PE, peak during E and ovulation, and drop back down to baseline levels by DE (Fig. 1.3B) (Allen 1922; Goldman et al. 2007; Christian and Moenter 2010). The mouse estrus cycle is 4 to 5 days with a one or two day estrus phase (Allen 1922; Byers et al. 2012).

In studies focused on GFAP changes during estrus without injury in rodents, GFAP concentrations typically increase during PE or E compared with DE concentrations (Garcia-Segura et al. 1994; Kohama et al. 1995; Stone et al. 1998; Fujishiro et al. 2002; Martinez et al. 2006; Arias et al. 2009). Regional location of the astrocytes seemed to play a role in E2 response with Stone et al. (1998) showing a significant increase in GFAP within the hypothalamus, but a decrease in the hippocampus during PE. After injury, Cordeau et al. (2008) showed female mice in PE or E had a significantly decreased GFAP response and a reduced ischemic injury size compared to female in ME or DE and males. While it is unclear why there are differences in GFAP response to E2, location and type of astrocyte as well as injury, timing, and E2 concentration may influence the response of GFAP to endogenous E2 (Arevalo et al. 2010).

E2 is synthesized in the brain (neuroestrogen), though it is unknown if synthesis is regulated by the estrus cycle or is independently regulated within the brain (Akwa et al. 1992; Giatti et al. 2012). Comparison of E2 concentration in brain and blood reveal no correlation in rodents (Tagawa et al. 2006; Konkle and McCarthy 2011) and song birds (Schlinger and Remage-Healey 2012). Neuroestrogen synthesis is dependent on cytochrome P450 aromatase activity (Veyrac and Bakker 2011; Toda et al. 2012). After injury, cytochrome P450 aromatase activity increases especially in astrocytes (Garcia-Segura et al. 1999; Peterson et al. 2004;

Duncan and Saldanha 2011; Duncan et al. 2013), suggesting neuroestrogen synthesis may increase immediately following injury though this has not been specifically monitored. Knock-out of cytochrome P450 aromatase or aromatase inhibition does exacerbate ischemic injury (McCullough et al. 2003; Cincioglu et al. 2012; Jazbutyte et al. 2012) indirectly indicating that increased neuroestrogen synthesis is neuroprotective (Garcia-Segura et al. 1999; Peterson et al. 2004).

Estrogen Receptors

E2 acts through classical and non-classical pathways by binding estrogen receptors (ERs) to control gene expression which may be important for the mediation of its neuroprotective role. There are several different ERs, but the main two are estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Both are found in the cytoplasm as well as in the membrane of cells. ERs are composed of three different domains known as the amino-terminal domain (NTD), the DNA binding domain (DBD), and the ligand binding domain (LBD) (Nilsson et al. 2001; Yakimchuk et al. in press). The NTD recruits co-regulator proteins after ligand and DNA binding and is very active in ERα (Cowley and Parker 1999). The DBD is highly conserved between both ERα and ERβ. After ligand binding, ERs form homodimer or heterodimers and then the DBD recognizes and binds estrogen receptor elements (EREs) in the promoter region (Yakimchuk et al. in press). Comparison of ERα and ERβ reveals ERα is more effective at inducing transcription after binding EREs compared with ERβ (Mosselman et al. 1996; McInerney et al. 1998; Cowley and Parker 1999; Foster 2012). Additionally, ERβ has been shown to reduce ERα activity through moderating the magnitude of ERα expression and moderating the proliferation stimulation induced by ERα after binding E2 (Wang et al. 2006; Gottfried-Blackmore et al. 2007; Gonzales et al. 2008; Minutolo et al. 2011; Foster 2012). The third domain of ERs, the LBD, binds ligands such as E2. After binding of the ligand, the ERs

change conformation and begin to interact with co-regulatory factors to enhance transcription. Comparison of the LBD in both ERα and ERβ reveal only minor differences which allows both selective and non-selective binding of ligands. For example, E2 can bind both ERα and ERβ and has similar affinity to both while propyl-pyrazole-triol (PPT) is a specific ERα agonist and diarylpropiolnitrile (DPN) is a specific ERβ agonist (Stauffer et al. 2000; Meyers et al. 2001; Harrington et al. 2003; Carroll et al. 2012; Foster 2012). These different agonists can be used to determine the specific contribution of ERα or ERβ to estrogen induced transcription.

E2 can bind ERs and regulate gene transcription through two different pathways, the classical and non-classical pathways. In the classical pathway, E2 binds cytoplasmic receptors such as ERα and ERβ. The receptor-hormone complex then binds to EREs located in the promoter regions to control gene expression. Both ERα and ERβ bind EREs with similar affinity. After ERs bind to the EREs found in the promoter region, ERs recruit co-regulators, chromatin remodeling proteins, and transcription factors to induce transcription (Wilson et al. 1995; Roepke et al. 2011; Foster 2012; Yakimchuk et al. in press). E2 may also act through the non-classical pathway in which E2 binds a membrane-bound receptor such as G-protein coupled receptor 30 (GPR30) or metabotropic glutamate receptors (mGluRs) (Meldrum 2007; Micevych and Mermelstein 2008; Zarate and Seilicovich 2010). During membrane ER activation, signaling proteins such as protein kinase C, protein kinase A, mitogen-activated protein kinase (MAPK), and extracellular signal-related kinase (ERK) are activated and may regulate signal transduction, protein phosphorylation, and other pathways which may alter gene expression (Kelly and Ronnekleiv 2009; Roepke et al. 2009; Roepke et al. 2011; Yakimchuk et al. in press). Both classical and non-classical actions of E2 control several response pathways which include but are not limited to genes involved in growth hormone secretion, inflammation, antioxidant activity and apoptotic regulation (Green and Simpkins 2000; Spence and Voskuhl 2012).

E2 may also control GFAP concentrations through either or both of these pathways. Astrocytes express both classical and non-classical ERs (Milner et al. 2005; Kim et al. 2007; Mermelstein and Micevych 2008; Carbonaro et al. 2009; Heron et al. 2009; Sakuma et al. 2009; Micevych et al. 2010). After injury, ER expression increases in astrocytes which may vary regionally and in different types of astrocytes (Kim et al. 2007; Carbonaro et al. 2009; Sakuma et al. 2009; Giraud et al. 2010). Knock-out of ERα specifically in astrocytes reduces ERα agonist protective effects in EAE mice (Spence et al. 2011), suggesting E2 neuroprotection and GFAP regulation may be mediated by ERs in astrocytes. Additionally, previous studies indicate ERα but not ERβ is important for GFAP regulation and negative correlates to GFAP concentrations (Dubal et al. 2001; Kim et al. 2007; Crawford et al. 2010; Giraud et al. 2010; Spence et al. 2011). Together, these results indicate E2 treatment may reduce GFAP concentrations possibly through ERs, suggesting E2 might be a good treatment for AxD.

Chapter Overviews

In the second chapter, we explored *Gfap* promoter activity using a luciferase reporter and GFAP in CSF to determine their potential as biomarkers in the brains of mouse models of AxD. Firefly luciferase activity accurately reflects *Gfap* promoter activity, mRNA and protein levels in wild-type mice. In the $Gfap^{R236H/+}$ mice, *Gfap* promoter activity has an early, sustained increase which mimics *Gfap* mRNA and varies regionally. GFAP protein levels in $Gfap^{R236H/+}$ mice varies regionally with higher fold increases compared with both luciferase and mRNA fold increases in most brain regions. GFAP protein in mouse CSF significantly increased in all mouse models of AxD tested ($Gfap^{R76H/+}$, $Gfap^{R236H/+}$, and $GFAP^{Tg}$) in a manner corresponding to concentrations of GFAP seen in the brain. Overall, these data showed *Gfap* promoter activity and GFAP in CSF have a sustained increase in mouse models of AxD which could be exploited for use as a biomarker during studies of potential therapies.

In the third chapter, we determined the efficacy of GFAP as a biomarker for human AxD patients by quantifying GFAP in plasma and CSF of AxD patients compared with controls. GFAP was detectable in most of the control CSF samples and was elevated in almost all the CSF samples from individuals with AxD. For the plasma samples, GFAP was detectable in approximately 60% of the control plasma samples and elevated in most of the AxD patients. No correlation between GFAP concentrations and the age at collection was found in CSF or plasma. Overall, this study showed detectable and elevated GFAP concentrations in AxD patients both in CSF and plasma which may be sites where we can monitor GFAP changes for future drug studies in humans.

In the fourth chapter, we wanted to explore whether E2 treatment decreased GFAP concentrations in the *Gfap*$^{R236H/+}$ mouse model of AxD, providing a potential treatment for AxD. E2 treatment did not alter GFAP mRNA or protein concentrations in both control and *Gfap*$^{R236H/+}$ mice. Gonadectomy also did not increase GFAP concentrations in mice of the B6 background, but did significantly increase GFAP in FVB/N mice, suggesting strain effects in GFAP regulation. During the various stages of estrus, quantification of GFAP revealed no changes in mRNA or protein. These results suggest caution in the implementation of E2 treatment for AxD due to inconsistent results in mice.

Overall, analysis of luciferase activity as an indirect monitor of *Gfap* promoter activity reveals a useful biomarker candidate for longitudinal studies of drug effects in mouse models of AxD. Quantification of GFAP in CSF and blood reveals increased GFAP in AxD patients and mouse models of AxD compared with controls. In mice, the increase of GFAP in CSF was similar to brain GFAP concentrations, suggesting GFAP in CSF reflects brain levels and may be a good biomarker for AxD. Finally, E2 revealed minimal effects on GFAP, indicating caution in the implementation of estrogen-based treatments for AxD. Overall, the conclusions from these

studies reveal GFAP is a useful biomarker for AxD which can be utilized in future clinical trials to determine drug efficacy.

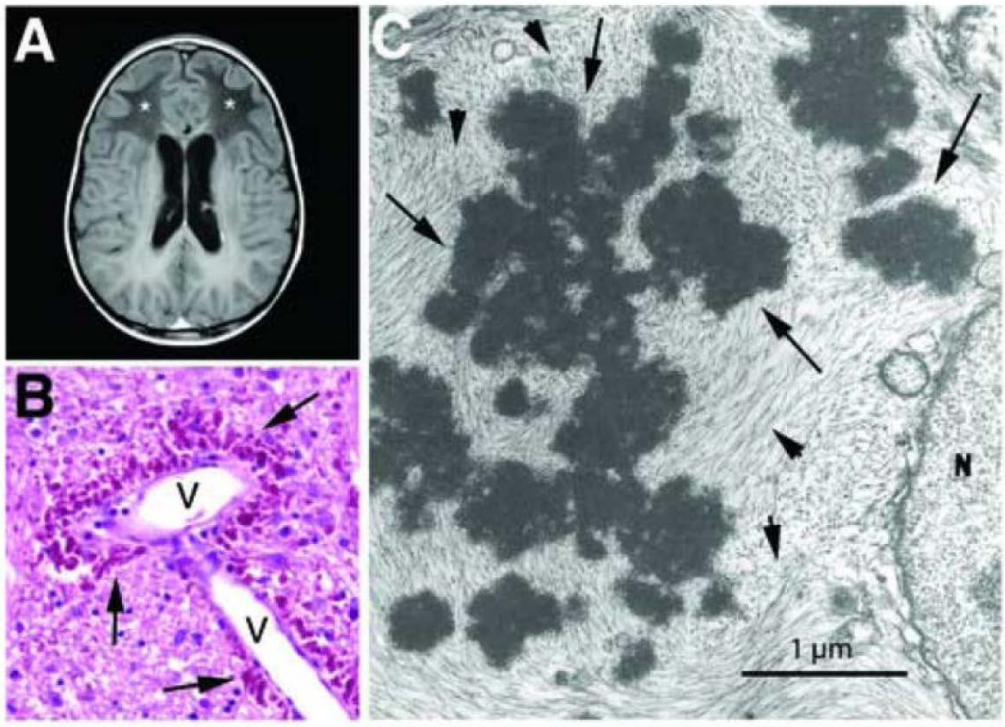

A
B
V
V
C
N
1 µm

Figure 1.1: Features of Alexander disease. (A) MRI image showing extensive white matter deficits (*) in an AxD patient. Rosenthal fibers located in AxD patients are eosinophilic (B) and electron dense (C) structures. Here RFs are shown surrounding blood vessels (V). Previously published as Fig. 1 inQuinlan et al. (2007).

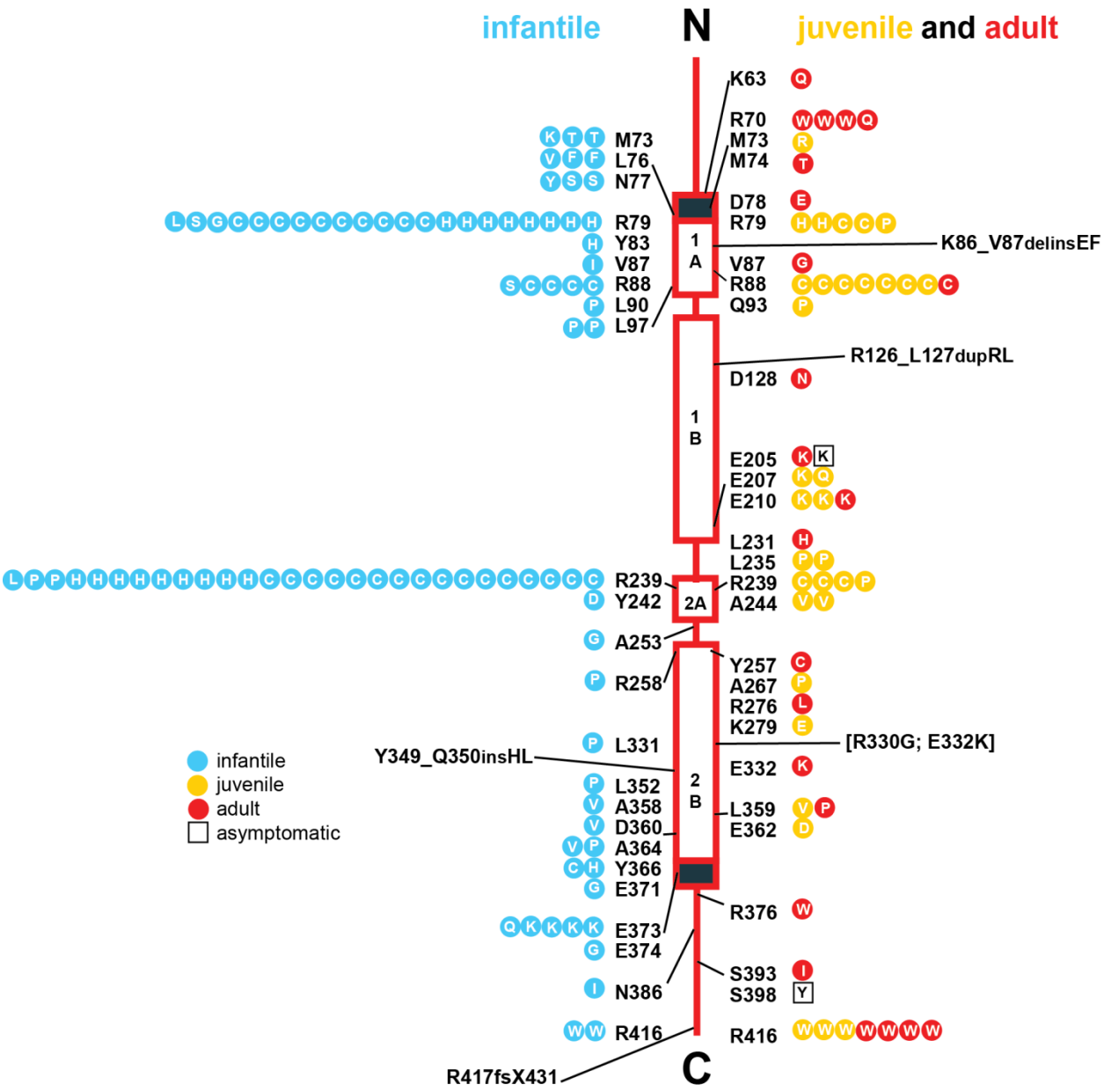
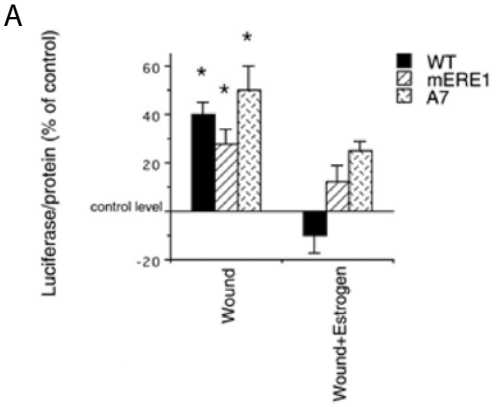

infantile
N
juvenile and adult
K63 Q
R70 WWWQ
M73 R
M74 T
M73
L76
N77
D78 E
R79 HHCCP
R79
Y83
V87
R88
L90
L97
1
A
K86_V87delinsEF
V87 G
R88 CCCCCCCC
Q93 P
R126_L127dupRL
D128 N
1
B
E205 KK
E207 KQ
E210 KKK
L231 H
L235 PP
R239 CCCP
A244 VV
R239
Y242
2A
A253
R258
Y257 C
A267 P
R276 L
K279 E
L331
[R330G; E332K]
Y349_Q350insHL
E332 K
L352
A358
D360
A364
Y366
E371
2
B
L359 VP
E362 D
R376 W
E373
E374
S393 I
S398 Y
N386
R416
R416 WWWWWWW
R417fsX431
C
infantile
juvenile
adult
asymptomatic

Figure 1.2: Known mutations within GFAP from AxD Patients. Mutations in AxD patients can occur throughout GFAP. Each circle represents the form of AxD (infantile – blue; juvenile – yellow; adult – red) and its associated mutation at various locations in the GFAP protein. Asymptomatic patients (□) contain mutations in GFAP, but have none of the symptoms of AxD Previously published as Fig. 24.4 in Brenner et al. (2009).

A


Luciferase/protein (% of control)
60
40
20
control level
-20
WT
mERE1
A7
Wound
Wound+Estrogen

B

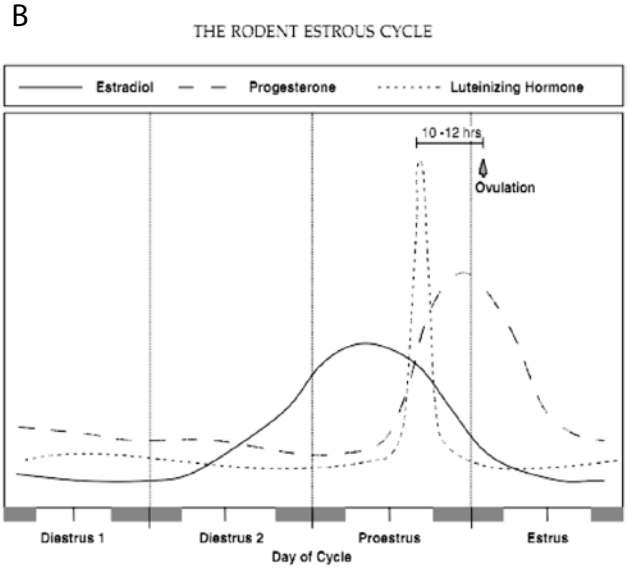

THE RODENT ESTROUS CYCLE
Estradiol
Progesterone
Luteinizing Hormone
10 -12 hrs
Ovulation
Diestrus 1
Diestrus 2
Proestrus
Estrus
Day of Cycle

Figure 1.3: Estrogen response elements in the *Gfap* promoter and hormonal fluctuations during the estrus cycle. (A) Estradiol (E2) regulation of GFAP after wound injury in culture. Astrocytes were transfected with rat *Gfap* promoter which was either full length (WT), contained mutations in estrogen response element 1 (mERE1), or where estrogen response element 2 was removed (A7). E2 significantly reduced astrocyte response to injury. Mutation or removal of either estrogen response element reduced astrocytic response to E2. *, $p<0.05$ compared with control levels. Previously published as Fig. 3 in Rozovsky et al. (2002). (B) Hormonal concentrations during a 4-day estrus cycle in rat serum. Shaded blocks indicate dark portions of a 14:10 hour light:dark day. Previously published as Fig. 3 in Goldman et al. (2007).

					Brain Region								E2 Treatment			Treatment Offset					
Injury	Gonadectomy (Gdx)	Gdx VS Sham	Gdx + E2 VS Gdx	Sham + E2	Whole Brain	Olfactory Bulb	Hippocampus	Cortex	Cerebellum	Hypothalamus	Amygdala	Spinal Cord	E2 Method	E2 Concentration	E2 Duration	From Gdx	From Injury	Species	Sex	Age (mos)	Reference
stab	Y		↓				X						s.c.	3x 100 ug/kg	3d	Post 15d	-	rat	F	2,8, 18	(Barreto et al. 2009)
MCAO	Y	-	↓		X								implt		14d	-	Pre 7d	mouse	M & F	2-3	(Cordeau et al. 2008)
EAE	N			↓								X	pel.	5mg	14-16d		Post 14d	mouse	F		(Giraud et al. 2010)
SCI	N			↑								X	IP	1x 0.1-4 mg/kg	28d		-	rat	M		(Ritz and Hausmann 2008)
ECL	Y		↓				X						pel.	0.72 mg	7d	Post 7d	-	rat	F	3	(Rozovsky et al. 2002)
	Y		-				X	X					s.c.	1,10 ug/ rat/day	14d	-		rat	F	3	(Camacho-Arroyo et al. 2011)
	Y		-				X	X					pel.	0.05,0.5 mg	18 wks	-		rat	F	3	(Camacho-Arroyo et al. 2011)
	Y	↑ -	↓ -				X	X		X			implt		3 wks	-		rat	M	3	(Day et al. 1993)
	Y		↑							X			s.c.	1x 1,10,300 ug/rat	2d	Post 1 mo		rat	F	2	(Garcia-Segura et al. 1994)

					Brain Region								E2 Treatment			Treatment Offset					
Injury	Gonadectomy (Gdx)	Gdx VS Sham	Gdx + E2 VS Gdx	Sham + E2	Whole Brain	Olfactory Bulb	Hippocampus	Cortex	Cerebellum	Hypothalamus	Amygdala	Spinal Cord	E2 Method	E2 Concentration	E2 Duration	From Gdx	From Injury	Species	Sex	Age (mos)	Reference
	Y		↓				X						pel.	1.7 mg	60d	-		mouse	F	20-24	(Lei et al. 2003)
	Y		↑ -				X	X					pel.		5d	Post 2 wks		mouse	F	1	(Levin-Allerhand et al. 2001)
	Y		↑								X		s.c.	3x 10ug/ 0.1mL	4d	Post 1wk		rat	F	3-5	(Martinez et al. 2006)
	Y		↓			X	X	X	X				pel.	0.36 mg	49d	-		mouse	F	2-3	(McAsey et al. 2006)

Table 1.1: GFAP regulation by E2 treatment. GFAP response to gonadectomy (Gdx) and estrogen treatment (E2) both with and without injury. Arrows indicate GFAP response (↑, GFAP increase; ↓, GFAP decrease; -, GFAP unaltered). Brain region for observations (X). If injury field is blank, then there was no injury. E2 treatment method indicates the type of treatment used in each study (s.c., subcutaneous injection; pel., pellet implantation from Innovative Research of America; implt, silastic implant created by researcher; IP, intraperitoneal injection). EAE, experimental autoimmune encephalomyelitis; ECL, entorhinal cortex lesion; MCAO, middle cerebral artery occlusion; SCI, spinal cord injury.

REFERENCE

Ahlsen, G., L. Rosengren, M. Belfrage, A. Palm, K. Haglid, A. Hamberger and C. Gillberg (1993). "Glial fibrillary acidic protein in the cerebrospinal fluid of children with autism and other neuropsychiatric disorders." Biol Psychiatry **33**(10): 734-743.

Akwa, Y., R. F. Morfin, P. Robel and E. E. Baulieu (1992). "Neurosteroid metabolism. 7 alpha-Hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes." Biochem J **288 (Pt 3)**: 959-964.

Albers, K. and E. Fuchs (1987). "The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines." J Cell Biol **105**(2): 791-806.

Alexander, W. S. (1949). "Progressive fibrinoid degeneration of fibrillary astrocytes associated with mental retardation in a hydrocephalic infant." Brain **72**(3): 373-381, 373 pl.

Allen, E. (1922). "The oestrous cycle in the mouse." American Journal of Anatomy **30**(3): 297-371.

Andriezen, W. L. (1893). "The Neuroglia Elements in the Human Brain." Br Med J **2**(1700): 227-230.

Araque, A., V. Parpura, R. P. Sanzgiri and P. G. Haydon (1998). "Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons." Eur J Neurosci **10**(6): 2129-2142.

Arevalo, M. A., M. Santos-Galindo, M. J. Bellini, I. Azcoitia and L. M. Garcia-Segura (2010). "Actions of estrogens on glial cells: Implications for neuroprotection." Biochim Biophys Acta **1800**(10): 1106-1112.

Arias, C., A. Zepeda, K. Hernandez-Ortega, P. Leal-Galicia, C. Lojero and I. Camacho-Arroyo (2009). "Sex and estrous cycle-dependent differences in glial fibrillary acidic protein immunoreactivity in the adult rat hippocampus." Horm Behav **55**(1): 257-263.

Atack, J. R., S. I. Rapoport and M. B. Schapiro (1998). "Cerebrospinal fluid production is normal in Down syndrome." Neurobiol Aging **19**(4): 307-309.

Aurell, A., L. E. Rosengren, B. Karlsson, J. E. Olsson, V. Zbornikova and K. G. Haglid (1991). "Determination of S-100 and glial fibrillary acidic protein concentrations in cerebrospinal fluid after brain infarction." Stroke **22**(10): 1254-1258.

Axelsson, M., C. Malmestrom, S. Nilsson, S. Haghighi, L. Rosengren and J. Lycke (2011). "Glial fibrillary acidic protein: a potential biomarker for progression in multiple sclerosis." J Neurol **258**: 882-888.

Bai, X., A. S. Saab, W. Huang, I. K. Hoberg, F. Kirchhoff and A. Scheller (in press). "Genetic background affects human glial fibrillary acidic protein promoter activity." PLoS One.

Bal-Price, A. and G. C. Brown (2001). "Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity." J Neurosci **21**(17): 6480-6491.

Baraka, A. M., A. A. Korish, G. A. Soliman and H. Kamal (2011). "The possible role of estrogen and selective estrogen receptor modulators in a rat model of Parkinson's disease." Life Sci **88**(19-20): 879-885.

Barkho, B. Z., H. Song, J. B. Aimone, R. D. Smrt, T. Kuwabara, K. Nakashima, . . . X. Zhao (2006). "Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation." Stem Cells Dev **15**(3): 407-421.

Barreto, G., M. Santos-Galindo, Y. Diz-Chaves, O. Pernia, P. Carrero, I. Azcoitia and L. M. Garcia-Segura (2009). "Selective estrogen receptor modulators decrease reactive astrogliosis in the injured brain: effects of aging and prolonged depletion of ovarian hormones." Endocrinology **150**(11): 5010-5015.

Bebo, B. F., Jr., A. Fyfe-Johnson, K. Adlard, A. G. Beam, A. A. Vandenbark and H. Offner (2001). "Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains." J Immunol **166**(3): 2080-2089.

Benington, J. H., S. K. Kodali and H. C. Heller (1995). "Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation." Brain Res **692**(1-2): 79-85.

Besnard, F., M. Brenner, Y. Nakatani, R. Chao, H. J. Purohit and E. Freese (1991). "Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein." J Biol Chem **266**(28): 18877-18883.

Brambilla, R., A. Hurtado, T. Persaud, K. Esham, D. D. Pearse, M. Oudega and J. R. Bethea (2009). "Transgenic inhibition of astroglial NF-kappa B leads to increased axonal sparing and sprouting following spinal cord injury." J Neurochem **110**(2): 765-778.

Brambilla, R., T. Persaud, X. Hu, S. Karmally, V. I. Shestopalov, G. Dvoriantchikova, . . . J. R. Bethea (2009). "Transgenic inhibition of astroglial NF-kappa B improves functional outcome in experimental autoimmune encephalomyelitis by suppressing chronic central nervous system inflammation." J Immunol **182**(5): 2628-2640.

Brenner, M. (1994). "Structure and transcriptional regulation of the GFAP gene." Brain Pathol **4**(3): 245-257.

Brenner, M., J. Goldman, R. Quinlan and A. Messing (2009). Alexander Disease: A Genetic Disorder of Astrocytes. In: Astrocytes in (Patho)Physiology of the Nervous System. P. G. Haydon and V. Parpura, Springer US**:** 591-648.

Brenner, M., A. B. Johnson, O. Boespflug-Tanguy, D. Rodriguez, J. E. Goldman and A. Messing (2001). "Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease." Nat Genet **27**(1): 117-120.

Brenner, M., W. C. Kisseberth, Y. Su, F. Besnard and A. Messing (1994). "GFAP promoter directs astrocyte-specific expression in transgenic mice." J Neurosci **14**(3 Pt 1): 1030-1037.

Brenner, M., K. Lampel, Y. Nakatani, J. Mill, C. Banner, K. Mearow, . . . E. Freese (1990). "Characterization of human cDNA and genomic clones for glial fibrillary acidic protein." Brain Res Mol Brain Res **7**(4): 277-286.

Brightman, M. W. and T. S. Reese (1969). "Junctions between intimately apposed cell membranes in the vertebrate brain." J Cell Biol **40**(3): 648-677.

Bush, T. G., N. Puvanachandra, C. H. Horner, A. Polito, T. Ostenfeld, C. N. Svendsen, . . . M. V. Sofroniew (1999). "Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice." Neuron **23**(2): 297-308.

Byers, S. L., M. V. Wiles, S. L. Dunn and R. A. Taft (2012). "Mouse estrous cycle identification tool and images." PLoS One **7**(4): e35538.

Camacho-Arroyo, I., A. Gonzalez-Arenas, J. Espinosa-Raya, A. G. Pina-Medina and O. Picazo (2011). "Short- and long-term treatment with estradiol or progesterone modifies the expression of GFAP, MAP2 and Tau in prefrontal cortex and hippocampus." Life Sci **89**(3-4): 123-128.

Carbonaro, V., F. Caraci, M. L. Giuffrida, S. Merlo, P. L. Canonico, F. Drago, . . . M. A. Sortino (2009). "Enhanced expression of ERalpha in astrocytes modifies the response of cortical neurons to beta-amyloid toxicity." Neurobiol Dis **33**(3): 415-421.

Carroll, V. M., M. Jeyakumar, K. E. Carlson and J. A. Katzenellenbogen (2012). "Diarylpropionitrile (DPN) enantiomers: synthesis and evaluation of estrogen receptor beta-selective ligands." J Med Chem **55**(1): 528-537.

Chen, H., F. Cabon, P. Sun, E. Parmantier, P. Dupouey, C. Jacque and B. Zalc (1993). "Regional and developmental variations of GFAP and actin mRNA levels in the CNS of jimpy and shiverer mutant mice." J Mol Neurosci **4**(2): 89-96.

Chiu, F. C. and J. E. Goldman (1984). "Synthesis and turnover of cytoskeletal proteins in cultured astrocytes." J Neurochem **42**(1): 166-174.

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Cho, W., T. L. Hagemann, D. A. Johnson, J. A. Johnson and A. Messing (2009). "Dual transgenic reporter mice as a tool for monitoring expression of glial fibrillary acidic protein." J Neurochem **110**(1): 343-351.

Christian, C. A. and S. M. Moenter (2010). "The neurobiology of preovulatory and estradiol-induced gonadotropin-releasing hormone surges." Endocr Rev **31**(4): 544-577.

Cincioglu, M., G. Kismali, S. A. Ugur and P. Kelicen-Ugur (2012). "Indinavir inhibits the expression of cytoplasmic aromatase and nuclear SREBP in the hippocampus of reperfusion injury-induced ischemic rats." J Steroid Biochem Mol Biol **130**(1-2): 81-89.

Cizas, P., A. Jekabsone, V. Borutaite and R. Morkuniene (2011). "Prevention of amyloid-beta oligomer-induced neuronal death by EGTA, estradiol, and endocytosis inhibitor." Medicina (Kaunas) **47**(2): 107-112.

Constantinescu, R., H. Zetterberg, B. Holmberg and L. Rosengren (2009). "Levels of brain related proteins in cerebrospinal fluid: an aid in the differential diagnosis of parkinsonian disorders." Parkinsonism Relat Disord **15**(3): 205-212.

Conway, J. F. and D. A. Parry (1990). "Structural features in the heptad substructure and longer range repeats of two-stranded alpha-fibrous proteins." Int J Biol Macromol **12**(5): 328-334.

Cordeau, P., Jr., M. Lalancette-Hebert, Y. C. Weng and J. Kriz (2008). "Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury." Stroke **39**(3): 935-942.

Coulombe, P. A. and P. Wong (2004). "Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds." Nat Cell Biol **6**(8): 699-706.

Cowley, S. M. and M. G. Parker (1999). "A comparison of transcriptional activation by ER alpha and ER beta." J Steroid Biochem Mol Biol **69**(1-6): 165-175.

Crawford, D. K., M. Mangiardi, B. Song, R. Patel, S. Du, M. V. Sofroniew, . . . S. K. Tiwari-Woodruff (2010). "Oestrogen receptor beta ligand: a novel treatment to enhance endogenous functional remyelination." Brain **133**(10): 2999-3016.

Czeiter, E., S. Mondello, N. Kovacs, J. Sandor, A. Gabrielli, K. Schmid, . . . A. Buki (2012). "Brain injury biomarkers may improve the predictive power of the IMPACT outcome calculator." J Neurotrauma **29**(9): 1770-1778.

Dandy, W. E. (1919). "Experimental Hydrocephalus." Ann Surg **70**(2): 129-142.

Day, J. R., N. J. Laping, M. Lampert-Etchells, S. A. Brown, J. P. O'Callaghan, T. H. McNeill and C. E. Finch (1993). "Gonadal steroids regulate the expression of glial fibrillary acidic protein in the adult male rat hippocampus." Neuroscience **55**(2): 435-443.

DeArmond, S. J., Y. L. Lee, H. A. Kretzschmar and L. F. Eng (1986). "Turnover of glial filaments in mouse spinal cord." J Neurochem **47**(6): 1749-1753.

DeMattos, R. B., K. R. Bales, M. Parsadanian, M. A. O'Dell, E. M. Foss, S. M. Paul and D. M. Holtzman (2002). "Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease." J Neurochem **81**(2): 229-236.

Dubal, D. B., H. Zhu, J. Yu, S. W. Rau, P. J. Shughrue, I. Merchenthaler, . . . P. M. Wise (2001). "Estrogen receptor alpha, not beta, is a critical link in estradiol-mediated protection against brain injury." Proc Natl Acad Sci U S A **98**(4): 1952-1957.

Duncan, K. A. and C. J. Saldanha (2011). "Neuroinflammation induces glial aromatase expression in the uninjured songbird brain." J Neuroinflammation **8**: 81.

Duncan, K. A., B. J. Walters and C. J. Saldanha (2013). "Traumatized and inflamed--but resilient: glial aromatization and the avian brain." Horm Behav **63**(2): 208-215.

Eliasson, C., C. Sahlgren, C. H. Berthold, J. Stakeberg, J. E. Celis, C. Betsholtz, . . . M. Pekny (1999). "Intermediate filament protein partnership in astrocytes." J Biol Chem **274**(34): 23996-24006.

Faulkner, J. R., J. E. Herrmann, M. J. Woo, K. E. Tansey, N. B. Doan and M. V. Sofroniew (2004). "Reactive astrocytes protect tissue and preserve function after spinal cord injury." J Neurosci **24**(9): 2143-2155.

Fernandes, C., J. L. Paya-Cano, F. Sluyter, U. D'Souza, R. Plomin and L. C. Schalkwyk (2004). "Hippocampal gene expression profiling across eight mouse inbred strains: towards understanding the molecular basis for behaviour." Eur J Neurosci **19**(9): 2576-2582.

Fitting, S., S. Zou, W. Chen, P. Vo, K. F. Hauser and P. E. Knapp (2010). "Regional heterogeneity and diversity in cytokine and chemokine production by astroglia: differential responses to HIV-1 Tat, gp120, and morphine revealed by multiplex analysis." J Proteome Res **9**(4): 1795-1804.

Fluker, M. R., L. A. Marshall, S. E. Monroe and R. B. Jaffe (1991). "Variable ovarian response to gonadotropin-releasing hormone antagonist-induced gonadotropin deprivation during different phases of the menstrual cycle." J Clin Endocrinol Metab **72**(4): 912-919.

Foerch, C., M. Niessner, T. Back, M. Bauerle, G. M. De Marchis, A. Ferbert, . . . M. Sitzer (2012). "Diagnostic Accuracy of Plasma Glial Fibrillary Acidic Protein for Differentiating Intracerebral Hemorrhage and Cerebral Ischemia in Patients with Symptoms of Acute Stroke." Clin Chem **58**(1): 237-245.

Foster, T. C. (2012). "Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging." Hippocampus **22**(4): 656-669.

Friede, R. L. (1964). "Alexander's Disease." Arch Neurol **11**: 414-422.

Friedman, W. J., S. Thakur, L. Seidman and A. B. Rabson (1996). "Regulation of nerve growth factor mRNA by interleukin-1 in rat hippocampal astrocytes is mediated by NFkappaB." J Biol Chem **271**(49): 31115-31120.

Fuchs, E. and K. Weber (1994). "Intermediate filaments: structure, dynamics, function, and disease." Annu Rev Biochem **63**: 345-382.

Fujishiro, J., T. Imanishi, K. Onozawa and M. Tsushima (2002). "Comparison of the anticholinergic effects of the serotonergic antidepressants, paroxetine, fluvoxamine and clomipramine." Eur J Pharmacol **454**(2-3): 183-188.

Fukuyama, R., T. Izumoto and S. Fushiki (2001). "The cerebrospinal fluid level of glial fibrillary acidic protein is increased in cerebrospinal fluid from Alzheimer's disease patients and correlates with severity of dementia." Eur Neurol **46**(1): 35-38.

Garcia-Segura, L. M., S. Luquin, A. Parducz and F. Naftolin (1994). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity and glial ultrastructure in the rat neuroendocrine hypothalamus." Glia **10**(1): 59-69.

Garcia-Segura, L. M., A. Wozniak, I. Azcoitia, J. R. Rodriguez, R. E. Hutchison and J. B. Hutchison (1999). "Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair." Neuroscience **89**(2): 567-578.

Geisler, N. and K. Weber (1982). "The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins." EMBO J **1**(12): 1649-1656.

Giatti, S., M. Boraso, R. C. Melcangi and B. Viviani (2012). "Neuroactive steroids, their metabolites, and neuroinflammation." J Mol Endocrinol **49**(3): R125-134.

Gideon, P., C. Thomsen, F. Stahlberg and O. Henriksen (1994). "Cerebrospinal fluid production and dynamics in normal aging: a MRI phase-mapping study." Acta Neurol Scand **89**(5): 362-366.

Giraud, S. N., C. M. Caron, D. Pham-Dinh, P. Kitabgi and A. B. Nicot (2010). "Estradiol inhibits ongoing autoimmune neuroinflammation and NFkappaB-dependent CCL2 expression in reactive astrocytes." Proc Natl Acad Sci U S A **107**(18): 8416-8421.

Goldman, J. E. and E. Corbin (1988). "Isolation of a major protein component of Rosenthal fibers." Am J Pathol **130**(3): 569-578.

Goldman, J. M., A. S. Murr and R. L. Cooper (2007). "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Res B Dev Reprod Toxicol **80**(2): 84-97.

Gomi, H., T. Yokoyama, K. Fujimoto, T. Ikeda, A. Katoh, T. Itoh and S. Itohara (1995). "Mice devoid of the glial fibrillary acidic protein develop normally and are susceptible to scrapie prions." Neuron **14**(1): 29-41.

Gonzales, K. L., M. J. Tetel and C. K. Wagner (2008). "Estrogen receptor (ER) beta modulates ERalpha responses to estrogens in the developing rat ventromedial nucleus of the hypothalamus." Endocrinology **149**(9): 4615-4621.

Goss, J. R., C. E. Finch and D. G. Morgan (1991). "Age-related changes in glial fibrillary acidic protein mRNA in the mouse brain." Neurobiol Aging **12**(2): 165-170.

Gottfried-Blackmore, A., G. Croft, B. S. McEwen and K. Bulloch (2007). "Transcriptional activity of estrogen receptors ERalpha and ERbeta in the EtC.1 cerebellar granule cell line." Brain Res **1186**: 41-47.

Green, P. S. and J. W. Simpkins (2000). "Neuroprotective effects of estrogens: potential mechanisms of action." Int J Dev Neurosci **18**(4-5): 347-358.

Gurnett, C. A., M. Landt and M. Wong (2003). "Analysis of cerebrospinal fluid glial fibrillary acidic protein after seizures in children." Epilepsia **44**(11): 1455-1458.

Hagemann, T. L., W. C. Boelens, E. F. Wawrousek and A. Messing (2009). "Suppression of GFAP toxicity by alphaB-crystallin in mouse models of Alexander disease." Hum Mol Genet **18**(7): 1190-1199.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

Hagemann, T. L., S. A. Gaeta, M. A. Smith, D. A. Johnson, J. A. Johnson and A. Messing (2005). "Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction." Hum Mol Genet **14**(16): 2443-2458.

Halassa, M. M., C. Florian, T. Fellin, J. R. Munoz, S. Y. Lee, T. Abel, . . . M. G. Frank (2009). "Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss." Neuron **61**(2): 213-219.

Haluzik, M., C. Colombo, O. Gavrilova, S. Chua, N. Wolf, M. Chen, . . . M. L. Reitman (2004). "Genetic background (C57BL/6J versus FVB/N) strongly influences the severity of diabetes and insulin resistance in ob/ob mice." Endocrinology **145**(7): 3258-3264.

Hansson, E. (1990). "Regional heterogeneity among astrocytes in the central nervous system." Neurochem Int **16**(3): 237-245.

Harrington, W. R., S. Sheng, D. H. Barnett, L. N. Petz, J. A. Katzenellenbogen and B. S. Katzenellenbogen (2003). "Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression." Mol Cell Endocrinol **206**(1-2): 13-22.

Hatzfeld, M. and K. Weber (1991). "Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain." J Cell Sci **99 (Pt 2)**: 351-362.

Head, M. W., E. Corbin and J. E. Goldman (1993). "Overexpression and abnormal modification of the stress proteins alpha B-crystallin and HSP27 in Alexander disease." Am J Pathol **143**(6): 1743-1753.

Head, M. W., E. Corbin and J. E. Goldman (1994). "Coordinate and independent regulation of alpha B-crystallin and hsp27 expression in response to physiological stress." J Cell Physiol **159**(1): 41-50.

Heisey, S. R., D. Held and J. R. Pappenheimer (1962). "Bulk flow and diffusion in the cerebrospinal fluid system of the goat." Am J Physiol **203**: 775-781.

Heneka, M. T., M. Sastre, L. Dumitrescu-Ozimek, I. Dewachter, J. Walter, T. Klockgether and F. Van Leuven (2005). "Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice." J Neuroinflammation **2**: 22.

Heneka, M. T., H. Wiesinger, L. Dumitrescu-Ozimek, P. Riederer, D. L. Feinstein and T. Klockgether (2001). "Neuronal and glial coexpression of argininosuccinate synthetase

and inducible nitric oxide synthase in Alzheimer disease." J Neuropathol Exp Neurol **60**(9): 906-916.

Herndon, R. M., L. J. Rubinstein, J. M. Freeman and G. Mathieson (1970). "Light and electron microscopic observations on Rosenthal fibers in Alexander's disease and in multiple sclerosis." J Neuropathol Exp Neurol **29**(4): 524-551.

Heron, P. M., J. Turchan-Cholewo, A. J. Bruce-Keller and M. E. Wilson (2009). "Estrogen receptor alpha inhibits the estrogen-mediated suppression of HIV transcription in astrocytes: implications for estrogen neuroprotection in HIV dementia." AIDS Res Hum Retroviruses **25**(11): 1071-1081.

Herrmann, M., P. Vos, M. T. Wunderlich, C. H. de Bruijn and K. J. Lamers (2000). "Release of glial tissue-specific proteins after acute stroke: A comparative analysis of serum concentrations of protein S-100B and glial fibrillary acidic protein." Stroke **31**(11): 2670-2677.

Hirrlinger, J., S. Hulsmann and F. Kirchhoff (2004). "Astroglial processes show spontaneous motility at active synaptic terminals in situ." Eur J Neurosci **20**(8): 2235-2239.

Hochwald, G. M. and M. Wallenstein (1967). "Exchange of albumin between blood, cerebrospinal fluid, and brain in the cat." Am J Physiol **212**(5): 1199-1204.

Honda, M., R. Tsuruta, T. Kaneko, S. Kasaoka, T. Yagi, M. Todani, . . . T. Maekawa (2010). "Serum glial fibrillary acidic protein is a highly specific biomarker for traumatic brain injury in humans compared with S-100B and neuron-specific enolase." J Trauma **69**(1): 104-109.

Hovatta, I., M. A. Zapala, R. S. Broide, E. E. Schadt, O. Libiger, N. J. Schork, . . . C. Barlow (2007). "DNA variation and brain region-specific expression profiles exhibit different relationships between inbred mouse strains: implications for eQTL mapping studies." Genome Biol **8**(2): R25.

Hsiao, V. C., R. Tian, H. Long, M. Der Perng, M. Brenner, R. A. Quinlan and J. E. Goldman (2005). "Alexander-disease mutation of GFAP causes filament disorganization and decreased solubility of GFAP." J Cell Sci **118**(Pt 9): 2057-2065.

Iliff, J. J., M. Wang, Y. Liao, B. A. Plogg, W. Peng, G. A. Gundersen, . . . M. Nedergaard (2012). "A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta." Sci Transl Med **4**(147): 147ra111.

Inagaki, M., Y. Nakamura, M. Takeda, T. Nishimura and N. Inagaki (1994). "Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation." Brain Pathol **4**(3): 239-243.

Iwaki, T., A. Iwaki, J. Tateishi and J. E. Goldman (1994). "Sense and antisense modification of glial alpha B-crystallin production results in alterations of stress fiber formation and thermoresistance." J Cell Biol **125**(6): 1385-1393.

Iwaki, T., A. Kume-Iwaki, R. K. Liem and J. E. Goldman (1989). "Alpha B-crystallin is expressed in non-lenticular tissues and accumulates in Alexander's disease brain." Cell **57**(1): 71-78.

Iwaki, T., T. Wisniewski, A. Iwaki, E. Corbin, N. Tomokane, J. Tateishi and J. E. Goldman (1992). "Accumulation of alpha B-crystallin in central nervous system glia and neurons in pathologic conditions." Am J Pathol **140**(2): 345-356.

Jackson, S. o. t. J. (2013). Mouse Phenome Database, Jackson Laboratories.

Jazbutyte, V., J. Stumpner, A. Redel, J. M. Lorenzen, N. Roewer, T. Thum and F. Kehl (2012). "Aromatase inhibition attenuates desflurane-induced preconditioning against acute myocardial infarction in male mouse heart in vivo." PLoS One **7**(8): e42032.

Jesse, S., P. Steinacker, L. Cepek, C. A. von Arnim, H. Tumani, S. Lehnert, . . . M. Otto (2009). "Glial fibrillary acidic protein and protein S-100B: different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease." J Alzheimers Dis **17**(3): 541-551.

Johanson, C. E., J. A. Duncan, 3rd, P. M. Klinge, T. Brinker, E. G. Stopa and G. D. Silverberg (2008). "Multiplicity of cerebrospinal fluid functions: New challenges in health and disease." Cerebrospinal Fluid Res **5**: 10.

Johnson, W. B., M. D. Ruppe, E. M. Rockenstein, J. Price, V. P. Sarthy, L. C. Verderber and L. Mucke (1995). "Indicator expression directed by regulatory sequences of the glial fibrillary acidic protein (GFAP) gene: in vivo comparison of distinct GFAP-lacZ transgenes." Glia **13**(3): 174-184.

Kadurugamuwa, J. L., K. Modi, O. Coquoz, B. Rice, S. Smith, P. R. Contag and T. Purchio (2005). "Reduction of astrogliosis by early treatment of pneumococcal meningitis measured by simultaneous imaging, in vivo, of the pathogen and host response." Infect Immun **73**(12): 7836-7843.

Kaneko, R., N. Hagiwara, K. Leader and N. Sueoka (1994). "Glial-specific cAMP response of the glial fibrillary acidic protein gene cell lines." Proc Natl Acad Sci U S A **91**(10): 4529-4533.

Keller, A. F., M. Gravel and J. Kriz (2009). "Live imaging of amyotrophic lateral sclerosis pathogenesis: disease onset is characterized by marked induction of GFAP in Schwann cells." Glia **57**(10): 1130-1142.

Kelly, M. J. and O. K. Ronnekleiv (2009). "Control of CNS neuronal excitability by estrogens via membrane-initiated signaling." Mol Cell Endocrinol **308**(1-2): 17-25.

Kida, S., A. Pantazis and R. O. Weller (1993). "CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance." Neuropathol Appl Neurobiol **19**(6): 480-488.

Kim, E. J., C. S. Oh, J. Kim, W. H. Kim, Y. H. Chung and D. H. Shin (2007). "Reactive astrocytes expressing intense estrogen receptor-alpha immunoreactivities have much elongated cytoplasmic processes: an autopsy case of human cerebellar tissue with multiple genitourinary and gastrointestinal anomalies." J Korean Med Sci **22**(5): 936-941.

Kim, S., S. Lee, S. Ryu, J. Suk and C. Park (2002). "Comparative analysis of the anxiety-related behaviors in four inbred mice." Behav Processes **60**(2): 181-190.

Kohama, S. G., J. R. Goss, T. H. McNeill and C. E. Finch (1995). "Glial fibrillary acidic protein mRNA increases at proestrus in the arcuate nucleus of mice." Neurosci Lett **183**(3): 164-166.

Konkle, A. T. and M. M. McCarthy (2011). "Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain." Endocrinology **152**(1): 223-235.

Koyama, Y. and J. E. Goldman (1999). "Formation of GFAP cytoplasmic inclusions in astrocytes and their disaggregation by alphaB-crystallin." Am J Pathol **154**(5): 1563-1572.

Kress, Y., F. Gaskin, D. S. Horoupian and C. Brosnan (1981). "Nickel induction of Rosenthal fibers in rat brain." Brain Res **210**(1-2): 419-425.

Kwon, B. K., A. M. Stammers, L. M. Belanger, A. Bernardo, D. Chan, C. M. Bishop, . . . M. F. Dvorak (2010). "Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury." J Neurotrauma **27**(4): 669-682.

Kyllerman, M., L. Rosengren, L. M. Wiklund and E. Holmberg (2005). "Increased levels of GFAP in the cerebrospinal fluid in three subtypes of genetically confirmed Alexander disease." Neuropediatrics **36**(5): 319-323.

Lafon-Cazal, M., O. Adjali, N. Galeotti, J. Poncet, P. Jouin, V. Homburger, . . . P. Marin (2003). "Proteomic analysis of astrocytic secretion in the mouse. Comparison with the cerebrospinal fluid proteome." J Biol Chem **278**(27): 24438-24448.

Lamers, K. J., B. G. van Engelen, F. J. Gabreels, O. R. Hommes, G. F. Borm and R. A. Wevers (1995). "Cerebrospinal neuron-specific enolase, S-100 and myelin basic protein in neurological disorders." Acta Neurol Scand **92**(3): 247-251.

Lamers, K. J., P. Vos, M. M. Verbeek, F. Rosmalen, W. J. van Geel and B. G. van Engelen (2003). "Protein S-100B, neuron-specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) in cerebrospinal fluid (CSF) and blood of neurological patients." Brain Res Bull **61**(3): 261-264.

Laping, N. J., B. Teter, C. P. Anderson, H. H. Osterburg, J. P. O'Callaghan, S. A. Johnson and C. E. Finch (1994). "Age-related increases in glial fibrillary acidic protein do not show proportionate changes in transcription rates or DNA methylation in the cerebral cortex and hippocampus of male rats." J Neurosci Res **39**(6): 710-717.

Laping, N. J., B. Teter, N. R. Nichols, I. Rozovsky and C. E. Finch (1994). "Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors." Brain Pathol **4**(3): 259-275.

Lara, H. E., J. K. McDonald, C. E. Ahmed and S. R. Ojeda (1990). "Guanethidine-mediated destruction of ovarian sympathetic nerves disrupts ovarian development and function in rats." Endocrinology **127**(5): 2199-2209.

Lee, Y., A. Messing, M. Su and M. Brenner (2008). "GFAP promoter elements required for region-specific and astrocyte-specific expression." Glia **56**(5): 481-493.

Lee, Y., M. Su, A. Messing and M. Brenner (2006). "Astrocyte heterogeneity revealed by expression of a GFAP-LacZ transgene." Glia **53**(7): 677-687.

Lei, D. L., J. M. Long, J. Hengemihle, J. O'Neill, K. F. Manaye, D. K. Ingram and P. R. Mouton (2003). "Effects of estrogen and raloxifene on neuroglia number and morphology in the hippocampus of aged female mice." Neuroscience **121**(3): 659-666.

Lein, E. S., M. J. Hawrylycz, N. Ao, M. Ayres, A. Bensinger, A. Bernard, . . . A. R. Jones (2007). "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124): 168-176.

Lelu, K., L. Delpy, V. Robert, E. Foulon, S. Laffont, L. Pelletier, . . . J. C. Guery (2010). "Endogenous estrogens, through estrogen receptor alpha, constrain autoimmune inflammation in female mice by limiting CD4+ T-cell homing into the CNS." Eur J Immunol **40**(12): 3489-3498.

Letai, A., P. A. Coulombe and E. Fuchs (1992). "Do the ends justify the mean? Proline mutations at the ends of the keratin coiled-coil rod segment are more disruptive than internal mutations." J Cell Biol **116**(5): 1181-1195.

Levin-Allerhand, J., B. S. McEwen, C. E. Lominska, D. B. Lubahn, K. S. Korach and J. D. Smith (2001). "Brain region-specific up-regulation of mouse apolipoprotein E by pharmacological estrogen treatments." J Neurochem **79**(4): 796-803.

Li, L., A. Lundkvist, D. Andersson, U. Wilhelmsson, N. Nagai, A. C. Pardo, . . . M. Pekny (2008). "Protective role of reactive astrocytes in brain ischemia." J Cereb Blood Flow Metab **28**(3): 468-481.

Li, R., A. B. Johnson, G. Salomons, J. E. Goldman, S. Naidu, R. Quinlan, . . . M. Brenner (2005). "Glial fibrillary acidic protein mutations in infantile, juvenile, and adult forms of Alexander disease." Ann Neurol **57**(3): 310-326.

Li, R., A. B. Johnson, G. S. Salomons, M. S. van der Knaap, D. Rodriguez, O. Boespflug-Tanguy, . . . M. Brenner (2006). "Propensity for paternal inheritance of de novo mutations in Alexander disease." Hum Genet **119**(1-2): 137-144.

Li, R., A. Messing, J. E. Goldman and M. Brenner (2002). "GFAP mutations in Alexander disease." Int J Dev Neurosci **20**(3-5): 259-268.

Loo, D. T., M. C. Althoen and C. W. Cotman (1994). "Down regulation of nestin by TGF-beta or serum in SFME cells accompanies differentiation into astrocytes." Neuroreport **5**(13): 1585-1588.

Lovatt, D., U. Sonnewald, H. S. Waagepetersen, A. Schousboe, W. He, J. H. Lin, . . . M. Nedergaard (2007). "The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex." J Neurosci **27**(45): 12255-12266.

Lu, A., R. Q. Ran, J. Clark, M. Reilly, A. Nee and F. R. Sharp (2002). "17-beta-estradiol induces heat shock proteins in brain arteries and potentiates ischemic heat shock protein induction in glia and neurons." J Cereb Blood Flow Metab **22**(2): 183-195.

Lu, X. and E. B. Lane (1990). "Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation." Cell **62**(4): 681-696.

Luo, J., P. Ho, L. Steinman and T. Wyss-Coray (2008). "Bioluminescence in vivo imaging of autoimmune encephalomyelitis predicts disease." J Neuroinflammation **5**: 6.

Macauley, S. L., M. Pekny and M. S. Sands (2011). "The role of attenuated astrocyte activation in infantile neuronal ceroid lipofuscinosis." J Neurosci **31**(43): 15575-15585.

MacKenzie-Graham, A. J., G. A. Rinek, A. Avedisian, L. B. Morales, E. Umeda, B. Boulat, . . . R. R. Voskuhl (2012). "Estrogen treatment prevents gray matter atrophy in experimental autoimmune encephalomyelitis." J Neurosci Res **90**(7): 1310-1323.

Mamber, C., W. Kamphuis, N. L. Haring, N. Peprah, J. Middeldorp and E. M. Hol (2012). "GFAPdelta Expression in Glia of the Developmental and Adolescent Mouse Brain." PLoS One **7**(12): e52659.

Marchuk, D., S. McCrohon and E. Fuchs (1984). "Remarkable conservation of structure among intermediate filament genes." Cell **39**(3 Pt 2): 491-498.

Marcondes, F. K., F. J. Bianchi and A. P. Tanno (2002). "Determination of the estrous cycle phases of rats: some helpful considerations." Braz J Biol **62**(4A): 609-614.

Martin, P. M. and J. P. O'Callaghan (1995). "A direct comparison of GFAP immunocytochemistry and GFAP concentration in various regions of ethanol-fixed rat and mouse brain." J Neurosci Methods **58**(1-2): 181-192.

Martinez, F. G., E. E. Hermel, L. L. Xavier, G. G. Viola, J. Riboldi, A. A. Rasia-Filho and M. Achaval (2006). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats." Brain Res **1108**(1): 117-126.

Martinez, L. and S. de Lacalle (2007). "Astrocytic reaction to a lesion, under hormonal deprivation." Neurosci Lett **415**(2): 190-193.

Matsumae, M., R. Kikinis, I. A. Morocz, A. V. Lorenzo, T. Sandor, M. S. Albert, . . . F. A. Jolesz (1996). "Age-related changes in intracranial compartment volumes in normal adults assessed by magnetic resonance imaging." J Neurosurg **84**(6): 982-991.

McAsey, M. E., C. Cady, L. M. Jackson, M. Li, S. Randall, B. P. Nathan and R. G. Struble (2006). "Time course of response to estradiol replacement in ovariectomized mice: brain apolipoprotein E and synaptophysin transiently increase and glial fibrillary acidic protein is suppressed." Exp Neurol **197**(1): 197-205.

McCullough, L. D., K. Blizzard, E. R. Simpson, O. K. Oz and P. D. Hurn (2003). "Aromatase cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection." J Neurosci **23**(25): 8701-8705.

McInerney, E. M., K. E. Weis, J. Sun, S. Mosselman and B. S. Katzenellenbogen (1998). "Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras." Endocrinology **139**(11): 4513-4522.

McLachlan, A. D. (1978). "Coiled coil formation and sequence regularities in the helical regions of alpha-keratin." J Mol Biol **124**(1): 297-304.

Mehlen, P., X. Preville, P. Chareyron, J. Briolay, R. Klemenz and A. P. Arrigo (1995). "Constitutive expression of human hsp27, Drosophila hsp27, or human alpha B-crystallin

confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts." J Immunol **154**(1): 363-374.

Meins, M., K. Brockmann, S. Yadav, M. Haupt, J. Sperner, U. Stephani and F. Hanefeld (2002). "Infantile Alexander disease: a GFAP mutation in monozygotic twins and novel mutations in two other patients." Neuropediatrics **33**(4): 194-198.

Meldrum, D. R. (2007). "G-protein-coupled receptor 30 mediates estrogen's nongenomic effects after hemorrhagic shock and trauma." Am J Pathol **170**(4): 1148-1151.

Mermelstein, P. G. and P. E. Micevych (2008). "Nervous system physiology regulated by membrane estrogen receptors." Rev Neurosci **19**(6): 413-424.

Messing, A., J. E. Goldman, A. B. Johnson and M. Brenner (2001). "Alexander disease: new insights from genetics." J Neuropathol Exp Neurol **60**(6): 563-573.

Messing, A., M. W. Head, K. Galles, E. J. Galbreath, J. E. Goldman and M. Brenner (1998). "Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice." Am J Pathol **152**(2): 391-398.

Messing, A., C. M. LaPash Daniels and T. L. Hagemann (2010). "Strategies for treatment in Alexander disease." Neurotherapeutics **7**(4): 507-515.

Messing, A., R. Li, S. Naidu, J. P. Taylor, L. Silverman, D. Flint, . . . M. Brenner (2012). "Archetypal and new families with Alexander disease and novel mutations in GFAP." Arch Neurol **69**(2): 208-214.

Meyers, M. J., J. Sun, K. E. Carlson, G. A. Marriner, B. S. Katzenellenbogen and J. A. Katzenellenbogen (2001). "Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues." J Med Chem **44**(24): 4230-4251.

Micevych, P., G. Bondar and J. Kuo (2010). "Estrogen actions on neuroendocrine glia." Neuroendocrinology **91**(3): 211-222.

Micevych, P. E. and P. G. Mermelstein (2008). "Membrane estrogen receptors acting through metabotropic glutamate receptors: an emerging mechanism of estrogen action in brain." Mol Neurobiol **38**(1): 66-77.

Middeldorp, J. and E. M. Hol (2011). "GFAP in health and disease." Prog Neurobiol **93**(3): 421-443.

Milhorat, T. H., M. K. Hammock, J. D. Fenstermacher and V. A. Levin (1971). "Cerebrospinal fluid production by the choroid plexus and brain." Science **173**(3994): 330-332.

Milner, T. A., K. Ayoola, C. T. Drake, S. P. Herrick, N. E. Tabori, B. S. McEwen, . . . S. E. Alves (2005). "Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation." J Comp Neurol **491**(2): 81-95.

Minutolo, F., M. Macchia, B. S. Katzenellenbogen and J. A. Katzenellenbogen (2011). "Estrogen receptor beta ligands: recent advances and biomedical applications." Med Res Rev **31**(3): 364-442.

Morales, L. B., K. K. Loo, H. B. Liu, C. Peterson, S. Tiwari-Woodruff and R. R. Voskuhl (2006). "Treatment with an estrogen receptor alpha ligand is neuroprotective in experimental autoimmune encephalomyelitis." J Neurosci **26**(25): 6823-6833.

Morrison, R. S., J. De Vellis, Y. L. Lee, R. A. Bradshaw and L. F. Eng (1985). "Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes." J Neurosci Res **14**(2): 167-176.

Mosselman, S., J. Polman and R. Dijkema (1996). "ER beta: identification and characterization of a novel human estrogen receptor." FEBS Lett **392**(1): 49-53.

Nagra, G. and M. G. Johnston (2007). "Impact of ageing on lymphatic cerebrospinal fluid absorption in the rat." Neuropathol Appl Neurobiol **33**(6): 684-691.

Namekawa, M., Y. Takiyama, Y. Aoki, N. Takayashiki, K. Sakoe, H. Shimazaki, . . . I. Nakano (2002). "Identification of *GFAP* gene mutation in hereditary adult-onset Alexander's disease." Annals of Neurology **52**(6): 779-785.

Nilsson, S., S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, . . . J. A. Gustafsson (2001). "Mechanisms of estrogen action." Physiol Rev **81**(4): 1535-1565.

Nishie, M., F. Mori, M. Ogawa, S. Sannohe, K. Tanno, K. Kurahashi, . . . K. Wakabayashi (2004). "Multinucleated astrocytes in old demyelinated plaques in a patient with multiple sclerosis." Neuropathology **24**(3): 248-253.

Norgren, N., P. Sundstrom, A. Svenningsson, L. Rosengren, T. Stigbrand and M. Gunnarsson (2004). "Neurofilament and glial fibrillary acidic protein in multiple sclerosis." Neurology **63**(9): 1586-1590.

Notturno, F., C. M. Caporale, A. De Lauretis and A. Uncini (2008). "Glial fibrillary acidic protein: a marker of axonal Guillain-Barre syndrome and outcome." Muscle Nerve **38**(1): 899-903.

Nylen, K., M. Ost, L. Z. Csajbok, I. Nilsson, K. Blennow, B. Nellgard and L. Rosengren (2006). "Increased serum-GFAP in patients with severe traumatic brain injury is related to outcome." J Neurol Sci **240**(1-2): 85-91.

O'Callaghan, J. P. and D. B. Miller (1991). "The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain." Neurobiol Aging **12**(2): 171-174.

Offner, H. and M. Polanczyk (2006). "A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis." Ann N Y Acad Sci **1089**: 343-372.

Okada, S., M. Nakamura, H. Katoh, T. Miyao, T. Shimazaki, K. Ishii, . . . H. Okano (2006). "Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury." Nat Med **12**(7): 829-834.

Oliet, S. H., R. Piet and D. A. Poulain (2001). "Control of glutamate clearance and synaptic efficacy by glial coverage of neurons." Science **292**(5518): 923-926.

Oshio, K., H. Watanabe, Y. Song, A. S. Verkman and G. T. Manley (2005). "Reduced cerebrospinal fluid production and intracranial pressure in mice lacking choroid plexus water channel Aquaporin-1." FASEB J **19**(1): 76-78.

Palfreyman, J. W., D. G. Thomas, J. G. Ratcliffe and D. I. Graham (1979). "Glial fibrillary acidic protein (GFAP): purification from human fibrillary astrocytoma, development and validation of a radioimmunoassay for GFAP-like immunoactivity." J Neurol Sci **41**(1): 101-113.

Papaiconomou, C., R. Bozanovic-Sosic, A. Zakharov and M. Johnston (2002). "Does neonatal cerebrospinal fluid absorption occur via arachnoid projections or extracranial lymphatics?" Am J Physiol Regul Integr Comp Physiol **283**(4): R869-876.

Pauling, L. and R. B. Corey (1953). "Compound helical configurations of polypeptide chains: structure of proteins of the alpha-keratin type." Nature **171**(4341): 59-61.

Pekny, M., C. Eliasson, R. Siushansian, M. Ding, S. J. Dixon, M. Pekna, . . . A. Hamberger (1999). "The impact of genetic removal of GFAP and/or vimentin on glutamine levels and transport of glucose and ascorbate in astrocytes." Neurochem Res **24**(11): 1357-1362.

Pekny, M., P. Leveen, M. Pekna, C. Eliasson, C. H. Berthold, B. Westermark and C. Betsholtz (1995). "Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally." EMBO J **14**(8): 1590-1598.

Pekny, M. and M. Nilsson (2005). "Astrocyte activation and reactive gliosis." Glia **50**(4): 427-434.

Pekny, M. and M. Pekna (2004). "Astrocyte intermediate filaments in CNS pathologies and regeneration." J Pathol **204**(4): 428-437.

Perng, M. D., L. Cairns, I. P. van den, A. Prescott, A. M. Hutcheson and R. A. Quinlan (1999). "Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin." J Cell Sci **112 (Pt 13)**: 2099-2112.

Peterson, R. S., D. W. Lee, G. Fernando and B. A. Schlinger (2004). "Radial glia express aromatase in the injured zebra finch brain." J Comp Neurol **475**(2): 261-269.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Pratico, D., Y. Yao, J. Rokach, M. Mayo, G. G. Silverberg and D. McGuire (2004). "Reduction of brain lipid peroxidation by CSF drainage in Alzheimer's disease patients." J Alzheimers Dis **6**(4): 385-389; discussion 443-389.

Price, J. C., S. Guan, A. Burlingame, S. B. Prusiner and S. Ghaemmaghami (2010). "Analysis of proteome dynamics in the mouse brain." Proc Natl Acad Sci U S A **107**(32): 14508-14513.

Probst, E. N., C. Hagel, V. Weisz, S. Nagel, O. Wittkugel, H. Zeumer and A. Kohlschutter (2003). "Atypical focal MRI lesions in a case of juvenile Alexander's disease." Ann Neurol **53**(1): 118-120.

Prust, M., J. Wang, H. Morizono, A. Messing, M. Brenner, E. Gordon, . . . A. Vanderver (2011). "GFAP mutations, age at onset, and clinical subtypes in Alexander disease." Neurology **77**(13): 1287-1294.

Quinlan, R. A., M. Brenner, J. E. Goldman and A. Messing (2007). "GFAP and its role in Alexander disease." Exp Cell Res **313**(10): 2077-2087.

Raval, A. P., N. Hirsch, K. R. Dave, D. R. Yavagal, H. Bramlett and I. Saul (2011). "Nicotine and estrogen synergistically exacerbate cerebral ischemic injury." Neuroscience **181**: 216-225.

Regan, M. R., Y. H. Huang, Y. S. Kim, M. I. Dykes-Hoberg, L. Jin, A. M. Watkins, . . . J. D. Rothstein (2007). "Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS." J Neurosci **27**(25): 6607-6619.

Ritz, M. F. and O. N. Hausmann (2008). "Effect of 17beta-estradiol on functional outcome, release of cytokines, astrocyte reactivity and inflammatory spreading after spinal cord injury in male rats." Brain Res **1203**: 177-188.

Roepke, T. A., J. Qiu, M. A. Bosch, O. K. Ronnekleiv and M. J. Kelly (2009). "Cross-talk between membrane-initiated and nuclear-initiated oestrogen signalling in the hypothalamus." J Neuroendocrinol **21**(4): 263-270.

Roepke, T. A., O. K. Ronnekleiv and M. J. Kelly (2011). "Physiological consequences of membrane-initiated estrogen signaling in the brain." Front Biosci **16**: 1560-1573.

Rosengren, L. E., G. Ahlsen, M. Belfrage, C. Gillberg, K. G. Haglid and A. Hamberger (1992). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of children." J Neurosci Methods **44**(2-3): 113-119.

Rosengren, L. E., C. Wikkelso and L. Hagberg (1994). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of adults." J Neurosci Methods **51**(2): 197-204.

Rothstein, J. D., M. Dykes-Hoberg, C. A. Pardo, L. A. Bristol, L. Jin, R. W. Kuncl, . . . D. F. Welty (1996). "Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate." Neuron **16**(3): 675-686.

Rozovsky, I., M. Wei, D. J. Stone, H. Zanjani, C. P. Anderson, T. E. Morgan and C. E. Finch (2002). "Estradiol (E2) enhances neurite outgrowth by repressing glial fibrillary acidic protein expression and reorganizing laminin." Endocrinology **143**(2): 636-646.

Rubin, R. C., E. S. Henderson, A. K. Ommaya, M. D. Walker and D. P. Rall (1966). "The production of cerebrospinal fluid in man and its modification by acetazolamide." J Neurosurg **25**(4): 430-436.

Rubinsztein, D. C. (2006). "The roles of intracellular protein-degradation pathways in neurodegeneration." Nature **443**(7113): 780-786.

Rudick, R. A., D. K. Zirretta and R. M. Herndon (1982). "Clearance of albumin from mouse subarachnoid space: a measure of CSF bulk flow." J Neurosci Methods **6**(3): 253-259.

Sakuma, S., D. Tokuhara, H. Hattori, O. Matsuoka and T. Yamano (2009). "Expression of estrogen receptor alpha and beta in reactive astrocytes at the male rat hippocampus after status epilepticus." Neuropathology **29**(1): 55-62.

Santos, J. B. and P. E. Schauwecker (2003). "Protection provided by cyclosporin A against excitotoxic neuronal death is genotype dependent." Epilepsia **44**(8): 995-1002.

Sarid, J. (1991). "Identification of a cis-acting positive regulatory element of the glial fibrillary acidic protein gene." J Neurosci Res **28**(2): 217-228.

Sarkar, S. and N. J. Cowan (1991). "Intragenic sequences affect the expression of the gene encoding glial fibrillary acidic protein." J Neurochem **57**(2): 675-684.

Schauwecker, P. E. (2010). "Neuroprotection by glutamate receptor antagonists against seizure-induced excitotoxic cell death in the aging brain." Exp Neurol **224**(1): 207-218.

Schlinger, B. A. and L. Remage-Healey (2012). "Neurosteroidogenesis: insights from studies of songbirds." J Neuroendocrinol **24**(1): 16-21.

Schnitzer, J., W. W. Franke and M. Schachner (1981). "Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system." J Cell Biol **90**(2): 435-447.

Shih, A. Y., D. A. Johnson, G. Wong, A. D. Kraft, L. Jiang, H. Erb, . . . T. H. Murphy (2003). "Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress." J Neurosci **23**(8): 3394-3406.

Shiroma, N., N. Kanazawa, M. Izumi, K. Sugai, M. Fukumizu, M. Sasaki, . . . S. Tsujino (2001). "Diagnosis of Alexander disease in a Japanese patient by molecular genetic analysis." J Hum Genet **46**(10): 579-582.

Silverberg, G. D., G. Heit, S. Huhn, R. A. Jaffe, S. D. Chang, H. Bronte-Stewart, . . . T. A. Saul (2001). "The cerebrospinal fluid production rate is reduced in dementia of the Alzheimer's type." Neurology **57**(10): 1763-1766.

Sofroniew, M. V. (2009). "Molecular dissection of reactive astrogliosis and glial scar formation." Trends Neurosci **32**(12): 638-647.

Sofroniew, M. V. and H. V. Vinters (2010). "Astrocytes: biology and pathology." Acta Neuropathol **119**(1): 7-35.

Song, H., C. F. Stevens and F. H. Gage (2002). "Astroglia induce neurogenesis from adult neural stem cells." Nature **417**(6884): 39-44.

Spence, R. D., M. E. Hamby, E. Umeda, N. Itoh, S. Du, A. J. Wisdom, . . . R. R. Voskuhl (2011). "Neuroprotection mediated through estrogen receptor-{alpha} in astrocytes." Proc Natl Acad Sci U S A **108**(21): 8867-8872.

Spence, R. D. and R. R. Voskuhl (2012). "Neuroprotective effects of estrogens and androgens in CNS inflammation and neurodegeneration." Front Neuroendocrinol **33**(1): 105-115.

Stauffer, S. R., C. J. Coletta, R. Tedesco, G. Nishiguchi, K. Carlson, J. Sun, . . . J. A. Katzenellenbogen (2000). "Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists." J Med Chem **43**(26): 4934-4947.

Stone, D. J., Y. Song, C. P. Anderson, K. K. Krohn, C. E. Finch and I. Rozovsky (1998). "Bidirectional transcription regulation of glial fibrillary acidic protein by estradiol in vivo and in vitro." Endocrinology **139**(7): 3202-3209.

Su, W., H. B. Chen, S. H. Li and D. Y. Wu (2012). "Correlational study of the serum levels of the glial fibrillary acidic protein and neurofilament proteins in Parkinson's disease patients." Clin Neurol Neurosurg **114**(4): 372-375.

Sugita, Y., S. Nakashima, K. Ohshima, M. Terasaki and M. Morioka (2013). "Anaplastic astrocytomas with abundant Rosenthal fibers in elderly patients: A diagnostic pitfall of high-grade gliomas." Neuropathology.

Suzuki, S., C. M. Brown, C. D. Dela Cruz, E. Yang, D. A. Bridwell and P. M. Wise (2007). "Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions." Proc Natl Acad Sci U S A **104**(14): 6013-6018.

Sweet, W. H. and H. B. Locksley (1953). "Formation, flow, and reabsorption of cerebrospinal fluid in man." Proc Soc Exp Biol Med **84**(2): 397-402.

Tagawa, N., Y. Sugimoto, J. Yamada and Y. Kobayashi (2006). "Strain differences of neurosteroid levels in mouse brain." Steroids **71**(9): 776-784.

Takalo, M., A. Salminen, H. Soininen, M. Hiltunen and A. Haapasalo (2013). "Protein aggregation and degradation mechanisms in neurodegenerative diseases." Am J Neurodegener Dis **2**(1): 1-14.

Takeda, S. and T. Matsuzawa (1984). "Brain atrophy during aging: a quantitative study using computed tomography." J Am Geriatr Soc **32**(7): 520-524.

Takizawa, T., K. Nakashima, M. Namihira, W. Ochiai, A. Uemura, M. Yanagisawa, . . . T. Taga (2001). "DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain." Dev Cell **1**(6): 749-758.

Tamguney, G., K. P. Francis, K. Giles, A. Lemus, S. J. DeArmond and S. B. Prusiner (2009). "Measuring prions by bioluminescence imaging." Proc Natl Acad Sci U S A **106**(35): 15002-15006.

Tang, G., Z. Xu and J. E. Goldman (2006). "Synergistic effects of the SAPK/JNK and the proteasome pathway on glial fibrillary acidic protein (GFAP) accumulation in Alexander disease." J Biol Chem **281**(50): 38634-38643.

Thakkar, M. M., R. A. Delgiacco, R. E. Strecker and R. W. McCarley (2003). "Adenosinergic inhibition of basal forebrain wakefulness-active neurons: a simultaneous unit recording and microdialysis study in freely behaving cats." Neuroscience **122**(4): 1107-1113.

Thakkar, M. M., S. Winston and R. W. McCarley (2003). "A1 receptor and adenosinergic homeostatic regulation of sleep-wakefulness: effects of antisense to the A1 receptor in the cholinergic basal forebrain." J Neurosci **23**(10): 4278-4287.

Tian, R., M. Gregor, G. Wiche and J. E. Goldman (2006). "Plectin regulates the organization of glial fibrillary acidic protein in Alexander disease." Am J Pathol **168**(3): 888-897.

Toda, K., Y. Hayashi, M. Ono and T. Saibara (2012). "Impact of ovarian sex steroids on ovulation and ovulatory gene induction in aromatase-null mice." Endocrinology **153**(1): 386-394.

Tomokane, N., T. Iwaki, J. Tateishi, A. Iwaki and J. E. Goldman (1991). "Rosenthal fibers share epitopes with alpha B-crystallin, glial fibrillary acidic protein, and ubiquitin, but not with vimentin. Immunoelectron microscopy with colloidal gold." Am J Pathol **138**(4): 875-885.

van der Knaap, M. S., S. Naidu, S. N. Breiter, S. Blaser, H. Stroink, S. Springer, . . . J. M. Powers (2001). "Alexander disease: diagnosis with MR imaging." AJNR Am J Neuroradiol **22**(3): 541-552.

Veyrac, A. and J. Bakker (2011). "Postnatal and adult exposure to estradiol differentially influences adult neurogenesis in the main and accessory olfactory bulb of female mice." FASEB J **25**(3): 1048-1057.

Vissers, J. L., M. E. Mersch, C. F. Rosmalen, M. J. van Heumen, W. J. van Geel, K. J. Lamers, . . . M. Herrmann (2006). "Rapid immunoassay for the determination of glial fibrillary acidic protein (GFAP) in serum." Clin Chim Acta **366**(1-2): 336-340.

Von Visger, J. R., D. S. Yeon, T. H. Oh and G. J. Markelonis (1994). "Differentiation and maturation of astrocytes derived from neuroepithelial progenitor cells in culture." Exp Neurol **128**(1): 34-40.

Vos, P. E., K. J. Lamers, J. C. Hendriks, M. van Haaren, T. Beems, C. Zimmerman, . . . M. M. Verbeek (2004). "Glial and neuronal proteins in serum predict outcome after severe traumatic brain injury." Neurology **62**(8): 1303-1310.

Voskuhl, R. R., R. S. Peterson, B. Song, Y. Ao, L. B. Morales, S. Tiwari-Woodruff and M. V. Sofroniew (2009). "Reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS." J Neurosci **29**(37): 11511-11522.

Wahlund, L. O., O. Almkvist, H. Basun and P. Julin (1996). "MRI in successful aging, a 5-year follow-up study from the eighth to ninth decade of life." Magn Reson Imaging **14**(6): 601-608.

Wang, J. M., R. W. Irwin and R. D. Brinton (2006). "Activation of estrogen receptor alpha increases and estrogen receptor beta decreases apolipoprotein E expression in hippocampus in vitro and in vivo." Proc Natl Acad Sci U S A **103**(45): 16983-16988.

Watts, J. C., K. Giles, S. K. Grillo, A. Lemus, S. J. DeArmond and S. B. Prusiner (2011). "Bioluminescence imaging of Abeta deposition in bigenic mouse models of Alzheimer's disease." Proc Natl Acad Sci U S A **108**(6): 2528-2533.

Wilhelmsson, U., L. Li, M. Pekna, C. H. Berthold, S. Blom, C. Eliasson, . . . M. Pekny (2004). "Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration." J Neurosci **24**(21): 5016-5021.

Wilson, C. A., S. A. Mrose and D. W. Thomas (1995). "Enhanced production of B lymphocytes after castration." Blood **85**(6): 1535-1539.

Wippold, F. J., 2nd, A. Perry and J. Lennerz (2006). "Neuropathology for the neuroradiologist: Rosenthal fibers." AJNR Am J Neuroradiol **27**(5): 958-961.

Wong, P. C. and D. W. Cleveland (1990). "Characterization of dominant and recessive assembly-defective mutations in mouse neurofilament NF-M." J Cell Biol **111**(5 Pt 1): 1987-2003.

Wunderlich, M. T., C. W. Wallesch and M. Goertler (2006). "Release of glial fibrillary acidic protein is related to the neurovascular status in acute ischemic stroke." Eur J Neurol **13**(10): 1118-1123.

Xu, L., R. M. Sapolsky and R. G. Giffard (2001). "Differential sensitivity of murine astrocytes and neurons from different brain regions to injury." Exp Neurol **169**(2): 416-424.

Yachnis, A. T., L. B. Rorke, V. M. Lee and J. Q. Trojanowski (1993). "Expression of neuronal and glial polypeptides during histogenesis of the human cerebellar cortex including observations on the dentate nucleus." J Comp Neurol **334**(3): 356-369.

Yakimchuk, K., M. Jondal and S. Okret (in press). "Estrogen receptor alpha and beta in the normal immune system and in lymphoid malignancies." Mol Cell Endocrinol.

Yano, H., N. Nakayama, Y. Hirose, N. Ohe, J. Shinoda, S. Yoshimura and T. Iwama (2011). "Intraventricular glioneuronal tumor with disseminated lesions at diagnosis--a case report." Diagn Pathol **6**: 119.

Yeh, T. H., Y. Lee da, S. M. Gianino and D. H. Gutmann (2009). "Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation." Glia **57**(11): 1239-1249.

Zarate, S. and A. Seilicovich (2010). "Estrogen receptors and signaling pathways in lactotropes and somatotropes." Neuroendocrinology **92**(4): 215-223.

Zhao, X., A. Ahram, R. F. Berman, J. P. Muizelaar and B. G. Lyeth (2003). "Early loss of astrocytes after experimental traumatic brain injury." Glia **44**(2): 140-152.

Zhu, L., S. Ramboz, D. Hewitt, L. Boring, D. S. Grass and A. F. Purchio (2004). "Non-invasive imaging of GFAP expression after neuronal damage in mice." Neurosci Lett **367**(2): 210-212.

Zoltewicz, J. S., D. Scharf, B. Yang, A. Chawla, K. J. Newsom and L. Fang (2012). "Characterization of Antibodies that Detect Human GFAP after Traumatic Brain Injury." Biomark Insights **7**: 71-79.

Chapter 2:

GFAP expression as an indicator of disease severity in mouse models of Alexander disease

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AUTHORS CONTRIBUTIONS

TLH quantified the GFAP concentrations in the brain regions and contributed to the writing and editing of the chapter. PLJ contributed the remainder of the data and contributed to the design of the experiments (with the guidance of AM) and writing.

ABSTRACT

Alexander Disease (AxD) is a rare disorder caused by heterozygous mutations in glial fibrillary acidic protein (GFAP) resulting in accumulation of the GFAP protein and elevation of *Gfap* mRNA. To test whether GFAP itself can serve as a biomarker of disease status or progression, we investigated two independent measures of GFAP expression in AxD mouse models, one using a genetic reporter of promoter activity and the other quantifying GFAP protein directly in a manner that could also be employed in human studies. Using a transgenic reporter line that expresses firefly luciferase under the control of the murine *Gfap* promoter (*Gfap-luc*), we found that luciferase activity reflected the regional CNS variability of *Gfap* mRNA in $Gfap^{+/+}$ mice, and increased in mice containing a point mutation in *Gfap* that mimics a common human mutation in AxD (R239H in the human sequence, and R236H in the murine sequence). In a second set of studies, we quantified GFAP protein in CSF taken from three different AxD mouse models and littermate controls. GFAP levels in CSF were increased in all three AxD models, in a manner corresponding to the concentrations of GFAP in brain. These studies demonstrate that transactivation of the *Gfap* promoter is an early and sustained indicator of the disease process in the mouse. Furthermore, GFAP in CSF serves as a potential biomarker that is comparable between mouse models and human patients.

INTRODUCTION

Alexander disease (AxD) is a rare neurodegenerative disorder caused by heterozygous mutations in the gene encoding glial fibrillary acidic protein (GFAP), the major intermediate filament protein of astrocytes (Brenner et al. 2001). The mutations appear to act in a gain-of-function fashion, with a rise in GFAP levels above a toxic threshold serving as a key step in pathogenesis (Brenner et al. 2009). However, the mechanisms underlying the accumulation of GFAP are not completely understood. Studies from cell culture models implicate impaired degradation through the proteasomal pathway as one cause of GFAP increase, despite a near simultaneous increase in autophagy (Tang et al. 2006; Tang et al. 2008). In addition, analysis of both human tissues and mouse models reveal increases in GFAP mRNA (Hagemann et al. 2005; Hagemann et al. 2006), suggesting that increased synthesis could also contribute to an accumulation of GFAP protein. Given the very slow turnover rate for GFAP protein (Chiu and Goldman 1984; Morrison et al. 1985; DeArmond et al. 1986; Price et al. 2010), any changes in GFAP promoter activity and increased synthesis would have long-lasting effects. GFAP accumulation may also exacerbate pathology through the formation of positive feedback loops that impact both synthesis and degradation (Messing et al. 2012).

Regulation of GFAP expression occurs primarily at the level of GFAP transcription, rather than through alterations in mRNA stability or translational efficiency (Brenner 1994). Whether the changes in GFAP mRNA noted above reflect feedback stimulation of *Gfap* promoter activity is not known. Methods for investigating promoter activity *in vivo* have been greatly facilitated by the development of transgenic models that carry reporter genes under the control of cell-specific regulatory elements (Cui et al. 1994). One such reporter is the *Gfap-luc* mouse, which expresses the firefly luciferase gene under the control of a 12kb 5' flanking region from the murine *Gfap* gene (Zhu et al. 2004). These mice have previously been shown to exhibit increases in luciferase activity in response to a variety of insults or diseases that also

result in astrogliosis and raised levels of GFAP mRNA, including kainic acid-induced seizures (Zhu et al. 2004), bacterial infection (Kadurugamuwa et al. 2005), inflammation (Luo et al. 2008), stroke (Cordeau et al. 2008), scrapie (Tamguney et al. 2009), motor neuron degeneration (Keller et al. 2009), and expression of mutant amyloid precursor protein (Watts et al. 2011).

With the goal of identifying indicators of disease severity and progression that could be easily monitored in mouse models of AxD, we sought to test whether the *Gfap-luc* reporter is responsive to the novel genetic injury represented by the expression of mutant GFAP. We also tested the hypothesis that the elevations in GFAP protein previously detected in brain parenchyma are also reflected at the level of cerebrospinal fluid (CSF), a site that is readily amenable to biopsy in human patients and in which increased GFAP has been observed in Alexander disease patients (Kyllerman et al. 2005). We found distinct variations in GFAP expression and response to AxD mutations in different brain regions of the mouse models. Increased activity from the *Gfap-luc* reporter is evident at the whole brain level as soon as 14 days after birth, and this increase is sustained through at least 6 months of age. We also found that GFAP is detectable at low levels in the CSF of control mice, but is elevated in three different AxD models to degrees corresponding to the amount of GFAP accumulation in brain.

MATERIALS AND METHODS

Mice

The experiments described here were approved by the Animal Care and Use Committee for the Graduate School at the University of Wisconsin, Madison. The $Gfap^{R236H/+}$ and $Gfap^{R76H/+}$ lines of mice contain knock-in mutations at the endogenous *Gfap* locus that are homologous to common human Alexander disease-associated mutations (R239H and R79H respectively) (Hagemann et al. 2006). The $GFAP^{Tg}$ mice are a transgenic line (Tg73.7) that over-expresses

wild-type human GFAP (Messing et al. 1998). The *Gfap-luc* mice are a transgenic line that expresses firefly luciferase under the control of a 12 kb murine *Gfap* promoter (Zhu et al. 2004). *Gfap*^{tm1Mes} mice carry a null mutation at the *Gfap* locus (McCall et al. 1996), and were used as negative controls in the validation of the GFAP ELISA (see below). All mice were maintained in the FVB/N background and were housed under a 14-10 light-dark cycle with ad libitum access to food. Samples were collected from mice at 8 weeks of age. Brains were either divided sagittally into two equal halves, or dissected into individual regions (olfactory bulb, frontal cortex [anterior to Bregma, dorsal gray matter containing little or no white matter], hippocampus, cerebellum, and brain stem) along with cervical spinal cord. Tissues were immediately frozen in liquid nitrogen and stored at -80^{o} C until further processing.

Quantitation of *Gfap* promoter activity

Gfap-luc transgenic mice were used as indirect reporters of *Gfap* promoter activity. Firefly luciferase activity was quantified using the Dual-Glo luciferase kit (Promega), according to the manufacturer's directions. Briefly, tissues were homogenized in reporter lysis buffer (125 mg tissue per mL buffer), centrifuged at 17,500 *x g* for 20 minutes at 4^{o} C, and the supernatant taken for analysis. An aliquot of this supernatant (40 µL for half brains, 20 µL for smaller pieces) was diluted 1:1 in firefly luciferase reagent and allowed to incubate at room temperature (20-25^{o}C) for 12 minutes. The signal intensity was then determined with a GloRunner Microplate Luminometer (Turner Biosystems). A separate aliquot was assayed for total protein using a BCA Protein Assay kit with bovine serum albumin (BSA) as a standard (Thermo Scientific). Values are expressed in arbitrary firefly luminescent units (fLuc) per mg protein.

Quantitation of *Gfap* mRNA

Tissues were homogenized in TRIzol Reagent (Invitrogen) according to the manufacturer's directions. cDNA was synthesized using Superscript III (Invitrogen) according to

the manufacturer's protocol. Specific transcripts were then quantified by real time PCR using Power SYBR Green Master Mix and an Applied Biosystem 7500 Real-Time PCR system as described previously (Hagemann et al. 2005). Primer sets were the following: *Gfap* (forward: 5' CAA CGT TAA GCT AGC CCT GGA CAT 3', reverse: 5' CTC ACC ATC CCG CAT CTC CAC AGT), 18S ribosomal RNA (forward: 5' CGC CGC TAG AGG TGA AAT TCT 3', reverse: 5' CGA ACC TCC GAC TTT CGT TCT 3'), and TATA-binding protein (forward 5' GCA CAG GAG CCA AGA GTG A 3', reverse 5' CCC ACC ATG TTC TGG ATC TT 3'). *Gfap* transcript levels were normalized to both the 18S ribosomal and TATA-binding protein RNA's. Both normalizations yielded similar results. Data shown was normalized to the 18S ribosomal RNA.

Collection of Cerebrospinal Fluid

CSF was collected from mice according to the method of DeMattos et al. (2002). Briefly, mice were anesthetized with avertin (400-600 mg/kg i.p.). A midline sagittal incision was made over the dorsal aspect of the hindbrain and three muscle layers carefully peeled back to expose the cisterna magna. The membrane covering the cisterna magna was pierced with a 30 gauge needle, and CSF collected immediately using a flexible plastic pipette. Approximately 10 µL of CSF was collected per animal, and stored at -80^{o} C until further processing.

Quantitation of GFAP protein

Brain samples were homogenized (w/v 1:16) in 2% SDS, 50mM Tris-HCl (pH 7.4), 5mM EDTA (pH 7.4), 1mM PefablocSC (Sigma-Aldrich), and 1x Complete Proteinase Inhibitor (Roche) using a Geno/Grinder tissue homogenizer (SPEX CertiPrep). After homogenization, samples were boiled for 15 minutes. Samples were then diluted in phosphate buffered saline (Fisher, #BP399-4) and protein concentration determined using a BCA Protein Assay kit with BSA as a standard (Pierce). This same diluted sample was then used for the GFAP ELISA as described below.

GFAP protein was quantitated using a sandwich ELISA as previously described (Petzold et al. 2004; Hagemann et al. 2009), with minor modifications. Briefly, a microtiter plate (Nunc MaxiSorp) was coated with a cocktail of monoclonal antibodies (Covance, SMI-26R) diluted in PBS (Fisher). Plates were blocked with 5% milk in PBS before addition of samples or standards diluted in PBS with 0.05% Tween 20 (Tw20) and 1% bovine albumin serum (BSA) (Sigma, #A7030). Antibody incubation steps were performed in 5% milk-PBS, and washing steps were performed in PBS-Tw20 (without BSA). Standard curves were generated using bovine GFAP (Fitzgerald Industries International, # RDI-PRO62007) diluted in PBS-1% BSA. Assay volumes consisted of 100 µL/well when analyzing brain samples or 50 µL/well when analyzing CSF. Samples were diluted with PBS-Tw20-BSA as needed to bring their GFAP values within the linear range of the standard curve.

In the case of CSF samples from controls, which typically had low levels of GFAP, the dilutions required to bring the reaction volume up to the minimum 100 µL for duplicate wells often meant that the values fell below the biological limit of detection (see below). We therefore used pooled samples for controls, replicated as four independent sets derived from 3-4 animals per group, and diluted a minimal amount (36 µL CSF + 64 µL ELISA buffer). Mice from the AxD models had high enough levels of GFAP to allow dilutions from individual samples, typically 1:16-1:120. After washing, a rabbit polyclonal antibody (Dako, # Z334) was used to detect the GFAP followed by a peroxidase conjugated anti-rabbit IgG secondary antibody (Sigma, # A6154). The peroxidase activity was detected with SuperSignal ELISA Pico Chemiluminescent Substrate (Pierce, Thermo Scientific) and quantified with a GloRunner Microplate Luminometer (Turner Biosystems). GFAP values for brain samples were expressed per mg total protein, and for CSF samples as ng/L.

Under these conditions the lower limit of detection (defined as 3 standard deviations above the mean of replicate blank samples) was 11 ng/L, and the biological limit of detection

(BLD) (defined as the lower limit of detection plus 3 standard deviations of a known low concentration sample) (Westgard and Barry 2008) was 21 ng/L for a 50 µL reaction volume. The intra-assay coefficient of variation (COV), determined using the bovine GFAP standard at 33 ng/L in 10 sets of triplicate wells, was 13%. The inter-assay COV, determined from pooled CSF samples taken from *GFAP*^{Tg} mice (with values higher on the standard curve) divided into multiple identical aliquots to allow 10 independent assays on different days, was 11%. Samples from *Gfap*-null mice give readings that are below the BLD in this assay (data not shown), thus validating its specificity.

Statistical Analysis

For the comparisons of *Gfap* promoter activity, mRNA, and protein shown in Figure 2.2, the data was first log transformed [$Y = \log(Y)$] to equalize the standard deviations (figure shows the original untransformed data) and then analyzed using a 2-way ANOVA with post Bonferonni-corrected t-tests. All other data analyses used a 1-way ANOVA with post Bonferonni-corrected t-tests between *Gfap*$^{R236H/+}$ and *Gfap*$^{+/+}$. The error shown for the fold-changes between *Gfap*$^{R236H/+}$ and *Gfap*$^{+/+}$ mice in Figure 2.2 is the calculated propagated error for each data point. The CSF analysis also required log transformation of the data to equalize the standard deviations and was then analyzed using a 1-way ANOVA with post Bonferonni-corrected t-tests.

RESULTS

Gfap promoter activity, mRNA, and protein vary by brain regions in *Gfap*$^{+/+}$ mice.

We sought to test whether *Gfap-luc* transgenic reporter mice, which express the firefly luciferase under the control of the murine *Gfap* promoter, thus serving as an indirect monitor of promoter activity, faithfully reflect the regional variation in GFAP expression that is known to exist in the rodent central nervous system. In *Gfap-luc* transgenics that are wild-type at the

Gfap locus (*Gfap*$^{+/+}$), luciferase activity was highest in the spinal cord, intermediate in olfactory bulb, hippocampus and brain stem, and lowest in cerebral cortex and cerebellum (Fig. 2.1A). For comparison, we measured *Gfap* mRNA levels in these same regions, and found that spinal cord and brain stem had the highest levels, and cerebellum and cerebral cortex the lowest (Fig. 2.1B). Total GFAP protein levels, as measured by ELISA, followed essentially the same pattern as mRNA (Fig. 2.1C). Hence, luciferase activity from the reporter mice mirrored relative mRNA and protein levels for all regions that were examined, with the exception of brain stem.

Regional *Gfap* gene activity in *Gfap*$^{R236H/+}$ mice

Mice carrying point mutations at the *Gfap* locus mimicking those found in Alexander disease patients have elevated levels of *Gfap* mRNA and protein (Hagemann et al. 2006; LaPash Daniels et al. 2012), although not uniformly in all regions of the CNS. To test whether these changes in GFAP expression are mirrored by the *Gfap-luc* reporter, we crossed the reporter with a knock-in of the R236H mutation (*Gfap*$^{R236H/+}$), and examined all three measures of GFAP expression in various CNS regions of 8 week old mice (Fig. 2.2).

Gfap promoter activity significantly increased in all six regions of *Gfap-luc*;*Gfap*$^{R236H/+}$ mice compared to *Gfap-luc*;*Gfap*$^{+/+}$ littermates. The largest fold-increase of *Gfap* promoter activity was in the olfactory bulb (7.6 ± 1.2), and the smallest in cerebellum (2.1 ± 0.4), with intermediate changes for the other regions. Quantitation of *Gfap* mRNA levels in the same mice revealed fold changes similar to those found for promoter activity in olfactory bulb, hippocampus, cerebral cortex, and cerebellum, and more modest increases in brain stem and spinal cord.

We then measured total GFAP protein levels in the same brain regions from *Gfap* mutant vs. wild type mice. As previously described (Hagemann et al. 2006; LaPash Daniels et al. 2012), both olfactory bulb and hippocampus displayed substantially increased levels of

GFAP (40-fold ± 5 for olfactory bulb; 30 ± 3 for hippocampus), but well beyond the fold changes observed for promoter activity and mRNA. Cerebellar GFAP increased a modest amount, commensurate with the changes in promoter activity and mRNA. Interestingly, the two regions of the CNS with the highest basal levels of GFAP protein in wild type mice, brain stem and spinal cord (see Fig. 2.1C), displayed marked discrepancies between promoter activity/mRNA levels and protein levels when evaluated in the *Gfap*$^{R236H/+}$ mutants. Although promoter activity increased ~5-fold in both brain stem and spinal cord, mRNA levels increased a lesser amount, and protein remained unchanged in brain stem and actually decreased in spinal cord. Hence, when examined in the context of the injury response associated with expression of mutant GFAP, the precise relationships between promoter activity, transcription, and protein accumulation is complex and varies considerably between different regions of the CNS.

Induction of *Gfap* promoter and increase in mRNA is not affected by gender

Previous studies have implicated estrogens in the hormonal regulation of GFAP expression (Laping et al. 1994; Stone et al. 1998; Levin-Allerhand et al. 2001; McAsey et al. 2006; Cho et al. 2010). We therefore tested whether the changes in *Gfap* promoter activity and mRNA observed in the context of the R236H point mutation were influenced by gender. However, none of the regions examined showed any differences between males and females (olfactory bulb illustrated in Fig. 2.3, other regions – data not shown).

Time course of induction of *Gfap* promoter activity in *Gfap*$^{+/+}$ *and Gfap*$^{R236H/+}$ mice

To determine when *Gfap* promoter activity becomes elevated in *Gfap* mutant mice during development, we analyzed luciferase activity in whole brains (including olfactory bulbs) from *Gfap-luc*;*Gfap*$^{R236H/+}$ mice compared to *Gfap-luc* littermate controls at various ages beginning at post-natal day 1 (p1). Luciferase activity displayed a ~4-fold increase in *Gfap*$^{R236H/+}$ mice as early as p14, and remained significantly elevated through 8 weeks of age (Fig. 2.4).

Using a different luciferase assay we examined mice at 6 months of age, and found that elevated promoter activity was still evident (data not shown).

GFAP in CSF of *Gfap* mutant and *GFAP* transgenic mice

In pilot studies of CSF from individual control mice using standard dilutions required to reach minimal assay reaction volumes (see Methods), one third of the samples yielded values that were below the biological limit of detection (BLD) of the assay. To permit an accurate determination of the values in controls, we therefore created four separate sets of pooled samples, each composed of CSF taken from 3-4 $Gfap^{+/+}$ mice. In this manner the volume of CSF was sufficient for use with minimal dilution in the GFAP ELISA, thus increasing the sensitivity for detection. We then measured GFAP in CSF from individual mice of three AxD models that have previously been found to have varying levels of GFAP accumulation in brain parenchyma: the $Gfap^{R236H/+}$ mice described above, the $Gfap^{R76H/+}$ knock-in model (homologous to the human R79H mutation and intermediate in levels of GFAP between control and $Gfap^{R236H/+}$ mice), and the transgenic that over-expresses human wild type GFAP to very high levels (Hagemann et al., 2006). We found that GFAP in the CSF of $Gfap^{+/+}$ mice was detectable in all four pooled samples (354 ± 217 ng/L, mean ± SD), whereas GFAP in the CSF of all three AxD models was significantly elevated compared to controls, and in the same rank order as expected from brain (1264 ± 596 for $Gfap^{R76H/+}$; 2637 ± 1001 for $Gfap^{R236H/+}$; 46,676 ± 40,533 for $GFAP^{Tg}$ mice; n = 9-14 in each group). In addition, the differences in CSF levels between the AxD model groups were statistically significant in all pair-wise comparisons (Fig. 2.5).

Brain levels of GFAP were then determined in each individual from which the CSF samples were taken in the three AxD models. Although the between-group comparisons showed distinct differences, within each group the correlation between CSF and brain levels for

individuals was not significant (*Gfap*$^{R76H/+}$, r = 0.27; *Gfap*$^{R236H/+}$, r = 0.38; *GFAP*^{Tg}, r = 0.07; analyzed by the Spearman's Rank-Order Correlation) (Fig. 2.6).

DISCUSSION

While mutations in *GFAP* are clearly the initiating event in AxD, ultimately the total amount of GFAP rises with secondary consequences for a multitude of astrocyte functions. What accounts for this increase is not clearly understood, although changes in the rates of both degradation and synthesis have been proposed (Messing and Brenner 2013). Whether documented elevations in *GFAP* mRNA can be accounted for by increased activity of the *GFAP* promoter has not previously been addressed, and whether the putative changes in promoter activity and increases in protein can be exploited for drug discovery and clinical studies is also not known. Here we demonstrate, using several mouse models of AxD, that the activity of the murine *Gfap* promoter is indeed increased as an early and sustained response following expression of mutant GFAP *in vivo*. Furthermore, we find that the levels of GFAP in CSF increase, roughly in parallel with the degree of increase present in brain. These results provide a foundation on which to build future studies seeking to interfere with the toxic increase in GFAP as a therapeutic goal (Messing et al. 2010).

Activation of the *GFAP* promoter with resulting increase in synthesis is considered a fundamental property of reactive astrocytes. Although the precise mechanisms mediating this increase are still topics of active investigation, numerous studies suggest that at least some of the regulatory elements for the reactive response lie within a 2.2 kb fragment of the 5' region of the gene (Brenner 1994). Previous studies from our lab utilized this 2.2 kb human *GFAP* promoter construct to create dual luciferase reporter mice for monitoring of *GFAP* promoter activity, and in crosses of these luciferase reporters to the *Gfap*$^{R236H/+}$ mutants we found only a modest increase in luciferase activity which disappeared by 8 weeks of age (Cho et al. 2009).

In contrast, the *Gfap-luc* mouse utilized in the present study, which differs from our dual luciferase mouse in several respects, reports a much larger and sustained increase in promoter activity. In addition, the fold-increase in luciferase activity more closely resembles the fold change in mRNA levels, suggesting as well that the predominant regulation of GFAP expression occurs at the level of transcriptional activation.

The degree to which elevated levels of GFAP protein per se provide the initial stimulus leading to promoter activation is not yet clear. Future studies will examine the response of the *Gfap*-luc reporter in transgenic lines that over-express wild-type rather than mutant GFAP, although it will be important to closely match the levels of GFAP protein between lines. It is possible that mutant GFAP acts in essentially the same manner as wild-type, but more efficiently and at lower levels of over-expression.

That expression of GFAP varies considerably in different regions of the central nervous system and in different populations of astrocytes is well known. For instance, both mouse and human data reveal the highest levels of both mRNA and protein in spinal cord and brain stem, followed in descending order by hippocampus, olfactory bulb, cerebellum, and cerebral cortex (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007). In contrast, one study of rats found cerebellum to be higher than hippocampus (Martin and O'Callaghan 1995). The reasons for these differences are not clear, but could reflect variations in either the number or composition of astrocyte sub-types among regions. Recent studies of human astrocytes differentiated *in vitro* from embryonic stem cells reveal that those induced to a "caudalized" phenotype through exposure to retinoic acid express higher levels of GFAP than those following the default rostral pathway of differentiation (Krencik et al. 2011). Our studies support the idea that regulation of GFAP levels occurs primarily at the level of transcription, as originally proposed by Brenner (1994), with a notable exception being spinal cord of the GFAP mutants. It is interesting that while the infantile form of Alexander disease is characterized by

predominance of forebrain lesions, the adult form shifts to a hindbrain distribution, with many adult-onset patients experiencing atrophy of the medulla and cervical spinal cord (Prust et al. 2011; Messing et al. 2012).

Why GFAP should appear in the CSF is not at all clear. Studies in a wide variety of human disorders document elevations of GFAP that are typically attributed to cell death, although most of these studies also find detectable levels even in healthy controls (Liem and Messing 2009). One possibility is that GFAP, despite being a cytoskeletal protein, is normally secreted from astrocytes, as has been found to occur for vimentin from macrophages (Mor-Vaknin et al. 2003). Studies of the astrocyte secretome have largely utilized cell cultures and are complicated by the problem of correcting for contamination by cytoplasmic contents – most report finding vimentin but not GFAP is secreted (Delcourt et al. 2005; Keene et al. 2009; Greco et al. 2010). Dowell et al. (2009) did find GFAP in the supernatant from cultured mouse astrocytes, but considered it a contaminant. Moore et al. (2009), in a study using rat astrocyte cultures, found neither vimentin nor GFAP. Whether the GFAP mutations and brain over-expression that have been found in Alexander disease lead to astrocyte death, with consequent release of cytoplasmic contents into the extracellular space and eventually CSF, is also not known. Evidence for an increase in cell death following expression of mutant GFAP has been obtained from cell culture models (Mignot et al. 2007; Cho and Messing 2009), but no similar data have been obtained from the *in vivo* models of disease (Hagemann et al. 2006; Tanaka et al. 2007). The amount of GFAP present in the CSF of *GFAP*^{Tg} mice is sufficiently high as to be detectable by mass spectrometry, thus opening new possibilities for methods of quantitation and characterization (Cunningham et al. 2013).

A major problem facing therapeutic research is the identification of suitable biomarkers that reflect key pathways of disease and could be responsive to drugs or other types of treatment. The response of the *Gfap-luc* reporter mouse clearly demonstrates transactivation of

the *Gfap* promoter, perhaps as a final common pathway in the putative positive feedback loops that lead to toxic accumulation of GFAP (Messing et al. 2012). Activation of the *Gfap* promoter may also represent a step in pathogenesis that is amenable to drug discovery or screening efforts (Cho et al. 2010; Messing et al. 2010). In addition, the elevation of GFAP that occurs in CSF may prove useful as a biomarker of disease severity or progression in clinical studies of Alexander disease. Indeed, Kyllerman et al. (2005) studied three patients, each of whom exhibited markedly elevated levels in their CSF compared to controls. A study of CSF levels of GFAP in a larger cohort of AxD patients is currently underway, along with an evaluation of blood levels. The validation of biomarkers that occur both in human patients as well as animal models will greatly facilitate the evaluation of candidate therapies in the future.

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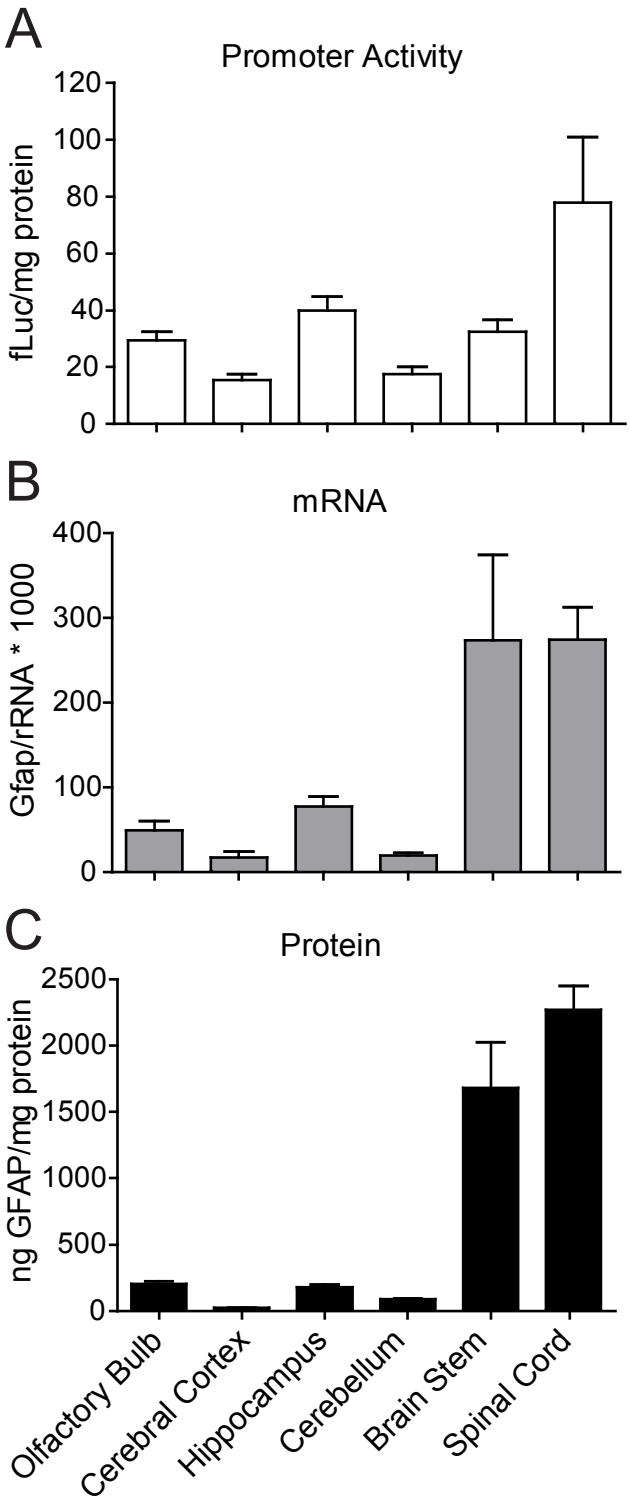
A
Promoter Activity
fLuc/mg protein
120
100
80
60
40
20
0
B
mRNA
Gfap/rRNA * 1000
400
300
200
100
0
C
Protein
ng GFAP/mg protein
2500
2000
1500
1000
500
0
Olfactory Bulb
Cerebral Cortex
Hippocampus
Cerebellum
Brain Stem
Spinal Cord

Figure 2.1: *Gfap* promoter activity, mRNA, and protein in *Gfap*$^{+/+}$ mice. *Gfap* promoter activity (A), mRNA (B), and protein (C) concentrations vary among regions, with the highest levels in spinal cord for all three measures of *Gfap*. Presented as mean ± 1 standard deviation (SD) (n=3-6, males). fLuc, firefly luciferase.

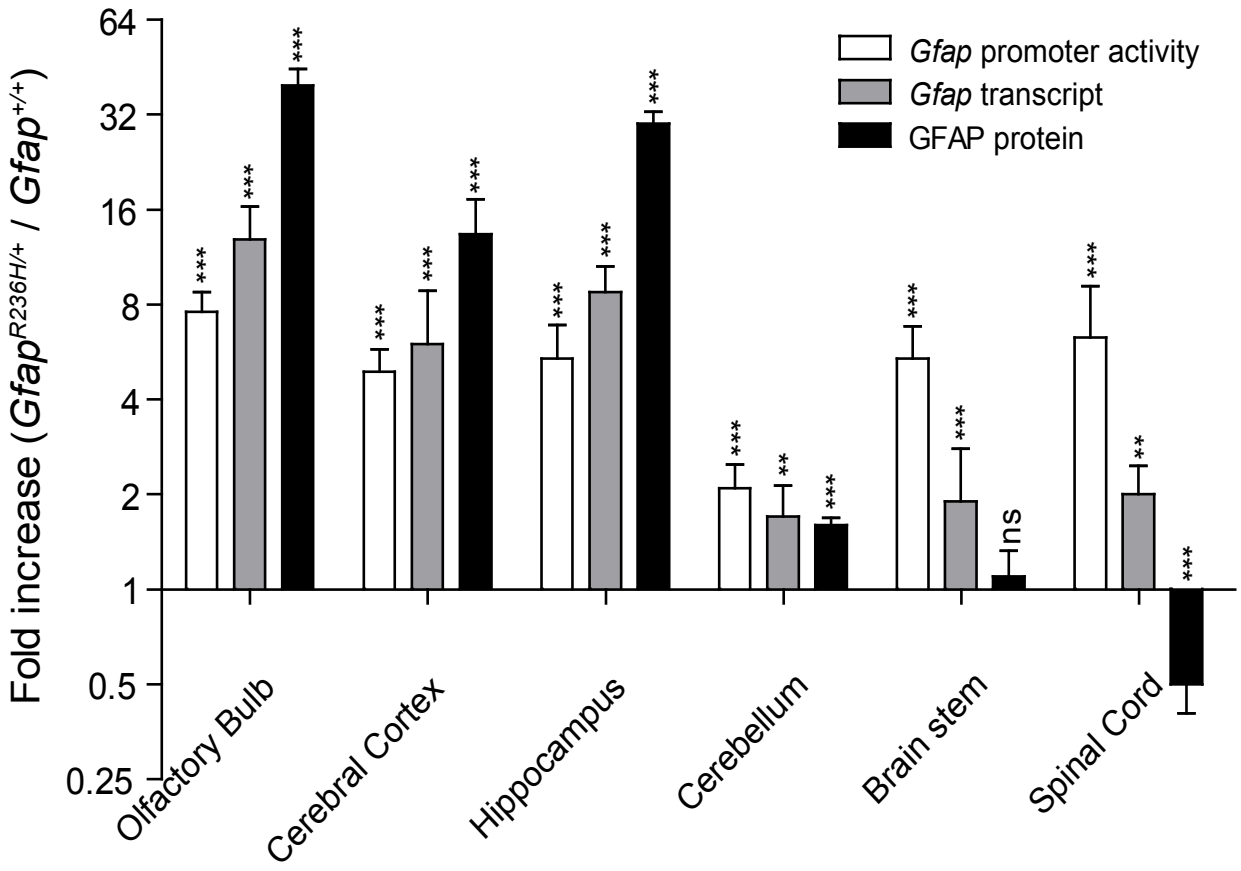
Fold increase (Gfap^R236H/+ / Gfap^+/+)
64
32
16
8
4
2
1
0.5
0.25
Gfap promoter activity
Gfap transcript
GFAP protein
***
***
***
***
***
***
***
***
***
***
**
***
***
***
ns
***
**
***
Olfactory Bulb
Cerebral Cortex
Hippocampus
Cerebellum
Brain stem
Spinal Cord

Figure 2.2: Levels of *Gfap* promoter activity, mRNA, and protein in *Gfap*$^{R236H/+}$ mice compared to *Gfap*$^{+/+}$ mice. A comparison of the fold changes seen in *Gfap* promoter activity, mRNA, and protein shows that all three are reflective of the other except in spinal cord and brain stem (2-way ANOVA with post-hoc Bonferroni t-test; ns, not significant; ** $p<0.01$;*** $p<0.001$ compared to wild-type). Presented as average fold-change ± 1 SD on log 2 scale (n=3-6 per genotype, males).

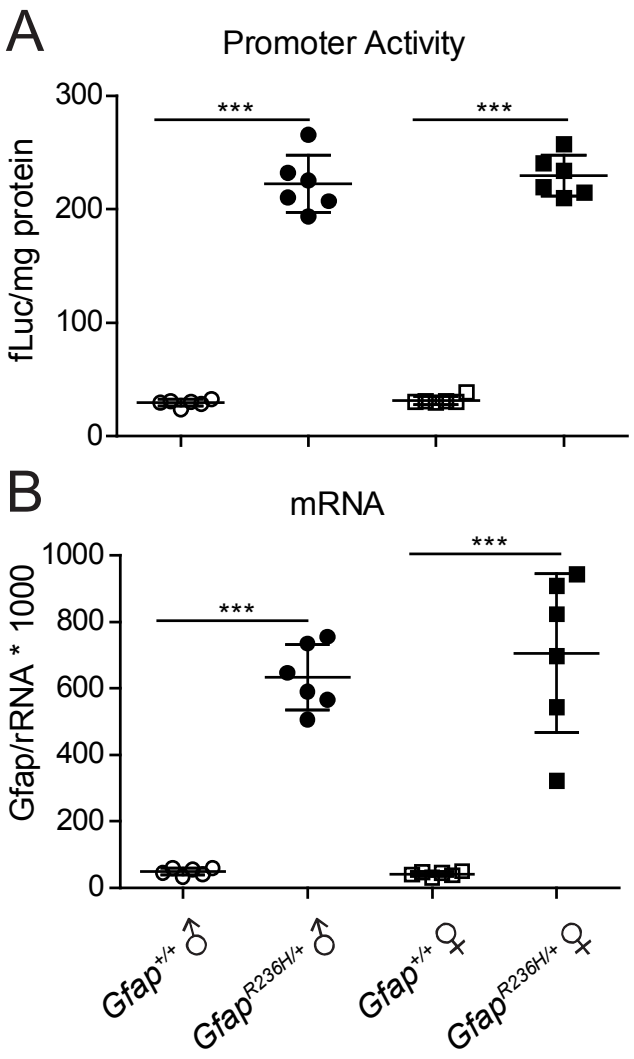
A
Promoter Activity
fLuc/mg protein
300
200
100
0
***
***
B
mRNA
Gfap/rRNA * 1000
1000
800
600
400
200
0
***
***
Gfap+/+ ♂
GfapR236H/+ ♂
Gfap+/+ ♀
GfapR236H/+ ♀

Figure 2.3: *Gfap* promoter and mRNA levels by gender. While *Gfap* promoter activity (A) and mRNA (B) are elevated in $Gfap^{R236H/+}$ mice compared to $Gfap^{+/+}$, the effect is similar in males and females in olfactory bulb (illustrated) as well as in other brain regions (data not shown). (One-way ANOVA with post-hoc Bonferroni t-test; *** $p<0.001$). Presented as mean ± 1 SD (n=6 male and 6 female mice per genotype).

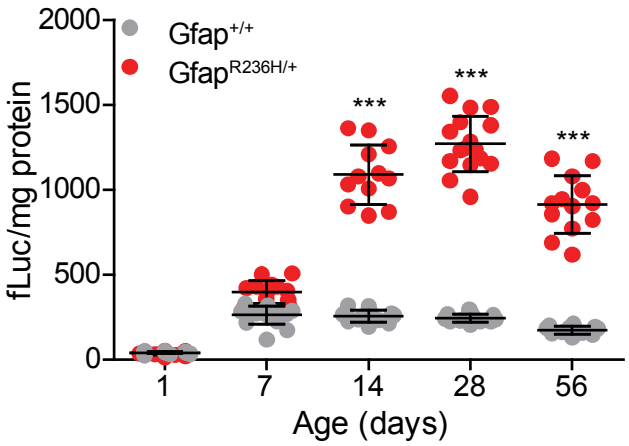
2000
1500
1000
500
0
fLuc/mg protein
Gfap+/+
GfapR236H/+
***
***
***
1
7
14
28
56
Age (days)

Figure 2.4: Developmental time course of *Gfap* promoter activity in *Gfap*$^{R236H/+}$ brain. *Gfap* promoter activity is significantly elevated in *Gfap*$^{R236H/+}$ mice over *Gfap*$^{+/+}$ controls as early as postnatal day 14 (p14) and remains elevated through 8 weeks of age (p56). (One-way ANOVA with post-hoc Bonferroni t-test; *** $p<0.001$). Presented as mean ± 1 SD (n=12-25).

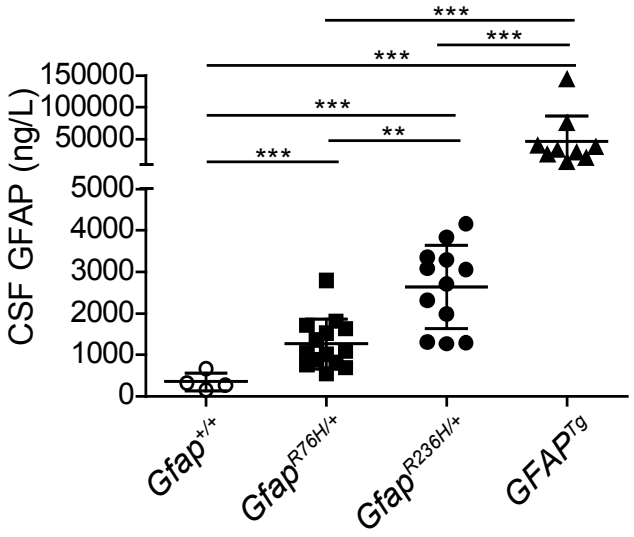
***
***
***
***
**
***
150000
100000
50000
5000
4000
3000
2000
1000
0
CSF GFAP (ng/L)
Gfap+/+
GfapR76H/+
GfapR236H/+
GFAPTg

Figure 2.5: GFAP protein in CSF of mouse models of AxD. GFAP is elevated in CSF of all three mouse models of AxD (*Gfap*$^{R76H/+}$,*Gfap*$^{R236H/+}$, and *GFAP*^{Tg}) compared to *Gfap*$^{+/+}$ controls. (One-way ANOVA with post-hoc Bonferroni t-test; ** $p<0.01$; *** $p<0.001$). Presented as mean ± 1 SD on a split linear scale (n=9-14, males).

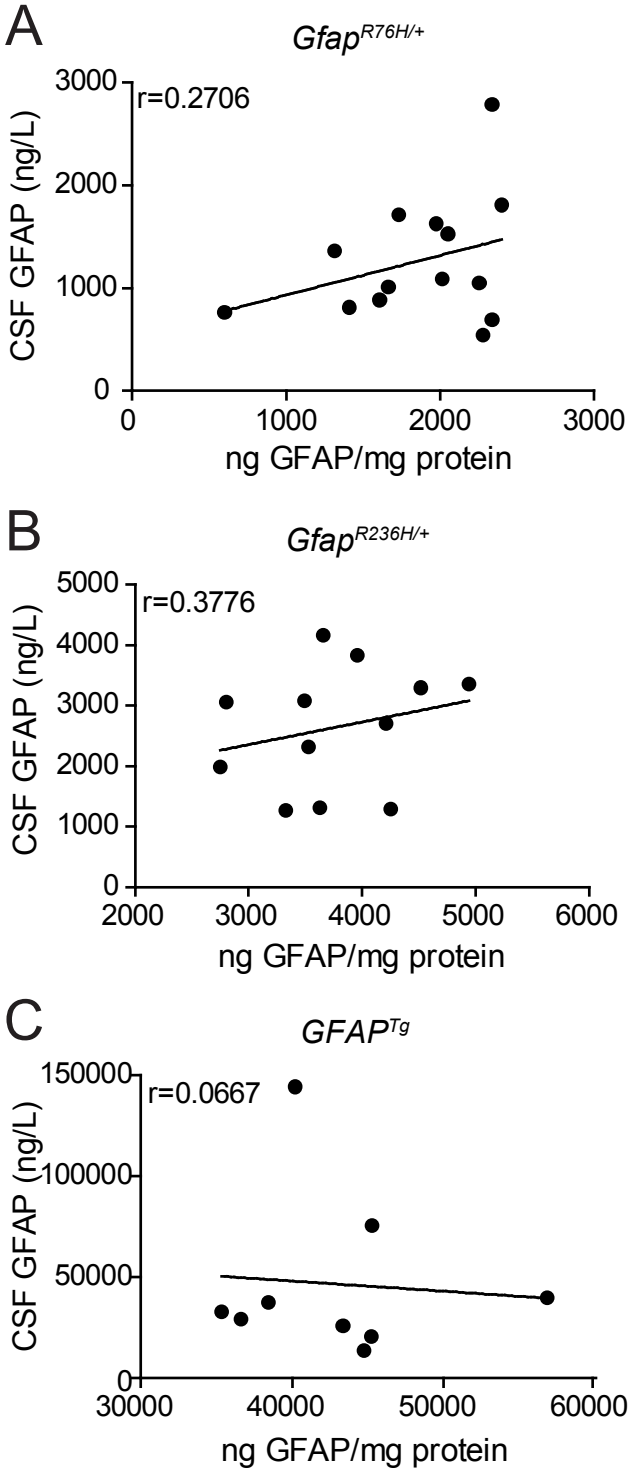
A
Gfap R76H/+
r=0.2706
CSF GFAP (ng/L)
3000
2000
1000
0
0
1000
2000
3000
ng GFAP/mg protein
B
Gfap R236H/+
r=0.3776
CSF GFAP (ng/L)
5000
4000
3000
2000
1000
0
2000
3000
4000
5000
6000
ng GFAP/mg protein
C
GFAP Tg
r=0.0667
CSF GFAP (ng/L)
150000
100000
50000
0
30000
40000
50000
60000
ng GFAP/mg protein

Figure 2.6: Correlation between GFAP levels in CSF and GFAP levels in the brain. Within each of the AxD mouse model groups, there was no correlation between the concentration of GFAP in CSF (ng/L GFAP) and in the brain (ng GFAP/mg protein) in individual mice. (A) *Gfap*$^{R76H/+}$ (*n*=14), (B) *Gfap*$^{R236H/+}$ (*n*=12), or (C) *GFAP*^{Tg} (*n*=9). (Spearman's Rank-Order Correlation). Each data point represents one mouse.

REFERENCE

Brenner, M. (1994). "Structure and transcriptional regulation of the GFAP gene." Brain Pathol **4**(3): 245-257.

Brenner, M., J. Goldman, R. Quinlan and A. Messing (2009). Alexander Disease: A Genetic Disorder of Astrocytes. In: Astrocytes in (Patho)Physiology of the Nervous System. P. G. Haydon and V. Parpura, Springer US**:** 591-648.

Brenner, M., A. B. Johnson, O. Boespflug-Tanguy, D. Rodriguez, J. E. Goldman and A. Messing (2001). "Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease." Nat Genet **27**(1): 117-120.

Chen, H., F. Cabon, P. Sun, E. Parmantier, P. Dupouey, C. Jacque and B. Zalc (1993). "Regional and developmental variations of GFAP and actin mRNA levels in the CNS of jimpy and shiverer mutant mice." J Mol Neurosci **4**(2): 89-96.

Chiu, F. C. and J. E. Goldman (1984). "Synthesis and turnover of cytoskeletal proteins in cultured astrocytes." J Neurochem **42**(1): 166-174.

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Cho, W., T. L. Hagemann, D. A. Johnson, J. A. Johnson and A. Messing (2009). "Dual transgenic reporter mice as a tool for monitoring expression of glial fibrillary acidic protein." J Neurochem **110**(1): 343-351.

Cho, W. and A. Messing (2009). "Properties of astrocytes cultured from GFAP over-expressing and GFAP mutant mice." Exp Cell Res **315**(7): 1260-1272.

Cordeau, P., Jr., M. Lalancette-Hebert, Y. C. Weng and J. Kriz (2008). "Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury." Stroke **39**(3): 935-942.

Cui, C., M. A. Wani, D. Wight, J. Kopchick and P. J. Stambrook (1994). "Reporter genes in transgenic mice." Transgenic Res **3**(3): 182-194.

Cunningham, R., P. Jany, A. Messing and L. Li (2013). "Protein changes in immunodepleted cerebrospinal fluid from a transgenic mouse model of alexander disease detected using mass spectrometry." J Proteome Res **12**(2): 719-728.

DeArmond, S. J., Y. L. Lee, H. A. Kretzschmar and L. F. Eng (1986). "Turnover of glial filaments in mouse spinal cord." J Neurochem **47**(6): 1749-1753.

Delcourt, N., P. Jouin, J. Poncet, E. Demey, E. Mauger, J. Bockaert, . . . N. Galeotti (2005). "Difference in mass analysis using labeled lysines (DIMAL-K): a new, efficient proteomic quantification method applied to the analysis of astrocytic secretomes." Mol Cell Proteomics **4**(8): 1085-1094.

DeMattos, R. B., K. R. Bales, M. Parsadanian, M. A. O'Dell, E. M. Foss, S. M. Paul and D. M. Holtzman (2002). "Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease." J Neurochem **81**(2): 229-236.

Dowell, J. A., J. A. Johnson and L. Li (2009). "Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics." J Proteome Res **8**(8): 4135-4143.

Greco, T. M., S. H. Seeholzer, A. Mak, L. Spruce and H. Ischiropoulos (2010). "Quantitative mass spectrometry-based proteomics reveals the dynamic range of primary mouse astrocyte protein secretion." J Proteome Res **9**(5): 2764-2774.

Hagemann, T. L., W. C. Boelens, E. F. Wawrousek and A. Messing (2009). "Suppression of GFAP toxicity by alphaB-crystallin in mouse models of Alexander disease." Hum Mol Genet **18**(7): 1190-1199.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

Hagemann, T. L., S. A. Gaeta, M. A. Smith, D. A. Johnson, J. A. Johnson and A. Messing (2005). "Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction." Hum Mol Genet **14**(16): 2443-2458.

Kadurugamuwa, J. L., K. Modi, O. Coquoz, B. Rice, S. Smith, P. R. Contag and T. Purchio (2005). "Reduction of astrogliosis by early treatment of pneumococcal meningitis measured by simultaneous imaging, in vivo, of the pathogen and host response." Infect Immun **73**(12): 7836-7843.

Keene, S. D., T. M. Greco, I. Parastatidis, S. H. Lee, E. G. Hughes, R. J. Balice-Gordon, . . . H. Ischiropoulos (2009). "Mass spectrometric and computational analysis of cytokine-induced alterations in the astrocyte secretome." Proteomics **9**(3): 768-782.

Keller, A. F., M. Gravel and J. Kriz (2009). "Live imaging of amyotrophic lateral sclerosis pathogenesis: disease onset is characterized by marked induction of GFAP in Schwann cells." Glia **57**(10): 1130-1142.

Krencik, R., J. P. Weick, Y. Liu, Z. J. Zhang and S. C. Zhang (2011). "Specification of transplantable astroglial subtypes from human pluripotent stem cells." Nat Biotechnol **29**(6): 528-534.

Kyllerman, M., L. Rosengren, L. M. Wiklund and E. Holmberg (2005). "Increased levels of GFAP in the cerebrospinal fluid in three subtypes of genetically confirmed Alexander disease." Neuropediatrics **36**(5): 319-323.

LaPash Daniels, C. M., E. V. Austin, D. E. Rockney, E. M. Jacka, T. L. Hagemann, D. A. Johnson, . . . A. Messing (2012). "Beneficial effects of nrf2 overexpression in a mouse model of alexander disease." J Neurosci **32**(31): 10507-10515.

Laping, N. J., B. Teter, N. R. Nichols, I. Rozovsky and C. E. Finch (1994). "Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors." Brain Pathol **4**(3): 259-275.

Lein, E. S., M. J. Hawrylycz, N. Ao, M. Ayres, A. Bensinger, A. Bernard, . . . A. R. Jones (2007). "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124): 168-176.

Levin-Allerhand, J., B. S. McEwen, C. E. Lominska, D. B. Lubahn, K. S. Korach and J. D. Smith (2001). "Brain region-specific up-regulation of mouse apolipoprotein E by pharmacological estrogen treatments." J Neurochem **79**(4): 796-803.

Liem, R. K. and A. Messing (2009). "Dysfunctions of neuronal and glial intermediate filaments in disease." J Clin Invest **119**(7): 1814-1824.

Luo, J., P. Ho, L. Steinman and T. Wyss-Coray (2008). "Bioluminescence in vivo imaging of autoimmune encephalomyelitis predicts disease." J Neuroinflammation **5**: 6.

Martin, P. M. and J. P. O'Callaghan (1995). "A direct comparison of GFAP immunocytochemistry and GFAP concentration in various regions of ethanol-fixed rat and mouse brain." J Neurosci Methods **58**(1-2): 181-192.

McAsey, M. E., C. Cady, L. M. Jackson, M. Li, S. Randall, B. P. Nathan and R. G. Struble (2006). "Time course of response to estradiol replacement in ovariectomized mice: brain apolipoprotein E and synaptophysin transiently increase and glial fibrillary acidic protein is suppressed." Exp Neurol **197**(1): 197-205.

McCall, M. A., R. G. Gregg, R. R. Behringer, M. Brenner, C. L. Delaney, E. J. Galbreath, . . . A. Messing (1996). "Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology." Proc Natl Acad Sci U S A **93**(13): 6361-6366.

Messing, A. and M. Brenner (2013). Genetic disorders affecting astrocytes. Neuroglia. H. Kettenmann and B. R. Ransom. New York, Oxford University Press**:** 884-895.

Messing, A., M. Brenner, M. B. Feany, M. Nedergaard and J. E. Goldman (2012). "Alexander disease." J Neurosci **32**(15): 5017-5023.

Messing, A., M. W. Head, K. Galles, E. J. Galbreath, J. E. Goldman and M. Brenner (1998). "Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice." Am J Pathol **152**(2): 391-398.

Messing, A., C. M. LaPash Daniels and T. L. Hagemann (2010). "Strategies for treatment in Alexander disease." Neurotherapeutics **7**(4): 507-515.

Mignot, C., C. Delarasse, S. Escaich, B. Della Gaspera, E. Noe, E. Colucci-Guyon, . . . D. Pham-Dinh (2007). "Dynamics of mutated GFAP aggregates revealed by real-time imaging of an astrocyte model of Alexander disease." Exp Cell Res **313**(13): 2766-2779.

Moore, N. H., L. G. Costa, S. A. Shaffer, D. R. Goodlett and M. Guizzetti (2009). "Shotgun proteomics implicates extracellular matrix proteins and protease systems in neuronal development induced by astrocyte cholinergic stimulation." J Neurochem **108**(4): 891-908.

Mor-Vaknin, N., A. Punturieri, K. Sitwala and D. M. Markovitz (2003). "Vimentin is secreted by activated macrophages." Nat Cell Biol **5**(1): 59-63.

Morrison, R. S., J. De Vellis, Y. L. Lee, R. A. Bradshaw and L. F. Eng (1985). "Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes." J Neurosci Res **14**(2): 167-176.

Palfreyman, J. W., D. G. Thomas, J. G. Ratcliffe and D. I. Graham (1979). "Glial fibrillary acidic protein (GFAP): purification from human fibrillary astrocytoma, development and validation of a radioimmunoassay for GFAP-like immunoactivity." J Neurol Sci **41**(1): 101-113.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Price, J. C., S. Guan, A. Burlingame, S. B. Prusiner and S. Ghaemmaghami (2010). "Analysis of proteome dynamics in the mouse brain." Proc Natl Acad Sci U S A **107**(32): 14508-14513.

Prust, M., J. Wang, H. Morizono, A. Messing, M. Brenner, E. Gordon, . . . A. Vanderver (2011). "GFAP mutations, age at onset, and clinical subtypes in Alexander disease." Neurology **77**(13): 1287-1294.

Stone, D. J., Y. Song, C. P. Anderson, K. K. Krohn, C. E. Finch and I. Rozovsky (1998). "Bidirectional transcription regulation of glial fibrillary acidic protein by estradiol in vivo and in vitro." Endocrinology **139**(7): 3202-3209.

Tamguney, G., K. P. Francis, K. Giles, A. Lemus, S. J. DeArmond and S. B. Prusiner (2009). "Measuring prions by bioluminescence imaging." Proc Natl Acad Sci U S A **106**(35): 15002-15006.

Tanaka, K. F., H. Takebayashi, Y. Yamazaki, K. Ono, M. Naruse, T. Iwasato, . . . K. Ikenaka (2007). "Murine model of Alexander disease: analysis of GFAP aggregate formation and its pathological significance." Glia **55**(6): 617-631.

Tang, G., Z. Xu and J. E. Goldman (2006). "Synergistic effects of the SAPK/JNK and the proteasome pathway on glial fibrillary acidic protein (GFAP) accumulation in Alexander disease." J Biol Chem **281**(50): 38634-38643.

Tang, G., Z. Yue, Z. Talloczy, T. Hagemann, W. Cho, A. Messing, . . . J. E. Goldman (2008). "Autophagy induced by Alexander disease-mutant GFAP accumulation is regulated by p38/MAPK and mTOR signaling pathways." Hum Mol Genet **17**(11): 1540-1555.

Watts, J. C., K. Giles, S. K. Grillo, A. Lemus, S. J. DeArmond and S. B. Prusiner (2011). "Bioluminescence imaging of Abeta deposition in bigenic mouse models of Alzheimer's disease." Proc Natl Acad Sci U S A **108**(6): 2528-2533.

Westgard, J. O. and P. L. Barry (2008). Basic method validation. Madison, WI, Westgard QC.

Zhu, L., S. Ramboz, D. Hewitt, L. Boring, D. S. Grass and A. F. Purchio (2004). "Non-invasive imaging of GFAP expression after neuronal damage in mice." Neurosci Lett **367**(2): 210-212.

Chapter 3:

Plasma and cerebrospinal fluid levels of GFAP in Alexander disease

Portions of this chapter derived from:

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AUTHORS CONTRIBUTIONS

PLJ performed the assays and calculated the GFAP values. AM recruited the patients, designed the experiments, and organized the tables. JCE did most of the statistical analysis. MSvdK and FE participated in planning and analysis of clinical information. All other authors helped obtain samples and clinical information. PLJ and AM both contributed to writing the manuscript.

ABSTRACT

Alexander disease (AxD) is a rare and usually fatal disease caused by mutations in glial fibrillary acidic protein (GFAP). While there is no treatment for AxD, current strategies for potential treatments focus on reducing GFAP concentrations below its toxic threshold or interfering with other downstream effects of GFAP toxicity. In anticipation of experimental therapies, we need to identify and evaluate suitable biomarkers which can act as indicators of the potential response to therapy. One potential biomarker is GFAP itself, which is normally present in both cerebrospinal fluid (CSF) and plasma at low levels and elevated after injury or in neurological diseases. While GFAP has not been quantified in plasma from AxD patients, a recent study did evaluate GFAP concentrations in AxD patient CSF. In the previous study, GFAP concentrations were significantly elevated in the CSF of all three AxD patients tested, though these results need to be confirmed in a larger cohort. In the current study, we analyzed GFAP concentrations in CSF and plasma from AxD patients compared with controls. We found GFAP was significantly elevated in both CSF and plasma from AxD patients, suggesting GFAP is a reliable biomarker and may be utilized in future clinical trials.

INTRODUCTION

Alexander disease (AxD) is a progressive and generally fatal neurogenetic disorder with ages of onset ranging from fetal through late adulthood. The hallmark feature of the pathology is cytoplasmic aggregates known as Rosenthal fibers (RFs) within astrocytes of the central nervous system (CNS). These aggregates are composed primarily of the astrocyte-specific intermediate filament, glial fibrillary acidic protein (GFAP), along with several small stress proteins. In 2001, we reported the discovery that heterozygous missense mutations in GFAP itself are responsible for nearly all cases of AxD (Brenner et al. 2001), a finding which has now been widely replicated and has led to the rapid adoption of GFAP sequencing as the standard diagnostic test worldwide. Nearly half of all patients carry mutations in either of two amino acids (R79 or R239), although it appears that mutations distributed throughout the protein produce essentially identical RFs and similar disease (Li et al. 2005; Messing et al. 2012). Mouse models have been created via both transgenic and knock-in approaches that reproduce key aspects of the Alexander phenotype, particularly the formation of RFs identical to those found in the human disease (Messing et al. 1998) and increased seizure susceptibility (Hagemann et al. 2006; Tanaka et al. 2007). These and other studies point to the idea that the GFAP mutations, which are genetically dominant, act in a gain-of-function fashion, and that elevations of total GFAP levels may be a major factor in pathogenesis. Several strategies are now being discussed as potential treatments for Alexander disease, chief among them being pharmacologic approaches for suppressing expression of GFAP below its toxic threshold or interfering with other downstream effects of GFAP toxicity (Messing et al. 2010).

The prospect of experimental therapies now highlights the need to begin planning the design of clinical trials. The patients can serve as their own controls, by comparing status pre- and post-treatment. However, though existing MRI criteria are highly reliable as diagnostic tools, they are not suitable for quantifying disease severity or monitoring disease progression.

In anticipation of the prospect for any clinical trial (ours or others), there is an urgent need to identify and evaluate biomarkers that can serve as surrogate indicators of the potential response to therapy.

One potential biomarker is GFAP itself. GFAP is normally present only at low levels in plasma and CSF, fluids that are often collected as part of routine diagnostic workups. Increased levels of GFAP in plasma and CSF have been found in a number of clinical settings (Liem and Messing 2009). Additionally, quantification of GFAP in CSF from mouse models of AxD revealed GFAP concentrations in CSF increased in a manner corresponding to brain GFAP concentrations (Jany et al. 2013), suggesting GFAP concentrations in CSF reflect GFAP concentrations in the brain. While GFAP has not been quantified in plasma from AxD patients, a recent study did evaluate GFAP concentrations in AxD patient CSF. Kyllerman et al. (2005) found that GFAP was elevated in the CSF of AxD patients, though only three patients were examined and only one determination was made for each patient. These results are in need of replication in a larger cohort of patients. In the current study, we quantified GFAP in AxD patient plasma and CSF and found GFAP was significantly elevated in both compared with controls. These results suggest GFAP is a reliable biomarker in plasma and CSF of AxD patients.

MATERIALS AND METHODS

AxD patient samples

The sole inclusion criterion for participation in this study was genetic confirmation of the diagnosis by sequencing of GFAP. Informed consent for studies of CSF was obtained following protocols approved by the Institutional Review Boards (IRBs) at the University of Wisconsin-Madison, the Children's National Medical Center (CNMC), and the Mayo Clinic. Only leftover samples from a previous clinical use were permitted for study. Informed consent for studies of

blood was obtained following protocols approved by IRB's at the University of Wisconsin-Madison, CNMC, Massachusetts General Hospital, Washington University in St. Louis, and Mayo Clinic. Again, consents were obtained either through in-person interview or by telephone, with written confirmation. For samples collected in The Netherlands, Italy, and Germany, the principles outlined in the Declaration of Helsinki were followed.

Control samples

Controls for CSF studies consisted of 24 de-identified samples collected by lumbar puncture (LP) at CNMC for various purposes but were considered within the range of normal for protein, glucose, and cell counts. The CSF controls were exempted from the requirement for consent. Additional information about the CSF control group (age, sex, reason for LP) is given in Table 3.1. Controls for blood were obtained from apparently healthy adults of both sexes who were asked to exclude themselves if they had specific conditions (neurologic or psychiatric disorders, head or brain trauma within the past 12 months, type I diabetes, inflammatory bowel disease) or were taking specific medications within the past 3 months (clomipramine, amitriptyline, prednisone, dexamethasone, or tamoxifen). The exclusion criteria for plasma controls were based on a literature review of conditions known to influence GFAP levels in CSF and blood (Liem and Messing 2009), and a study specifically aimed at identifying pharmacological modifiers of GFAP expression (Cho et al. 2010).

Plasma preparation

Fresh samples of venous blood were collected into lavender-topped tubes that contained K_2-EDTA as anti-coagulant to allow preparation of plasma. The samples were centrifuged within 60 min of collection at 2500 rcf for 15 min at room temperature (RT), and the supernatant immediately placed in a polypropylene tube and stored either on dry ice for shipping or in a

-20°C freezer until shipping could be arranged. In Wisconsin, the samples were then thawed, divided into aliquots, and stored at -80°C until further analysis.

Quantitation of GFAP protein

GFAP protein levels in CSF and plasma were quantitated using a sandwich ELISA as previously described (Jany et al. 2013). The capture antibodies consisted of a cocktail of monoclonal antibodies (Covance, SMI-26R) diluted in phosphate buffered saline (PBS) (Fisher, # BP3994). Plates were blocked with 5% milk in PBS before addition of samples or standards diluted in PBS with 0.05% Tween 20 (Tw20) and 1% bovine albumin serum (BSA) (Sigma, # A7030), with each sample analyzed in triplicate. Antibody incubation steps were performed in 5% milk-PBS, and washing steps were performed in PBS-Tw20. Standard curves were generated using bovine GFAP (Fitzgerald Industries International, # RDI-PRO62007), and reaction volumes consisted of 100 µL/well for both CSF and plasma. CSF and plasma samples were initially diluted 1:1 with ELISA buffer, though in some cases higher dilutions were necessary to bring the values within the linear range of the assay. GFAP values were expressed as ng/L. Under these conditions the lower limit of detection was 11 ng/L, and the biological limit of detection (BLD) (after accounting for the minimum 1:1 dilution with reaction buffer) was 46 ng/L. Any samples below the BLD were given a value of 46 ng/L for all statistical analysis. The intra-assay coefficient of variation (COV), determined using the bovine GFAP standard at 33 ng/L in 10 sets of triplicate wells, was 13%. The inter-assay COV, determined using pooled CSF samples taken from *GFAP*^{Tg} mice that over-express wild type human GFAP, was 11%. CSF and plasma samples from Gfap-null mice give readings that are below the BLD in this assay (data not shown), thus validating its specificity. In addition, plasma samples from Gfap-null mice were spiked with known concentrations of purified bovine GFAP to verify that the

1:1 dilutions of plasma used here did not interfere with the sensitivity of the assay (data not shown).

RESULTS

Patient Population

Samples were collected from AxD patients with confirmed mutations in GFAP. Those for whom leftover clinical CSF samples were available included 4 females and 6 males, ranging in age from 2 to 46. Those for whom plasma samples were collected included 19 females and 14 males, ranging in age from 1 to 65. Both plasma and CSF samples were available for 8 of these patients. Information for each patient regarding gender, specific mutation, age of first symptom, age at collection of samples, and age at death (if applicable) is provided in Table 3.2.

GFAP levels in Cerebrospinal Fluid

We established a reference range for controls in our assay using a set of 24 samples obtained at the Children's National Medical Center, and found the mean GFAP level to be 249 ± 316 ng/L (range = 46 to 1386 ng/L)(see Table 3.1 and Fig. 3.1 for additional data including GFAP in relation to age). CSF samples were available for 10 AxD patients, and in a single assay, all of the AxD samples were run alongside 12 of the controls (Fig. 3.1) Considered as a group, GFAP levels in the AxD patients were significantly elevated compared with the controls ($p<0.001$). Considered as individuals, ten of the eleven AxD samples were elevated compared to the control population. Because of the paucity of samples, this experiment was replicated once, with similar results. Individual GFAP values for each AxD patient in relation to age, gender, genotype, and category of disease are shown in Table 3.3.

GFAP levels in Blood

For plasma GFAP concentrations, we established a reference range for controls using a set of 111 samples obtained from healthy volunteers. We found GFAP concentrations ranged from 46 to 1118 ng/L with an average of 155 ± 200 ng/L (see Fig. 3.2 for comparisons between GFAP in age or gender). Plasma samples were available from 33 AxD patients. In a single assay, all of the AxD samples were run alongside a subset of 12 controls. GFAP concentrations in the subset compared to the full 111 plasma control samples revealed no statically significant differences (data not shown). As a group, AxD patient GFAP concentrations in plasma were significantly elevated compared with controls (Fig. 3.3). These results were confirmed with two replicate assays. In some AxD patients, both plasma and CSF samples were collected. Comparison of GFAP concentrations in CSF and plasma from the same individual revealed GFAP concentrations were significantly higher in CSF compared with plasma (p=0.0002, data not shown). Correlation analysis between CSF and plasma revealed a positive correlation, though it did not reach statistical significance (r=0.68, p=0.0503) (Fig. 3.4).

DISCUSSION

AxD is caused by mutations in GFAP which lead to protein upregulation above an unknown toxic threshold. While there is no cure for AxD, reduction of GFAP concentrations may alleviate AxD patient symptoms. Drug studies are currently underway in mouse models of AxD to determine potential therapies which may be translated to AxD patients. In anticipation of clinical trials, we analyzed GFAP as a biomarker in CSF and plasma of AxD patients compared with controls. We found GFAP was significantly elevated in both CSF and plasma of AxD patients, suggesting GFAP is a reliable biomarker for AxD. These results indicate GFAP may be a useful tool for analyzing drug efficacy during future clinical trials.

GFAP has been analyzed as a biomarker in CSF and plasma for several diseases and injuries (Liem and Messing 2009). After brain ischemic injury, GFAP concentrations in CSF and plasma correlate with infarct size and clinical classification (Aurell et al. 1991; Herrmann et al. 2000; Wunderlich et al. 2006), suggesting GFAP may correlate with severity and/or progression in AxD patients. In a preliminary study of three AxD patients, GFAP concentrations in CSF were significantly elevated compared with control CSF, though no correlation was found between GFAP and disease severity (Kyllerman et al. 2005).

We analyzed GFAP in CSF from a larger cohort of AxD patients (n=11) to determine if GFAP is increased in all AxD patients and elucidate the range of GFAP concentrations in CSF. In addition, we quantified GFAP in AxD patient plasma (n=33). We found GFAP was significantly elevated in AxD patient CSF and plasma compared with controls. There are conflicting results on the correlation between GFAP in CSF and age (Table 3.4) (Rosengren et al. 1992; Ahlsen et al. 1993; Rosengren et al. 1994; Fukuyama et al. 2001; Petzold et al. 2004; Axelsson et al. 2011). We found no correlation between age and GFAP concentration in plasma in our control samples, similar to previous studies (Herrmann et al. 2000; Vos et al. 2006; Dvorak et al. 2009). However, a negative correlation was found between age and GFAP concentration in AxD patient plasma samples, which likely reflects disease severity rather than a change in GFAP concentration with age. While we have not finished the correlation analyses between GFAP concentrations and disease severity or progression, we did find a significant difference in plasma GFAP concentrations when comparing infantile to adult forms of AxD (Fig. 3.5), suggesting GFAP will correlate to disease severity.

In the current study, comparisons of GFAP concentrations between AxD patients and controls were complicated by the lack of age matched healthy controls. Control patient CSF samples were de-identified samples from CNMC who were considered to have normal protein, glucose, and cell counts in CSF. GFAP concentrations were detectable but low in

approximately 90% of the control patients and were comparable to previous reports (Jesse et al. 2009; Misu et al. 2009; Axelsson et al. 2011), suggesting the current CSF controls are sufficient for analysis. For plasma samples, AxD ages ranged from less than one year to 65 years of age with a median age of 15. Conversely, control plasma samples were from volunteers aged 18 or older with a median age of 30. While the absence of age-matched controls may be a caveat, the current and previous studies reveal no correlation between GFAP and age in control plasma samples (Herrmann et al. 2000; Vos et al. 2006; Dvorak et al. 2009). This lack of correlation between GFAP and age suggests the addition of age matched plasma controls samples may not alter the results seen in this study.

For the few AxD patients where both CSF and plasma were collected, comparison of GFAP concentrations in plasma and CSF samples from the same individuals revealed a positive correlation. GFAP concentrations in CSF were also higher than plasma, similar to previous reports (Beems et al. 2003; Steiner et al. 2006; Kwon et al. 2010). Since GFAP concentrations are lower in plasma samples, further analysis of plasma GFAP concentrations in AxD patients is required to elucidate the sensitivity of GFAP as a biomarker in plasma.

Overall, we found GFAP concentrations were significantly elevated in AxD patient CSF and plasma compared with controls. Similarly, mouse models of AxD have elevated GFAP concentrations in CSF which reflect brain GFAP concentrations (Jany et al. 2013). Together, these results suggest GFAP may be a reliable biomarker for analysis of severity and/or progression in AxD patients as well as drug effects during future therapies. In future studies, detailed clinical information will be obtained from AxD patients to allow correlation analysis between GFAP and disease severity and/or progression.

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Table 3.1 - Control CSF samples (sorted by age at collection)

age at collection	sex	reason for lumbar puncture
0.08	M	RSV bronchiolitis
0.08	F	acute respiratory failure
0.12	F	Acute life-threatening event
0.66	M	Vomiting
2.16	F	Gastrointestinal virus
2.25	M	Lymphoma
3.08	M	DiGeorge syndrome
3.75	F	acute lymphocytic leukemia/chemotherapy evaluation
3.92	M	acute lymphocytic leukemia/chemotherapy evaluation
4.08	M	acute lymphocytic leukemia/chemotherapy evaluation
4.33	M	acute lymphocytic leukemia/chemotherapy evaluation
5.25	M	acute lymphocytic leukemia/chemotherapy evaluation
5.33	M	acute lymphocytic leukemia
5.75	M	acute lymphocytic leukemia/chemotherapy evaluation
6.92	F	acute lymphocytic leukemia/chemotherapy evaluation
9.25	M	acute lymphocytic leukemia/chemotherapy evaluation
9.58	F	Ehlers-Danlos syndrome, mental status change
12.33	M	acute lymphocytic leukemia/chemotherapy evaluation
14.16	F	acute lymphocytic leukemia/chemotherapy evaluation
14.75	F	acute lymphocytic leukemia/chemotherapy evaluation
15.33	F	idiopathic intracranial hypertension
16.66	M	acute lymphocytic leukemia/chemotherapy evaluation
17.33	M	acute lymphocytic leukemia/chemotherapy evaluation
19.00	M	acute lymphocytic leukemia/chemotherapy evaluation

Table 3.1: Control CSF Samples. Control CSF samples were de-identified from individuals requiring CSF evaluation. Age at collection is given in years. Abbreviations: RSV, respiratory syncytial virus.

Table 3.2 – Alexander disease patient samples

ID#	mutation (blocked for privacy reasons)	sex	age of onset	age at CSF collection	age at blood collection	age of death
1		F	40.5		44.67	
2		M	0.16		1.75	
3		F	1		3.26	
4		F	0.58		19.19	
5		M	0.5	17.28	20.84	
6		M	0.25		6.20	
7		F	4*	4*		
8		F	0.5		2.02	
9		F	0.29		2.25	2.65
10		F	0.58	6.27	7.11	
11		F	0.5		1.92	
12		F	1.25		10.51	
13		F	0		3.07	
14		M	0.5	4.65	4.65	
15		M	4		13.36	
16		M	0.75		2.24	
17		M	2		5.77	
18		M	10	15.78	17.87	
19		F	10		40.95	
20		M	10	17.32	21.62	
21		F	6		15.74	
22		M	50		56.72	
23		M	10.5		22.11	22.52
24		M	50		64.88	
25		M	N/A		34.85	
26		M	0.5		2.50	
27		F	1.5		2.10	
28		F	7.00		9.20	
29		F	1.67		1.90	
30		F	0.29		0.72	1.14
31		F	0		0.96	1.00
32		M	2	23.85	23.85	
33		M	1.5		3.25	

ID#		sex	age of onset	age at CSF collection	age at blood collection	age of death
34		M	10		36.68	
35		M	0		2.61	
36		F	0.25*		3.93	
37		F	12		13.82	
38		F	5		20.52	
39		M	<1*		9.78	
40		F	34		36.60	
41		F	0	1.91		12.82
42		F	45	45.59	45.84	
43		F	51*		56.67	
44		F	40		52.22	
45		F	4.00		19.46	
46		M	14	31.47	31.19	33.63
47		M	13		31.68	
48		M	6		8.14	
49		F	16		25.93	
50		F	30	44.15	44.15	

***** Estimated age of onset
◊ Unsure if mutation is disease causing/pathogenic

Table 3.2: Alexander disease patient samples. CSF and blood samples from Alexander disease (AxD) patients were obtained with consent from patients containing a mutation within their GFAP sequence. All blood samples are plasma, except for 9, 16, and 50 which are serum. ID is the unique ID number used in the current study. For some patients, age of onset was estimated (*) or the patient was asymptomatic but had familial history of AxD (N/A). If no age of collection is identified, then CSF or plasma was not collected from that individual. All ages are given in years. Abbreviations: CSF, cerebrospinal fluid.

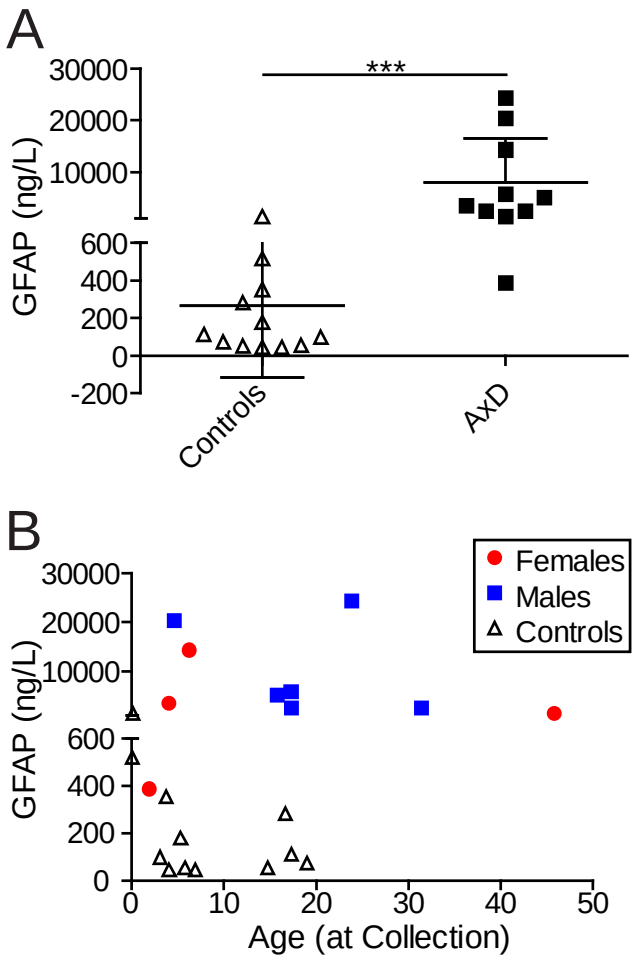
A
30000
20000
10000
600
400
200
0
-200
GFAP (ng/L)
***
Controls
AxD
B
30000
20000
10000
600
400
200
0
GFAP (ng/L)
0
10
20
30
40
50
Age (at Collection)
Females
Males
Controls

Figure 3.1: GFAP levels in CSF of both control and AxD patients. (A) GFAP is significantly elevated in AxD patient CSF compared with controls. (Wilcoxon Rank Sum test, *** $p<0.001$). Presented as mean ± 1 standard deviation (SD) on a split linear scale. (B) GFAP concentrations in CSF do not correlate with age of collection. (Spearman's Rank-Order Correlation). Each data point represents one patient (n=7♂, 5♀ controls and 6♂, 4♀ AxD patients).

Table 3.3 - GFAP concentrations in CSF and plasma in AxD patients.

ID#	mutation (blocked for privacy reasons)	sex	CSF (ng/L)	blood (ng/L)	disease classification	duration of illness (CSF)	duration of illness (blood)
1		F		64	Adult		4.17
2		M		1640	Infantile		1.59
3		F		802	Infantile		2.26
4		F		480	Infantile		18.61
5		M	5803	256	Infantile	16.78	20.34
6		M		1864	Infantile		5.95
7		F	3489		Juvenile	0.10	
8		F		426	Infantile		1.52
9		F		1201	Infantile		1.96
10		F	14290	1154	Infantile	5.69	6.53
11		F		255	Infantile		1.42
12		F		302	Infantile		9.26
13		F		572	Infantile		3.07
14		M	20355	1925	Infantile	4.15	4.15
15		M		238	Juvenile		9.36
16		M		46	Infantile		1.49
17		M		329	Infantile		3.77
18		M	5095	132	Juvenile	5.78	7.87
19		F		1068	Juvenile		30.95
20		M	2493	122	Juvenile	7.32	11.62
21		F		105	Juvenile		9.74
22		M		219	Adult		6.72
23		M		751	Juvenile		11.61
24		M		95	Adult		14.88
25		M		243			N/A
26		M		1007	Infantile		2.00
27		F		791	Infantile		0.60
28		F		504	Juvenile		2.20
29		F		237	Infantile		0.23
30		F		169	Infantile		0.43
31		F		711	Infantile		0.96
32		M	24272	713	Infantile	21.85	21.85
33		M		461	Infantile		1.75

Table 3.3 continued - GFAP concentrations in CSF and plasma in AxD patients.

ID#	mutation	sex	CSF (ng/L)	blood (ng/L)	disease classification	duration of illness (CSF)	duration of illness (blood)
34		M		248	Juvenile		26.68
35		M		750	Infantile		2.61
36		F		1314	Infantile		3.68†
37		F		446	Juvenile		1.82
38		F		322	Juvenile		15.52
39		M		704	Infantile		8.88†
40		F		187	Adult		2.60
41		F	387		Infantile	1.91	
42		F	1402	505	Adult	0.59	0.84
43		F		112	Adult		5.67†
44		F		46	Adult		12.22
45		F		571	Juvenile		15.46
46		M	2478	62	Adult	17.47	17.19
47		M		545	Adult		18.68
48		M		712	Juvenile		2.14
49		F		64	Adult		9.93
50		F	1749	114	Adult	14.15	14.15

† Estimate duration of illness
Duration of illness is the age of onset subtracted from the age at collection (see Table 3.2)

Table 3.3: GFAP concentrations in CSF and plasma in AxD patients. GFAP concentrations (ng/L) in CSF and plasma of individual AxD patients. Patient 25 was asymptomatic at the time of collection (N/A). Disease classification refers to the form of AxD: Infantile, Juvenile, or Adult. Duration of illness is defined as the age at onset subtracted from age at collection.

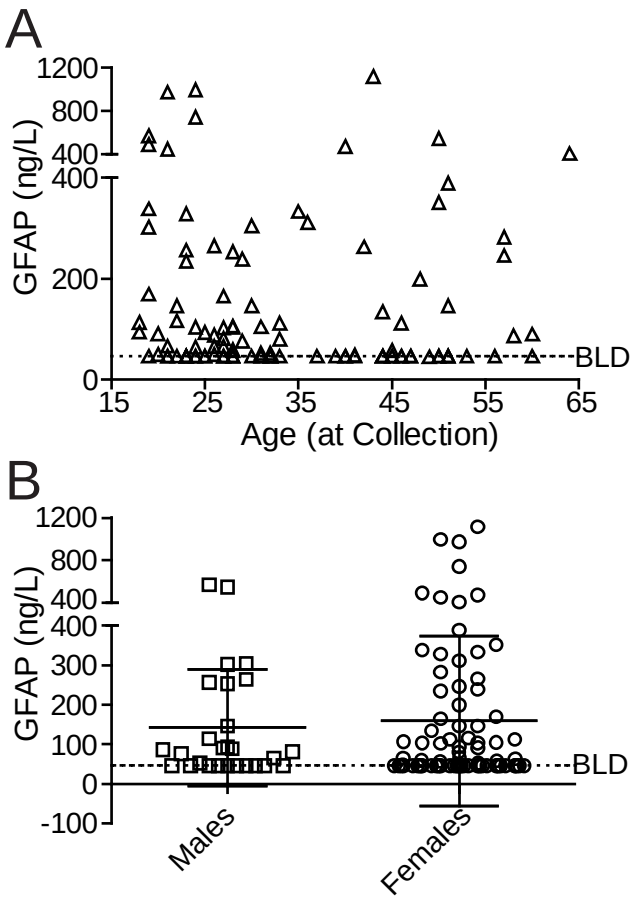
A
1200
800
400
400
200
0
GFAP (ng/L)
BLD
15
25
35
45
55
65
Age (at Collection)
B
1200
800
400
400
300
200
100
0
-100
GFAP (ng/L)
BLD
Males
Females

Figure 3.2: Comparison of GFAP concentrations to age and gender in all plasma control samples. (A) GFAP concentrations in plasma control samples do not correlate with age at collection. (Spearman's Rank-Order Correlation). Each data point represents one patient. (B) There are no differences in GFAP concentration between male and female controls. (Wilcoxon Rank Sum test). Presented as mean ± 1 SD on a split linear scale (n=111).

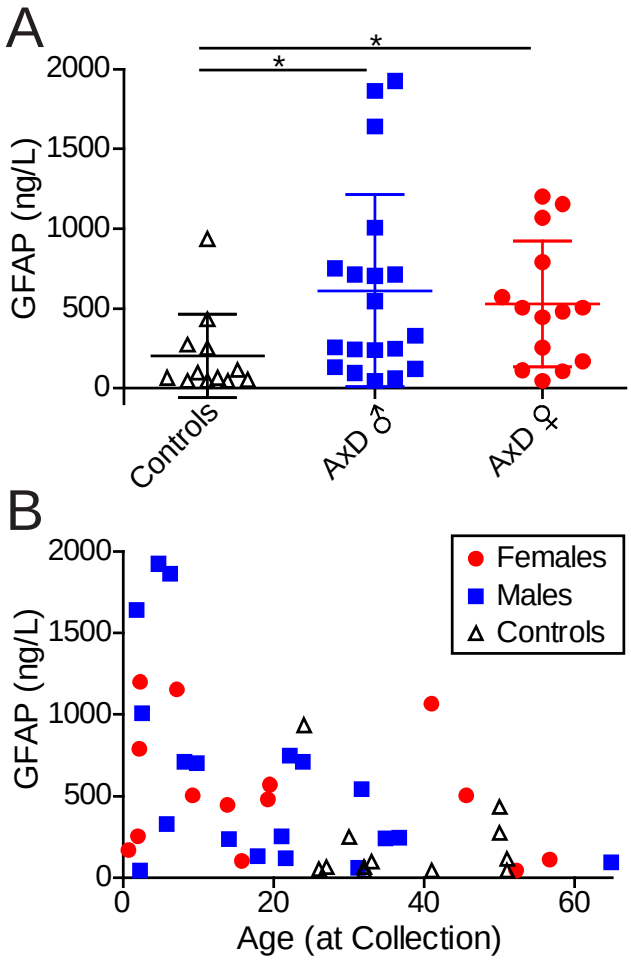
A
*
*
2000
1500
1000
500
0
GFAP (ng/L)
Controls
AxD ♂
AxD ♀
B
2000
1500
1000
500
0
GFAP (ng/L)
Females
Males
Controls
0
20
40
60
Age (at Collection)

Figure 3.3: AxD patient and control plasma GFAP concentrations and its correlation with age. (A) In AxD patient plasma, GFAP concentrations are significantly elevated compared with control plasma. (Kruskal-Wallis One-way ANOVA with Dunns multiple comparison, * $p<0.05$). Presented as mean ± 1 SD. (B) No correlation between GFAP and age at collection was found in controls. In AxD patients, GFAP negatively correlated with age. (Spearman's Rank-Order Correlation, r=-0.38, $p=0.0281$). Each data point represents one patient (n=12 controls, 19 AxD males, and 14 AxD females).

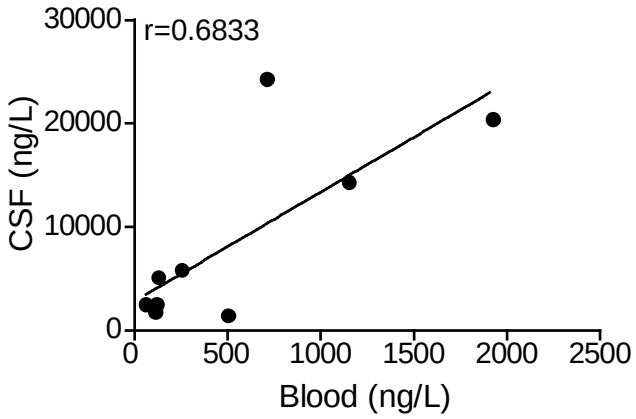
r=0.6833
CSF (ng/L)
30000
20000
10000
0
0
500
1000
1500
2000
2500
Blood (ng/L)

Figure 3.4: A direct comparison of GFAP concentrations in CSF and plasma of AxD patients. In AxD patients where both CSF and plasma were collected (n=8), there was a non-significant positive correlation between GFAP concentrations in plasma and CSF. (Spearman's Rank-Order Correlation, p=0.0503). Each data point represents one patient.

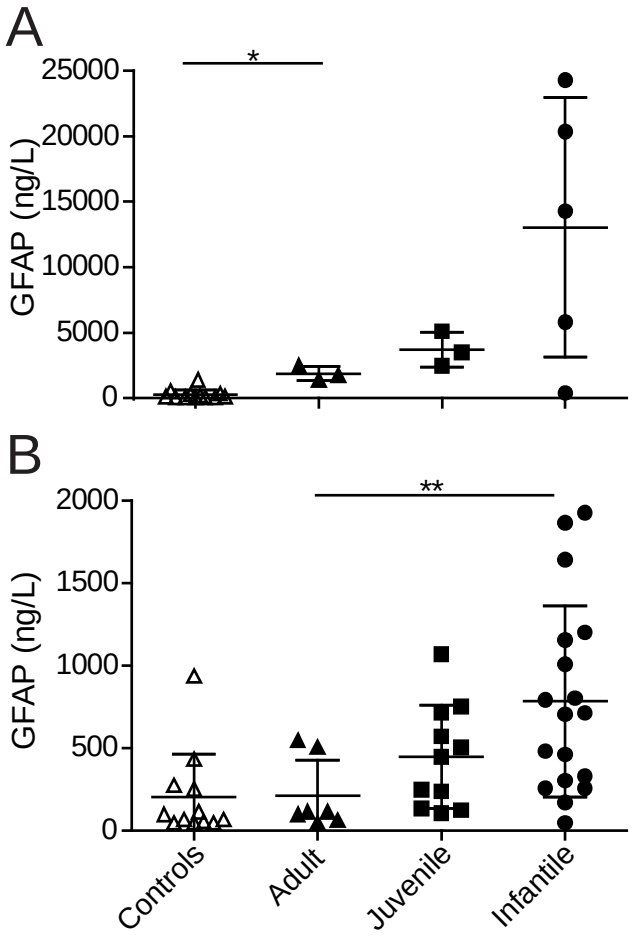
A
*
25000
20000
15000
10000
5000
0
GFAP (ng/L)
B
**
2000
1500
1000
500
0
GFAP (ng/L)
Controls
Adult
Juvenile
Infantile

Figure 3.5: Correlation between GFAP levels in CSF and blood and the disease category of AxD in patients. (A) GFAP concentrations in CSF do not correlate with the AxD disease category (Infantile, Juvenile, Adult). All three disease categories of AxD showed GFAP concentrations which were significantly elevated compared with controls (only adult compared with control shown) (n=12 controls and 3 adult, 3 juvenile, and 4 infantile AxD patients). (B) Infantile AxD patients have GFAP concentrations in blood which are significantly elevated compared with adult AxD patients. Juvenile AxD patients have intermediate values of GFAP in blood which are not significantly different from either infantile or adult AxD patients. GFAP concentrations in adult AxD patients were not significantly elevated compared with controls (n=12 controls and 7 adult, 11 juvenile, and 18 infantile AxD patients). (Data was first log transformed [$Y = \log(Y)$] to equalize the standard deviations (figure shows the original untransformed data) and then analyzed using a 2-way ANOVA with post Bonferonni-corrected t-tests, * $p<0.05$; ** $p<0.01$). Presented as mean ± 1 SD.

Table 3.4 - GFAP correlation with age in control CSF.

reference	control population	age	n	GFAP (ng/L)	correlation with age
(Ahlsen et al. 1993)	individuals undergoing elective non-neurological surgery	10.8 (1.3-18)	10	61 ± 17 (SEM)	ns, r=0.50
(Axelsson et al. 2011)	volunteers, collected at 2 different time points approximately 9 years apart	33 (18-53)	28	358 ± 122[a]	**, r=0.50
(Fukuyama et al. 2001)	individuals with no abnormal biochemical or cytological CSF analyses	14 (1-25)	13	2960 ± 1040	ns
		41.6 (26-55)	9	2800 ± 1460	ns
		65.4 (56+)	8	3990 ± 1550	ns
(Petzold et al. 2004)	from sample library of neurologic patients undergoing routine lumbar puncture	44.3 ± 15.9	315	≤ 9	ns, r=-0.02
(Rosengren et al. 1994)	volunteers/individuals undergoing elective non-neurological surgery	≤ 20 (16-77)	25	≤ 200	***
		75+ (16-77)		500-1300	
(Rosengren et al. 1992)	individuals undergoing elective non-neurological surgery	1.3-18	10	66.6 ± 17.2 (SEM)	**
		28-29	3		

[a] saw 6.5 ± 5.9 annual increase in GFAP

Table 3.4: GFAP correlation with age in control CSF. Literature comparison of the correlation between GFAP (ng/L) in CSF and age. Age is given as the mean age in years with the range in parentheses. GFAP is given as the average ± 1 SD (unless otherwise indicated). (Correlation Analysis, ** $p<0.01$; *** $p<0.001$). Abbreviations: CSF, cerebrospinal fluid; n, number of patients in that study.

REFERENCE

Ahlsen, G., L. Rosengren, M. Belfrage, A. Palm, K. Haglid, A. Hamberger and C. Gillberg (1993). "Glial fibrillary acidic protein in the cerebrospinal fluid of children with autism and other neuropsychiatric disorders." Biol Psychiatry **33**(10): 734-743.

Aurell, A., L. E. Rosengren, B. Karlsson, J. E. Olsson, V. Zbornikova and K. G. Haglid (1991). "Determination of S-100 and glial fibrillary acidic protein concentrations in cerebrospinal fluid after brain infarction." Stroke **22**(10): 1254-1258.

Axelsson, M., C. Malmestrom, S. Nilsson, S. Haghighi, L. Rosengren and J. Lycke (2011). "Glial fibrillary acidic protein: a potential biomarker for progression in multiple sclerosis." J Neurol **258**: 882-888.

Beems, T., K. S. Simons, W. J. Van Geel, H. P. De Reus, P. E. Vos and M. M. Verbeek (2003). "Serum- and CSF-concentrations of brain specific proteins in hydrocephalus." Acta Neurochir (Wien) **145**(1): 37-43.

Brenner, M., A. B. Johnson, O. Boespflug-Tanguy, D. Rodriguez, J. E. Goldman and A. Messing (2001). "Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease." Nat Genet **27**(1): 117-120.

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Dvorak, F., I. Haberer, M. Sitzer and C. Foerch (2009). "Characterisation of the diagnostic window of serum glial fibrillary acidic protein for the differentiation of intracerebral haemorrhage and ischaemic stroke." Cerebrovasc Dis **27**(1): 37-41.

Fukuyama, R., T. Izumoto and S. Fushiki (2001). "The cerebrospinal fluid level of glial fibrillary acidic protein is increased in cerebrospinal fluid from Alzheimer's disease patients and correlates with severity of dementia." Eur Neurol **46**(1): 35-38.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

Herrmann, M., P. Vos, M. T. Wunderlich, C. H. de Bruijn and K. J. Lamers (2000). "Release of glial tissue-specific proteins after acute stroke: A comparative analysis of serum concentrations of protein S-100B and glial fibrillary acidic protein." Stroke **31**(11): 2670-2677.

Jany, P. L., T. L. Hagemann and A. Messing (2013). "GFAP expression as an indicator of disease severity in mouse models of Alexander disease." ASN Neuro **5**(1): e00109.

Jesse, S., P. Steinacker, L. Cepek, C. A. von Arnim, H. Tumani, S. Lehnert, . . . M. Otto (2009). "Glial fibrillary acidic protein and protein S-100B: different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease." J Alzheimers Dis **17**(3): 541-551.

Kwon, B. K., A. M. Stammers, L. M. Belanger, A. Bernardo, D. Chan, C. M. Bishop, . . . M. F. Dvorak (2010). "Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury." J Neurotrauma **27**(4): 669-682.

Kyllerman, M., L. Rosengren, L. M. Wiklund and E. Holmberg (2005). "Increased levels of GFAP in the cerebrospinal fluid in three subtypes of genetically confirmed Alexander disease." Neuropediatrics **36**(5): 319-323.

Li, R., A. B. Johnson, G. Salomons, J. E. Goldman, S. Naidu, R. Quinlan, . . . M. Brenner (2005). "Glial fibrillary acidic protein mutations in infantile, juvenile, and adult forms of Alexander disease." Ann Neurol **57**(3): 310-326.

Liem, R. K. and A. Messing (2009). "Dysfunctions of neuronal and glial intermediate filaments in disease." J Clin Invest **119**(7): 1814-1824.

Messing, A., M. Brenner, M. B. Feany, M. Nedergaard and J. E. Goldman (2012). "Alexander disease." J Neurosci **32**(15): 5017-5023.

Messing, A., M. W. Head, K. Galles, E. J. Galbreath, J. E. Goldman and M. Brenner (1998). "Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice." Am J Pathol **152**(2): 391-398.

Messing, A., C. M. LaPash Daniels and T. L. Hagemann (2010). "Strategies for treatment in Alexander disease." Neurotherapeutics **7**(4): 507-515.

Misu, T., R. Takano, K. Fujihara, T. Takahashi, S. Sato and Y. Itoyama (2009). "Marked increase in cerebrospinal fluid glial fibrillar acidic protein in neuromyelitis optica: an astrocytic damage marker." J Neurol Neurosurg Psychiatry **80**(5): 575-577.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Rosengren, L. E., G. Ahlsen, M. Belfrage, C. Gillberg, K. G. Haglid and A. Hamberger (1992). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of children." J Neurosci Methods **44**(2-3): 113-119.

Rosengren, L. E., C. Wikkelso and L. Hagberg (1994). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of adults." J Neurosci Methods **51**(2): 197-204.

Steiner, J., H. Bielau, H. G. Bernstein, B. Bogerts and M. T. Wunderlich (2006). "Increased cerebrospinal fluid and serum levels of S100B in first-onset schizophrenia are not related to a degenerative release of glial fibrillar acidic protein, myelin basic protein and neurone-specific enolase from glia or neurones." J Neurol Neurosurg Psychiatry **77**(11): 1284-1287.

Tanaka, K. F., H. Takebayashi, Y. Yamazaki, K. Ono, M. Naruse, T. Iwasato, . . . K. Ikenaka (2007). "Murine model of Alexander disease: analysis of GFAP aggregate formation and its pathological significance." Glia **55**(6): 617-631.

Vos, P. E., M. van Gils, T. Beems, C. Zimmerman and M. M. Verbeek (2006). "Increased GFAP and S100beta but not NSE serum levels after subarachnoid haemorrhage are associated with clinical severity." Eur J Neurol **13**(6): 632-638.

Wunderlich, M. T., C. W. Wallesch and M. Goertler (2006). "Release of glial fibrillary acidic protein is related to the neurovascular status in acute ischemic stroke." Eur J Neurol **13**(10): 1118-1123.

Chapter 4:

Estrogens and GFAP expression in a mouse model of Alexander Disease

This chapter is derived from:

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ABSTRACT

Alexander disease (AxD) is a rare neurodegenerative disorder caused by mutations within glial fibrillary acidic protein (GFAP). These mutations lead to upregulation of GFAP through unknown mechanisms. There is no cure for AxD, but reducing GFAP protein levels through drug treatments may alleviate AxD symptoms. A previous *in vitro* drug screen of FDA-approved drugs identified estradiol (E2) as a potential treatment for AxD. To determine if E2 decreases GFAP levels *in vivo*, we investigated GFAP concentrations during hormone deprivation through ovariectomy (OVX), E2 treatment, and the estrus cycle in a mouse model of AxD, *Gfap*$^{R236H/+}$. We found that E2 had minimal effects on GFAP in mice. E2 treatment decreased GFAP after OVX in the olfactory bulb and hippocampus of wild-type mice and the hippocampus and brain of *Gfap*$^{R236H/+}$ mice, but not in any of the brain regions of sham-operated mice. *Gfap* mRNA was unaltered by surgical procedures or treatment in all mice. We also found GFAP response to OVX varied in different background strains of *Gfap*$^{R236H/+}$ mice. The FVB/N background strain increased GFAP concentrations after OVX, while mice of the B6 strain had no change in GFAP concentrations. In addition, GFAP protein and mRNA were unaltered by the stage of estrus in *Gfap*$^{R236H/+}$ mice of the FVB/N background. Overall, these results indicate E2 regulation of GFAP may vary with brain region, stress, and mouse background strain, indicating caution in the implementation of estrogen-based treatments for AxD.

INTRODUCTION

Alexander Disease (AxD) is a rare and generally fatal neurodegenerative disorder caused by mutations within the gene encoding glial fibrillary acidic protein (GFAP), the major intermediate filament of astrocytes (Brenner et al. 2001). These mutations, which occur at multiple sites throughout the central rod domain and tail domains, activate multiple pathways that converge on one or more positive feedback loops leading to accumulation of GFAP in nearly all regions of the central nervous system (CNS). The increased levels of GFAP are hypothesized to be toxic for astrocytes, and contribute in some as yet undefined fashion to astrocyte dysfunction (Messing et al. 2012).

One approach proposed as a therapeutic strategy for Alexander disease is to suppress the expression of GFAP, thereby reducing levels below the toxic threshold. Using primary cultures of cerebral cortical astrocytes, Cho et al. (2010) screened libraries of FDA-approved drugs in search of compounds that reduced either promoter activity (monitored through a GFAP-luciferase reporter) or protein. One compound identified through this screen was estradiol benzoate. Astrocytes do express estrogen receptors, both of the α and β type, though with variations based on particular location and degree of reactivity in response to injury or disease (Carbonaro et al. 2009; Sakuma et al. 2009; Giraud et al. 2010). In addition, estrogens are neuroprotective in several models of neurological disease (Dhandapani and Brann 2007; Arevalo et al. 2013). In experimental autoimmune encephalomyelitis, the protective effects of estrogens in spinal cord were shown to be mediated primarily through the ERα receptors (Spence et al. 2011).

Previous studies both in cell culture and in vivo have implicated gonadal hormones in the regulation of GFAP expression, although often with conflicting conclusions. For instance, Stone et al. (1998) found that primary rat astrocytes in culture respond to estradiol (E2) by decreasing their expression of GFAP, but only when co-cultured with neurons, though the same group later

found that scratch-wound stimulation of GFAP transcription was suppressed even in the absence of neurons (Rozovsky et al. 2002). In vivo, McAsey et al. (2006) found that depletion of endogenous estrogens through ovariectomy (OVX) led to an increase in GFAP protein at 7 weeks post-surgery, an effect that was suppressed by supplementation with exogenous E2. Similarly, treatment with E2 suppressed the GFAP increase seen in hippocampus following lesioning of the entorhinal cortex (Rozovsky et al. 2002). In contrast, Levin-Allerhand et al. (2001) found that E2 treatment following OVX further increased GFAP levels in hippocampus. Hence, the GFAP response to OVX and hormone replacement remains a matter of debate.

We therefore sought to test whether gonadal hormones regulate the expression of GFAP in the context of disease, specifically missense mutations in GFAP that cause AxD and that spontaneously lead to excessive accumulation of GFAP protein. Mouse models have been engineered to carry point mutations in the endogenous *Gfap* gene that are identical to common and severe mutations found in human patients (Hagemann et al. 2006). These mice exhibit several features similar to AxD patients, including increased expression of GFAP that is manifest in multiple brain regions as well as the CSF (Jany et al. 2013). We hypothesized that depletion of gonadal hormones through ovariectomy would increase expression of GFAP and exacerbate disease, whereas hormonal replacement or supplementation via administration of 17β-estradiol would mitigate these effects. However, we found minimal changes in GFAP expression resulting from either surgical or pharmacological manipulation of estradiol levels. In addition, no fluctuation in GFAP expression was evident during the natural variations in endogenous estrogens occurring during the estrous cycle of female mice. These results suggest caution in the implementation of estrogen-based treatments for AxD.

MATERIALS AND METHODS

Mice

Gfap$^{R236H/+}$ mice carry a point mutation homologous to a common mutation site in Alexander disease patients (R239H in the human sequence) (Hagemann et al. 2006). Mice were maintained as heterozygotes, congenic in either FVB/N and C57BL/6J (B6) backgrounds, and housed under a 14-10 light-dark cycle with ad libitum access to food (LabDiet 5015) and water. All mice were allowed to acclimate to a 12-12 light-dark cycle one week before surgery (described below). All experiments were approved by the Institutional Animal Care and Use Committee of the Graduate School of the University of Wisconsin-Madison.

Ovariectomy

At 8-9 weeks of age, female mice underwent either a bilateral ovariectomy (OVX) or sham surgery under sterile conditions. A flank incision was used to expose each ovary, the oviduct was cauterized, and the ovary removed. The abdominal wall was sutured and the skin incision closed with wound clips that were removed ten days after surgery. In sham-operated mice, the same procedures were performed without cauterization or removal of the ovaries. At the same time, mice were implanted with either one estradiol 60-day release pellet (0.36 mg)(E2) or a placebo pellet (PL)(Innovative Research of America, SE-121 and SC-111 respectively) subcutaneously via a trochar. Pellet implantation did not require antibiotics or sutures. After surgery, mice were given metacam (150mg/kg) for analgesia. In the first set of experiments, *Gfap*$^{+/+}$ and *Gfap*$^{R236H/+}$ mice that were congenic in the B6 background underwent either OVX or sham surgeries and were given either placebo or estrogen-containing pellets. In the second set of experiments, *Gfap*$^{R236H/+}$ mice that were congenic in the FVB/N background underwent either OVX or sham surgeries only, without pellet implantation. At seven weeks

post-surgery, mice were euthanized for collection of tissues, CSF, and plasma (as described below).

Stages of Estrus

Stages of estrus were determined by vaginal cytology (Allen 1922; Laboratory and Green 1966; Byers et al. 2012) in a cohort of *Gfap*$^{R236H/+}$ mice congenic in the FVB/N background. To establish the normal cycle length, mice were first monitored for at least one month with daily scoring, beginning at 8-9 weeks of age. The vaginal lavage was performed between 8 and 9 am each morning. Mice were restrained in a supine position while approximately 40 µL of sterile saline (0.9% NaCl filtered) was gently flushed into the vaginal orifice via disposable transfer pipettes (Fisher, # 13-711-9AM). The lavage solution was withdrawn and transferred to a glass slide for viewing. The stages of estrus were defined using the following criteria: proestrus (PE) consisting mainly of nucleated epithelial cells, estrus (E) containing anucleated cornified cells with jagged irregular edges, and diestrus (DE) primarily consisting of leukocytes (Laboratory and Green 1966; Byers et al. 2012). Mice were euthanized for collection of tissues (CNS and CSF, see below) between 8 am-12 pm for the PE am, E, and DE stages of estrus, and between 8-9 pm for the PE pm stage.

Collection of cerebrospinal fluid (CSF), plasma, and tissues

CSF was collected according to the method of DeMattos et al. (2002). Briefly, mice were anesthetized with avertin (400-600 mg/kg i.p.) and a midline sagittal incision made over the dorsal aspect of the hindbrain. After three muscle layers were carefully peeled back, the cisterna magna was exposed and pierced with a 30 gauge needle. CSF was collected immediately using a flexible plastic pipette with approximately 10 µL of CSF collected per animal, and stored at -80°C until further processing. For some mice, after CSF taps, approximately 0.5 ml of blood was collected from the axillary plexus using disposable transfer

pipettes and placed into K_2-EDTA coated tubes (BD Diagnostic, # 365974). Within 60 minutes of collection, samples were centrifuged for 15 minutes at 2500 *x g* at room temperature (22-24°C). Plasma was transferred into clean low retention tubes (Fisher, # 02-681-311) and stored at -20^{0}C until analysis. After collection of fluids, mice were euthanized and the brains were removed and bisected sagitally into two equal halves, in some cases with further dissection into individual regions (olfactory bulb, hippocampus, parietal cortex, cerebellum, and brain stem). Cervical spinal cord was collected as well. Samples of brain and spinal cord were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Plasma estrogen determinations

E2 levels in plasma samples were determined at the Ligand Assay and Analysis Core at the University of Virginia using an ELISA (Calbiotech), following the manufacturer's procedures. Results are expressed as pg/ml.

GFAP protein and mRNA quantification

Quantitation of GFAP protein was performed essentially as previously described (Jany et al. 2013). Briefly, brain or spinal cord samples were homogenized in a 2% SDS buffer, diluted, and evaluated using a sandwich ELISA. CSF samples were diluted directly in ELISA buffer. Results were expressed as ng/mg total protein for brain and spinal cord, and ng/L for CSF, using purified bovine GFAP as a standard. Quantitation of mRNA was performed as described previously (Hagemann et al. 2005). In this case, samples were homogenized in TRIzol Reagent (Invitrogen) and analyzed by RT-PCR. Results for *Gfap* were normalized to both the 18S ribosomal RNA and TATA-binding protein. Both normalizations yielded similar results. Data as shown was normalized to the 18S ribosomal RNA.

RESULTS

GFAP protein and mRNA after OVX and estradiol replacement or supplementation

We sought to test whether estrogen, either through addition or removal, regulates GFAP expression or levels in female mice. We initially attempted to replicate the study of McAsey et al. (2006), performing ovariectomy (OVX) or sham surgeries in B6 females and then treating these mice with either placebo (PL) or estrogen (E2) pellets. A comparison of the OVX and sham-operated mice with PL treatments showed no change in GFAP concentrations in any of the brain regions examined. When OVX mice were treated with the E2 pellet, GFAP concentrations decreased in the hippocampus and olfactory bulb, but E2 supplementation did not reduce GFAP concentrations in sham-operated mice (Fig. 4.1A-B). Since GFAP protein has a slow turnover rate *in vivo* (Chiu and Goldman 1984; DeArmond et al. 1986; Price et al. 2010), we considered the possibility that estrogen regulation may be more apparent at the level of *Gfap* mRNA. However, *Gfap* transcript levels did not change following any of the surgical or pellet treatments (hippocampus and olfactory bulb are shown in Fig. 4.1C-D – other data not shown).

Response to OVX in *Gfap*$^{R236H/+}$ mutant mice

We considered whether E2 might influence GFAP levels in the context of mutations associated with AxD, where GFAP levels spontaneously rise (Hagemann et al. 2006). We therefore performed OVX and sham surgeries on *Gfap*$^{R236H/+}$ mutant mice, and then treated each group with either E2 or PL pellets (all in the B6 background). PL treatment caused no change in GFAP concentrations, either in OVX or sham-operated mice. In contrast, E2 treatment did reduce GFAP, at the level of either whole brain or hippocampus, but only in mice that had undergone OVX (Fig. 4.2A-B). E2 treatment had no effect on GFAP concentrations in sham-operated mice in any of the brain regions tested. These results again indicate that E2 treatment only alters GFAP concentrations in mice that have experienced OVX.

To test for potential effects of genetic background on the response to OVX, we repeated these experiments using *Gfap*$^{R236H/+}$ mice that were congenic in the FVB/N strain. In this background, OVX caused a significant increase in GFAP levels in the hippocampus, cortex, and brain stem, and a trend towards an increase that did not reach statistical significance in the olfactory bulb (Fig. 4.3A). Considering GFAP levels as measured in whole brain rather than in specific regions, OVX did cause a significant increase, but this was not manifested in the levels of GFAP in CSF (likely due to higher variability in the CSF)(Fig. 4.3B).

Validation of surgical and treatment procedures

Given the minor effects noted above, we sought independent validation of the surgical and treatment procedures employed in these experiments, by directly measuring E2 levels in plasma, and monitoring body weight, a property known to be sensitive to estrogen manipulations. Plasma E2 levels derived from endogenous stores did not show a statistically significant change following ovariectomy (Fig. 4.4A, compare sham+PL vs. OVX+PL) , which is not surprising given previous studies using gas chromatography/tandem mass spectrometry (the gold standard for E2 blood analysis) and this ELISA in mice (Haisenleder et al. 2011). Nevertheless, mice subjected to OVX did display an increase in body weight compared to mice given sham-surgeries (Fig. 4.4B, compare sham+PL vs. OVX+PL), as predicted for depletion of endogenous E2 (Witte et al. 2010). Treatment with E2-pellets caused a significant increase in plasma E2 levels, but this only reached statistical significance in mice subjected to OVX (Fig. 4.4A, compare OVX+PL vs. OVX+E2), and mitigated the increase in body weight (Fig 4.4B). Similar results were obtained in *Gfap*$^{R236H/+}$ mutants congenic in both the B6 and FVB/N backgrounds (B6 data illustrated, FVB/N data not shown).

GFAP and the stages of estrus in *Gfap*$^{R236H/+}$ mutant mice

Previous studies in mice and rats have shown that GFAP protein and mRNA levels are affected by the stage of estrus (Stone et al. 1998; Struble et al. 2006; Cordeau et al. 2008). In particular, a mouse stroke model showed decreased *Gfap* promoter activity when mice were in proestrus (PE) and estrus (E) compared to mice in metestrus (ME) and diestrus (DE) (Cordeau et al. 2008). To test whether estrus impacts GFAP expression and levels in the *Gfap*$^{R236H/+}$ mutants, a genetic model of injury, we measured GFAP protein and mRNA in various brain regions of mice classified by vaginal cytology as being either in PE, E or DE. In addition, since E2 concentrations are known to increase on the morning of PE and reduce to baseline levels by the same evening, we divided PE into both morning (PE am) and evening (PE pm) categories (Goldman et al. 2007; Christian and Moenter 2010). Neither GFAP protein (Fig. 4.5A-B) nor mRNA (Fig. 4.5C-D) showed any change during the various stages of estrus in all brain regions examined (hippocampus and olfactory bulb shown – other data not shown). The stages of estrus also did not impact the levels of GFAP protein detected in CSF from the same mice (Fig. 4.5E), where such levels have been proposed as a biomarker applicable for clinical studies. Thus, normal physiological variations in endogenous E2 do not appear to regulate the spontaneously elevated levels of GFAP expression observed in the *Gfap*$^{R236H/+}$ mutants.

DISCUSSION

While estrogenic effects on GFAP have been shown by several researchers (Day et al. 1993; Levin-Allerhand et al. 2001; Martinez et al. 2006; McAsey et al. 2006), we found E2 had minimal effects on GFAP in mouse models of AxD. After OVX, E2 decreased GFAP concentrations in some brain regions both in wild-type and *Gfap*$^{R236H/+}$ mice. Conversely, E2 had no effects on GFAP protein or mRNA in sham-operated mice. GFAP response to OVX in *Gfap*$^{R236H/+}$ mice also differed by mouse background strain with the B6 background showing no

change in GFAP and the FVB/N background increasing GFAP after OVX. Analysis of GFAP protein and mRNA during the different stages of estrus revealed no changes in GFAP in any of the observed brain regions. Overall, these results indicate E2 regulation of GFAP may vary with brain region, stress, and mouse background strain, and thus E2 may not be a reliable treatment for AxD.

In *Gfap*$^{+/+}$ and *Gfap*$^{R236H/+}$ mice, GFAP concentrations were unaltered by E2 treatment after the sham operation. After OVX, E2 treatment decreased GFAP concentrations in the olfactory bulb and hippocampus of *Gfap*$^{+/+}$ mice and the hippocampus and whole brain of *Gfap*$^{R236H/+}$ mice. These results suggest OVX may indirectly regulate GFAP concentrations possibly through inducing stress and activating inflammatory pathways. E2 reduces inflammatory cytokine and chemokine expression such as nuclear factor-kappa B (NFκB), tumor necrosis factor alpha (TNFα), and transforming growth factor beta 1 (TGFβ1) both in astrocytes and microglia (Arevalo et al. 2012; Giatti et al. 2012). E2 also alters mitochondrial responses to injury by reducing reactive oxygen species, cell death, and altering ATP response in primary astrocytic cultures (Guo et al. 2012). GFAP is regulated by similar stress pathways (Zhang et al. 2000; Makwana et al. 2007; Hwang et al. 2010; Kang and Hebert 2011), suggesting E2 may indirectly alter GFAP after injury or in neurodegenerative diseases. Analysis of stress pathways in AxD revealed several pathways are activated which may act to exacerbate GFAP accumulation (Brenner et al. 2009). Comparison of stress pathways mediated by E2 and active in AxD reveal little overlap, suggesting E2 does not mediate the stress pathways activated in AxD.

Interestingly, stress pathways activated by OVX may vary in different mouse background strains in *Gfap*$^{R236H/+}$ mice. While removal of ovaries did not increase GFAP within the B6 background strain, GFAP in the FVB/N background increased significantly in response to OVX. Previous studies indicate hormone concentrations differ between mouse strains (Phan et al.

2002; Tagawa et al. 2006; Lee et al. 2007), though no comparison of E2 in B6 and FVB/N mice has been documented. Comparison of mating schedules, litter size, and maternal instincts between B6 and FVB/N mice reveal drastic differences (Mouse Phenome Project, http://phenome.jax.org/), suggesting hormonal regulation varies in the different mouse strains. These hormonal differences may be important not only for breeding but also for E2 neuroprotective actions and stress pathway regulation.

While OVX increased GFAP concentrations in *Gfap*$^{R236H/+}$ mice of the FVB/N strain, the stage of estrus did not alter GFAP concentrations. During the different stages of estrus, E2 plasma levels fluctuate with a peak in E2 concentrations on the morning of PE (Goldman et al. 2007). In the brain, it is unknown if E2 concentrations reflect plasma E2 fluctuations. E2 is synthesized in ovaries and the brain (Hojo et al. 2008; Konkle and McCarthy 2011). The neuroactive form of E2 (neuroestrogen) is synthesized by aromatase in neurons and astrocytes and may not be regulated by the estrus cycle (Duncan et al. 2013). Previous studies indicate brain E2 concentrations do not correlate with blood E2 levels in rodents (Tagawa et al. 2006; Konkle and McCarthy 2011) and song birds (Schlinger and Remage-Healey 2012). If neuroestrogen is regulating GFAP concentrations and is not controlled by the estrus cycle, GFAP concentrations in the brain may not fluctuate during the estrus cycle. Analysis of brain E2 concentrations during the different stages of estrus may reveal a role for neuroestrogen in regulating GFAP.

A caveat to these experiments may be the method of E2 treatment. The E2 pellet (Innovative Research of America) steadily releases E2 over 60 days after pellet implantation. Conversely, Ingberg et al. (2012) found the E2 blood concentrations from the pellets (Innovative Research of America) peak early after implantation and then gradually drop off by the end of the 60 day release cycle. Analysis of plasma E2 concentrations in our samples indicate E2 levels

are elevated but variable after 49 days of E2 treatment, suggesting the lack of E2 effects on GFAP are not caused by diminished E2 concentrations.

Overall, we found minimal changes in GFAP expression with surgical or pharmacological manipulations of E2 and no response of GFAP to natural E2 fluctuations seen during the estrus cycle. E2 regulation of GFAP may involve regulation of stress pathways which indirectly act on GFAP. In AxD, a positive feedback loop is proposed between stress pathways and GFAP, which may exacerbate GFAP accumulation (Brenner et al. 2009; Messing and Brenner 2013). The results from the current study suggest E2 does not regulate stress pathways active in AxD, and thus is not a suitable treatment for AxD patients.

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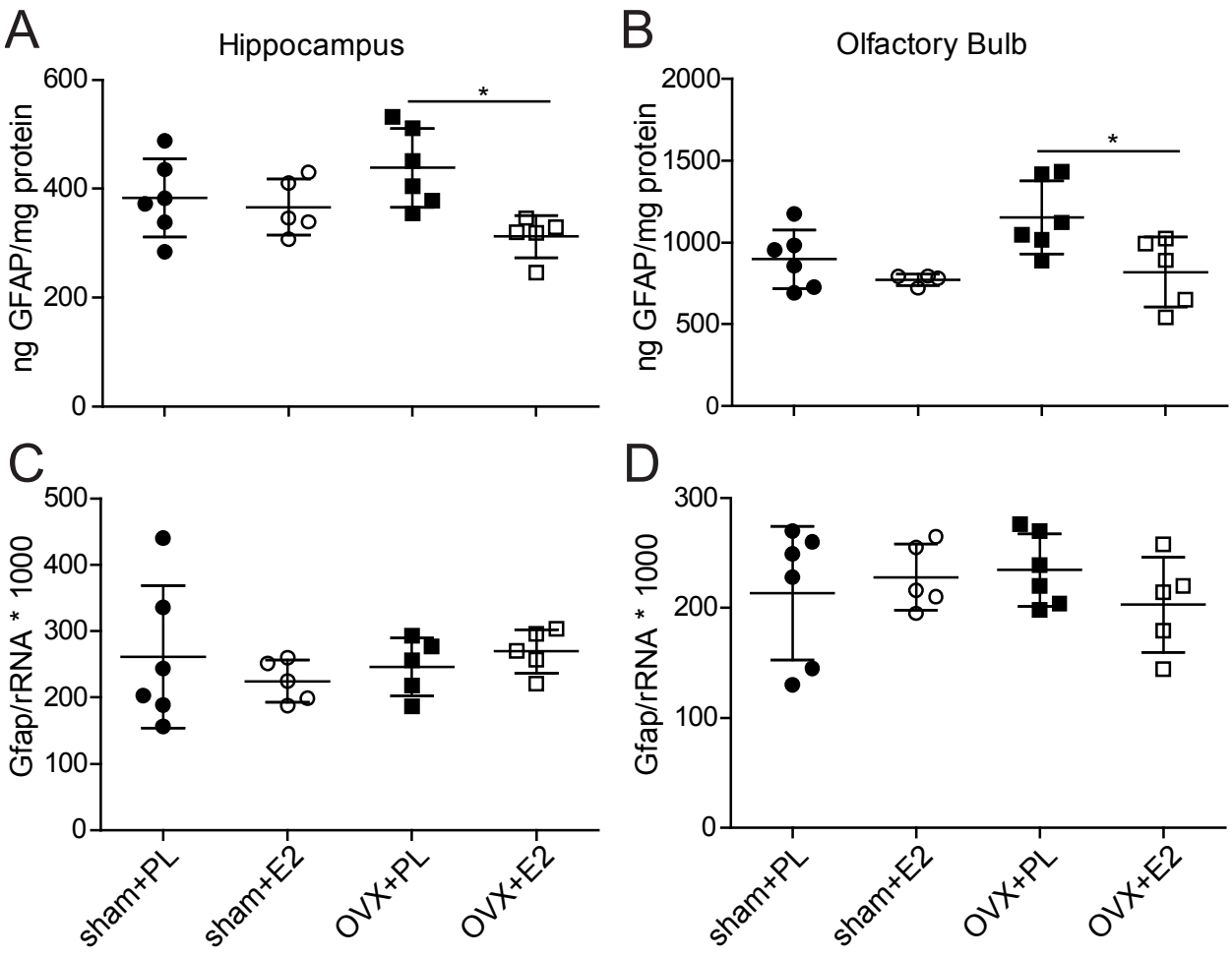
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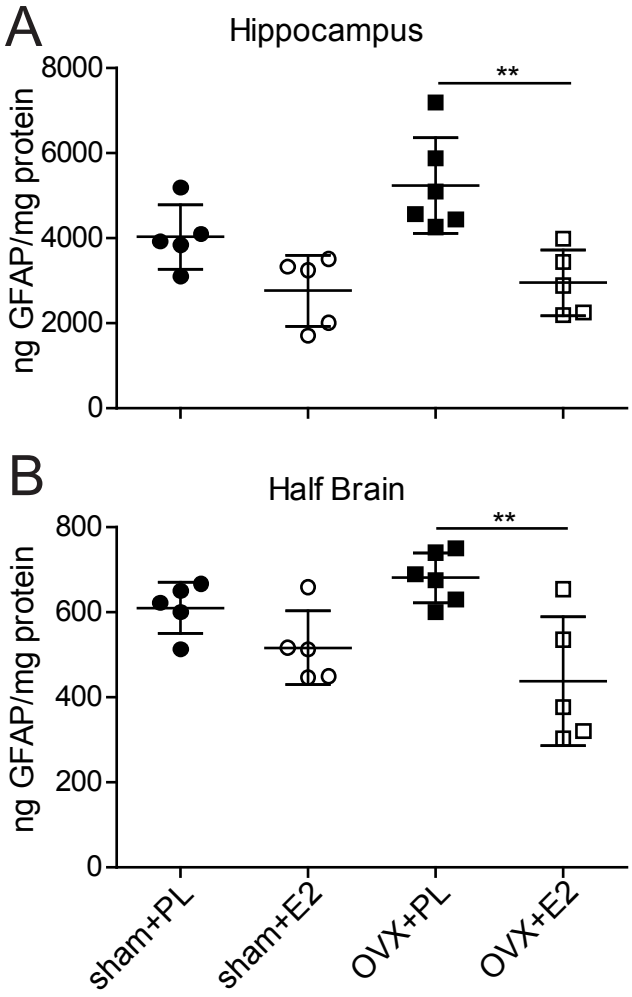
Figure 4.1: GFAP protein and mRNA response to OVX and E2 treatment in wild type mice. GFAP protein levels in B6 mice subjected to OVX decreased in response to estrogen (E2) treatment compared to placebo-treated OVX mice in the hippocampus (A) and olfactory bulb (B), while sham-operated mice showed no change in any of the brain regions examined. None of the brain regions showed a change in *Gfap* mRNA levels with any treatment conditions (C, D - hippocampus and olfactory bulb, other data not shown). (One-way ANOVA with post-hoc Bonferroni t-test; * $p<0.05$). Presented as average ± standard deviation (SD) *(*n=5-6 per procedure). PL, placebo; E2, estrogen; OVX, ovariectomy.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OVX+PL
OVX+E2

Figure 4.2: E2 effects on GFAP after OVX in *Gfap*$^{R236H/+}$ mice (B6 background). In *Gfap*$^{R236H/+}$ mice subjected to OVX, E2 significantly decreased GFAP concentrations in the hippocampus (A) and half-brain (B) compared to placebo-treated OVX mice. E2 did not alter GFAP concentrations in sham-operated mice in any brain regions tested. (One-way ANOVA with post-hoc Bonferroni t-test; ** $p<0.01$). Presented as average ± SD (n=5-6 per procedure). PL, placebo; E2, estrogen; OVX, ovariectomy.

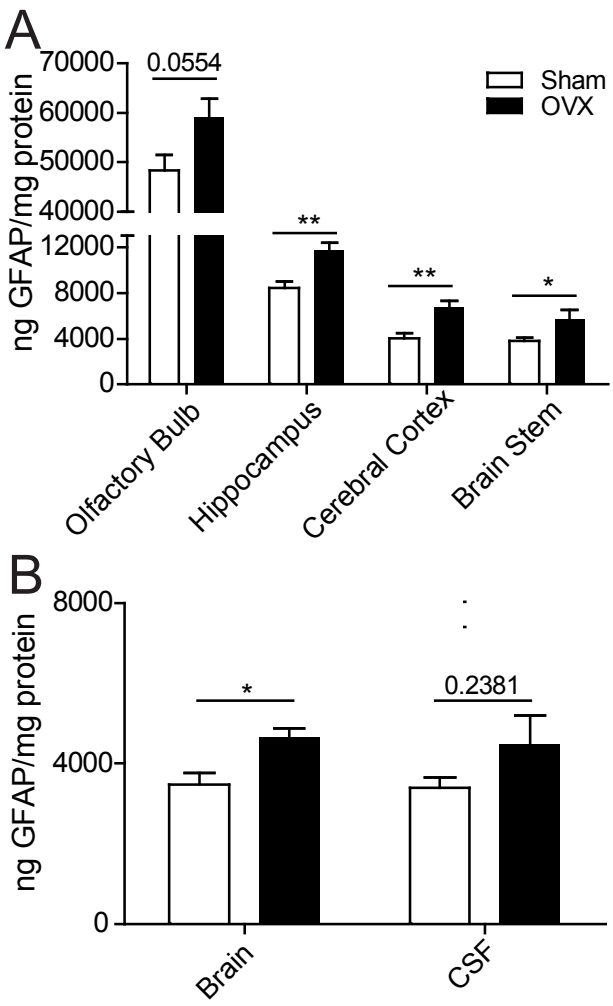
A
ng GFAP/mg protein
70000
60000
50000
40000
12000
8000
4000
0
0.0554
**
**
*
Sham
OVX
Olfactory Bulb
Hippocampus
Cerebral Cortex
Brain Stem
B
ng GFAP/mg protein
8000
4000
0
*
0.2381
Brain
CSF

Figure 4.3: GFAP concentrations in response to OVX in *Gfap*$^{R236H/+}$ mice (FVB/N background). (A) GFAP protein levels significantly increased in *Gfap*$^{R236H/+}$ mice (FVB/N background) subjected to OVX in the hippocampus, parietal cortex, and brain stem, while olfactory bulb showed a trend towards an increase. (B) GFAP concentrations also increased following OVX when measured at the level of whole brain, but CSF levels did not change. (Student's t-test; * $p<0.05$; ** $p<0.01$). Presented as average ± SD *(*n=5-6 per procedure). OVX, ovariectomy.

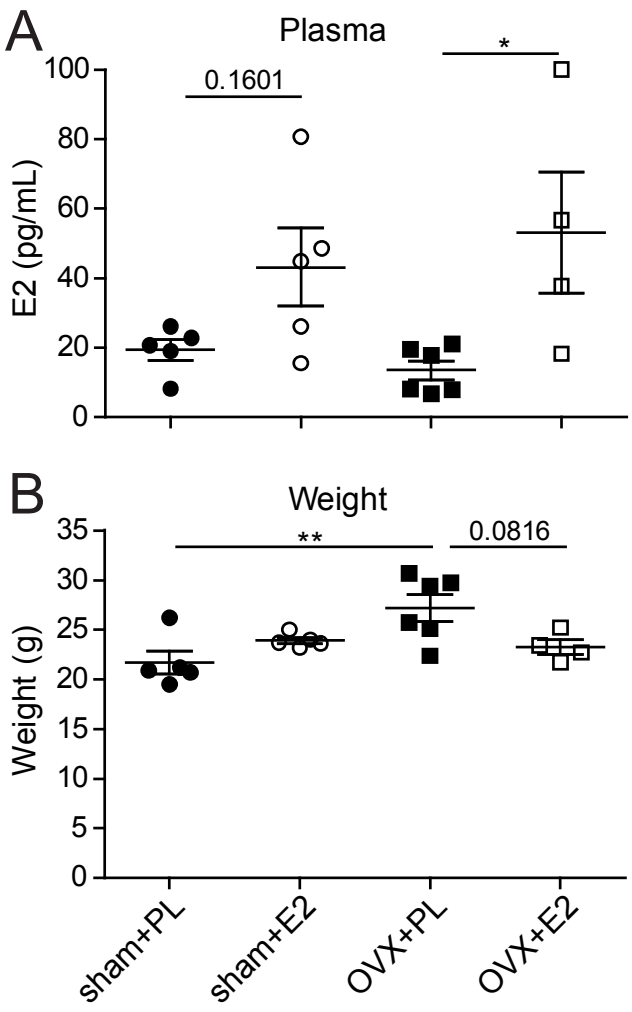
A
Plasma
E2 (pg/mL)
100
80
60
40
20
0
0.1601
*
B
Weight
Weight (g)
35
30
25
20
15
10
5
0
**
0.0816
sham+PL
sham+E2
OVX+PL
OVX+E2

Figure 4.4: Plasma E2 and body weight in *Gfap*$^{R236H/+}$ mice following OVX and pellet implantation. (A) E2 plasma levels significantly increased with treatment only in mice subjected to OVX (OVX+E2 vs OVX+PL, * $p<0.05$). (B) Body weight significantly increased after OVX (sham+PL vs OVX+PL, ** $p<0.01$). (One-way ANOVA with post-hoc Bonferroni t-test). Presented as average ± SD *(*n=5-6 per group). PL, placebo; E2, estradiol; OVX, ovariectomy.

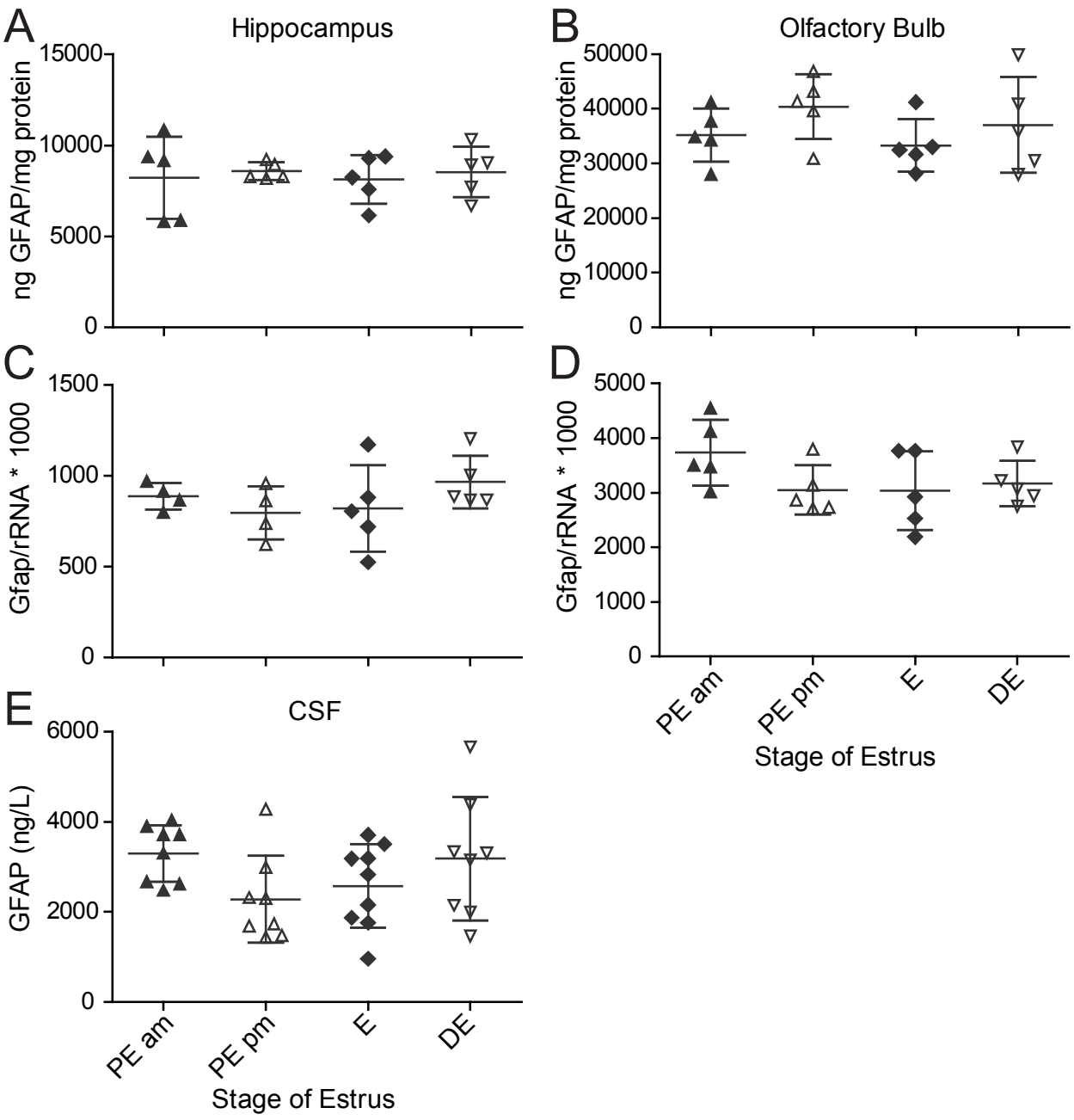
A
Hippocampus
ng GFAP/mg protein
15000
10000
5000
0
B
Olfactory Bulb
ng GFAP/mg protein
50000
40000
30000
20000
10000
0
C
Gfap/rRNA * 1000
1500
1000
500
0
D
Gfap/rRNA * 1000
5000
4000
3000
2000
1000
0
E
CSF
GFAP (ng/L)
6000
4000
2000
0
PE am
PE pm
E
DE
Stage of Estrus

Figure 4.5: The impact of estrus cycle on GFAP expression in *Gfap*$^{R236H/+}$ mice (FVB/N background). GFAP protein levels showed no significant changes as a function of the stages of estrus in either hippocampus (A) or olfactory bulb (B). Levels of *Gfap* mRNA (C-D) in these same regions, as well as in CSF (E), also showed no change. (One-way ANOVA with post-hoc Bonferroni t-test). Presented as average ± SD (n=4-5 in A-D, n=8-9 in E). PE, proestrus; E, estrus; DE, diestrus.

REFERENCE

Allen, E. (1922). "The oestrous cycle in the mouse." American Journal of Anatomy **30**(3): 297-371.

Arevalo, M. A., Y. Diz-Chaves, M. Santos-Galindo, M. J. Bellini and L. M. Garcia-Segura (2012). "Selective oestrogen receptor modulators decrease the inflammatory response of glial cells." J Neuroendocrinol **24**(1): 183-190.

Arevalo, M. A., M. Santos-Galindo, E. Acaz-Fonseca, I. Azcoitia and L. M. Garcia-Segura (2013). "Gonadal hormones and the control of reactive gliosis." Horm Behav **63**(2): 216-221.

Brenner, M., J. Goldman, R. Quinlan and A. Messing (2009). Alexander Disease: A Genetic Disorder of Astrocytes. In: Astrocytes in (Patho)Physiology of the Nervous System. P. G. Haydon and V. Parpura, Springer US**:** 591-648.

Brenner, M., A. B. Johnson, O. Boespflug-Tanguy, D. Rodriguez, J. E. Goldman and A. Messing (2001). "Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease." Nat Genet **27**(1): 117-120.

Byers, S. L., M. V. Wiles, S. L. Dunn and R. A. Taft (2012). "Mouse estrous cycle identification tool and images." PLoS One **7**(4): e35538.

Carbonaro, V., F. Caraci, M. L. Giuffrida, S. Merlo, P. L. Canonico, F. Drago, . . . M. A. Sortino (2009). "Enhanced expression of ERalpha in astrocytes modifies the response of cortical neurons to beta-amyloid toxicity." Neurobiol Dis **33**(3): 415-421.

Chiu, F. C. and J. E. Goldman (1984). "Synthesis and turnover of cytoskeletal proteins in cultured astrocytes." J Neurochem **42**(1): 166-174.

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Christian, C. A. and S. M. Moenter (2010). "The neurobiology of preovulatory and estradiol-induced gonadotropin-releasing hormone surges." Endocr Rev **31**(4): 544-577.

Cordeau, P., Jr., M. Lalancette-Hebert, Y. C. Weng and J. Kriz (2008). "Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury." Stroke **39**(3): 935-942.

Day, J. R., N. J. Laping, M. Lampert-Etchells, S. A. Brown, J. P. O'Callaghan, T. H. McNeill and C. E. Finch (1993). "Gonadal steroids regulate the expression of glial fibrillary acidic protein in the adult male rat hippocampus." Neuroscience **55**(2): 435-443.

DeArmond, S. J., Y. L. Lee, H. A. Kretzschmar and L. F. Eng (1986). "Turnover of glial filaments in mouse spinal cord." J Neurochem **47**(6): 1749-1753.

DeMattos, R. B., K. R. Bales, M. Parsadanian, M. A. O'Dell, E. M. Foss, S. M. Paul and D. M. Holtzman (2002). "Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease." J Neurochem **81**(2): 229-236.

Dhandapani, K. M. and D. W. Brann (2007). "Role of astrocytes in estrogen-mediated neuroprotection." Exp Gerontol **42**(1-2): 70-75.

Duncan, K. A., B. J. Walters and C. J. Saldanha (2013). "Traumatized and inflamed--but resilient: glial aromatization and the avian brain." Horm Behav **63**(2): 208-215.

Giatti, S., M. Boraso, R. C. Melcangi and B. Viviani (2012). "Neuroactive steroids, their metabolites, and neuroinflammation." J Mol Endocrinol **49**(3): R125-134.

Giraud, S. N., C. M. Caron, D. Pham-Dinh, P. Kitabgi and A. B. Nicot (2010). "Estradiol inhibits ongoing autoimmune neuroinflammation and NFkappaB-dependent CCL2 expression in reactive astrocytes." Proc Natl Acad Sci U S A **107**(18): 8416-8421.

Goldman, J. M., A. S. Murr and R. L. Cooper (2007). "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Res B Dev Reprod Toxicol **80**(2): 84-97.

Guo, J., S. P. Duckles, J. H. Weiss, X. Li and D. N. Krause (2012). "17beta-Estradiol prevents cell death and mitochondrial dysfunction by an estrogen receptor-dependent mechanism in astrocytes after oxygen-glucose deprivation/reperfusion." Free Radic Biol Med **52**(11-12): 2151-2160.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

Hagemann, T. L., S. A. Gaeta, M. A. Smith, D. A. Johnson, J. A. Johnson and A. Messing (2005). "Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction." Hum Mol Genet **14**(16): 2443-2458.

Haisenleder, D. J., A. H. Schoenfelder, E. S. Marcinko, L. M. Geddis and J. C. Marshall (2011). "Estimation of estradiol in mouse serum samples: evaluation of commercial estradiol immunoassays." Endocrinology **152**(11): 4443-4447.

Hojo, Y., G. Murakami, H. Mukai, S. Higo, Y. Hatanaka, M. Ogiue-Ikeda, . . . S. Kawato (2008). "Estrogen synthesis in the brain--role in synaptic plasticity and memory." Mol Cell Endocrinol **290**(1-2): 31-43.

Hwang, J., H. J. Lee, W. H. Lee and K. Suk (2010). "NF-kappaB as a common signaling pathway in ganglioside-induced autophagic cell death and activation of astrocytes." J Neuroimmunol **226**(1-2): 66-72.

Ingberg, E., A. Theodorsson, E. Theodorsson and J. O. Strom (2012). "Methods for long-term 17beta-estradiol administration to mice." Gen Comp Endocrinol **175**(1): 188-193.

Jackson, S. o. t. J. (2013). Mouse Phenome Database, Jackson Laboratories.

Jany, P. L., T. L. Hagemann and A. Messing (2013). "GFAP expression as an indicator of disease severity in mouse models of Alexander disease." ASN Neuro **5**(1): e00109.

Kang, W. and J. M. Hebert (2011). "Signaling pathways in reactive astrocytes, a genetic perspective." Mol Neurobiol **43**(3): 147-154.

Konkle, A. T. and M. M. McCarthy (2011). "Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain." Endocrinology **152**(1): 223-235.

Laboratory, S. o. t. J. and E. L. Green (1966). Biology of the laboratory mouse. New York,, Blakiston Division.

Lee, S. N., B. Peng, R. Desjardins, J. E. Pintar, R. Day and I. Lindberg (2007). "Strain-specific steroidal control of pituitary function." J Endocrinol **192**(3): 515-525.

Levin-Allerhand, J., B. S. McEwen, C. E. Lominska, D. B. Lubahn, K. S. Korach and J. D. Smith (2001). "Brain region-specific up-regulation of mouse apolipoprotein E by pharmacological estrogen treatments." J Neurochem **79**(4): 796-803.

Makwana, M., L. L. Jones, D. Cuthill, H. Heuer, M. Bohatschek, M. Hristova, . . . G. Raivich (2007). "Endogenous transforming growth factor beta 1 suppresses inflammation and promotes survival in adult CNS." J Neurosci **27**(42): 11201-11213.

Martinez, F. G., E. E. Hermel, L. L. Xavier, G. G. Viola, J. Riboldi, A. A. Rasia-Filho and M. Achaval (2006). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats." Brain Res **1108**(1): 117-126.

McAsey, M. E., C. Cady, L. M. Jackson, M. Li, S. Randall, B. P. Nathan and R. G. Struble (2006). "Time course of response to estradiol replacement in ovariectomized mice: brain

apolipoprotein E and synaptophysin transiently increase and glial fibrillary acidic protein is suppressed." Exp Neurol **197**(1): 197-205.

Messing, A. and M. Brenner (2013). Genetic disorders affecting astrocytes. Neuroglia. H. Kettenmann and B. R. Ransom. New York, Oxford University Press**:** 884-895.

Messing, A., M. Brenner, M. B. Feany, M. Nedergaard and J. E. Goldman (2012). "Alexander disease." J Neurosci **32**(15): 5017-5023.

Phan, V. L., A. Urani, P. Romieu and T. Maurice (2002). "Strain differences in sigma(1) receptor-mediated behaviours are related to neurosteroid levels." Eur J Neurosci **15**(9): 1523-1534.

Price, J. C., S. Guan, A. Burlingame, S. B. Prusiner and S. Ghaemmaghami (2010). "Analysis of proteome dynamics in the mouse brain." Proc Natl Acad Sci U S A **107**(32): 14508-14513.

Rozovsky, I., M. Wei, D. J. Stone, H. Zanjani, C. P. Anderson, T. E. Morgan and C. E. Finch (2002). "Estradiol (E2) enhances neurite outgrowth by repressing glial fibrillary acidic protein expression and reorganizing laminin." Endocrinology **143**(2): 636-646.

Sakuma, S., D. Tokuhara, H. Hattori, O. Matsuoka and T. Yamano (2009). "Expression of estrogen receptor alpha and beta in reactive astrocytes at the male rat hippocampus after status epilepticus." Neuropathology **29**(1): 55-62.

Schlinger, B. A. and L. Remage-Healey (2012). "Neurosteroidogenesis: insights from studies of songbirds." J Neuroendocrinol **24**(1): 16-21.

Spence, R. D., M. E. Hamby, E. Umeda, N. Itoh, S. Du, A. J. Wisdom, . . . R. R. Voskuhl (2011). "Neuroprotection mediated through estrogen receptor-{alpha} in astrocytes." Proc Natl Acad Sci U S A **108**(21): 8867-8872.

Stone, D. J., Y. Song, C. P. Anderson, K. K. Krohn, C. E. Finch and I. Rozovsky (1998). "Bidirectional transcription regulation of glial fibrillary acidic protein by estradiol in vivo and in vitro." Endocrinology **139**(7): 3202-3209.

Struble, R. G., S. Afridi, S. Beckman-Randall, M. Li, C. Cady, B. Nathan and M. E. McAsey (2006). "Neocortical and hippocampal glial fibrillary acidic protein immunoreactivity shows region-specific variation during the mouse estrous cycle." Neuroendocrinology **83**(5-6): 325-335.

Tagawa, N., Y. Sugimoto, J. Yamada and Y. Kobayashi (2006). "Strain differences of neurosteroid levels in mouse brain." Steroids **71**(9): 776-784.

Witte, M. M., D. Resuehr, A. R. Chandler, A. K. Mehle and J. M. Overton (2010). "Female mice and rats exhibit species-specific metabolic and behavioral responses to ovariectomy." Gen Comp Endocrinol **166**(3): 520-528.

Zhang, L., W. Zhao, B. Li, D. L. Alkon, J. L. Barker, Y. H. Chang, . . . D. R. Rubinow (2000). "TNF-alpha induced over-expression of GFAP is associated with MAPKs." Neuroreport **11**(2): 409-412.

Chapter 5:

Discussion and future directions

In Alexander disease (AxD), mutations in glial fibrillary acidic protein (GFAP) lead to GFAP accumulation above an unknown toxic threshold possibly through positive feedback loops (Tang et al. 2006; Tang et al. 2010; Jany et al. 2013; Messing and Brenner 2013). There is currently no cure for AxD, but reduction of GFAP protein levels may alleviate AxD symptoms. For analysis of drug effects in both human patients and mouse models of AxD, we need reliable biomarkers. Quantification of *Gfap* promoter activity in mice and protein in cerebrospinal fluid (CSF) and plasma of mice and humans may provide reliable biomarkers for analysis of drug effects on GFAP. From the previous chapters, we have shown luciferase activity accurately monitors *Gfap* promoter activity providing a biomarker for longitudinal studies in mice. We also found GFAP is detectable and elevated in CSF of mouse models of AxD and in plasma and CSF of AxD patients. In addition, GFAP in CSF of mice mimics brain concentration of GFAP, suggesting GFAP in CSF and possibly plasma may give an indirect indication of GFAP in the brain. Analysis of GFAP in CSF and plasma may therefore be utilized during future drug treatments to determine drug effects on GFAP in the brain. We also analyzed estradiol (E2) effects on GFAP. In a previous drug screen of FDA-approved drugs, E2 decreased human *GFAP* promoter activity by 26% in primary astrocyte cultures (Cho et al. 2010). Quantification of E2 effects on GFAP *in vivo* revealed E2 regulation varies with stress, brain region, and mouse background strain, suggesting caution in the implementation of estrogen-based treatments for AxD. Taken together, we found E2 may not be an effective treatment for AxD, but we did identify GFAP as a useful biomarker for AxD which can be utilized in future clinical trials to determine drug efficacy.

GFAP Promoter Activity

In chapter 2, we analyzed firefly luciferase efficacy as a biomarker for *Gfap* promoter activity in mouse models of AxD, through the use of a mouse reporter which expresses firefly

luciferase under the control of a 12 kilobase (kb) murine *Gfap* promoter (*Gfap*-luc) (Zhu et al. 2004). In *Gfap*$^{R236H/+}$ mice, which contain a heterozygous mutation homologous to a common mutation seen in AxD patients (R239H), we found luciferase activity increased by postnatal day 14 and remained elevated out to 6 months of age. Analysis of different brain regions at 8 weeks of age revealed similar increases in both *Gfap* promoter activity and mRNA concentrations in all regions. Conversely, GFAP protein showed a greater fold increase in olfactory bulb, cerebral cortex, and hippocampus when compared with both *Gfap* promoter activity and mRNA concentrations. In both the brain stem and spinal cord, GFAP concentrations were unaltered or decreased while both *Gfap* promoter activity and mRNA increased compared to wild-type littermate controls. These results suggest luciferase activity accurately reflects *Gfap* promoter activity and mRNA, but further regulation is involved in GFAP protein accumulation in AxD. Luciferase activity may therefore be utilized for analysis of drug effects of *Gfap* promoter activity during future drug studies, but is not a suitable surrogate to evaluate GFAP protein accumulation in affected tissues.

Regional Activity

We first analyzed luciferase activity in *Gfap*$^{+/+}$ littermate controls and *Gfap*$^{R236H/+}$ mice to discern if luciferase activity mimicked *Gfap* mRNA and protein levels in various brain regions. Previous studies revealed regional variability in GFAP protein and mRNA concentrations (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007), especially when comparing white and gray matter (Lewis and Cowan 1985; Kretzschmar et al. 1986). In mice and humans, both mRNA and protein concentrations of GFAP are highest in the spinal cord and brain stem, followed in descending order by hippocampus, olfactory bulb, cerebral cortex, and cerebellum (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007). Similarly, the current study found regional variations in

Gfap promoter activity, mRNA, and protein in both *Gfap*$^{+/+}$ and *Gfap*$^{R236H/+}$ mice. In *Gfap*$^{+/+}$ mice, *Gfap* promoter activity, mRNA, and protein concentrations were similar to each other in each individual brain region. Analysis of all three between brain regions revealed the highest concentrations were in the spinal cord and the lowest concentrations were observed in the cerebral cortex and cerebellum (Fig. 2.1). These results suggest luciferase activity not only mimics both *Gfap* mRNA and protein levels in various brain regions of *Gfap*$^{+/+}$ mice, but also confirms regional variability of *Gfap* mRNA and protein levels found in previous studies (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007).

In *Gfap*$^{R236H/+}$ mice, analysis of the fold increases of *Gfap* promoter activity and mRNA compared with wild-type littermates revealed similar fold increases for both in all of the examined brain regions (Fig. 2.2). This is consistent with previous studies showing that both luciferase activity and *Gfap* mRNA increases are similar in *Gfap-luc* mice after injury (Zhu et al. 2004; Kadurugamuwa et al. 2005; Cordeau et al. 2008) or in neurological disease (Luo et al. 2008; Keller et al. 2009; Tamguney et al. 2009; Watts et al. 2011). Conversely, the fold increase of GFAP protein compared with both *Gfap* promoter activity and mRNA was greater in olfactory bulb, hippocampus and cerebral cortex. In addition, GFAP protein concentrations were unaltered in the brain stem and decreased in the spinal cord compared with *Gfap*$^{+/+}$ mice while the fold increases for both *Gfap* promoter activity and mRNA were significantly elevated (Fig. 2.2).

The differences seen in *Gfap* expression and accumulation may reflect astrocyte heterogeneity. Astrocytes have regional variations in astrocyte sub-type (protoplasmic versus fibrous), cytokine and protein expression, as well as response to injury (Andriezen 1893; Hansson 1990; Malhotra et al. 1993; Xu et al. 2001; Geiger et al. 2006; Regan et al. 2007; Yeh et al. 2009; Fitting et al. 2010). Astrocyte density also varies regionally, though an early study found no correlation between astrocyte density and GFAP (Martin and O'Callaghan 1995).

Interestingly, GFAP accumulation in *Gfap*$^{R236H/+}$ mice varies regionally with some regions showing no GFAP accumulation or decreases in GFAP concentrations, suggesting astrocyte response to disease is also heterogeneous. In primary astrocyte cultures, GFAP mutations alter degradation machinery activity (Tang et al. 2006; Tang et al. 2008), suggesting *in vivo* analysis of degradation machinery in different brain regions may reveal an important mechanism leading to GFAP accumulation. Additionally, GFAP may be regulated by differential gene expression in brain regions. Analysis of human astrocyte development *in vitro* reveals astrocytes with a "caudalized" phenotype through exposure to retinoic acid express higher concentrations of GFAP compared to the default rostral pathway of differentiation (Krencik et al. 2011), suggesting regional variations in GFAP may reflect changes in regulatory signals. Analysis of gene expression and degradation machinery in different brain regions may reveal important regulators of *Gfap* promoter activity, mRNA, and protein expression in mouse models of AxD.

Time Course

We also wanted to know when elevations in *Gfap* promoter activity begin in *Gfap*$^{R236H/+}$ mice. During injury, infection, and neurological diseases, astrocytes become reactive resulting in upregulation of the *Gfap* promoter (Andriezen 1893; Eng et al. 2000). In mouse models of AxD, *Gfap* mRNA and protein levels increase significantly in adult (8 week old) mice compared with littermate controls (Hagemann et al. 2006). It was previously unclear if *Gfap* promoter activity had an early, sustained increase which may contribute to GFAP protein accumulation. In the current study, we found luciferase activity significantly increased at postnatal day 14 in *Gfap*$^{R236H/+}$ mice compared with littermate controls, and this increase was sustained through 6 months of age (Fig. 2.4). These results suggest luciferase activity in *Gfap*-luc mice mimics the sustained increase in *Gfap* mRNA (Hagemann et al. 2006). Conversely, in a previous study utilizing a mouse reporter line which expresses firefly luciferase under the control of a 2.2 kb

human *GFAP* promoter (Cho et al. 2009), we found *GFAP* promoter activity increased early in development but was not sustained out to 8 weeks of age. These results indicate the 2.2 kb human *GFAP* promoter activity does not accurately reflect the changes in endogenous *Gfap* mRNA in adult $Gfap^{R236H/+}$ mice and thus is not a sufficient surrogate for AxD pathogenesis.

Gfap Promoter Regulation

The 2.2 kb human *GFAP* promoter is known to have several regulatory elements important for GFAP expression in astrocytes and upregulation of GFAP in response to injury (Brenner 1994; Brenner et al. 1994; Johnson et al. 1995; Lee et al. 2006; Lee et al. 2008; Cho et al. 2009). Analysis of the 2.2 kb *GFAP* promoter reveals a TATA-like sequence required for transcription located 29 bp upstream of the transcription start site as well as several positive and negative regulators of transcription (Sarid 1991; Sarkar and Cowan 1991; Brenner 1994; Kaneko et al. 1994). The *GFAP* promoter is positively regulated by activator protein 1 (AP-1) and transcription factor II D at sites that are essential for transcription (Nakatani et al. 1990; Brenner 1994; Lee et al. 2008; Middeldorp and Hol 2011) as well as by signal transducer and activator of transcription 3 (STAT3), transforming growth factor beta (TGFβ), and tumor necrosis factor α (TNFα) which induce GFAP upregulation after injury or toxic insult (Galbreath et al. 1995; Zhang et al. 2000; Moon and Fawcett 2001; Takizawa et al. 2001; Herrmann et al. 2008; Lee et al. 2008; Hwang et al. 2010; Barcia et al. 2011; Kang and Hebert 2011; Shu et al. 2011; Urayama et al. 2013). Additionally, STAT3, nuclear factor 1 (NF-1), and activator protein 2 (AP-2) are important for astrocyte differentiation and cell specific expression of GFAP (Miura et al. 1990; Bonni et al. 1997; Nakashima et al. 1999; Cebolla and Vallejo 2006; Middeldorp and Hol 2011; Urayama et al. 2013). Other transcription factors which may also contribute to *Gfap* expression include but are not limited to CCAAT/enhancer binding protein, specificity protein 1 (Sp-1), AP-1, and cAMP response element (CRE) (Brenner 1994; Lee et al. 2008). Negative

regulators of *GFAP* promoter activity include interferon gamma (IFNγ) and β1 integrin (Brenner 1994; Kang and Hebert 2011) as well as nuclear factor kappa B (NFκB) and globin transcription factor (GATA) which specifically inhibit GFAP expression in neurons (Lee et al. 2008). NFκB can also induce GFAP expression in astrocytes after injury or insult, suggesting the role of NFκB may vary in different cell types (Brambilla et al. 2005; Hwang et al. 2010). Additionally, chromatin accessibility and DNA methylation in the 2.2 kb *GFAP* promoter have been shown to alter *GFAP* transcription rates in astrocytoma cell lines (Restrepo et al. 2011) and embryonic stem cells (Urayama et al. 2013). In fact, few studies have looked for regulatory elements outside the 2.2 kb *GFAP* promoter. In one study using a 10.8 kb rat *Gfap* promoter, progressive deletion of the promoter lead to a gradual decline in luciferase activity *in vitro*, but direct comparison of a 2 kb section of the *Gfap* promoter to the 10.8 kb *Gfap* promoter revealed only a slight decrease in luciferase activity (Kaneko et al. 1994). While it is unclear what additional regulatory elements are located in the 12 kb *Gfap* promoter, the results from our current study suggest they are important for maintained upregulation of *Gfap* promoter activity in mouse models of AxD. Analysis of these regulatory elements may elucidate mechanisms important for *Gfap* promoter upregulation both after injury and in mouse models of AxD.

In addition, differences seen between *Gfap* promoter activity and *Gfap* mRNA specifically in the brain stem of wild-type mice may be due to regulatory elements outside of the 12 kb *Gfap* promoter (Fig. 2.1). Nestin promoter activity is regulated by introns in the nestin gene known as intragenic regulatory sequences (Zimmerman et al. 1994). These intragenic regulatory sequences control and enhance nestin cell-specific gene expression (Zimmerman et al. 1994). Additionally, promoter activity can be regulated by distal transcriptional regulators which are located far from the promoter which they regulate (Heintzman and Ren 2009; Natarajan et al. 2012; Nora et al. 2012). While no studies have looked at distal transcriptional regulators of the *Gfap* promoter, two studies did look at intragenic regulatory sequences which

may alter *Gfap* promoter activity (Sarkar and Cowan 1991; Johnson et al. 1995). Johnson et al. (1995) analyzed the effects of intragenic regulatory sequences on a 2 kb murine *Gfap* promoter by ligating the promoter to the murine *Gfap* gene and comparing the promoter activity between the ligated and non-ligated form of the *Gfap* promoter. They found no change in *Gfap* promoter activity between the ligated and non-ligated forms both with and without injury. In another study, Sarkar and Cowan (1991) identified a negative regulatory sequence in the first intron of the *Gfap* gene which is important for cell type-specific expression. This intragenic regulatory sequence suppresses *Gfap* promoter activity in non-astrocytic cells, but does not alter *Gfap* promoter activity in astrocytes. In the current study, *Gfap* promoter activity in the brain stem is decreased compared with *Gfap* mRNA concentrations (Fig. 2.1). If the lack of intragenic regulator sequences is altering *Gfap* promoter activity in *Gfap*-luc mice, we would expect to see increased *Gfap* promoter activity compared with *Gfap* mRNA concentrations. Therefore, it is unlikely that the differences seen when comparing *Gfap* promoter activity and mRNA are due to intragenic regulatory sequences.

The experiments from the current study reveal that luciferase activity from the *Gfap*-luc transgene may be a good biomarker in mouse models of AxD for analysis of *Gfap* promoter activity changes throughout disease and in response to drug treatments. Analysis of *Gfap* promoter activity, mRNA, and protein in $Gfap^{+/+}$ mice reveal regional variations in all three which agree with previous literature for *Gfap* mRNA and protein expression in both mice and humans (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007). In $Gfap^{R236H/+}$ mice, both *Gfap* promoter activity and mRNA have similar fold increases in all observed brain regions. Conversely, GFAP protein fold increases vary in different brain regions compared with *Gfap* promoter activity and mRNA, suggesting GFAP mutations alter protein accumulation through additional mechanisms. Furthermore, the differences seen in luciferase activity between the 12 kb murine *Gfap* promoter and the 2.2 kb human *GFAP* promoter provide

insight into regulatory mechanisms important for the sustained increase of *Gfap* promoter activity in *Gfap*$^{R236H/+}$ mice. Overall, these results indicate luciferase activity in *Gfap*-luc reporter mice accurately mimics *Gfap* promoter activity which can be utilized as a biomarker for future analyses.

GFAP in Mouse Cerebrospinal Fluid

While brain concentrations of GFAP increase significantly in mouse models of AxD as well as AxD patients, no biomarker is currently available to monitor AxD severity or changes during future drug treatments which directly translate to human applications. GFAP is detectable in human cerebrospinal fluid (CSF) and elevated after injury (Aurell et al. 1991; Kwon et al. 2010; Ahmed et al. 2012; Zoltewicz et al. 2012) and in neurological diseases (Norgren et al. 2004; Kyllerman et al. 2005; Jesse et al. 2009; Takano et al. 2010; Axelsson et al. 2011), suggesting quantification of GFAP in CSF may be a reliable biomarker. In the second part of chapter 2, we quantified GFAP in CSF of three different mouse models of AxD; *Gfap*$^{R76H/+}$, *Gfap*$^{R236H/+}$, and *GFAP*^{Tg} mice compared with wild-type littermate controls. These three mouse models of AxD have varying severities which include differences in GFAP accumulation and Rosenthal fiber (RF) density. The *Gfap*$^{R76H/+}$ mice are the least severely affected and have the lowest GFAP concentrations while *GFAP*^{Tg} are the most severely affected with the highest GFAP concentration (Messing et al. 1998; Hagemann et al. 2006; Hagemann et al. 2009). Quantification of GFAP in mouse CSF revealed GFAP was detectable in wild-type mice at low levels and elevated in all three mouse models of AxD in a manner corresponding to brain GFAP concentrations (Fig. 2.5), though no direct correlation was found between brain and CSF GFAP concentrations (Fig. 2.6). These results suggest GFAP is a reliable biomarker which might be useful for analysis of drug effects on GFAP in mouse models of AxD and eventually human AxD patients.

GFAP Secretion

It is well established that GFAP is detectable in human CSF and the current study confirmed GFAP is present in mouse CSF as well, but the reason GFAP is detectable in CSF is unclear. Several possible explanations for GFAP presence in CSF include astrocyte turnover, death, or secretion of GFAP. In the adult mouse central nervous system (CNS), astrocytes have a slow but constant turnover of approximately 0.4% of cells per day (McCarthy and Leblond 1988). In AxD, GFAP mutations result in increased GFAP accumulation in astrocytes and as astrocytes are recycled during the turnover process a greater concentration of GFAP may be released from the dying astrocytes into the CSF, indicating increased GFAP in mouse CSF may reflect astrocyte turnover. This scenario seems unlikely due to the slow turnover and the lack of direct correlation with brain levels of GFAP (Fig. 2.6). Another explanation may be increased astrocytic death. In primary astrocyte cultures containing GFAP mutations, astrocyte death is increased (Tang et al. 2006; Cho and Messing 2009) though these results have not been confirmed in mouse models of AxD (Hagemann et al. 2005; Hagemann et al. 2006; Tanaka et al. 2007). Analysis of astrocyte death in mouse models of AxD may reveal increases which could correlate with GFAP concentrations in CSF, though preliminary experiments suggest little to no change in astrocyte death in mouse models of AxD (unpublished observation, T.L. Hagemann; C. LaPash Daniels).

Another possibility is secretion of GFAP from astrocytes. Using primary astrocyte cultures, previous studies analyzed astrocyte secretion products and found little evidence GFAP is secreted, though quantification is complicated by cell death (Lafon-Cazal et al. 2003; Delcourt et al. 2005; Dowell et al. 2009; Keene et al. 2009; Greco et al. 2010). One study found increased GFAP in media after treatment with TNFα (Edwards and Robinson 2006), but these results have not been replicated by other researchers. A different type III intermediate filament, vimentin, is secreted by macrophages (Mor-Vaknin et al. 2003). Vimentin is also expressed in

astrocytes. Analysis of astrocyte conditioned media reveals vimentin concentrations increase after lipopolysaccharide (LPS) or cytokine stimulation (Delcourt et al. 2005; Keene et al. 2009) and are enriched in astrocyte conditioned media compared with cell lysates (Greco et al. 2010), suggesting astrocytes secrete vimentin. Secretion of vimentin both in macrophages and astrocytes suggests GFAP may also be secreted by astrocytes but further studies are required.

Variability of GFAP in CSF

Quantification of GFAP in mouse CSF reveals high variability in GFAP concentrations from individual mice (Fig. 2.5) for reasons that are unclear. In human CSF, GFAP may be highly variable due to genetic diversity and differences in gene expression. Conversely, mice are congenic in the FVB/N background and should have little variability in *Gfap* gene or protein expression. Quantification of GFAP in the brains of mouse models of AxD reveal little inter-mouse variability with a coefficient of variation ranging from 15-27% (Fig. 2.6, coefficient of variation unpublished) (Hagemann et al. 2006; LaPash Daniels et al. 2012), suggesting GFAP concentrations in mouse CSF should be similar. In the same mice, we found CSF concentrations of GFAP were highly variable with a coefficient of variation ranging from 38-87% (Fig. 2.6, coefficient of variation unpublished), suggesting other factors may be influencing GFAP concentrations in mouse CSF.

The high variability of GFAP in mouse CSF may be due to blood contamination, stress from the collection process, time of collection, and variations in volume of CSF collected. Quantification of GFAP in plasma (see Appendix 2) reveals GFAP is undetectable in wild-type mice, low in $Gfap^{R236H/+}$ mice, and elevated in $GFAP^{Tg}$ mice (Fig. 6.1A). Comparison of GFAP in plasma and CSF both in this study and a previous study (Kwon et al. 2010) reveals GFAP concentrations are significantly lower in plasma compared with GFAP concentrations in CSF (Fig. 6.1B), suggesting blood contamination plays little role in GFAP variability in CSF from

mouse models of AxD (see Appendix 2). Stress due to the collection process may also contribute to GFAP variability in mouse CSF. After injury or invasive procedures, such as those employed during CSF collection, GFAP concentration in CSF increases within six hours of injury (Ahmed et al. 2012). In the current study, CSF is collected from mice within 5 to 10 minutes after surgery, suggesting GFAP upregulation due to stress should be minimal. Time of day may also alter GFAP concentration in CSF. GFAP immunoreactivity increases at night compared with day in the visual cortex and thalamus but not in the hippocampus (Hajos 2008), suggesting time of collection may alter GFAP concentrations in mouse CSF. Conversely, GFAP has a slow turnover of several days, indicating time of collection may only minimally contribute to GFAP variability in CSF (Chiu and Goldman 1984; Morrison et al. 1985; DeArmond et al. 1986; Rolland et al. 1990; Price et al. 2010). In the current study, CSF collections occurred throughout the day (9am-5pm). In future studies, comparison of GFAP concentration in CSF at various time points throughout both the day and night may elucidate the contribution of collection time to GFAP variability in CSF.

Volume of CSF collected from the mice may also contribute to the variability of GFAP concentrations seen in CSF. Proteins within CSF have a rostrocaudal concentration gradient which alters protein concentration depending upon the collection volume (Wurster 1988; Martino et al. 1990; Blennow et al. 1993; Teunissen et al. 2009). Martino et al. (1990) monitored protein concentrations in the first and last one milliliter (mL) fractions of a 10 mL CSF collection and found protein concentrations were altered when compared the two fractions of CSF. Immunoglobulin G (IgG) and albumin concentrations in CSF also decline in a linear fashion when comparing CSF from six successive fractions containing 2.5 mL per fraction (Wurster 1988). Blennow et al. (1993) found similar results when comparing six successive fractions containing 4 mL each, revealing protein concentrations are altered by the volume of CSF collected. To prevent alterations in human CSF protein concentration, a defined volume is

collected from the same location (Teunissen et al. 2009). Due to variations in CSF collection success in mice, the total volume collected from each mouse varied from 5 µL to 20 µL with an average of 10 µL, which may contribute to the variability in GFAP concentrations seen between mouse CSF samples. Analysis of volume collected versus GFAP protein concentration may provide insight into this phenomenon.

Overall, these experiments indicate detection of GFAP in mouse CSF may effectively monitor changes in brain GFAP concentration though the variability of GFAP in CSF may mask small changes in brain GFAP concentrations. We found GFAP is not only detectable in mouse CSF but is also elevated in all three mouse models of AxD in a manner corresponding to the concentrations of GFAP in brain. These results suggest GFAP in CSF is a useful biomarker for AxD which may be utilized for determination of drug efficacy during clinical trials of potential therapies.

GFAP in Human AxD CSF

In Chapter 3, we measured the levels of GFAP in CSF of human AxD patients to determine if GFAP is elevated in all AxD patients and elucidate the range of GFAP concentrations in AxD patients. We found GFAP was significantly elevated in greater than 90% of the AxD patients compared with controls. While there are conflicting results on the correlation between GFAP in CSF and age (Table 3.4) (Rosengren et al. 1992; Ahlsen et al. 1993; Rosengren et al. 1994; Fukuyama et al. 2001; Petzold et al. 2004; Axelsson et al. 2011), we found no correlation between GFAP and age in CSF samples from control and AxD patients. Overall, these results show GFAP is detectable and upregulated in AxD patient CSF samples, which may provide a biomarker for disease severity as well as a way to monitor future drug studies in AxD patients.

GFAP has been analyzed as a biomarker in CSF for several diseases and injuries (Aurell et al. 1991; Rosengren et al. 1994; Norgren et al. 2004; Petzold et al. 2004; Notturno et al. 2008; Misu et al. 2009; Kwon et al. 2010; Axelsson et al. 2011; Zoltewicz et al. 2012). After injury or in neurological diseases, GFAP concentrations in CSF are elevated compared to control patients. Additionally, GFAP concentrations in CSF correlate with infarct size and clinical classification after brain ischemic injury (Aurell et al. 1991) as well as to severity and progression rate of multiple sclerosis (MS) (Norgren et al. 2004), suggesting GFAP may correlate with severity in AxD patients. In a preliminary study of three AxD patients, GFAP concentrations in CSF were significantly higher compared with control CSF (Kyllerman et al. 2005). Though no correlation was found between GFAP and disease severity, the individual with rapid deterioration had the highest concentration of GFAP, consistent with the possibility that GFAP in CSF may correlate with disease severity.

We analyzed GFAP in CSF from a larger cohort of AxD patients (n=11) to determine if GFAP was increased in all AxD patients and elucidate the range of GFAP concentrations in CSF (Tables 3.2 and 3.3). We found GFAP was significantly elevated in AxD patients compared with controls with no correlation between age and GFAP concentrations (Fig. 3.1). We have not finished the correlation analysis between GFAP concentrations and severity, though GFAP concentrations were not significantly different when comparing the different forms of AxD (Fig. 3.5). Future analyses will provide insight into the correlation between GFAP and AxD severity.

A caveat to the current study is the limited clinical information on AxD patients and the lack of healthy control patients. Control patient CSF samples were from the Children's National Medical Center and were from individuals requiring CSF analysis to rule out neurological disorder or infection (Table 3.1), but were considered within the range of normal for protein, glucose, and cell counts. We obtained limited clinical information from these individuals, but

found GFAP concentrations were detectable but low in approximately 90% of the control patients. Comparisons of GFAP concentrations in CSF from the current and previous studies reveal similar concentrations (Jesse et al. 2009; Misu et al. 2009; Axelsson et al. 2011), suggesting the current CSF control are sufficient for analysis.

Overall, we found GFAP concentrations were significantly higher in AxD patient CSF compared with control CSF, suggesting GFAP may be a reliable biomarker of drug effects during future therapies. Additionally, while GFAP concentrations in CSF did not correlate to the form of AxD, GFAP concentrations may correlate to disease severity once an analysis of disease severity is completed. In future studies, an increased population of AxD patients as well as analysis of GFAP changes during future drug therapies will provide further insight into GFAP and its potential as a biomarker in AxD patient CSF.

GFAP in Human AxD Blood

In the second part of Chapter 3, we analyzed GFAP concentrations in plasma from AxD patients and healthy human controls to determine if GFAP is elevated in the plasma of AxD patients. Analysis of GFAP in plasma is preferable to CSF since blood collection is less invasive than CSF collections and may be easier for young patients. Similar to CSF results, plasma concentrations of GFAP were significantly elevated in AxD patients compared with healthy controls with no correlation between GFAP and age.

Quantification of GFAP in blood samples such as serum or plasma is another highly analyzed biomarker for brain and spinal injuries as well as neurodegenerative diseases (Missler et al. 1999; Herrmann et al. 2000; Vos et al. 2004; Nylen et al. 2006; Wunderlich et al. 2006; Jung et al. 2007; Notturno et al. 2008; Axelsson et al. 2011). After injury, GFAP in blood samples positively correlate with outcome of ischemic stroke patients (Herrmann et al. 2000; Wunderlich et al. 2006) as well as in traumatic brain injury patients (Vos et al. 2004; Nylen et al.

2006; Vos et al. 2010), suggesting blood sample concentrations of GFAP may correlate with AxD severity in patients. Again, we have not completed correlation analysis between GFAP and disease severity. No correlation was seen between age and GFAP concentration when comparing control samples (Fig. 3.2), similar to previous studies (Herrmann et al. 2000; Vos et al. 2006; Dvorak et al. 2009). In the current study, a negative correlation was found between age and GFAP concentration in AxD patient plasma samples (Fig. 3.3), which likely reflects disease severity rather than a change in GFAP concentration with age. Specific analysis of disease severity and GFAP concentrations may confirm these results in future analyses.

In the current study, comparisons of GFAP concentrations between AxD patients and controls were complicated by the lack of age matched healthy controls. The AxD patient plasma samples ranged in age from less than one year to 65 years of age with a median age of 15 (Table 3.2). Conversely, control plasma samples were from volunteers aged 18 or older with a median age of 30. While the absence of age-matched controls may be a caveat, the current and previous studies reveal no correlation between GFAP and age in control samples in the current study or in previous studies (Herrmann et al. 2000; Vos et al. 2006; Dvorak et al. 2009). This lack of correlation between GFAP and age suggests the addition of age matched plasma controls samples may not alter the results seen in this study, which will be confirmed in future experiments.

Overall, we found GFAP concentrations in both plasma and CSF samples from AxD patients were significantly elevated compared with control. For the few AxD patients where both CSF and plasma were collected, comparison of GFAP concentrations in plasma and CSF samples from the same individuals revealed a non-significant positive correlation (Fig. 3.4). GFAP concentrations in CSF were also higher than plasma, similar to previous reports (Beems et al. 2003; Steiner et al. 2006; Kwon et al. 2010). Use of GFAP in plasma is preferable to CSF due to the ease of collection especially in young children, the largest demographic of AxD

sufferers. Since GFAP concentrations are lower in plasma samples, further analysis of plasma GFAP concentrations in mouse models of AxD as well as AxD patients will elucidate the sensitivity of GFAP as a biomarker in plasma.

Estradiol Treatment in Mouse Models of AxD

In Chapter 4, we analyzed estradiol (E2) regulatory effects on GFAP to elucidate the potential of E2 as a treatment for AxD through hormone depletion by ovariectomy (OVX), treatment with E2 or placebo (PL) pellets, and monitoring GFAP during the stages of estrus in wild-type and *Gfap*$^{R236H/+}$ mice. There is no cure for AxD, but since mutations in GFAP result in GFAP upregulation in AxD drugs which decrease GFAP concentration may alleviate AxD symptoms. A screen of FDA-approved drugs revealed E2 decreased *GFAP* promoter activity by 26% *in vitro* (Cho et al. 2010). E2, a naturally occurring hormone, has neuroprotective qualities and may regulate GFAP protein concentrations (Table 1.1) (Dubal et al. 1998; McAsey et al. 2006; Wise et al. 2009; Arevalo et al. 2010; Raval et al. 2011). In the current study, following the same methods as McAsey et al. (2006), we performed OVX and sham surgeries on C57BL/6J (B6) mice and treated with either E2 or PL subcutaneous pellet implants. McAsey et al. (2006) found GFAP significantly increased after OVX but E2 treatment prevented this increase. Conversely, we found GFAP concentrations were comparable in OVX mice treated with both E2 and PL (Fig. 4.1). E2 did reduce GFAP concentrations significantly after OVX in the olfactory bulb and hippocampus of *Gfap*$^{+/+}$ mice (Fig. 4.1) and in the hippocampus and brain of *Gfap*$^{R236H/+}$ mice (Fig. 4.2). E2 treatment in sham-operated mice in both *Gfap*$^{+/+}$ and *Gfap*$^{R236H/+}$ mice had no effect on GFAP concentrations. *Gfap* mRNA concentrations were also unaltered by E2 or OVX, suggesting E2 may decrease GFAP after injury or stress such as OVX but does not directly regulate GFAP in mouse models of AxD. Interestingly, GFAP response to OVX varied in FVB/N and B6 mouse background strains, with GFAP concentrations increasing

in the FVB/N strain after OVX (Fig. 4.3) while GFAP concentrations in the B6 strain were unaltered by OVX (Fig. 4.2), indicating E2 effects on GFAP may be altered by stress and mouse background strain. Analysis of GFAP concentrations during the different stages of estrus revealed no changes (Fig. 4.5). Overall, these results suggest E2 regulation of GFAP is complex and may vary with brain region, stress, and mouse background strain.

While previous studies agree E2 is neuroprotective in rodents after injury (Lu et al. 2002; Suzuki et al. 2007; Raval et al. 2011) and in neurological diseases (Bebo et al. 2001; Morales et al. 2006; Offner and Polanczyk 2006; Lelu et al. 2010; Baraka et al. 2011; Cizas et al. 2011; MacKenzie-Graham et al. 2012), E2 regulation of GFAP is unclear. The *Gfap* promoter contains two estrogen response elements (EREs) which regulate GFAP expression *in vitro* (Stone et al. 1998; Rozovsky et al. 2002). Mutations within either of the estrogen response elements prevent E2 downregulation of *Gfap* expression after injury *in vitro* (Rozovsky et al. 2002). Interestingly, *in vivo* analysis of E2 treatments revealed both increases and decreases in GFAP concentration which may vary by injury or brain region (Table 1.1) (Day et al. 1993; Levin-Allerhand et al. 2001; Rozovsky et al. 2002; Lei et al. 2003; Martinez et al. 2006; Cordeau et al. 2008; Ritz and Hausmann 2008; Barreto et al. 2009; Giraud et al. 2010; Camacho-Arroyo et al. 2011).

In the current study, we found regulation of GFAP through E2 treatment varied by brain region in both $Gfap^{+/+}$ and $Gfap^{R236H/+}$ mice (B6 strain) after OVX, but E2 treatment was unable to alter GFAP concentrations in sham-operated mice. The presence of E2 effects on GFAP after OVX but not in sham-operated mice may suggest E2 is acting to reduce stress and inflammatory pathways triggered by OVX which indirectly act to reduce GFAP concentrations. In addition, the differential regulation of GFAP by OVX in the two different mouse background strains may indicate E2 hormonal or estrogen receptor (ER) concentrations vary by mouse background strain. Finally, the lack of E2 effects on GFAP in sham-operated mice may indicate

the E2 concentrations used in the current study are not sufficient for GFAP regulation. Though we found E2 treatment did not downregulate GFAP in mouse models of AxD, we did find E2 regulation of GFAP is altered by stress (caused by OVX) and mouse background strain. Further characterization of E2 effects on GFAP may elucidate other mechanisms and pathways important for GFAP regulation.

Estrogen Response Elements and Receptors

E2 acts on ERs, both ERα and ERβ, to regulate gene transcription (Roepke et al. 2011; Foster 2012; Yakimchuk et al. in press). After E2 binds to the ligand binding domain (LBD), ERs dimerize and then bind estrogen response elements (EREs) located in the promoter regions of various genes (Foster 2012; Yakimchuk et al. in press). The *Gfap* promoter contains two EREs, ERE-1 (-149 bp) and ERE-2 (-1817 bp) (Laping et al. 1994; Stone et al. 1998; Rozovsky et al. 2002), which are conserved among mouse, rat, and human *GFAP* promoters (Laping et al. 1994). These two EREs have been shown to decrease rat *Gfap* promoter activity and GFAP concentrations after wounding in both primary rat cortical astrocyte cultures and astrocyte-neuron co-cultures (Rozovsky et al. 2002). Conversely, Stone et al. (1998) found E2 treatment increased *Gfap* mRNA in primary cortical astrocyte cultures, but decreased *Gfap* mRNA concentrations when astrocytes were co-cultured with neurons. In addition, Stone et al. (1998) found ERE-2 may inhibit ERE-1 activity, while Rozovsky et al. (2002) found both EREs worked synergistically to decrease *Gfap* promoter activity and protein levels after wounding in both primary and co-cultures. The reason for these discrepancies may be differences in wounding and stress to the primary astrocyte cultures. This agrees with the results from the current study showing E2 treatment does not alter GFAP concentrations until after the OVX induced stress.

Interestingly, E2 effects on GFAP also differed by brain region which may reflect ER anatomical distributions. ER concentrations and ERα/ERβ ratios change in different brain regions (Table 5.1) (Kuiper et al. 1997; Shughrue et al. 1997; Osterlund et al. 2000; Taylor and Al-Azzawi 2000; Mitra et al. 2003; Perez et al. 2003; Milner et al. 2005; Gonzalez et al. 2007; Mitterling et al. 2010). Additionally, ER distribution in the brain varies in different species with both humans and rats showing a predominance of ERβ in the hippocampus (Shughrue et al. 1997; Gonzalez et al. 2007; Foster 2012) where mice have a predominance of ERα (Mitra et al. 2003; Foster 2012). ER expression may also be altered by injury or disease. Sakuma et al. (2009) and Carbonaro et al. (2009) both found that astrocyte expression of ERα and ERβ increase after injury in the hippocampus and spinal cord respectively.

Comparisons of the effects of E2 on different regions in the current study are difficult to reconcile with the known distribution of ER's. For instance, E2 treatment decreased GFAP concentrations in the hippocampus and olfactory bulb of *Gfap*$^{+/+}$ mice. In the mouse hippocampus, ERα is predominant but in the olfactory bulb ERβ is higher (Mitra et al. 2003), suggesting ER contribution to GFAP regulation may depend on the brain region. In previous studies comparing ERα and ERβ effects on GFAP, it was found that ERα specifically acts to reduce GFAP concentrations after injury with little contribution by ERβ (Carbonaro et al. 2009; Crawford et al. 2010; Spence et al. 2011). ERα and ERβ are also known to moderate the activity of each other, which may mask E2 effects on GFAP (Wang et al. 2006; Gottfried-Blackmore et al. 2007; Gonzales et al. 2008; Minutolo et al. 2011; Foster 2012).

Future analysis of ERα and ERβ distribution and specific contributions could be addressed through the use of ER specific agonists (Table 5.2)(Shiau et al. 1998; Acarin et al. 2000; Stauffer et al. 2000; Meyers et al. 2001; Nilsson et al. 2001; Harrington et al. 2003; Harris et al. 2003; Morales et al. 2006; Wang et al. 2006; Lewis et al. 2008; Crawford et al. 2010; Leitman et al. 2010; Ma et al. 2010; Baraka et al. 2011; Das et al. 2011; Minutolo et al. 2011;

Sarvari et al. 2011; Yakimchuk et al. 2011; Carroll et al. 2012; Foster 2012; MacKenzie-Graham et al. 2012; Yakimchuk et al. in press). ERα and ERβ agonists have gained a lot of attention in the past few years for potential treatments of cancer, injury, and aging effects (Harris et al. 2003; Cvoro et al. 2007; Lewis et al. 2008; Leitman et al. 2010; Sarvari et al. 2010; Minutolo et al. 2011; Sarvari et al. 2011; Yakimchuk et al. 2011; Yakimchuk et al. in press), however due to the similarities in the LBD, finding ERα or ERβ specific agonists which do not affect the other ER is difficult. Since ERα and ERβ can have opposing effects on proliferation, apoptosis, inflammatory responses, as well as other mechanisms important for cell survival and response to injury, finding ERα and ERβ specific agonists to elucidate the role of both ERs is important (Harris et al. 2003; Cvoro et al. 2007; Lewis et al. 2008; Leitman et al. 2010; Sarvari et al. 2010; Minutolo et al. 2011; Sarvari et al. 2011; Yakimchuk et al. 2011; Yakimchuk et al. in press). Some ERα and ERβ agonists have dual function. For example, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and R,R-tetrahydrochrysene (R,R-THC) can act both as an ERα agonist and as an ERβ antagonist (Sun et al. 1999; Harrington et al. 2003; Lewis et al. 2008). While several ERα and ERβ agonists are synthetic, current research has found several ERβ specific agonists which are plant based. These include plant extracts such as genistein, MF101, liquiritigenin (LIQ), and coumestrol (Nilsson et al. 2001; Cvoro et al. 2007; Mersereau et al. 2008; Leitman et al. 2010; Minutolo et al. 2011). Currently, the most commonly used and well characterized ER specific agonists are propyl-pyrazole-triol (PPT) (a specifc ERα agonist) and diarylpropionitrile (DPN) (a specific ERβ agonist) (Harrington et al. 2003; Wang et al. 2006; Lewis et al. 2008; Leitman et al. 2010; Ma et al. 2010; Baraka et al. 2011; Das et al. 2011; Minutolo et al. 2011; MacKenzie-Graham et al. 2012). Use of any of these ER agonists in future experiments will elucidate the role of either ERα or ERβ in the regulation of GFAP.

E2 Regulation of Stress Pathways

GFAP concentrations decrease with E2 treatment after OVX but not in sham-operated mice, suggesting E2 may activate different mechanisms dependent on the type of stress or injury. E2 activation of stress pathways is important for E2 neuroprotection. NFκB, an important protein in regulating cell response to stress, is decreased by E2 treatment after LPS or amyloid beta insults *in vitro* (Dodel et al. 1999) and in mouse models of MS (Giraud et al. 2010). E2 also decreases TNFα, a cytokine involved in inflammation (Drew and Chavis 2000; Matejuk et al. 2001; Taylor et al. 2010), and upregulates other cytokines such as TGFβ1, which induces apoptosis or suppresses proliferation of cells (Dhandapani et al. 2005; Son et al. 2005; Kumar et al. 2012; Taylor et al. 2012). E2 treatment also may reduce reactive oxygen species *in vitro* through activation of mitogen-activated protein kinase (MAPK) (Numakawa et al. 2007; Razmara et al. 2007; Numakawa et al. 2011).

These same stress response pathways alter GFAP concentrations. Inhibition of NFκB decreases glial reactivity after spinal cord injury and LPS insult (Brambilla et al. 2005; Hwang et al. 2010; Wu et al. 2012). TNFα induces upregulation of GFAP protein expression *in vitro* (Zhang et al. 2000), and inhibition prevents glial response and GFAP upregulation in experimental models of Parkinson's disease (Barcia et al. 2011). For TGFβ1, knock-out increases GFAP exacerbating injury response (Makwana et al. 2007), and inhibition of TGFβ1 decreases GFAP response to injury (Lindholm et al. 1992; Vergeli et al. 1995; Moon and Fawcett 2001), suggesting E2 regulation of stress pathways may indirectly regulate GFAP concentrations. In AxD, GFAP mutations activate several stress pathways including c-Jun N-terminal kinase (JNK), heat shock proteins, and oxidative stress (Head et al. 1993; Hagemann et al. 2005; Tang et al. 2006; Wang et al. 2007; Tang et al. 2008; Hagemann et al. 2009; Wang et al. 2011), which may alter GFAP accumulation (Tang et al. 2006; Cho and Messing 2009). While E2 regulation of stress pathways may alter GFAP concentration, E2 appears to have

minimal effects on stress pathways induced in AxD, suggesting activation of different stress pathways may explain the difference in GFAP response to E2 treatment in OVX and sham-operated mice. Future analysis of stress pathway activation in mouse models of AxD after OVX and/or E2 treatment might reveal which stress pathways are involved in E2 regulation of GFAP, providing insight into E2 effects on GFAP.

E2 Hormonal Regulation

Variations in GFAP response to E2 in different mouse background strains may indicate hormone concentration changes in different strains of mice. GFAP response to OVX varies by the different background strains of *Gfap*$^{R236H/+}$ mice (Fig. 4.2 and 4.3), with GFAP increasing significantly in the FVB/N strain after OVX, but not after OVX in the B6 strain. In mouse models of AxD, background strain alters body weight and Rosenthal fiber concentration (unpublished observation, T.L. Hagemann; P.L. Jany; C. LaPash Daniels). The variations seen in GFAP concentration and promoter activity in different mouse strains in this and previous studies (Fernandes et al. 2004; Hovatta et al. 2007; Sun et al. 2012; Bai et al. in press) may be the result of hormone changes. Hormone concentrations are known to vary between mouse strains (Phan et al. 2002; Tagawa et al. 2006; Lee et al. 2007), though no comparison of E2 values in FVB/N and B6 mice have been made. Comparison of mating schedules, litter size, and maternal instincts between B6 and FVB/N mice reveal drastic differences (Mouse Phenome Project, http://phenome.jax.org/), suggesting hormonal regulation varies in the different mouse strains. These hormonal differences may be important not only for breeding but also for E2 neuroprotective actions.

Hormone variations due to stage of estrus have also been shown to regulate GFAP concentrations. During proestrus (PE), high E2 levels increase GFAP in the hippocampus (Arias et al. 2009), hypothalamus (Garcia-Segura et al. 1994; Kohama et al. 1995; Stone et al.

1998), and amygdala (Stone et al. 1998; Martinez et al. 2006) compared with other stages of estrus when E2 levels are low, including metestrus (ME) and diestrus (DE). After middle cerebral arterial occlusion, GFAP concentrations were significantly reduced during PE and estrus (E) compared with ME and DE (Cordeau et al. 2008), suggesting the stage of estrus may also be important after injury or neurological disease. We observed no changes in *Gfap* mRNA and protein during the different stages of estrus in hippocampus, olfactory bulb, cerebellum, brain stem, or spinal cord (Fig. 4.5). While we saw no hormonal regulation of GFAP with the stage of estrus, future analysis of GFAP in other brain regions used in previous studies (Garcia-Segura et al. 1994; Kohama et al. 1995; Stone et al. 1998; Martinez et al. 2006) may reveal regional variations in GFAP regulation by the estrus cycle.

E2 Concentrations

The lack of E2 regulation on GFAP both during treatment and OVX may reflect insufficient E2 concentrations. To validate surgical and treatment procedures, we quantified E2 plasma concentration and monitored body weight, a property known to be sensitive to E2 manipulation (Witte et al. 2010; Eckel 2011). The E2 pellet (Innovative Research of America) is reported to steadily release E2 over 60 to 90 days after implantation, depending upon the release rate of the pellet. Conversely, Ingberg et al. (2012) found the E2 pellets produced high E2 blood values shortly after administration with a steady decline during the next 60 days. E2 concentrations also had high variability especially in the 90 day release pellet (Ingberg et al. 2012). Measurement of E2 plasma concentrations in our experiments revealed a significant increase in E2 concentrations with E2 treatment in OVX mice (Fig. 4.4). Sham-operated mice treated with E2 pellets had increased E2 blood concentrations though it was not statistically significant compared to PL treated mice. E2 concentrations were also highly variable in E2 implanted mice, suggesting E2 release from these pellets may not be uniform either due to

pellet implantation procedures or pellet composition. E2 plasma concentrations were similar between OVX and sham mice which confirms results from a previous study (Haisenleder et al. 2011). Analysis of weight revealed OVX mice weighed significantly more compared to sham-operated mice as predicted for ovary removal (Witte et al. 2010; Eckel 2011). E2 treatment reduced the weight gain apparent in OVX mice, though the reduction was not statistically significant. Therefore, E2 concentrations in blood, while variable, were elevated through the 49 days of E2 treatment, suggesting E2 levels were not diminished and were not responsible for the lack of E2 regulation of GFAP.

Additionally, regulation of GFAP by E2 may be masked by the soy-based food product used throughout the experiments. Soy beans contain isoflavones such as genistein which are phytoestrogens, or compounds with E2 functions. After ischemic injury, genistein decreases production of reactive oxygen species and downregulates NFκB activation (Qian et al. 2012) similar to E2 treatment (Numakawa et al. 2007; Razmara et al. 2007; Numakawa et al. 2011), suggesting the soy-based food product may be masking E2 treatment effects. Quantification of GFAP changes with OVX and E2 treatment reveals GFAP is altered by OVX in FVB/N mouse strains and by E2 treatment after OVX in B6 mouse strains. Detection of changes in GFAP with E2 treatment suggests minimal contribution of genistein estrogenic regulation of GFAP in the current study.

E2 concentration may also vary in the brain which may alter E2 regulation of GFAP. E2 is synthesized in the brain (neuroestrogen) as well as in the ovaries. Neuroestrogen synthesis may be regulated by the estrus cycle or independently within the brain. Comparison of neuroestrogen concentrations to blood E2 reveals no correlation in rodents (Tagawa et al. 2006; Konkle and McCarthy 2011) and song birds (Schlinger and Remage-Healey 2012), suggesting neuroestrogen concentration may not be regulated by the estrus cycle. Neuroestrogen synthesis is regulated by cytochrome P450 aromatase activity (Veyrac and Bakker 2011; Toda

et al. 2012). After injury, cytochrome P450 aromatase levels are increased especially in astrocytes (Garcia-Segura et al. 1999; Peterson et al. 2004), suggesting neuroestrogen synthesis may increase immediately following injury. Knock-out of cytochrome P450 aromatase or aromatase inhibition exacerbates ischemic injury (McCullough et al. 2003), suggesting neuroestrogen may be neuroprotective after injury. Measurement of E2 in the brain in future experiments may reveal neuroestrogen regulation is independent of the estrus cycle and may change with signals, conditions, injury, or mouse background strain, suggesting neuroestrogen concentrations could be important for GFAP regulation normally and in mouse models of AxD.

Overall, these results indicate E2 regulation of GFAP may change with stress and brain region. Interestingly, E2 effects on GFAP were also altered by the background strain of the mouse, implicating the importance of strain during future drug testing and stress experiments, especially for E2 pathways. While E2 might not have an important regulatory role in AxD, it may have neuroprotective functions in other neurodegenerative diseases or injuries.

Future Directions

Drug Targets of AxD Treatment

There is currently no cure for AxD, only treatment of symptoms. Discovery of drugs which directly or indirectly decrease GFAP levels in the brain may cure or decrease debilitating symptoms of AxD patients (Messing et al. 2010). A previous drug screen of FDA-approved drugs yielded several possible drug candidates which decreased human *GFAP* promoter activity in primary cortical astrocytes (Cho et al. 2010). Future and ongoing studies (Cho et al. 2010; Hagemann et al. 2012; LaPash Daniels et al. 2012) are using genetic modifications and candidate drugs in mouse models of AxD to analyze how these modifications and/or drugs effect GFAP concentrations *in vivo*. Over-expression of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor which mediates antioxidant responses, reduces *Gfap* mRNA and

protein levels as well as RF quantity (LaPash Daniels et al. 2012), though not uniformly in all of the observed brain regions. The regional differences in GFAP and RF concentrations both in mouse models of AxD and after Nrf2 over-expression may be due to astrocyte heterogeneity which varies in astrocyte activity (Hansson 1990; Regan et al. 2007; Yeh et al. 2009; Fitting et al. 2010) and GFAP expression (Palfreyman et al. 1979; Chen et al. 1993; Martin and O'Callaghan 1995; Lein et al. 2007). In addition, regional differences may be due to differences in degradation machinery. Both proteasome function and autophagy are altered by GFAP mutations *in vitro* and have feedback loops which alter GFAP accumulation, suggesting degradation machinery may be an important mediator of astrocyte and GFAP regional diversity. Comparison of gene expression profiles and degradation machinery in various brain regions of mouse models of AxD might reveal other potential drug targets for AxD and elucidate mechanisms leading to GFAP accumulation.

To elucidate differences in gene expression which may be important for mouse models of AxD and possibly identify important drug targets, microarray analysis of gene expression profiles may be done in the different brain regions. Microarray analysis of olfactory bulb in *GFAP*^{Tg} mice revealed altered expression of genes involved in stress response, immune response, vesicular trafficking, neurogenesis, myelination, and neurotransmitter expression as well as cytoskeletal proteins (Hagemann et al. 2005). Comparison of expression profiles between brain regions with large protein fold increases in GFAP (olfactory bulb and hippocampus), moderate fold increases (cerebellum), and no increase (brain stem and spinal cord) (Fig. 2.2) (LaPash Daniels et al. 2012) may provide further insight into AxD and the mechanisms controlling GFAP protein accumulation and *Gfap* promoter activation.

Differences in protein degradation machinery, such as proteasome function and autophagy, may also yield potential drug targets and elucidate mechanisms involved in GFAP accumulation in mouse models of AxD. Proteasomes function to degrade proteins which are

identified by ubiquitin attachment (Kunjappu and Hochstrasser in press; Teixeira and Reed in press). In AxD, ubiquitin is found in RFs (Goldman and Corbin 1988; Lowe et al. 1989; Tomokane et al. 1991; Hagemann et al. 2006; Mignot et al. 2007), suggesting proteins such as mutant GFAP may be ubiquitinated but are sequestered into RFs before degradation can occur. Lack of degradation through sequestration of ubiquitinated GFAP into RFs may lead to elevated GFAP concentrations. Quantification of RF per area in different brain regions revealed RF density varies with corpus callosum and olfactory bulb having a high density of RFs and brain stem containing a low number of RFs (LaPash Daniels et al. 2012). Similarly, GFAP protein levels in *Gfap*$^{R236H/+}$ mice are highest in the olfactory bulb and lower in the brain stem, suggesting RF sequestration may be an important mechanism leading to GFAP accumulation which may vary regionally. Further analysis of RF density and GFAP accumulation as well as mechanisms leading to RF formation in different brain regions may reveal potential drug targets and elucidate mechanisms behind GFAP accumulation.

In addition, proteasome function may play a key role in GFAP protein accumulation which can be utilized for drug therapies of AxD patients. Mutations in GFAP *in vitro* decrease proteasome function resulting in increased GFAP concentrations (Tang et al. 2006; Cho and Messing 2009). In mouse models of AxD, analysis of proteasome function in different brain regions may indicate proteasome impairment varies regionally. Proteasome function is measured by quantification of proteasomal subunits (McNaught et al. 2003; Tang et al. 2008; Liggett et al. 2010; Um et al. 2010), proteasomic cleavage of a fluorgenic peptide substrate (Rivett et al. 2002; Rodgers and Dean 2003; Liggett et al. 2010), and ELISAs for proteasome core components (Wada et al. 1993; Majetschak and Sorell 2008; Liggett et al. 2010). Using these identified methods, we may be able to compare GFAP accumulation and proteasome function and find a positive correlation between the two, indicating drugs which enhance proteasome function as a useful treatment for AxD.

Autophagy is also an important pathway for protein degradation which is altered by mutant GFAP (Tang et al. 2008; Wang et al. 2011), and might be an important drug target for treatment of AxD. Autophagy clears proteins and organelles from cells through the use of lysosomes (Mortimore and Schworer 1977; Neely et al. 1977; Schworer et al. 1979; Schreiber and Peter in press). Once the desired protein is engulfed by the lysosome and fuses with the autophagosome, hydrolase enzymes degrade or recycle the protein. In AxD, autophagy pathways are induced indirectly through mutant GFAP (Tang et al. 2008; Wang et al. 2011), which act to inhibit mammalian target of rapamycin (mTOR), a negative regulator of autophagy. Inhibition of autophagy pathways increase GFAP protein accumulation (Tang et al. 2008) suggesting autophagy function is also an important regulator of GFAP accumulation. Monitoring proteins involved in autophagy such as LC3-II, a protein found in the autophagosomal membrane, may reveal regional differences especially in the brain stem and spinal cord where GFAP accumulation is lowest and thus potential targets for AxD treatment. In addition, increasing autophagy further through drugs may decrease GFAP protein concentrations (Tang et al. 2008) in mouse models of AxD, providing a potential therapy for AxD patients. Overall, analysis of mechanisms involved in protein degradation in various brain regions may elucidate mechanisms leading to differential GFAP accumulation in mouse models of AxD and provide other candidate drug targets.

Luciferase Activity as a Biomarker of Drug Efficacy

Analysis of the *Gfap*-luc reporter mouse line identified luciferase activity as a good biomarker for *Gfap* promoter activity, which may be useful during future drug studies in mouse models of AxD. Previous studies of *Gfap*-luc mice utilized *in vivo* imaging of luciferase signal to monitor *Gfap* promoter activity changes over time after infection or injury (Kadurugamuwa et al. 2005; Cordeau et al. 2008; Luo et al. 2008) and found activity correlated with mRNA (Tamguney

et al. 2009). These results suggest visualization of luciferase activity in *Gfap*$^{R236H/+}$ and *GFAP*^{Tg} mice is possible. In preliminary experiments of mouse models of AxD, no increase in luciferase activity was detectable in *GFAP*^{Tg} mice, though these results were complicated by luciferase signal expression in the ears and feet (unpublished data, Elizabeth Austin). For future experiments, the ears and feet will be obscured with black marker or black construction paper, though other methods of optimization may be necessary. Quantification of luciferase activity *in vivo* will provide longitudinal analysis of drug effects on *Gfap* promoter activity and may determine direct or indirect regulation, with indirect regulation of the *Gfap* promoter resulting in decreases in luciferase activity which occur after several days of treatment. Detection of luciferase activity in different brain regions may reveal region specific alteration in *Gfap* promoter activity, which could manifest into overt changes such as sensitivity to kainic acid induced seizures or behavior changes and which can then be tested for in mouse models of AxD.

Analysis of *Gfap* promoter activity should also be combined with protein quantification. Changes in promoter activity due to drug effects may not reflect changes in GFAP. Several proteins, cytokines, chemokines, and transcription factors regulate GFAP expression both during synthesis and breakdown (Eddleston and Mucke 1993; Eng et al. 2000; Edwards and Robinson 2006; Romao et al. 2008; Hwang et al. 2010; Kang and Hebert 2011). Drug actions on *Gfap* promoter may not immediately translate to decreases in protein concentrations especially given the slow turnover rate of GFAP (Chiu and Goldman 1984; DeArmond et al. 1986; Price et al. 2010), suggesting caution in *Gfap* promoter activity analysis, especially during short-term treatments.

GFAP in CSF and Blood

GFAP concentrations in CSF reveal similar concentrations to brain GFAP in mouse models of AxD, suggesting GFAP may be a useful biomarker for determination of severity of AxD. The mouse models of AxD used for the current study display varying severities of disease based on GFAP concentration, RF density, lethality, and reduction in body weight (Messing et al. 1998; Hagemann et al. 2006). Further characterization of severity and prognosis in mouse models of AxD may reveal a correlation between severity and GFAP in CSF of mice. The current human studies also had limited clinical information but revealed high variability in GFAP concentrations in CSF and blood of AxD patients. Future clinical trials may correlate GFAP in CSF or blood to severity.

Measurement of GFAP in CSF and blood, while reflecting brain concentrations of GFAP in mice, may not accurately reflect drug effects on GFAP in the brain. We found GFAP was detectable in both wild-type mice and all three different mouse models of AxD, with GFAP in CSF showing similar elevations as GFAP in brain. When treating mouse models of AxD with drug therapies, GFAP may decrease in the brain but remain unchanged or increase in CSF and blood, suggesting analysis of GFAP in CSF, blood, and brain during drug treatments is required. Small changes in GFAP levels in the brain may not be reflected immediately in CSF, as seen after OVX in *Gfap*$^{R236H/+}$ mice (Fig. 4.3), especially give the slow turnover rate of GFAP (Chiu and Goldman 1984; DeArmond et al. 1986; Price et al. 2010). It is also possible GFAP levels may increase in CSF while decreasing in the brain if RFs begin to breakdown during drug treatment. Over-expression of Nrf2 in *Gfap*$^{R236H/+}$ mice significantly reduced the number of RFs present in various brain regions (LaPash Daniels et al. 2012). Since RFs are composed of GFAP as well as other proteins (Iwaki et al. 1992; Head et al. 1993; Tang et al. 2006), GFAP concentrations may initially increase in CSF during drug treatment before eventually decreasing. Monitoring changes in RF density and GFAP concentrations in CSF at several time points

throughout the drug treatments may elucidate the contribution of RF degradation to GFAP in CSF. Overall, measurement of RF density as well as GFAP in CSF, blood, and brain may determine the efficacy of GFAP as a biomarker during drug treatments.

Other Biomarkers

Combining other biomarkers with GFAP may also provide improved diagnostic information both in analysis of severity and in future drug treatments. To identify other potential biomarkers of AxD, Cunningham, et al. (2012) performed mass spectrometry analysis on CSF from *GFAP^{Tg}* mice, revealing several upregulated and downregulated proteins in *GFAP^{Tg}* CSF compared to wild-type. Cathepsins B, S, and L1, peroxiredoxin-6, complement C4-B, and serine protease inhibitor A3N are upregulated, while contactin-1 and carboxypeptidase E are downregulated in CSF compared to wild-type mice (Cunningham et al. 2013), agreeing with a previous study in olfactory bulbs of *GFAP^{Tg}* mice (Hagemann et al. 2005). These seven proteins may elucidate mechanisms leading to AxD presentation and GFAP accumulation. In addition, if these seven proteins are detectable and altered in CSF and blood of both human and mouse models of AxD compared with controls, they may be candidate biomarkers which may be combined with GFAP to increase the sensitivity of the biomarkers for analysis of severity or drug efficacy.

GFAP Presence in CSF and Blood

While the current and previous studies confirm GFAP is detectable in CSF and blood and elevated after injury or neurological disease (Notturno et al. 2008; Axelsson et al. 2011), the process leading to GFAP presence in CSF and blood is unknown. Two main possibilities for GFAP presence in CSF and blood are astrocytic secretion of GFAP breakdown products or increased astrocytic death/turnover. To determine how GFAP is released into CSF, we can analyze GFAP breakdown products present in CSF and blood through western blots. Full

length, unmodified GFAP produces an approximate 50 kilodalton (kDa) band on a western blot (Eng 1985). Analysis of GFAP in CSF and serum after traumatic brain injury in human patients revealed a 38 kDa band corresponding to the calpain cleavage product of GFAP (Papa et al. 2012; Zoltewicz et al. 2012). Calpain is a cysteine protease important for regulation of apoptosis (Zatz and Starling 2005; Liu et al. 2011). Cathepsins B, L1, and S are also cysteine proteases (Turk et al. 2000; Cirman et al. 2004; Lamparska-Przybysz et al. 2005; Biniossek et al. 2011) and are upregulated in CSF and olfactory bulbs of *GFAP*^{Tg} mice (Hagemann et al. 2005; Cunningham et al. 2013). After spinal cord injury, cysteine proteases such as calpain, caspases, and cathepsin B activate and degrade cytoskeleton proteins as well as other proteins important for preventing death in neurons and glial cells (Ray et al. 2011). Preliminary experiments of CSF from *GFAP*^{Tg} mice (Fig. 6.3) reveal an approximate 37 kDa band but no full-length GFAP band. In future experiments, optimization of protein precipitation techniques, increased GFAP concentrations, and use of antibodies specific for breakdown products of GFAP may reveal further information about GFAP release into CSF from astrocytes, which might elucidate mechanisms involved in AxD.

Conclusion

Overall, the current study showed luciferase activity under the control of a 12 kb mouse *Gfap* promoter reliably reflects *Gfap* promoter activity in *Gfap*$^{R236H/+}$ mice, both early in development and through 6 months of age as well as in different brain regions. Interestingly, *Gfap* promoter activity does not mimic protein accumulation in all brain regions, suggesting a complex interaction between protein synthesis and degradation in mouse models of AxD. We also found GFAP is detectable and elevated in CSF from mouse models of AxD as well as CSF and blood of human AxD patients compared with controls. Analysis of GFAP concentration in CSF of mouse models of AxD reveals a similar increase in CSF and brain GFAP concentration,

suggesting GFAP may be a good biomarker for AxD and useful for determination of drug efficacy during future treatments. Finally, we monitored E2 regulation of GFAP in mouse models of AxD and found E2 regulation varied with stress, brain region, and mouse background strain, suggesting caution for E2 treatment in AxD. Overall, the conclusions of the work described in this report reveal GFAP is a useful biomarker for AxD and can be utilized in future clinical trials to determine drug efficacy.

Table 5.1 – Estrogen receptor anatomical distribution in the brain.

Strain	Brain Region						Reference
	Olfactory Bulb	Hippocampus	Cortex	Cerebellum	Brain Stem	Spinal Cord	
Mouse	β	α	β	β	αβ	-	(Mitra et al. 2003)
	-	αβ	-	-	-	-	(Mitterling et al. 2010)
Human	-	β	β	-	-	-	(Gonzalez et al. 2007)
	-	αβ	αβ	-	-	-	(Osterlund et al. 2000)
	-	α	αβ	αβ	β	-	(Taylor and Al-Azzawi 2000)
Rat	β	-	-	β	β	β	(Kuiper et al. 1997)
	-	αβ	-	-	-	-	(Milner et al. 2005)
	-	α	α	αβ	αβ	-	(Perez et al. 2003)
	β	β	-	β	β	αβ	(Shughrue et al. 1997)

α – ERα is the predominant form of ER
β – ERβ is the predominant form of ER
αβ – ERα and ERβ are roughly equal in quantity

Table 5.1: Estrogen receptor anatomical distribution in the brain. α indicates ERα is predominant while β indicates ERβ is predominant in that brain region. αβ indicates both ERα and ERβ are present in the brain region in almost equal concentrations. – indicates ER qas not measured in that particular brain region.

Table 5.2 – Estrogen receptor specific agonists.

Chemical name		Agonist Activity	References
Abbreviation	Full		
16α-LE2	16α-lactone-estradiol	ERα agonist	(Sarvari et al. 2011)
	chalcone	ERα agonist	(Leitman et al. 2010)
	coumestrol	ERβ agonist	(Kuiper et al. 1998; Minutolo et al. 2011)
DES	disethylstibestrol	ERα agonist	(Shiau et al. 1998; Nilsson et al. 2001)
DPN	diarylpropionitrile	ERβ agonist	(Meyers et al. 2001; Harrington et al. 2003; Wang et al. 2006; Lewis et al. 2008; Crawford et al. 2010; Leitman et al. 2010; Ma et al. 2010; Baraka et al. 2011; Das et al. 2011; Minutolo et al. 2011; Sarvari et al. 2011; Yakimchuk et al. 2011; Carroll et al. 2012; MacKenzie-Graham et al. 2012)
	ERB-041	ERβ agonist	(Harris et al. 2003; Minutolo et al. 2011)
	Genistein	ERβ agonist, partial	(Kuiper et al. 1998; Nilsson et al. 2001; Minutolo et al. 2011)
HPTE	2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane	ERα agonist ERβ antagonist	(Lewis et al. 2008)
LIQ	liquiritigenin	ERβ agonist	(Mersereau et al. 2008; Leitman et al. 2010)
	MF101	ERβ agonist	(Cvoro et al. 2007; Leitman et al. 2010)
PPT	propyl-pyrazole-triol	ERα agonist	(Stauffer et al. 2000; Harrington et al. 2003; Morales et al. 2006; Wang et al. 2006; Lewis et al. 2008; Leitman et al. 2010; Ma et al. 2010; Baraka et al. 2011; Das et al. 2011; Minutolo et al. 2011; MacKenzie-Graham et al. 2012)
R,R-THC	R,R-tetrahydrochrysene	ERα agonists ERβ antagonist	(Sun et al. 1999; Harrington et al. 2003)

Table 5.2: Estrogen receptor specific agonists. List of known estrogen receptor (ER) agonists. ERα agonists specifically activate ERα while ERβ agonists specifically activate ERβ. Antagonists specifically inhibit the identified ER. "Agonist, partial" indicates the identified agonist partially activates the ER.

REFERENCE

Acarin, L., B. Gonzalez and B. Castellano (2000). "STAT3 and NFkappaB activation precedes glial reactivity in the excitotoxically injured young cortex but not in the corresponding distal thalamic nuclei." J Neuropathol Exp Neurol **59**(2): 151-163.

Ahlsen, G., L. Rosengren, M. Belfrage, A. Palm, K. Haglid, A. Hamberger and C. Gillberg (1993). "Glial fibrillary acidic protein in the cerebrospinal fluid of children with autism and other neuropsychiatric disorders." Biol Psychiatry **33**(10): 734-743.

Ahmed, F., A. Gyorgy, A. Kamnaksh, G. Ling, L. Tong, S. Parks and D. Agoston (2012). "Time-dependent changes of protein biomarker levels in the cerebrospinal fluid after blast traumatic brain injury." Electrophoresis **33**(24): 3705-3711.

Andriezen, W. L. (1893). "The Neuroglia Elements in the Human Brain." Br Med J **2**(1700): 227-230.

Arevalo, M. A., M. Santos-Galindo, M. J. Bellini, I. Azcoitia and L. M. Garcia-Segura (2010). "Actions of estrogens on glial cells: Implications for neuroprotection." Biochim Biophys Acta **1800**(10): 1106-1112.

Arias, C., A. Zepeda, K. Hernandez-Ortega, P. Leal-Galicia, C. Lojero and I. Camacho-Arroyo (2009). "Sex and estrous cycle-dependent differences in glial fibrillary acidic protein immunoreactivity in the adult rat hippocampus." Horm Behav **55**(1): 257-263.

Aurell, A., L. E. Rosengren, B. Karlsson, J. E. Olsson, V. Zbornikova and K. G. Haglid (1991). "Determination of S-100 and glial fibrillary acidic protein concentrations in cerebrospinal fluid after brain infarction." Stroke **22**(10): 1254-1258.

Axelsson, M., C. Malmestrom, S. Nilsson, S. Haghighi, L. Rosengren and J. Lycke (2011). "Glial fibrillary acidic protein: a potential biomarker for progression in multiple sclerosis." J Neurol **258**: 882-888.

Bai, X., A. S. Saab, W. Huang, I. K. Hoberg, F. Kirchhoff and A. Scheller (in press). "Genetic background affects human glial fibrillary acidic protein promoter activity." PLoS One.

Baraka, A. M., A. A. Korish, G. A. Soliman and H. Kamal (2011). "The possible role of estrogen and selective estrogen receptor modulators in a rat model of Parkinson's disease." Life Sci **88**(19-20): 879-885.

Barcia, C., C. M. Ros, V. Annese, A. Gomez, F. Ros-Bernal, D. Aguado-Yera, . . . M. T. Herrero (2011). "IFN-gamma signaling, with the synergistic contribution of TNF-alpha, mediates

cell specific microglial and astroglial activation in experimental models of Parkinson's disease." Cell Death Dis **2**: e142.

Barreto, G., M. Santos-Galindo, Y. Diz-Chaves, O. Pernia, P. Carrero, I. Azcoitia and L. M. Garcia-Segura (2009). "Selective estrogen receptor modulators decrease reactive astrogliosis in the injured brain: effects of aging and prolonged depletion of ovarian hormones." Endocrinology **150**(11): 5010-5015.

Bebo, B. F., Jr., A. Fyfe-Johnson, K. Adlard, A. G. Beam, A. A. Vandenbark and H. Offner (2001). "Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains." J Immunol **166**(3): 2080-2089.

Beems, T., K. S. Simons, W. J. Van Geel, H. P. De Reus, P. E. Vos and M. M. Verbeek (2003). "Serum- and CSF-concentrations of brain specific proteins in hydrocephalus." Acta Neurochir (Wien) **145**(1): 37-43.

Biniossek, M. L., D. K. Nagler, C. Becker-Pauly and O. Schilling (2011). "Proteomic identification of protease cleavage sites characterizes prime and non-prime specificity of cysteine cathepsins B, L, and S." J Proteome Res **10**(12): 5363-5373.

Blennow, K., P. Fredman, A. Wallin, C. G. Gottfries, G. Langstrom and L. Svennerholm (1993). "Protein analyses in cerebrospinal fluid. I. Influence of concentration gradients for proteins on cerebrospinal fluid/serum albumin ratio." Eur Neurol **33**(2): 126-128.

Bonni, A., Y. Sun, M. Nadal-Vicens, A. Bhatt, D. A. Frank, I. Rozovsky, . . . M. E. Greenberg (1997). "Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway." Science **278**(5337): 477-483.

Brambilla, R., V. Bracchi-Ricard, W. H. Hu, B. Frydel, A. Bramwell, S. Karmally, . . . J. R. Bethea (2005). "Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury." J Exp Med **202**(1): 145-156.

Brenner, M. (1994). "Structure and transcriptional regulation of the GFAP gene." Brain Pathol **4**(3): 245-257.

Brenner, M., W. C. Kisseberth, Y. Su, F. Besnard and A. Messing (1994). "GFAP promoter directs astrocyte-specific expression in transgenic mice." J Neurosci **14**(3 Pt 1): 1030-1037.

Camacho-Arroyo, I., A. Gonzalez-Arenas, J. Espinosa-Raya, A. G. Pina-Medina and O. Picazo (2011). "Short- and long-term treatment with estradiol or progesterone modifies the expression of GFAP, MAP2 and Tau in prefrontal cortex and hippocampus." Life Sci **89**(3-4): 123-128.

Carbonaro, V., F. Caraci, M. L. Giuffrida, S. Merlo, P. L. Canonico, F. Drago, . . . M. A. Sortino (2009). "Enhanced expression of ERalpha in astrocytes modifies the response of cortical neurons to beta-amyloid toxicity." Neurobiol Dis **33**(3): 415-421.

Carroll, V. M., M. Jeyakumar, K. E. Carlson and J. A. Katzenellenbogen (2012). "Diarylpropionitrile (DPN) enantiomers: synthesis and evaluation of estrogen receptor beta-selective ligands." J Med Chem **55**(1): 528-537.

Cebolla, B. and M. Vallejo (2006). "Nuclear factor-I regulates glial fibrillary acidic protein gene expression in astrocytes differentiated from cortical precursor cells." J Neurochem **97**(4): 1057-1070.

Chen, H., F. Cabon, P. Sun, E. Parmantier, P. Dupouey, C. Jacque and B. Zalc (1993). "Regional and developmental variations of GFAP and actin mRNA levels in the CNS of jimpy and shiverer mutant mice." J Mol Neurosci **4**(2): 89-96.

Chiu, F. C. and J. E. Goldman (1984). "Synthesis and turnover of cytoskeletal proteins in cultured astrocytes." J Neurochem **42**(1): 166-174.

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Cho, W., T. L. Hagemann, D. A. Johnson, J. A. Johnson and A. Messing (2009). "Dual transgenic reporter mice as a tool for monitoring expression of glial fibrillary acidic protein." J Neurochem **110**(1): 343-351.

Cho, W. and A. Messing (2009). "Properties of astrocytes cultured from GFAP over-expressing and GFAP mutant mice." Exp Cell Res **315**(7): 1260-1272.

Cirman, T., K. Oresic, G. D. Mazovec, V. Turk, J. C. Reed, R. M. Myers, . . . B. Turk (2004). "Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins." J Biol Chem **279**(5): 3578-3587.

Cizas, P., A. Jekabsone, V. Borutaite and R. Morkuniene (2011). "Prevention of amyloid-beta oligomer-induced neuronal death by EGTA, estradiol, and endocytosis inhibitor." Medicina (Kaunas) **47**(2): 107-112.

Cordeau, P., Jr., M. Lalancette-Hebert, Y. C. Weng and J. Kriz (2008). "Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury." Stroke **39**(3): 935-942.

Crawford, D. K., M. Mangiardi, B. Song, R. Patel, S. Du, M. V. Sofroniew, . . . S. K. Tiwari-Woodruff (2010). "Oestrogen receptor beta ligand: a novel treatment to enhance endogenous functional remyelination." Brain **133**(10): 2999-3016.

Cunningham, R., P. Jany, A. Messing and L. Li (2013). "Protein changes in immunodepleted cerebrospinal fluid from a transgenic mouse model of alexander disease detected using mass spectrometry." J Proteome Res **12**(2): 719-728.

Cvoro, A., S. Paruthiyil, J. O. Jones, C. Tzagarakis-Foster, N. J. Clegg, D. Tatomer, . . . D. C. Leitman (2007). "Selective activation of estrogen receptor-beta transcriptional pathways by an herbal extract." Endocrinology **148**(2): 538-547.

Das, A., J. A. Smith, C. Gibson, A. K. Varma, S. K. Ray and N. L. Banik (2011). "Estrogen receptor agonists and estrogen attenuate TNF-{alpha}-induced apoptosis in VSC4.1 motoneurons." J Endocrinol **208**(2): 171-182.

Day, J. R., N. J. Laping, M. Lampert-Etchells, S. A. Brown, J. P. O'Callaghan, T. H. McNeill and C. E. Finch (1993). "Gonadal steroids regulate the expression of glial fibrillary acidic protein in the adult male rat hippocampus." Neuroscience **55**(2): 435-443.

DeArmond, S. J., Y. L. Lee, H. A. Kretzschmar and L. F. Eng (1986). "Turnover of glial filaments in mouse spinal cord." J Neurochem **47**(6): 1749-1753.

Delcourt, N., P. Jouin, J. Poncet, E. Demey, E. Mauger, J. Bockaert, . . . N. Galeotti (2005). "Difference in mass analysis using labeled lysines (DIMAL-K): a new, efficient proteomic quantification method applied to the analysis of astrocytic secretomes." Mol Cell Proteomics **4**(8): 1085-1094.

Dhandapani, K. M., F. M. Wade, V. B. Mahesh and D. W. Brann (2005). "Astrocyte-derived transforming growth factor-{beta} mediates the neuroprotective effects of 17{beta}-estradiol: involvement of nonclassical genomic signaling pathways." Endocrinology **146**(6): 2749-2759.

Dodel, R. C., Y. Du, K. R. Bales, F. Gao and S. M. Paul (1999). "Sodium salicylate and 17beta-estradiol attenuate nuclear transcription factor NF-kappaB translocation in cultured rat astroglial cultures following exposure to amyloid A beta(1-40) and lipopolysaccharides." J Neurochem **73**(4): 1453-1460.

Dowell, J. A., J. A. Johnson and L. Li (2009). "Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics." J Proteome Res **8**(8): 4135-4143.

Drew, P. D. and J. A. Chavis (2000). "Female sex steroids: effects upon microglial cell activation." J Neuroimmunol **111**(1-2): 77-85.

Dubal, D. B., M. L. Kashon, L. C. Pettigrew, J. M. Ren, S. P. Finklestein, S. W. Rau and P. M. Wise (1998). "Estradiol protects against ischemic injury." J Cereb Blood Flow Metab **18**(11): 1253-1258.

Dvorak, F., I. Haberer, M. Sitzer and C. Foerch (2009). "Characterisation of the diagnostic window of serum glial fibrillary acidic protein for the differentiation of intracerebral haemorrhage and ischaemic stroke." Cerebrovasc Dis **27**(1): 37-41.

Eckel, L. A. (2011). "The ovarian hormone estradiol plays a crucial role in the control of food intake in females." Physiol Behav **104**(4): 517-524.

Eddleston, M. and L. Mucke (1993). "Molecular profile of reactive astrocytes--implications for their role in neurologic disease." Neuroscience **54**(1): 15-36.

Edwards, M. M. and S. R. Robinson (2006). "TNF alpha affects the expression of GFAP and S100B: implications for Alzheimer's disease." J Neural Transm **113**(11): 1709-1715.

Eng, L. F. (1985). "Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes." J Neuroimmunol **8**(4-6): 203-214.

Eng, L. F., R. S. Ghirnikar and Y. L. Lee (2000). "Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000)." Neurochem Res **25**(9-10): 1439-1451.

Fernandes, C., J. L. Paya-Cano, F. Sluyter, U. D'Souza, R. Plomin and L. C. Schalkwyk (2004). "Hippocampal gene expression profiling across eight mouse inbred strains: towards understanding the molecular basis for behaviour." Eur J Neurosci **19**(9): 2576-2582.

Fitting, S., S. Zou, W. Chen, P. Vo, K. F. Hauser and P. E. Knapp (2010). "Regional heterogeneity and diversity in cytokine and chemokine production by astroglia: differential responses to HIV-1 Tat, gp120, and morphine revealed by multiplex analysis." J Proteome Res **9**(4): 1795-1804.

Foster, T. C. (2012). "Role of estrogen receptor alpha and beta expression and signaling on cognitive function during aging." Hippocampus **22**(4): 656-669.

Fukuyama, R., T. Izumoto and S. Fushiki (2001). "The cerebrospinal fluid level of glial fibrillary acidic protein is increased in cerebrospinal fluid from Alzheimer's disease patients and correlates with severity of dementia." Eur Neurol **46**(1): 35-38.

Galbreath, E., S. J. Kim, K. Park, M. Brenner and A. Messing (1995). "Overexpression of TGF-beta 1 in the central nervous system of transgenic mice results in hydrocephalus." J Neuropathol Exp Neurol **54**(3): 339-349.

Garcia-Segura, L. M., S. Luquin, A. Parducz and F. Naftolin (1994). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity and glial ultrastructure in the rat neuroendocrine hypothalamus." Glia **10**(1): 59-69.

Garcia-Segura, L. M., A. Wozniak, I. Azcoitia, J. R. Rodriguez, R. E. Hutchison and J. B. Hutchison (1999). "Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair." Neuroscience **89**(2): 567-578.

Geiger, K. D., P. Stoldt, W. Schlote and A. Derouiche (2006). "Ezrin immunoreactivity reveals specific astrocyte activation in cerebral HIV." J Neuropathol Exp Neurol **65**(1): 87-96.

Giraud, S. N., C. M. Caron, D. Pham-Dinh, P. Kitabgi and A. B. Nicot (2010). "Estradiol inhibits ongoing autoimmune neuroinflammation and NFkappaB-dependent CCL2 expression in reactive astrocytes." Proc Natl Acad Sci U S A **107**(18): 8416-8421.

Goldman, J. E. and E. Corbin (1988). "Isolation of a major protein component of Rosenthal fibers." Am J Pathol **130**(3): 569-578.

Gonzales, K. L., M. J. Tetel and C. K. Wagner (2008). "Estrogen receptor (ER) beta modulates ERalpha responses to estrogens in the developing rat ventromedial nucleus of the hypothalamus." Endocrinology **149**(9): 4615-4621.

Gonzalez, M., A. Cabrera-Socorro, C. G. Perez-Garcia, J. D. Fraser, F. J. Lopez, R. Alonso and G. Meyer (2007). "Distribution patterns of estrogen receptor alpha and beta in the human cortex and hippocampus during development and adulthood." J Comp Neurol **503**(6): 790-802.

Gottfried-Blackmore, A., G. Croft, B. S. McEwen and K. Bulloch (2007). "Transcriptional activity of estrogen receptors ERalpha and ERbeta in the EtC.1 cerebellar granule cell line." Brain Res **1186**: 41-47.

Greco, T. M., S. H. Seeholzer, A. Mak, L. Spruce and H. Ischiropoulos (2010). "Quantitative mass spectrometry-based proteomics reveals the dynamic range of primary mouse astrocyte protein secretion." J Proteome Res **9**(5): 2764-2774.

Hagemann, T. L., W. C. Boelens, E. F. Wawrousek and A. Messing (2009). "Suppression of GFAP toxicity by alphaB-crystallin in mouse models of Alexander disease." Hum Mol Genet **18**(7): 1190-1199.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

Hagemann, T. L., S. A. Gaeta, M. A. Smith, D. A. Johnson, J. A. Johnson and A. Messing (2005). "Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction." Hum Mol Genet **14**(16): 2443-2458.

Hagemann, T. L., E. M. Jobe and A. Messing (2012). "Genetic ablation of Nrf2/antioxidant response pathway in Alexander disease mice reduces hippocampal gliosis but does not impact survival." PLoS One **7**(5): e37304.

Haisenleder, D. J., A. H. Schoenfelder, E. S. Marcinko, L. M. Geddis and J. C. Marshall (2011). "Estimation of estradiol in mouse serum samples: evaluation of commercial estradiol immunoassays." Endocrinology **152**(11): 4443-4447.

Hajos, F. (2008). "Changes in glial fibrillary acidic protein (GFAP) immonureactivity reflect neuronal states." Neurochem Res **33**(8): 1643-1650.

Hansson, E. (1990). "Regional heterogeneity among astrocytes in the central nervous system." Neurochem Int **16**(3): 237-245.

Harrington, W. R., S. Sheng, D. H. Barnett, L. N. Petz, J. A. Katzenellenbogen and B. S. Katzenellenbogen (2003). "Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression." Mol Cell Endocrinol **206**(1-2): 13-22.

Harris, H. A., L. M. Albert, Y. Leathurby, M. S. Malamas, R. E. Mewshaw, C. P. Miller, . . . J. C. Keith, Jr. (2003). "Evaluation of an estrogen receptor-beta agonist in animal models of human disease." Endocrinology **144**(10): 4241-4249.

Head, M. W., E. Corbin and J. E. Goldman (1993). "Overexpression and abnormal modification of the stress proteins alpha B-crystallin and HSP27 in Alexander disease." Am J Pathol **143**(6): 1743-1753.

Heintzman, N. D. and B. Ren (2009). "Finding distal regulatory elements in the human genome." Curr Opin Genet Dev **19**(6): 541-549.

Herrmann, J. E., T. Imura, B. Song, J. Qi, Y. Ao, T. K. Nguyen, . . . M. V. Sofroniew (2008). "STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury." J Neurosci **28**(28): 7231-7243.

Herrmann, M., P. Vos, M. T. Wunderlich, C. H. de Bruijn and K. J. Lamers (2000). "Release of glial tissue-specific proteins after acute stroke: A comparative analysis of serum concentrations of protein S-100B and glial fibrillary acidic protein." Stroke **31**(11): 2670-2677.

Hovatta, I., M. A. Zapala, R. S. Broide, E. E. Schadt, O. Libiger, N. J. Schork, . . . C. Barlow (2007). "DNA variation and brain region-specific expression profiles exhibit different relationships between inbred mouse strains: implications for eQTL mapping studies." Genome Biol **8**(2): R25.

Hwang, J., H. J. Lee, W. H. Lee and K. Suk (2010). "NF-kappaB as a common signaling pathway in ganglioside-induced autophagic cell death and activation of astrocytes." J Neuroimmunol **226**(1-2): 66-72.

Ingberg, E., A. Theodorsson, E. Theodorsson and J. O. Strom (2012). "Methods for long-term 17beta-estradiol administration to mice." Gen Comp Endocrinol **175**(1): 188-193.

Iwaki, A., T. Iwaki, J. E. Goldman, K. Ogomori, J. Tateishi and Y. Sakaki (1992). "Accumulation of alpha B-crystallin in brains of patients with Alexander's disease is not due to an abnormality of the 5'-flanking and coding sequence of the genomic DNA." Neurosci Lett **140**(1): 89-92.

Jackson, S. o. t. J. (2013). Mouse Phenome Database, Jackson Laboratories.

Jany, P. L., T. L. Hagemann and A. Messing (2013). "GFAP expression as an indicator of disease severity in mouse models of Alexander disease." ASN Neuro **5**(1): e00109.

Jesse, S., P. Steinacker, L. Cepek, C. A. von Arnim, H. Tumani, S. Lehnert, . . . M. Otto (2009). "Glial fibrillary acidic protein and protein S-100B: different concentration pattern of glial proteins in cerebrospinal fluid of patients with Alzheimer's disease and Creutzfeldt-Jakob disease." J Alzheimers Dis **17**(3): 541-551.

Johnson, W. B., M. D. Ruppe, E. M. Rockenstein, J. Price, V. P. Sarthy, L. C. Verderber and L. Mucke (1995). "Indicator expression directed by regulatory sequences of the glial fibrillary acidic protein (GFAP) gene: in vivo comparison of distinct GFAP-lacZ transgenes." Glia **13**(3): 174-184.

Jung, C. S., C. Foerch, A. Schanzer, A. Heck, K. H. Plate, V. Seifert, . . . M. Sitzer (2007). "Serum GFAP is a diagnostic marker for glioblastoma multiforme." Brain **130**(Pt 12): 3336-3341.

Kadurugamuwa, J. L., K. Modi, O. Coquoz, B. Rice, S. Smith, P. R. Contag and T. Purchio (2005). "Reduction of astrogliosis by early treatment of pneumococcal meningitis measured by simultaneous imaging, in vivo, of the pathogen and host response." Infect Immun **73**(12): 7836-7843.

Kaneko, R., N. Hagiwara, K. Leader and N. Sueoka (1994). "Glial-specific cAMP response of the glial fibrillary acidic protein gene cell lines." Proc Natl Acad Sci U S A **91**(10): 4529-4533.

Kang, W. and J. M. Hebert (2011). "Signaling pathways in reactive astrocytes, a genetic perspective." Mol Neurobiol **43**(3): 147-154.

Keene, S. D., T. M. Greco, I. Parastatidis, S. H. Lee, E. G. Hughes, R. J. Balice-Gordon, . . . H. Ischiropoulos (2009). "Mass spectrometric and computational analysis of cytokine-induced alterations in the astrocyte secretome." Proteomics **9**(3): 768-782.

Keller, A. F., M. Gravel and J. Kriz (2009). "Live imaging of amyotrophic lateral sclerosis pathogenesis: disease onset is characterized by marked induction of GFAP in Schwann cells." Glia **57**(10): 1130-1142.

Kohama, S. G., J. R. Goss, T. H. McNeill and C. E. Finch (1995). "Glial fibrillary acidic protein mRNA increases at proestrus in the arcuate nucleus of mice." Neurosci Lett **183**(3): 164-166.

Konkle, A. T. and M. M. McCarthy (2011). "Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain." Endocrinology **152**(1): 223-235.

Krencik, R., J. P. Weick, Y. Liu, Z. J. Zhang and S. C. Zhang (2011). "Specification of transplantable astroglial subtypes from human pluripotent stem cells." Nat Biotechnol **29**(6): 528-534.

Kretzschmar, H. A., S. B. Prusiner, L. E. Stowring and S. J. DeArmond (1986). "Scrapie prion proteins are synthesized in neurons." Am J Pathol **122**(1): 1-5.

Kuiper, G. G., B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson and J. A. Gustafsson (1997). "Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta." Endocrinology **138**(3): 863-870.

Kuiper, G. G., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, . . . J. A. Gustafsson (1998). "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta." Endocrinology **139**(10): 4252-4263.

Kumar, A., M. Ruan, K. Clifton, F. Syed, S. Khosla and M. J. Oursler (2012). "TGF-beta mediates suppression of adipogenesis by estradiol through connective tissue growth factor induction." Endocrinology **153**(1): 254-263.

Kunjappu, M. J. and M. Hochstrasser (in press). "Assembly of the 20S proteasome." Biochim Biophys Acta.

Kwon, B. K., A. M. Stammers, L. M. Belanger, A. Bernardo, D. Chan, C. M. Bishop, . . . M. F. Dvorak (2010). "Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury." J Neurotrauma **27**(4): 669-682.

Kyllerman, M., L. Rosengren, L. M. Wiklund and E. Holmberg (2005). "Increased levels of GFAP in the cerebrospinal fluid in three subtypes of genetically confirmed Alexander disease." Neuropediatrics **36**(5): 319-323.

Lafon-Cazal, M., O. Adjali, N. Galeotti, J. Poncet, P. Jouin, V. Homburger, . . . P. Marin (2003). "Proteomic analysis of astrocytic secretion in the mouse. Comparison with the cerebrospinal fluid proteome." J Biol Chem **278**(27): 24438-24448.

Lamparska-Przybysz, M., B. Gajkowska and T. Motyl (2005). "Cathepsins and BID are involved in the molecular switch between apoptosis and autophagy in breast cancer MCF-7 cells exposed to camptothecin." J Physiol Pharmacol **56 Suppl 3**: 159-179.

LaPash Daniels, C. M., E. V. Austin, D. E. Rockney, E. M. Jacka, T. L. Hagemann, D. A. Johnson, . . . A. Messing (2012). "Beneficial effects of nrf2 overexpression in a mouse model of alexander disease." J Neurosci **32**(31): 10507-10515.

Laping, N. J., B. Teter, N. R. Nichols, I. Rozovsky and C. E. Finch (1994). "Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors." Brain Pathol **4**(3): 259-275.

Lee, S. N., B. Peng, R. Desjardins, J. E. Pintar, R. Day and I. Lindberg (2007). "Strain-specific steroidal control of pituitary function." J Endocrinol **192**(3): 515-525.

Lee, Y., A. Messing, M. Su and M. Brenner (2008). "GFAP promoter elements required for region-specific and astrocyte-specific expression." Glia **56**(5): 481-493.

Lee, Y., M. Su, A. Messing and M. Brenner (2006). "Astrocyte heterogeneity revealed by expression of a GFAP-LacZ transgene." Glia **53**(7): 677-687.

Lei, D. L., J. M. Long, J. Hengemihle, J. O'Neill, K. F. Manaye, D. K. Ingram and P. R. Mouton (2003). "Effects of estrogen and raloxifene on neuroglia number and morphology in the hippocampus of aged female mice." Neuroscience **121**(3): 659-666.

Lein, E. S., M. J. Hawrylycz, N. Ao, M. Ayres, A. Bensinger, A. Bernard, . . . A. R. Jones (2007). "Genome-wide atlas of gene expression in the adult mouse brain." Nature **445**(7124): 168-176.

Leitman, D. C., S. Paruthiyil, O. I. Vivar, E. F. Saunier, C. B. Herber, I. Cohen, . . . T. P. Speed (2010). "Regulation of specific target genes and biological responses by estrogen receptor subtype agonists." Curr Opin Pharmacol **10**(6): 629-636.

Lelu, K., L. Delpy, V. Robert, E. Foulon, S. Laffont, L. Pelletier, . . . J. C. Guery (2010). "Endogenous estrogens, through estrogen receptor alpha, constrain autoimmune

inflammation in female mice by limiting CD4+ T-cell homing into the CNS." Eur J Immunol **40**(12): 3489-3498.

Levin-Allerhand, J., B. S. McEwen, C. E. Lominska, D. B. Lubahn, K. S. Korach and J. D. Smith (2001). "Brain region-specific up-regulation of mouse apolipoprotein E by pharmacological estrogen treatments." J Neurochem **79**(4): 796-803.

Lewis, D. K., A. B. Johnson, S. Stohlgren, A. Harms and F. Sohrabji (2008). "Effects of estrogen receptor agonists on regulation of the inflammatory response in astrocytes from young adult and middle-aged female rats." J Neuroimmunol **195**(1-2): 47-59.

Lewis, S. A. and N. J. Cowan (1985). "Temporal expression of mouse glial fibrillary acidic protein mRNA studied by a rapid in situ hybridization procedure." J Neurochem **45**(3): 913-919.

Liggett, A., L. J. Crawford, B. Walker, T. C. Morris and A. E. Irvine (2010). "Methods for measuring proteasome activity: current limitations and future developments." Leuk Res **34**(11): 1403-1409.

Lindholm, D., E. Castren, R. Kiefer, F. Zafra and H. Thoenen (1992). "Transforming growth factor-beta 1 in the rat brain: increase after injury and inhibition of astrocyte proliferation." J Cell Biol **117**(2): 395-400.

Liu, Z., J. Cao, X. Gao, Q. Ma, J. Ren and Y. Xue (2011). "GPS-CCD: a novel computational program for the prediction of calpain cleavage sites." PLoS One **6**(4): e19001.

Lowe, J., K. Morrell, G. Lennox, M. Landon and R. J. Mayer (1989). "Rosenthal fibres are based on the ubiquitination of glial filaments." Neuropathol Appl Neurobiol **15**(1): 45-53.

Lu, A., R. Q. Ran, J. Clark, M. Reilly, A. Nee and F. R. Sharp (2002). "17-beta-estradiol induces heat shock proteins in brain arteries and potentiates ischemic heat shock protein induction in glia and neurons." J Cereb Blood Flow Metab **22**(2): 183-195.

Luo, J., P. Ho, L. Steinman and T. Wyss-Coray (2008). "Bioluminescence in vivo imaging of autoimmune encephalomyelitis predicts disease." J Neuroinflammation **5**: 6.

Ma, Y., X. Qiao, A. E. Falone, O. M. Reslan, S. J. Sheppard and R. A. Khalil (2010). "Gender-specific reduction in contraction is associated with increased estrogen receptor expression in single vascular smooth muscle cells of female rat." Cell Physiol Biochem **26**(3): 457-470.

MacKenzie-Graham, A. J., G. A. Rinek, A. Avedisian, L. B. Morales, E. Umeda, B. Boulat, . . . R. R. Voskuhl (2012). "Estrogen treatment prevents gray matter atrophy in experimental autoimmune encephalomyelitis." J Neurosci Res **90**(7): 1310-1323.

Majetschak, M. and L. T. Sorell (2008). "Immunological methods to quantify and characterize proteasome complexes: development and application." J Immunol Methods **334**(1-2): 91-103.

Makwana, M., L. L. Jones, D. Cuthill, H. Heuer, M. Bohatschek, M. Hristova, . . . G. Raivich (2007). "Endogenous transforming growth factor beta 1 suppresses inflammation and promotes survival in adult CNS." J Neurosci **27**(42): 11201-11213.

Malhotra, S. K., M. Svensson, H. Aldskogius, R. Bhatnagar, G. D. Das and T. K. Shnitka (1993). "Diversity among reactive astrocytes: proximal reactive astrocytes in lacerated spinal cord preferentially react with monoclonal antibody J1-31." Brain Res Bull **30**(3-4): 395-404.

Martin, P. M. and J. P. O'Callaghan (1995). "A direct comparison of GFAP immunocytochemistry and GFAP concentration in various regions of ethanol-fixed rat and mouse brain." J Neurosci Methods **58**(1-2): 181-192.

Martinez, F. G., E. E. Hermel, L. L. Xavier, G. G. Viola, J. Riboldi, A. A. Rasia-Filho and M. Achaval (2006). "Gonadal hormone regulation of glial fibrillary acidic protein immunoreactivity in the medial amygdala subnuclei across the estrous cycle and in castrated and treated female rats." Brain Res **1108**(1): 117-126.

Martino, G., L. M. Grimaldi, L. Moiola, M. Filippi, V. Martinelli, G. Comi and N. Canal (1990). "Discontinuous distribution of IgG oligoclonal bands in cerebrospinal fluid from multiple sclerosis patients." J Neuroimmunol **30**(2-3): 129-134.

Matejuk, A., K. Adlard, A. Zamora, M. Silverman, A. A. Vandenbark and H. Offner (2001). "17 beta-estradiol inhibits cytokine, chemokine, and chemokine receptor mRNA expression in the central nervous system of female mice with experimental autoimmune encephalomyelitis." J Neurosci Res **65**(6): 529-542.

McAsey, M. E., C. Cady, L. M. Jackson, M. Li, S. Randall, B. P. Nathan and R. G. Struble (2006). "Time course of response to estradiol replacement in ovariectomized mice: brain apolipoprotein E and synaptophysin transiently increase and glial fibrillary acidic protein is suppressed." Exp Neurol **197**(1): 197-205.

McCarthy, G. F. and C. P. Leblond (1988). "Radioautographic evidence for slow astrocyte turnover and modest oligodendrocyte production in the corpus callosum of adult mice infused with 3H-thymidine." J Comp Neurol **271**(4): 589-603.

McCullough, L. D., K. Blizzard, E. R. Simpson, O. K. Oz and P. D. Hurn (2003). "Aromatase cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection." J Neurosci **23**(25): 8701-8705.

McNaught, K. S., R. Belizaire, O. Isacson, P. Jenner and C. W. Olanow (2003). "Altered proteasomal function in sporadic Parkinson's disease." Exp Neurol **179**(1): 38-46.

Mersereau, J. E., N. Levy, R. E. Staub, S. Baggett, T. Zogovic, S. Chow, . . . D. C. Leitman (2008). "Liquiritigenin is a plant-derived highly selective estrogen receptor beta agonist." Mol Cell Endocrinol **283**(1-2): 49-57.

Messing, A. and M. Brenner (2013). Genetic disorders affecting astrocytes. Neuroglia. H. Kettenmann and B. R. Ransom. New York, Oxford University Press**:** 884-895.

Messing, A., M. W. Head, K. Galles, E. J. Galbreath, J. E. Goldman and M. Brenner (1998). "Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice." Am J Pathol **152**(2): 391-398.

Messing, A., C. M. LaPash Daniels and T. L. Hagemann (2010). "Strategies for treatment in Alexander disease." Neurotherapeutics **7**(4): 507-515.

Meyers, M. J., J. Sun, K. E. Carlson, G. A. Marriner, B. S. Katzenellenbogen and J. A. Katzenellenbogen (2001). "Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues." J Med Chem **44**(24): 4230-4251.

Middeldorp, J. and E. M. Hol (2011). "GFAP in health and disease." Prog Neurobiol **93**(3): 421-443.

Mignot, C., C. Delarasse, S. Escaich, B. Della Gaspera, E. Noe, E. Colucci-Guyon, . . . D. Pham-Dinh (2007). "Dynamics of mutated GFAP aggregates revealed by real-time imaging of an astrocyte model of Alexander disease." Exp Cell Res **313**(13): 2766-2779.

Milner, T. A., K. Ayoola, C. T. Drake, S. P. Herrick, N. E. Tabori, B. S. McEwen, . . . S. E. Alves (2005). "Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation." J Comp Neurol **491**(2): 81-95.

Minutolo, F., M. Macchia, B. S. Katzenellenbogen and J. A. Katzenellenbogen (2011). "Estrogen receptor beta ligands: recent advances and biomedical applications." Med Res Rev **31**(3): 364-442.

Missler, U., M. Wiesmann, G. Wittmann, O. Magerkurth and H. Hagenstrom (1999). "Measurement of glial fibrillary acidic protein in human blood: analytical method and preliminary clinical results." Clin Chem **45**(1): 138-141.

Misu, T., R. Takano, K. Fujihara, T. Takahashi, S. Sato and Y. Itoyama (2009). "Marked increase in cerebrospinal fluid glial fibrillar acidic protein in neuromyelitis optica: an astrocytic damage marker." J Neurol Neurosurg Psychiatry **80**(5): 575-577.

Mitra, S. W., E. Hoskin, J. Yudkovitz, L. Pear, H. A. Wilkinson, S. Hayashi, . . . S. E. Alves (2003). "Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha." Endocrinology **144**(5): 2055-2067.

Mitterling, K. L., J. L. Spencer, N. Dziedzic, S. Shenoy, K. McCarthy, E. M. Waters, . . . T. A. Milner (2010). "Cellular and subcellular localization of estrogen and progestin receptor immunoreactivities in the mouse hippocampus." J Comp Neurol **518**(14): 2729-2743.

Miura, M., T. Tamura and K. Mikoshiba (1990). "Cell-specific expression of the mouse glial fibrillary acidic protein gene: identification of the cis- and trans-acting promoter elements for astrocyte-specific expression." J Neurochem **55**(4): 1180-1188.

Moon, L. D. and J. W. Fawcett (2001). "Reduction in CNS scar formation without concomitant increase in axon regeneration following treatment of adult rat brain with a combination of antibodies to TGFbeta1 and beta2." Eur J Neurosci **14**(10): 1667-1677.

Mor-Vaknin, N., A. Punturieri, K. Sitwala and D. M. Markovitz (2003). "Vimentin is secreted by activated macrophages." Nat Cell Biol **5**(1): 59-63.

Morales, L. B., K. K. Loo, H. B. Liu, C. Peterson, S. Tiwari-Woodruff and R. R. Voskuhl (2006). "Treatment with an estrogen receptor alpha ligand is neuroprotective in experimental autoimmune encephalomyelitis." J Neurosci **26**(25): 6823-6833.

Morrison, R. S., J. De Vellis, Y. L. Lee, R. A. Bradshaw and L. F. Eng (1985). "Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes." J Neurosci Res **14**(2): 167-176.

Mortimore, G. E. and C. M. Schworer (1977). "Induction of autophagy by amino-acid deprivation in perfused rat liver." Nature **270**(5633): 174-176.

Nakashima, K., M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, M. Kawabata, . . . T. Taga (1999). "Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300." Science **284**(5413): 479-482.

Nakatani, Y., M. Brenner and E. Freese (1990). "An RNA polymerase II promoter containing sequences upstream and downstream from the RNA startpoint that direct initiation of transcription from the same site." Proc Natl Acad Sci U S A **87**(11): 4289-4293.

Natarajan, A., G. G. Yardimci, N. C. Sheffield, G. E. Crawford and U. Ohler (2012). "Predicting cell-type-specific gene expression from regions of open chromatin." Genome Res **22**(9): 1711-1722.

Neely, A. N., J. R. Cox, J. A. Fortney, C. M. Schworer and G. E. Mortimore (1977). "Alterations of lysosomal size and density during rat liver perfusion. Suppression by insulin and amino acids." J Biol Chem **252**(19): 6948-6954.

Nilsson, S., S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, . . . J. A. Gustafsson (2001). "Mechanisms of estrogen action." Physiol Rev **81**(4): 1535-1565.

Nora, E. P., B. R. Lajoie, E. G. Schulz, L. Giorgetti, I. Okamoto, N. Servant, . . . E. Heard (2012). "Spatial partitioning of the regulatory landscape of the X-inactivation centre." Nature **485**(7398): 381-385.

Norgren, N., P. Sundstrom, A. Svenningsson, L. Rosengren, T. Stigbrand and M. Gunnarsson (2004). "Neurofilament and glial fibrillary acidic protein in multiple sclerosis." Neurology **63**(9): 1586-1590.

Notturno, F., C. M. Caporale, A. De Lauretis and A. Uncini (2008). "Glial fibrillary acidic protein: a marker of axonal Guillain-Barre syndrome and outcome." Muscle Nerve **38**(1): 899-903.

Numakawa, T., T. Matsumoto, Y. Numakawa, M. Richards, S. Yamawaki and H. Kunugi (2011). "Protective Action of Neurotrophic Factors and Estrogen against Oxidative Stress-Mediated Neurodegeneration." J Toxicol **2011**: 405194.

Numakawa, Y., T. Matsumoto, D. Yokomaku, T. Taguchi, E. Niki, H. Hatanaka, . . . T. Numakawa (2007). "17beta-estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium." Endocrinology **148**(2): 627-637.

Nylen, K., M. Ost, L. Z. Csajbok, I. Nilsson, K. Blennow, B. Nellgard and L. Rosengren (2006). "Increased serum-GFAP in patients with severe traumatic brain injury is related to outcome." J Neurol Sci **240**(1-2): 85-91.

Offner, H. and M. Polanczyk (2006). "A potential role for estrogen in experimental autoimmune encephalomyelitis and multiple sclerosis." Ann N Y Acad Sci **1089**: 343-372.

Osterlund, M. K., J. A. Gustafsson, E. Keller and Y. L. Hurd (2000). "Estrogen receptor beta (ERbeta) messenger ribonucleic acid (mRNA) expression within the human forebrain: distinct distribution pattern to ERalpha mRNA." J Clin Endocrinol Metab **85**(10): 3840-3846.

Palfreyman, J. W., D. G. Thomas, J. G. Ratcliffe and D. I. Graham (1979). "Glial fibrillary acidic protein (GFAP): purification from human fibrillary astrocytoma, development and validation of a radioimmunoassay for GFAP-like immunoactivity." J Neurol Sci **41**(1): 101-113.

Papa, L., L. M. Lewis, J. L. Falk, Z. Zhang, S. Silvestri, P. Giordano, . . . K. K. Wang (2012). "Elevated levels of serum glial fibrillary acidic protein breakdown products in mild and moderate traumatic brain injury are associated with intracranial lesions and neurosurgical intervention." Ann Emerg Med **59**(6): 471-483.

Perez, S. E., E. Y. Chen and E. J. Mufson (2003). "Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain." Brain Res Dev Brain Res **145**(1): 117-139.

Peterson, R. S., D. W. Lee, G. Fernando and B. A. Schlinger (2004). "Radial glia express aromatase in the injured zebra finch brain." J Comp Neurol **475**(2): 261-269.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Phan, V. L., A. Urani, P. Romieu and T. Maurice (2002). "Strain differences in sigma(1) receptor-mediated behaviours are related to neurosteroid levels." Eur J Neurosci **15**(9): 1523-1534.

Price, J. C., S. Guan, A. Burlingame, S. B. Prusiner and S. Ghaemmaghami (2010). "Analysis of proteome dynamics in the mouse brain." Proc Natl Acad Sci U S A **107**(32): 14508-14513.

Qian, Y., T. Guan, M. Huang, L. Cao, Y. Li, H. Cheng, . . . D. Yu (2012). "Neuroprotection by the soy isoflavone, genistein, via inhibition of mitochondria-dependent apoptosis pathways and reactive oxygen induced-NF-kappaB activation in a cerebral ischemia mouse model." Neurochem Int **60**(8): 759-767.

Raval, A. P., N. Hirsch, K. R. Dave, D. R. Yavagal, H. Bramlett and I. Saul (2011). "Nicotine and estrogen synergistically exacerbate cerebral ischemic injury." Neuroscience **181**: 216-225.

Ray, S. K., S. Samantaray, J. A. Smith, D. D. Matzelle, A. Das and N. L. Banik (2011). "Inhibition of cysteine proteases in acute and chronic spinal cord injury." Neurotherapeutics **8**(2): 180-186.

Razmara, A., S. P. Duckles, D. N. Krause and V. Procaccio (2007). "Estrogen suppresses brain mitochondrial oxidative stress in female and male rats." Brain Res **1176**: 71-81.

Regan, M. R., Y. H. Huang, Y. S. Kim, M. I. Dykes-Hoberg, L. Jin, A. M. Watkins, . . . J. D. Rothstein (2007). "Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS." J Neurosci **27**(25): 6607-6619.

Restrepo, A., C. A. Smith, S. Agnihotri, M. Shekarforoush, P. N. Kongkham, H. J. Seol, . . . J. T. Rutka (2011). "Epigenetic regulation of glial fibrillary acidic protein by DNA methylation in human malignant gliomas." Neuro Oncol **13**(1): 42-50.

Ritz, M. F. and O. N. Hausmann (2008). "Effect of 17beta-estradiol on functional outcome, release of cytokines, astrocyte reactivity and inflammatory spreading after spinal cord injury in male rats." Brain Res **1203**: 177-188.

Rivett, A. J., S. Bose, A. J. Pemberton, P. Brooks, D. Onion, D. Shirley, . . . K. Forti (2002). "Assays of proteasome activity in relation to aging." Exp Gerontol **37**(10-11): 1217-1222.

Rodgers, K. J. and R. T. Dean (2003). "Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates." Int J Biochem Cell Biol **35**(5): 716-727.

Roepke, T. A., O. K. Ronnekleiv and M. J. Kelly (2011). "Physiological consequences of membrane-initiated estrogen signaling in the brain." Front Biosci **16**: 1560-1573.

Rolland, B., G. Le Prince, C. Fages, J. Nunez and M. Tardy (1990). "GFAP turnover during astroglial proliferation and differentiation." Brain Res Dev Brain Res **56**(1): 144-149.

Romao, L. F., O. Sousa Vde, V. M. Neto and F. C. Gomes (2008). "Glutamate activates GFAP gene promoter from cultured astrocytes through TGF-beta1 pathways." J Neurochem **106**(2): 746-756.

Rosengren, L. E., G. Ahlsen, M. Belfrage, C. Gillberg, K. G. Haglid and A. Hamberger (1992). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of children." J Neurosci Methods **44**(2-3): 113-119.

Rosengren, L. E., C. Wikkelso and L. Hagberg (1994). "A sensitive ELISA for glial fibrillary acidic protein: application in CSF of adults." J Neurosci Methods **51**(2): 197-204.

Rozovsky, I., M. Wei, D. J. Stone, H. Zanjani, C. P. Anderson, T. E. Morgan and C. E. Finch (2002). "Estradiol (E2) enhances neurite outgrowth by repressing glial fibrillary acidic protein expression and reorganizing laminin." Endocrinology **143**(2): 636-646.

Sakuma, S., D. Tokuhara, H. Hattori, O. Matsuoka and T. Yamano (2009). "Expression of estrogen receptor alpha and beta in reactive astrocytes at the male rat hippocampus after status epilepticus." Neuropathology **29**(1): 55-62.

Sarid, J. (1991). "Identification of a cis-acting positive regulatory element of the glial fibrillary acidic protein gene." J Neurosci Res **28**(2): 217-228.

Sarkar, S. and N. J. Cowan (1991). "Intragenic sequences affect the expression of the gene encoding glial fibrillary acidic protein." J Neurochem **57**(2): 675-684.

Sarvari, M., E. Hrabovszky, I. Kallo, N. Solymosi, K. Toth, I. Liko, . . . Z. Liposits (2011). "Estrogens regulate neuroinflammatory genes via estrogen receptors alpha and beta in the frontal cortex of middle-aged female rats." J Neuroinflammation **8**: 82.

Sarvari, M., I. Kallo, E. Hrabovszky, N. Solymosi, K. Toth, I. Liko, . . . Z. Liposits (2010). "Estradiol replacement alters expression of genes related to neurotransmission and immune surveillance in the frontal cortex of middle-aged, ovariectomized rats." Endocrinology **151**(8): 3847-3862.

Schlinger, B. A. and L. Remage-Healey (2012). "Neurosteroidogenesis: insights from studies of songbirds." J Neuroendocrinol **24**(1): 16-21.

Schreiber, A. and M. Peter (in press). "Substrate recognition in selective autophagy and the ubiquitin-proteasome system." Biochim Biophys Acta.

Schworer, C. M., J. R. Cox and G. E. Mortimore (1979). "Alteration of lysosomal density by sequestered glycogen during deprivation-induced autophagy in rat liver." Biochem Biophys Res Commun **87**(1): 163-170.

Shiau, A. K., D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard and G. L. Greene (1998). "The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen." Cell **95**(7): 927-937.

Shu, M., Y. Zhou, W. Zhu, S. Wu, X. Zheng and G. Yan (2011). "Activation of a pro-survival pathway IL-6/JAK2/STAT3 contributes to glial fibrillary acidic protein induction during the cholera toxin-induced differentiation of C6 malignant glioma cells." Mol Oncol **5**(3): 265-272.

Shughrue, P. J., M. V. Lane and I. Merchenthaler (1997). "Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system." J Comp Neurol **388**(4): 507-525.

Son, E. D., J. Y. Lee, S. Lee, M. S. Kim, B. G. Lee, I. S. Chang and J. H. Chung (2005). "Topical application of 17beta-estradiol increases extracellular matrix protein synthesis by stimulating tgf-Beta signaling in aged human skin in vivo." J Invest Dermatol **124**(6): 1149-1161.

Spence, R. D., M. E. Hamby, E. Umeda, N. Itoh, S. Du, A. J. Wisdom, . . . R. R. Voskuhl (2011). "Neuroprotection mediated through estrogen receptor-{alpha} in astrocytes." Proc Natl Acad Sci U S A **108**(21): 8867-8872.

Stauffer, S. R., C. J. Coletta, R. Tedesco, G. Nishiguchi, K. Carlson, J. Sun, . . . J. A. Katzenellenbogen (2000). "Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists." J Med Chem **43**(26): 4934-4947.

Steiner, J., H. Bielau, H. G. Bernstein, B. Bogerts and M. T. Wunderlich (2006). "Increased cerebrospinal fluid and serum levels of S100B in first-onset schizophrenia are not related to a degenerative release of glial fibrillar acidic protein, myelin basic protein and neurone-specific enolase from glia or neurones." J Neurol Neurosurg Psychiatry **77**(11): 1284-1287.

Stone, D. J., Y. Song, C. P. Anderson, K. K. Krohn, C. E. Finch and I. Rozovsky (1998). "Bidirectional transcription regulation of glial fibrillary acidic protein by estradiol in vivo and in vitro." Endocrinology **139**(7): 3202-3209.

Sun, J., M. J. Meyers, B. E. Fink, R. Rajendran, J. A. Katzenellenbogen and B. S. Katzenellenbogen (1999). "Novel Ligands that Function as Selective Estrogens or Antiestrogens for Estrogen Receptor-α or Estrogen Receptor-β." Endocrinology **140**(2): 800-804.

Sun, W., S. Lee, V. Zhabotynsky, F. Zou, F. A. Wright, J. J. Crowley, . . . P. F. Sullivan (2012). "Transcriptome atlases of mouse brain reveals differential expression across brain regions and genetic backgrounds." G3 (Bethesda) **2**(2): 203-211.

Suzuki, S., C. M. Brown, C. D. Dela Cruz, E. Yang, D. A. Bridwell and P. M. Wise (2007). "Timing of estrogen therapy after ovariectomy dictates the efficacy of its neuroprotective and antiinflammatory actions." Proc Natl Acad Sci U S A **104**(14): 6013-6018.

Tagawa, N., Y. Sugimoto, J. Yamada and Y. Kobayashi (2006). "Strain differences of neurosteroid levels in mouse brain." Steroids **71**(9): 776-784.

Takano, R., T. Misu, T. Takahashi, S. Sato, K. Fujihara and Y. Itoyama (2010). "Astrocytic damage is far more severe than demyelination in NMO: a clinical CSF biomarker study." Neurology **75**(3): 208-216.

Takizawa, T., K. Nakashima, M. Namihira, W. Ochiai, A. Uemura, M. Yanagisawa, . . . T. Taga (2001). "DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain." Dev Cell **1**(6): 749-758.

Tamguney, G., K. P. Francis, K. Giles, A. Lemus, S. J. DeArmond and S. B. Prusiner (2009). "Measuring prions by bioluminescence imaging." Proc Natl Acad Sci U S A **106**(35): 15002-15006.

Tanaka, K. F., H. Takebayashi, Y. Yamazaki, K. Ono, M. Naruse, T. Iwasato, . . . K. Ikenaka (2007). "Murine model of Alexander disease: analysis of GFAP aggregate formation and its pathological significance." Glia **55**(6): 617-631.

Tang, G., M. D. Perng, S. Wilk, R. Quinlan and J. E. Goldman (2010). "Oligomers of mutant glial fibrillary acidic protein (GFAP) Inhibit the proteasome system in alexander disease

astrocytes, and the small heat shock protein alphaB-crystallin reverses the inhibition." J Biol Chem **285**(14): 10527-10537.

Tang, G., Z. Xu and J. E. Goldman (2006). "Synergistic effects of the SAPK/JNK and the proteasome pathway on glial fibrillary acidic protein (GFAP) accumulation in Alexander disease." J Biol Chem **281**(50): 38634-38643.

Tang, G., Z. Yue, Z. Talloczy, T. Hagemann, W. Cho, A. Messing, . . . J. E. Goldman (2008). "Autophagy induced by Alexander disease-mutant GFAP accumulation is regulated by p38/MAPK and mTOR signaling pathways." Hum Mol Genet **17**(11): 1540-1555.

Taylor, A. H. and F. Al-Azzawi (2000). "Immunolocalisation of oestrogen receptor beta in human tissues." J Mol Endocrinol **24**(1): 145-155.

Taylor, J. A., C. A. Richter, A. Suzuki, H. Watanabe, T. Iguchi, K. R. Coser, . . . F. S. vom Saal (2012). "Dose-related estrogen effects on gene expression in fetal mouse prostate mesenchymal cells." PLoS One **7**(10): e48311.

Taylor, L. C., K. Puranam, W. Gilmore, J. P. Ting and G. K. Matsushima (2010). "17beta-estradiol protects male mice from cuprizone-induced demyelination and oligodendrocyte loss." Neurobiol Dis **39**(2): 127-137.

Teixeira, L. K. and S. I. Reed (in press). "Ubiquitin Ligases and Cell Cycle Control." Annu Rev Biochem.

Teunissen, C. E., A. Petzold, J. L. Bennett, F. S. Berven, L. Brundin, M. Comabella, . . . F. Deisenhammer (2009). "A consensus protocol for the standardization of cerebrospinal fluid collection and biobanking." Neurology **73**(22): 1914-1922.

Toda, K., Y. Hayashi, M. Ono and T. Saibara (2012). "Impact of ovarian sex steroids on ovulation and ovulatory gene induction in aromatase-null mice." Endocrinology **153**(1): 386-394.

Tomokane, N., T. Iwaki, J. Tateishi, A. Iwaki and J. E. Goldman (1991). "Rosenthal fibers share epitopes with alpha B-crystallin, glial fibrillary acidic protein, and ubiquitin, but not with vimentin. Immunoelectron microscopy with colloidal gold." Am J Pathol **138**(4): 875-885.

Turk, B., D. Turk and V. Turk (2000). "Lysosomal cysteine proteases: more than scavengers." Biochim Biophys Acta **1477**(1-2): 98-111.

Um, J. W., E. Im, H. J. Lee, B. Min, L. Yoo, J. Yoo, . . . K. C. Chung (2010). "Parkin directly modulates 26S proteasome activity." J Neurosci **30**(35): 11805-11814.

Urayama, S., K. Semi, T. Sanosaka, Y. Hori, M. Namihira, J. Kohyama, . . . K. Nakashima (2013). "Chromatin Accessibility at a STAT3 Target Site Is Altered Prior to Astrocyte Differentiation." Cell Struct Funct **38**(1): 55-66.

Vergeli, M., B. mazzanti, C. Ballerini, B. Gran, L. Amaducci and L. Massacesi (1995). "Transforming growth factor-beta 1 inhibits the proliferation of rat astrocytes induced by serum and growth factors." J Neurosci Res **40**(1): 127-133.

Veyrac, A. and J. Bakker (2011). "Postnatal and adult exposure to estradiol differentially influences adult neurogenesis in the main and accessory olfactory bulb of female mice." FASEB J **25**(3): 1048-1057.

Vos, P. E., B. Jacobs, T. M. Andriessen, K. J. Lamers, G. F. Borm, T. Beems, . . . J. L. Vissers (2010). "GFAP and S100B are biomarkers of traumatic brain injury: an observational cohort study." Neurology **75**(20): 1786-1793.

Vos, P. E., K. J. Lamers, J. C. Hendriks, M. van Haaren, T. Beems, C. Zimmerman, . . . M. M. Verbeek (2004). "Glial and neuronal proteins in serum predict outcome after severe traumatic brain injury." Neurology **62**(8): 1303-1310.

Vos, P. E., M. van Gils, T. Beems, C. Zimmerman and M. M. Verbeek (2006). "Increased GFAP and S100beta but not NSE serum levels after subarachnoid haemorrhage are associated with clinical severity." Eur J Neurol **13**(6): 632-638.

Wada, M., M. Kosaka, S. Saito, T. Sano, K. Tanaka and A. Ichihara (1993). "Serum concentration and localization in tumor cells of proteasomes in patients with hematologic malignancy and their pathophysiologic significance." J Lab Clin Med **121**(2): 215-223.

Wang, F., G. A. Murrell and M. X. Wang (2007). "Oxidative stress-induced c-Jun N-terminal kinase (JNK) activation in tendon cells upregulates MMP1 mRNA and protein expression." J Orthop Res **25**(3): 378-389.

Wang, J. M., R. W. Irwin and R. D. Brinton (2006). "Activation of estrogen receptor alpha increases and estrogen receptor beta decreases apolipoprotein E expression in hippocampus in vitro and in vivo." Proc Natl Acad Sci U S A **103**(45): 16983-16988.

Wang, L., K. J. Colodner and M. B. Feany (2011). "Protein misfolding and oxidative stress promote glial-mediated neurodegeneration in an Alexander disease model." J Neurosci **31**(8): 2868-2877.

Watts, J. C., K. Giles, S. K. Grillo, A. Lemus, S. J. DeArmond and S. B. Prusiner (2011). "Bioluminescence imaging of Abeta deposition in bigenic mouse models of Alzheimer's disease." Proc Natl Acad Sci U S A **108**(6): 2528-2533.

Wise, P. M., S. Suzuki and C. M. Brown (2009). "Estradiol: a hormone with diverse and contradictory neuroprotective actions." Dialogues Clin Neurosci **11**(3): 297-303.

Witte, M. M., D. Resuehr, A. R. Chandler, A. K. Mehle and J. M. Overton (2010). "Female mice and rats exhibit species-specific metabolic and behavioral responses to ovariectomy." Gen Comp Endocrinol **166**(3): 520-528.

Wu, Y., W. Li, C. Zhou, F. Lu, T. Gao, Y. Liu, . . . C. Zhou (2012). "Ketamine inhibits lipopolysaccharide-induced astrocytes activation by suppressing TLR4/NF-kB pathway." Cell Physiol Biochem **30**(3): 609-617.

Wunderlich, M. T., C. W. Wallesch and M. Goertler (2006). "Release of glial fibrillary acidic protein is related to the neurovascular status in acute ischemic stroke." Eur J Neurol **13**(10): 1118-1123.

Wurster, U. (1988). "Protein gradients in the cerebrospinal fluid and the calculation of intracerebral IgG synthesis." J Neuroimmunol **20**(2-3): 233-235.

Xu, L., R. M. Sapolsky and R. G. Giffard (2001). "Differential sensitivity of murine astrocytes and neurons from different brain regions to injury." Exp Neurol **169**(2): 416-424.

Yakimchuk, K., M. Iravani, M. S. Hasni, P. Rhonnstad, S. Nilsson, M. Jondal and S. Okret (2011). "Effect of ligand-activated estrogen receptor beta on lymphoma growth in vitro and in vivo." Leukemia **25**(7): 1103-1110.

Yakimchuk, K., M. Jondal and S. Okret (in press). "Estrogen receptor alpha and beta in the normal immune system and in lymphoid malignancies." Mol Cell Endocrinol.

Yeh, T. H., Y. Lee da, S. M. Gianino and D. H. Gutmann (2009). "Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation." Glia **57**(11): 1239-1249.

Zatz, M. and A. Starling (2005). "Calpains and disease." N Engl J Med **352**(23): 2413-2423.

Zhang, L., W. Zhao, B. Li, D. L. Alkon, J. L. Barker, Y. H. Chang, . . . D. R. Rubinow (2000). "TNF-alpha induced over-expression of GFAP is associated with MAPKs." Neuroreport **11**(2): 409-412.

Zhu, L., S. Ramboz, D. Hewitt, L. Boring, D. S. Grass and A. F. Purchio (2004). "Non-invasive imaging of GFAP expression after neuronal damage in mice." Neurosci Lett **367**(2): 210-212.

Zimmerman, L., B. Parr, U. Lendahl, M. Cunningham, R. McKay, B. Gavin, . . . A. McMahon (1994). "Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors." Neuron **12**(1): 11-24.

Zoltewicz, J. S., D. Scharf, B. Yang, A. Chawla, K. J. Newsom and L. Fang (2012). "Characterization of Antibodies that Detect Human GFAP after Traumatic Brain Injury." Biomark Insights **7**: 71-79.

Appendix 1:

Enzyme-linked Immuno Sorbent Assay (ELISA) optimization for detection of glial fibrillary acidic protein (GFAP) in biological fluids

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TLH provided technical assistance and participated in the design of the experiments. PLJ contributed the remainder of this chapter, including designing the experiments (with the guidance of AM), and writing and editing the appendix.

ABSTRACT

Mutations in glial fibrillary acidic protein (GFAP) result in accumulation of the protein above a toxic threshold in Alexander disease (AxD), a rare neurodegenerative disease. There is no cure for AxD, but reduction of GFAP concentrations may alleviate symptoms or treat AxD. Analysis of GFAP in biological fluids such as cerebrospinal fluid (CSF) and plasma may provide a biomarker for analysis of GFAP changes during drug treatments for AxD, but require a sensitive assay for detection. In this study, we optimized the enzyme-linked immuno sorbent assay (ELISA) to decrease the inter-assay variability from 43% to 11-19% for biological fluids through changes to antibody reaction volumes, plate brand, detergent, and antibody diluents.

INTRODUCTION

Glial fibrillary acidic protein (GFAP), the main intermediate filament in astrocytes, increases in Alexander disease (AxD) patients and in mouse models of AxD. There is no cure for AxD, but reducing GFAP concentrations may help alleviate symptoms. A screen of FDA-approved drugs found several candidates which reduce *GFAP* promoter activity and protein levels (Cho et al. 2010). In mice, drug effects on GFAP can be determined by quantification of GFAP in brain homogenates via an enzyme-linked immuno sorbent assay (ELISA) (Petzold et al. 2004; Hagemann et al. 2006; LaPash Daniels et al. 2012). Measurement of GFAP in biological samples such as cerebrospinal fluid (CSF) and plasma may also be useful and are directly applicable to future human clinical trials. Using the original ELISA which effectively measures GFAP in brain homogenates, we found GFAP concentrations varied daily with an inter-assay variability of 43% for biological fluids but an inter-assay variability of 25% for mouse tissue samples. For analysis of biological fluids from both human and mouse following drug treatments, a sensitive ELISA is required which has a low inter-assay variability. By optimizing the ELISA through changes to antibody volume per well, plate brand, and detergent concentration, we established a modified GFAP ELISA with an inter-assay variability of 11-19% as well as high sensitivity.

METHODS AND RESULTS

The original ELISA procedures followed those outlined in Hagemann et al. (2009). Briefly, flat-bottom 96-well microtiter plates (Corning) were coated overnight at 4^{o}C with a cocktail of monoclonal antibodies (Covance, SMI-26R) diluted in phosphate buffered saline (PBS). Plates were blocked with 5% milk for 2 hours at room temperature (RT), then washed with PBS before addition of samples and standards diluted in PBS with 0.5% Triton-X-100 (TX) and 1% (w/v) bovine serum albumin (BSA). Standard curves were generated using bovine

GFAP (Fitzgerald Industries International, # RDI-PRO62007). After sample incubation (2 hours, RT) and washing (three times with PBS-0.5% TX), a rabbit polyclonal antibody (DAKO, Z334) used to detect GFAP was added and allowed to incubate overnight at 4^{o}C. Following an additional washing with PBS-0.5% TX, a peroxidase conjugated anti-rabbit antibody IgG secondary antibody (Sigma, A6154) was allowed to incubate for 2 hours at RT. The plate was again washed three times with PBS-0.5% TX followed by two PBS washes before addition of the SuperSignal ELISA Femto Chemiluminescent Substrate (Pierce, Thermo Scientific) and quantification of peroxidase activity by a GloRunner Microplate Luminometer (Turner Biosystems).

Initial analysis of ELISA inter-assay variability with mouse brain tissue samples revealing 25% variability in GFAP concentrations when comparing one brain tissue over ten separate ELISAs, but a tight intra-assay variability of 10%. The GFAP standard revealed high variability in assay values over all ten ELISAs (30%). To reduce this variability, we changed the storage buffer for the GFAP standard. Originally, purified GFAP was stored in the ELISA diluent: phosphate buffer saline (PBS) with 0.5% TX and 1% BSA. Removing the TX from the 1 µg stock vial reduced standard variability from 30% to 15% for a 100 µL reaction volume (data not shown).

We then analyzed the detection limits of the assay using the original ELISA procedure with the new standard (ELISA B; no TX in 1 µg stock vial). The lower limit of detection (LLD), defined as the lowest quantity of substance distinguishable from a blank sample, was determined as 3 standard deviations of the mean of replicate blank samples. The biological limit of detection (BLD) is the lower limit of detection plus 3 standard deviations of a known low concentration sample and distinguishes a “spiked” sample from background, preventing the detection of a false positive. The LLD for ELISA B was 8 ng/L and the BLD was 16 ng/L for a 100 µL volume. Due to the limited volume of mouse CSF, analysis of a 50 µL reaction revealed

a 15 ng/L LLD and 29 ng/L BLD. We then determined the inter-assay variability of ELISA B using biological samples. To eliminate variation in GFAP due to freeze-thaw cycles, we used ten separate aliquots of both human control plasma and CSF from mice which over-express human wild-type GFAP *(GFAP^{Tg}*). We measured GFAP concentration in human control plasma sample at 100 µL and *GFAP^{Tg}* samples at a 50 µL reaction volumes. The inter-assay variability of both control human plasma and *GFAP^{Tg}* was 43% (Fig. 6.1A).

Finding different LLD and BLD for 50 and 100 µL volumes as well as the high inter-assay variability seen in biological samples suggested further optimization of ELISA procedures were required. We then began a series of changes to determine optimal assay parameters which yielded high sensitivity and low variability. We first began shaking the plates at a low speed (75 rpm) during all incubation steps to provide uniform distribution of antibody and sample in the well. Loading volume of antibody mixture was also increased from 50 µL per well to 100 µL per well to provide thorough antibody coating.

Next, different assay plates and antibody diluents were compared to determine their contribution to inter-assay variability. The original ELISA used a Corning Flat-bottom assay plate, but Nunc Maxisorp^{TM} plates are high protein-binding plates which were thought to decrease variability. The Maxisorp^{TM} surface is polystyrene which has high affinity for hydrophilic and hydrophobic domains such as proteins and antibodies (Nunc production information). In a previous study comparing several different plate types, Maxisorp^{TM} were found to have high reproducibility and a wide dynamic range (Lu et al. 2012). For antibody dilutions, the original ELISA used 5% milk with 0.5% TX for all antibody dilutions except SMI-26R (Covance) which was diluted in PBS. TX concentrations in the original ELISA were high compared to previous studies (Engvall and Perlmann 1972; Ming and Fan 1988; Steinitz 2000); therefore we reduced TX concentrations from 0.5% to 0.05% in all mixtures (diluents and washes). Comparison of milk, milk with 0.05% TX, PBS with 0.05% TX, and PBS alone as

antibody diluents revealed high background values and low sensitivity for PBS diluents with and without detergent (Fig. 6.2A-B). These results agree with previous studies which indicate antibody diluents require detergent or a non-relevant protein such as found in milk to reduce non-specific binding of antibodies to residual ligand-free surfaces (Engvall and Perlmann 1972; Steinitz 2000). Milk without TX had the lowest background values (Fig. 6.2B). Using the same antibody diluents on both the Corning and Nunc plates revealed lower background values using the Nunc plate (Fig. 6.2A). Other published ELISA procedures also use an alternative non-ionic detergent, Tween 20 (Tw20), commonly used in immunoassays (Engvall and Perlmann 1972; Ming and Fan 1988; Steinitz 2000; Cullen et al. 2012; Zhang et al. 2013). Comparison of milk with 0.05% TX or 0.05% Tw20 revealed Tw20 produced less variability than TX (Fig. 6.2C). We therefore replaced TX with 0.05% Tw20 in all washes and diluents. Milk without detergent improved the signal to noise ratio for the GFAP ELISA. The modified ELISA procedures now included using the Nunc Maxisorp^{TM} plates, diluting all antibodies except SMI-26 in milk, diluting samples and standards in PBS with 0.05% Tw20 and 1% BSA, washing plates with PBS-Tw20, increasing antibody volumes from 50 to 100 µL, and using GFAP stocks frozen in 1% BSA for the standard (Table 6.1).

Analysis of LLD and BLD with the modified ELISA revealed a LLD of 11 ng/L and a BLD of 23 ng/L for 100 µL reaction volumes and a LLD of 11 ng/L and a BLD of 21 ng/L for a 50 µL reaction volume, suggesting the new ELISA parameters are preferable compared with the original ELISA. The inter-assay variability determined with human control plasma and *GFAP*^{Tg} CSF was 19% and 11% respectively (Fig. 6.1B).

DISCUSSION

These results revealed the changes made to the ELISA increased sensitivity and lowered inter-assay variability providing new, preferable ELISA procedures for detection of

GFAP in biological fluids and tissues. The plate alteration resulted in increased antibody binding due to the unique polystyrene structure which is optimized for antibody binding (Nunc, http://alab.com.pl/public/content/File/do_pobrania/Nunc_Surfaces_for_passive_adsorption.pdf). Additionally, conversion from TX to Tw20 detergent reduced the variability in the ELISA. TX is a non-ionic polyoxyethylene surfactant and is a mild, non-denaturing detergent (Sigma-Aldrich, http://www.snowpure.com/docs/triton-x-100-sigma.pdf). Tw20 is a non-ionic polyoxyethylene sorbitol ester which is a popular detergent used as a blocking agent especially in western blots (Sigma-Aldrich, http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/1/p5927pis.Par.0001.File.tmp/p5927pis.pdf). Interestingly, TX absorbs ultraviolet light which can interfere with protein quantification (Sigma-Aldrich), and this may be the cause for the greater variability seen with TX, though other unknown mechanisms could also result in the variability. Overall, through various changes, we enhanced the ELISA sensitivity and lowered the inter-assay variability providing ELISA procedures which reliably detect GFAP in biological fluids.

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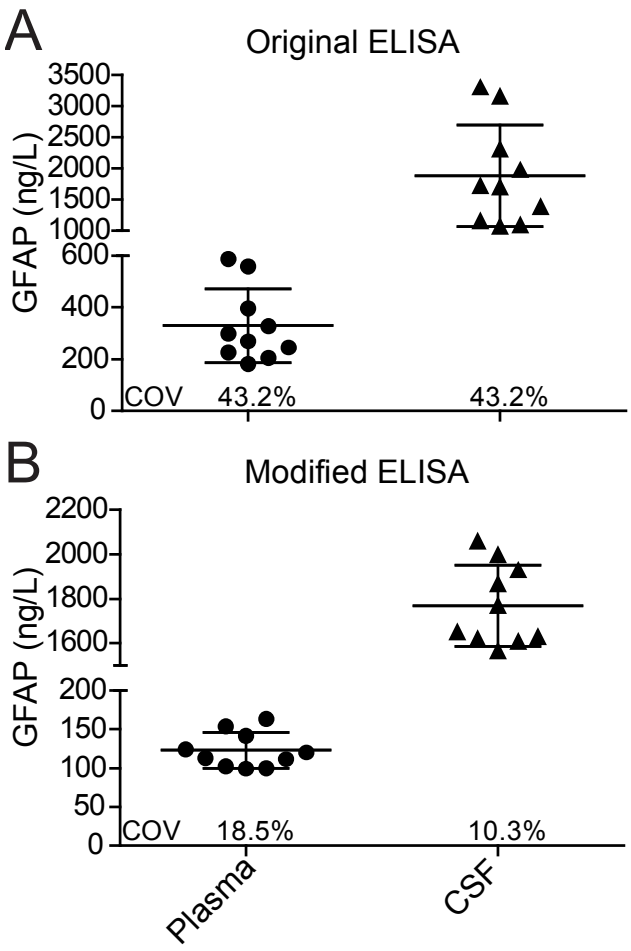
A
Original ELISA
GFAP (ng/L)
3500
3000
2500
2000
1500
1000
600
400
200
0
COV 43.2%
43.2%
B
Modified ELISA
GFAP (ng/L)
2200
2000
1800
1600
200
150
100
50
0
COV 18.5%
10.3%
Plasma
CSF

Figure 6.1: ELISA inter-assay variability of biological samples. (A) Inter-assay variability (COV) of human control plasma and *GFAP*^{Tg} CSF using the original ELISA procedures. (B) Inter-assay variability of human control plasma and *GFAP*^{Tg} CSF using the modified ELISA procedures. Each data point represents an independent assay. COV, coefficient of variation.

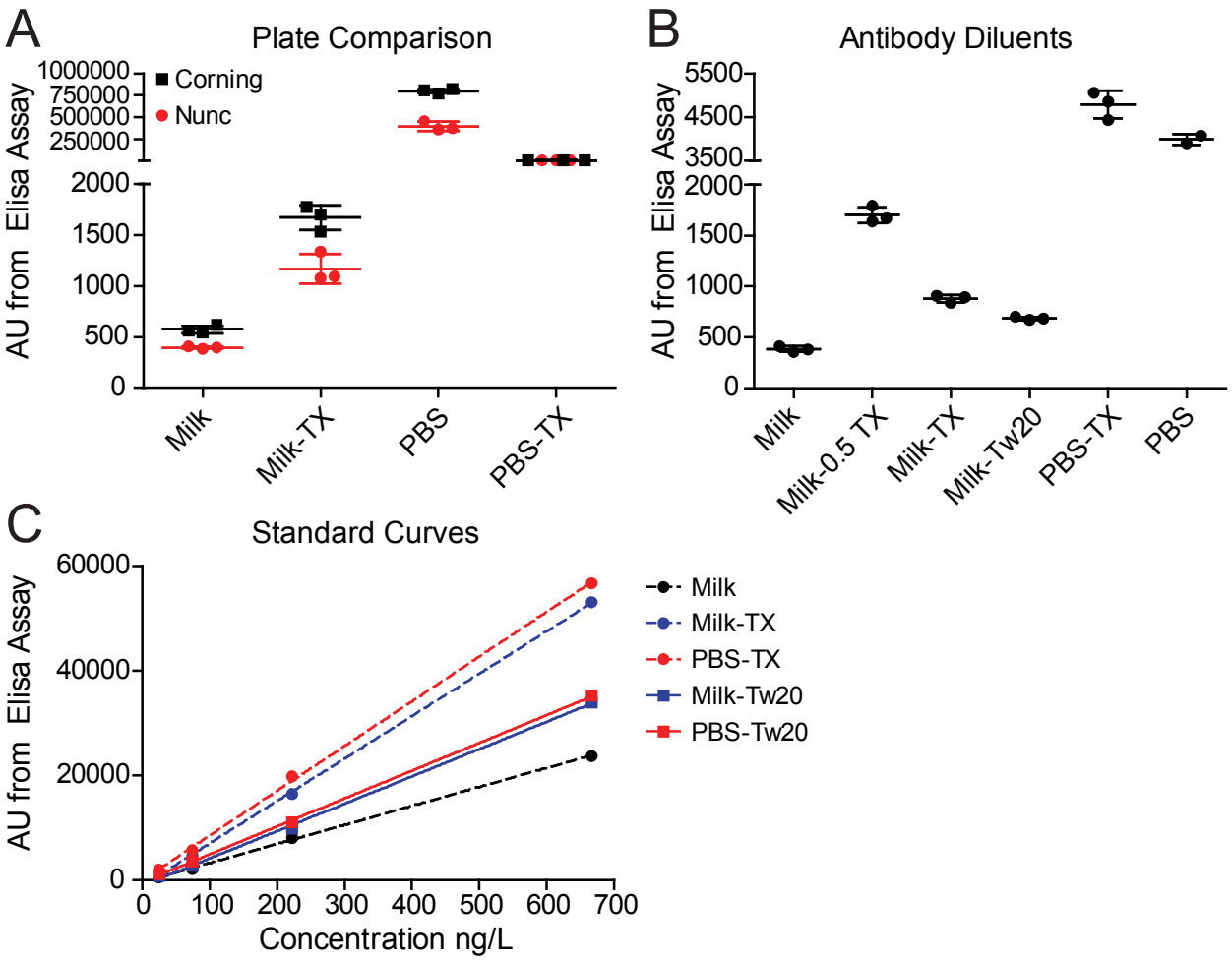
A
Plate Comparison
AU from Elisa Assay
1000000
750000
500000
250000
2000
1500
1000
500
0
Corning
Nunc
Milk
Milk-TX
PBS
PBS-TX
B
Antibody Diluents
AU from Elisa Assay
5500
4500
3500
2000
1500
1000
500
0
Milk
Milk-0.5 TX
Milk-TX
Milk-Tw20
PBS-TX
PBS
C
Standard Curves
AU from Elisa Assay
60000
40000
20000
0
0
100
200
300
400
500
600
700
Concentration ng/L
Milk
Milk-TX
PBS-TX
Milk-Tw20
PBS-Tw20

Figure 6.2: ELISA optimization. For ELISA optimization, different microtiter plates were compared (A). Comparison of Corning (from the original ELISA) and Nunc Maxisorp™ revealed Maxisorp plates had lower background values compared with Corning. (B) Comparison of different antibody diluents revealed lower background signals in samples with milk followed in increasing order by milk with 0.05% Tw20 (Milk-Tw20), milk with 0.05% TX (Milk-TX), milk with 0.5% TX (Milk-0.5 TX), PBS, and PBS with 0.05% TX (PBS-TX). Presented as mean ± 1 standard deviation (SD) on a split linear scale (n=2-3 per procedure). (C) Analysis of the GFAP standard curve using 5 different antibody diluents revealed low background for the skimmed milk diluent. TX, Triton X-100; Tw20, Tween 20; PBS, phosphate buffered saline; AU, arbitrary units.

	Original ELISA	**Modified ELISA**
Plate	Corning Flat-bottom	Nunc Maxisorp™
Antibody Volume	50 µL	100 µL
GFAP stock	PBS- 0.5% TX- 1% BSA	PBS- 1% BSA
Diluents		
SMI-26	PBS	PBS
DAKO, HRP	5% milk- 0.5% TX	5% milk
Sample, Standard	PBS- 0.5% TX- 1% BSA	PBS- 0.05% Tw20- 1% BSA
Wash Mixture		
1st Wash	PBS	PBS
Washes	PBS- 0.5% TX	PBS- 0.05% Tw20
Inter-assay variability	43%	11-19%

Table 6.1: Comparison of ELISA methods. For ELISA optimization, several parameters were changed (Modified ELISA). This table summarizes the various changes made to the ELISA to increase sensitivity and decrease inter-assay variability. TX, Triton X-100; Tw20, Tween 20; PBS, phosphate buffered saline; BSA, bovine serum albumin.

REFERENCE

Cho, W., M. Brenner, N. Peters and A. Messing (2010). "Drug screening to identify suppressors of GFAP expression." Hum Mol Genet **19**(16): 3169-3178.

Cullen, V. C., R. A. Fredenburg, C. Evans, P. R. Conliffe and M. E. Solomon (2012). "Development and advanced validation of an optimized method for the quantitation of Abeta42 in human cerebrospinal fluid." AAPS J **14**(3): 510-518.

Engvall, E. and P. Perlmann (1972). "Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes." J Immunol **109**(1): 129-135.

Hagemann, T. L., W. C. Boelens, E. F. Wawrousek and A. Messing (2009). "Suppression of GFAP toxicity by alphaB-crystallin in mouse models of Alexander disease." Hum Mol Genet **18**(7): 1190-1199.

Hagemann, T. L., J. X. Connor and A. Messing (2006). "Alexander disease-associated glial fibrillary acidic protein mutations in mice induce Rosenthal fiber formation and a white matter stress response." J Neurosci **26**(43): 11162-11173.

LaPash Daniels, C. M., E. V. Austin, D. E. Rockney, E. M. Jacka, T. L. Hagemann, D. A. Johnson, . . . A. Messing (2012). "Beneficial effects of nrf2 overexpression in a mouse model of alexander disease." J Neurosci **32**(31): 10507-10515.

Lu, W., R. Scott and J. Granchelli (2012). Accuracy and Dynamic Range of Thermo Scientific Nunc MaxiSorp Plates: Competitive Comparison. T. F. S. Inc.

Ming, C. H. and Y. S. Fan (1988). "Enhancement of anti-phospholipid antibody activity by Tween 20." J Immunol Methods **109**(2): 253-255.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Steinitz, M. (2000). "Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells." Anal Biochem **282**(2): 232-238.

Zhang, Y., A. H. Gao, B. Liu, W. Sheng, C. Tan, M. Yuan and S. Wang (2013). "A direct competitive enzyme-linked immunosorbent assay for rapid detection of anilofos residues in agricultural products and environmental samples." J Environ Sci Health B **48**(1): 1-8.

Appendix 2:

GFAP stability and degradation products in mouse CSF and mouse and human plasma samples

Jany, P.L., L.R. Moody, and A. Messing, GFAP stability and degradation products in mouse CSF and mouse and human plasma samples. Unpublished, 2013.

AUTHORS CONTRIBUTIONS

LRM provided technical assistance, and performed, designed, and analyzed the western blot, and reviewed and edited the appendix. PLJ contributed the remainder of this chapter, including designing the experiments (with the guidance of AM), and writing and editing the chapter.

ABTRACT

Glial fibrillary acidic protein (GFAP) is detectable and elevated in Alexander disease (AxD) in mouse cerebrospinal fluid (CSF) and human CSF and plasma. To determine if GFAP is also detectable in mouse plasma, we analyzed GFAP concentrations in plasma from wild-type mice as well as two mouse models of AxD, *Gfap*$^{R236H/+}$ and *GFAP*^{Tg}. *Gfap*$^{R236H/+}$ mice contain a GFAP mutation homologous to a mutation found in AxD (R239H) and *GFAP*^{Tg} mice overexpress human wild-type GFAP. GFAP was undetectable in plasma from wild-type mice, detectable in *Gfap*$^{R236H/+}$ mice, and elevated in all *GFAP*^{Tg} mice. Comparison of GFAP concentration in CSF and plasma in *GFAP*^{Tg} mice revealed higher concentrations in CSF compared with plasma. We next characterized GFAP in CSF and plasma by analyzing the stability of GFAP and GFAP degradation products to elucidate collection and storage condition requirements during future clinical trials. To examine the stability of GFAP in mouse CSF and human and mouse plasma, we quantified GFAP after 24 hour incubations at room temperature (RT), 4^{o}C, or -80^{o}C. In CSF samples, GFAP concentration significantly increased following the RT incubation compared with -80^{o}C and 4 ^{o}C while GFAP in plasma samples from both human and mice were unaltered by the RT incubation. In another set of experiments, we monitored GFAP degradation products in CSF from *GFAP*^{Tg} mice and found a 37 kilodalton (kDa) band. Overall, these studies indicate GFAP is detectable in plasma of mouse models of AxD, unstable in CSF possibly through active degradation by proteases, but is stable in plasma following a 24 hour RT incubation. These results suggest further characterization of GFAP is required to understand the collection and storage conditions necessary for future clinical trials using GFAP as a biomarker.

INTRODUCTION

Alexander disease (AxD) is a rare, fatal, neurodegenerative disease which has no cure. The etiology of AxD involves mutations in the main intermediate filament, glial fibrillary acidic protein (GFAP), which results in GFAP accumulation. In mice, overexpression of GFAP (*GFAP*^{Tg}) induces Rosenthal fiber formation, the pathological hallmark of AxD, increased fatality, and decreased body weight (Messing et al. 1998; Hagemann et al. 2005), suggesting GFAP accumulation is an important pathway in AxD. Reducing GFAP accumulation may alleviate AxD symptoms providing a potential drug treatment (Messing et al. 2010). Analysis of drug effects on GFAP requires a reliable biomarker which could be GFAP itself. We analyzed GFAP concentrations in mouse CSF and in human CSF and plasma and found GFAP is detectable in control samples and elevated in both human and mouse models of AxD (see Chapters 2 and 3), suggesting GFAP in CSF and plasma may be a reliable biomarker. In the current study, we analyzed GFAP in mouse plasma, determined the stability of GFAP in biological fluids such as CSF and plasma, and analyzed GFAP degradation products to further characterize GFAP in CSF and plasma.

The use of GFAP as a biomarker in biological fluids for future clinical trials of drug efficacy requires analysis of GFAP stability. Biological fluids will be collected from various institutions, thus a defined protocol is necessary to reduce inter-facility variations in biological fluid concentrations of GFAP. If GFAP is unstable in biological fluids and begins to degrade or breakdown under certain collection or storage conditions such as prolonged exposure to room temperature (RT), it may result in altered GFAP quantification with an ELISA suggesting a need to understand and prevent these effects on GFAP. Previous studies have shown GFAP in CSF is stable at both 4^{o}C and -80^{o}C storage conditions (Petzold et al. 2004). Mouse spinal cord stored at RT for up to 24 hours resulted in GFAP degradation or breakdown as evidenced by western blot, but degradation was prevented with the calcium chelator EGTA (DeArmond et al.

1983). Therefore, we need to evaluate the stability of GFAP in both CSF and plasma to elucidate the collection and storage conditions necessary for future clinical trials.

In addition, the reason GFAP is detectable in CSF and plasma is unclear. Analysis of GFAP in CSF and plasma after traumatic brain injury revealed a 37 kilodalton (kDa) band of GFAP which corresponds to the calpain cleavage product of GFAP (Papa et al. 2012; Zoltewicz et al. 2012). Calpain is a calcium-dependent cysteine protease involved in cytoskeletal remodeling, apoptosis, cell cycle regulation, and cell development (Zatz and Starling 2005; Liu et al. 2011). Other cysteine proteases such as cathepsins B, L1, and S are upregulated in *GFAP*^{Tg} mouse CSF (Cunningham et al. 2013) and olfactory bulbs (Hagemann et al. 2005) and may also degrade GFAP. Cathepsins are involved in autophagy and apoptosis pathways and are upregulated after injury (Turk et al. 2000; Cirman et al. 2004; Lamparska-Przybysz et al. 2005; Biniossek et al. 2011). Other proteases and caspases also cleave GFAP, such as caspase 3, which cleaves GFAP into 20 and 30 kDa products (Mouser et al. 2006; Acarin et al. 2007) and may actively degrade GFAP in AxD (Cho and Messing 2009; Chen et al. 2011). Cysteine proteases and caspase are intimately involved in cell death, suggesting GFAP presence in CSF and plasma may result from GFAP degradation during cell death.

To evaluate GFAP in CSF and plasma, we quantified GFAP in mouse plasma as well as monitored GFAP stability and degradation products in mouse CSF and mouse and human plasma. GFAP concentrations in mouse plasma were undetectable in wild-type mice and detectable and elevated in mouse models of AxD. We also found GFAP in mouse CSF increased significantly following a RT incubation for 24 hours compared with 4^{o}C and -80^{o}C incubations, suggesting GFAP is unstable in mouse CSF. Conversely, GFAP concentrations in human and mouse plasma were unaltered by the RT incubation. These results indicate GFAP is unstable in CSF but stable in plasma during a 24 hour RT incubation. Analysis of GFAP degradation products in mouse CSF revealed a 37 kDa band of GFAP, suggesting GFAP in

CSF may be degraded by the cysteine protease calpain. Overall, we found GFAP is detectable in plasma from mouse models of AxD, is unstable in CSF possibly due to calpain cleavage, and is stable in plasma, suggesting further characterization of GFAP in biological fluids in necessary.

RESULTS AND DISCUSSION

We previously found GFAP is detectable and elevated in CSF from mouse models of AxD and CSF and plasma from AxD patients (see Chapters 2 and 3), but had not measured GFAP in plasma from mouse models of AxD. We found GFAP is detectable in plasma from all *GFAP*^{Tg} mice tested, is detectable in *Gfap*$^{R236H/+}$ mice, and is undetectable in *Gfap*$^{+/+}$ mice (Fig. 7.1A). GFAP concentrations were considered undetectable if they were below the biological limit of detection (BLD) (see methods). In a previous study, GFAP concentrations are greater in CSF compared with plasma after spinal cord injury (Kwon et al. 2010). Therefore, we compared GFAP in CSF and plasma of *GFAP*^{Tg} mice and found higher concentrations of GFAP in CSF compared with plasma (Fig. 7.1B). To determine if GFAP is lower in plasma because of plasma inhibition on GFAP quantification, we spiked plasma from GFAP null mice into the GFAP standard. We found plasma did not alter GFAP quantification (Fig. 7.2), suggesting GFAP is detectable in plasma from mouse models of AxD but at lower concentrations than CSF. The lower concentration of GFAP in plasma seen in both mouse models of AxD (Fig. 7.1B) and human AxD patients (Fig. 3.4) may suggest GFAP in plasma is a less sensitive biomarker compared with GFAP in CSF. However, plasma collections are less invasive compared with CSF collections and may be easier for young patients such as those with AxD, indicating plasma analysis is preferred to CSF. Further characterization of GFAP in plasma is required to determine if it will be a suitable, sensitive biomarker in mouse models of AxD and human AxD patients.

Next, to elucidate the stability of GFAP in CSF and plasma samples, we analyzed GFAP concentrations in CSF and plasma after incubations at RT. If GFAP is unstable and begins to degrade or breakdown in CSF or plasma during certain collection or storage conditions, such as prolonged storage at RT, it may alter GFAP quantification via ELISA. Comparison of CSF from *GFAP*^{Tg} and *Gfap*$^{R236H/+}$ mice stored for 24 hours at RT, 4^{o}C, and -80^{o}C revealed RT incubations significantly increased GFAP concentrations in CSF compared with 4^{o}C and -80^{o}C incubations (Fig. 7.3A, 4^{o}C data not shown). The reason GFAP increases during RT incubations is unclear, but one possibility may be GFAP degradation. Degraded GFAP may increase the amount of GFAP quantified by ELISA due to less steric hindrance and/or an increase in available binding sites thus increasing antibody binding. Steric hindrance in an ELISA is caused by large proteins, such as full length GFAP, binding to a capture antibody (Covance, SMI-26R) and preventing nearby antibodies from binding other GFAP proteins in the solution. If GFAP is degraded, the smaller product may not produce as much steric hindrance allowing more GFAP to bind to the capture antibodies. In addition, more GFAP may be bound by the detection antibody (DAKO, Z334) if the GFAP is degraded. The detection antibody is a polyclonal antibody. GFAP degradation into smaller products may increase the number of recognition site or reveal sites with higher antibody binding affinity, resulting in increased signal from the ELISA.

To determine if the increase in GFAP signal seen in CFS after the RT incubation is due to GFAP degradation, we prevented GFAP degradation in mouse CSF through spinning and addition of protease inhibitors (PI). Spinning the CSF samples removes any cells present in the CSF which may release degradation enzymes and treating the CSF with the protease inhibitors, Complete Proteinase Inhibitor Cocktail (Roche) and Pefabloc SC (Sigma-Aldrich), prevents protease cleavage of GFAP. Pefabloc SC specifically inhibits serine proteases while Complete Proteinase Inhibitor Cocktail inhibits metalloproteases and serine and cysteine proteases (Roche and Sigma-Aldrich). Comparison of GFAP concentrations after RT and -80^{o}C

incubations revealed RT incubations increased GFAP even with the addition of spinning and/or inhibitors (Fig. 7.3B). Interestingly, Roche Applied Science found an approximate 20% reduction in the ability of Complete Proteinase to inhibit papain-like cysteine proteases, such as cathepsins B, L1, and S after only 60 minutes at RT (Roche, http://sevierlab.vet.cornell.edu/resources/ProteaseInhibitionGuide.pdf). In addition, while the Complete Proteinase Inhibitor Cocktail generally inhibits cysteine proteases, it does not contain a specific inhibitor for the calcium-dependent cysteine protease calpain (Roche). This lack of inhibition by the Complete Proteinase Inhibitor Cocktail on calpain may indicate GFAP in CSF is degraded by calpain during the 24 hour RT incubation.

GFAP concentrations were next measured in human and mouse plasma samples stored at RT or -80^{o}C for 24 hours. Human plasma samples showed no differences in GFAP concentrations when comparing RT and -80^{o}C incubations (Fig. 7.3C). Mouse plasma levels of GFAP were also unaltered by RT incubation for 24 hours (Fig. 7.3D). It is unclear why plasma and CSF responses differ to the RT incubation though a possible reason may be differences in the activation of proteases such as cathepsin. Cathepsins can act to degrade proteins both inside and outside the cell in biological fluids, suggesting GFAP may be degraded by cysteine proteases during the RT incubation. In plasma, proteases may not be active due to the EDTA used during plasma collection. EDTA is a calcium chelator which may act on cysteine proteases and inhibit their activity (Seiffert 1996; Wei et al. 2006). GFAP degradation by cathepsins in CSF may explain the reason GFAP in CSF is more variable compared with plasma and is degraded during the 24 hour RT incubation (Fig. 7.1B).

Measurement of GFAP in CSF by a GFAP ELISA does not distinguish breakdown products from full length GFAP. To evaluate breakdown products of GFAP, we precipitated protein from CSF of *GFAP*^{Tg} mice and analyzed GFAP product size with a western blot. We identified a 37 kDa product of GFAP, suggesting GFAP present in CSF is a breakdown product

(Fig. 7.4). Pooled CSF from wild-type mice and individual CSF samples from *GFAP*^{Tg} mice had no detectable GFAP present, indicating GFAP concentrations in CSF from pooled wild-type and individual *GFAP*^{Tg} mice are below the threshold of detection for the western blot. The 37 kDa band seen in mouse CSF samples may be a product of calpain cleavage (Papa et al. 2012; Zoltewicz et al. 2012). It is also possible other cysteine proteases such as cathepsins B, L1, and S, which were upregulated in *GFAP*^{Tg} CSF (Cunningham et al. 2013) and olfactory bulb (Hagemann et al. 2005), may degrade GFAP.

Overall, we found GFAP is detectable in plasma from mouse models of AxD and is unstable in CSF but stable in plasma during a 24 hour RT incubation. One reason GFAP may be degraded in CSF but not plasma could be differences in protease activity. Proteases may not be active in plasma due to the addition of EDTA, a calcium chelator. Treatment of CSF with specific calpain or cathepsin inhibitors may elucidate their involvement in GFAP degradation. In addition, we can analyze GFAP breakdown products in both CSF and plasma samples from mice and humans. We may also analyze the presence of cysteine proteases in mouse and human biological fluids to elucidate their role in GFAP degradation both with and without a 24 hour RT incubation. Future analysis and characterization of GFAP and GFAP degradation products in mouse and human biological fluids may provide further insight into GFAP, the collection and storage conditions necessary for future clinical trials, and the general reliability of GFAP as a biomarker for AxD.

MATERIALS AND METHODS

CSF and Plasma Collection and Modification

CSF was collected according to the method of DeMattos et al. (2002). Briefly, mice were anesthetized with avertin (400-600 mg/kg i.p.) and a midline sagittal incision was made over the dorsal aspect of the hindbrain. After three muscle layers were carefully peeled back,

the cisterna magna was exposed and pierced with a 30 gauge needle. CSF was collected immediately using a flexible plastic pipette with approximately 10 µL of CSF collected per animal and stored at -80°C until further processing. Some CSF samples were immediately pooled and half were spun at 2000 *x g* for 10 minutes at RT. Protease inhibitors Pefabloc SC (1mM) (Sigma-Aldrich) and Complete Proteinase Inhibitor Cocktail (1x) (Roche) were added to half of the spun samples and half of the unspun samples before storing at -80°C.

For plasma collection, approximately 0.5 ml of blood was collected from the axillary plexus using disposable transfer pipettes and placed into K_2-EDTA coated tubes (BD Diagnostic, # 365974). Within 60 minutes of collection, samples were centrifuged for 15 minutes at 2500 x g at room temperature (22-24°C). Plasma was transferred into clean low retention tubes (Fisher, #02-681-311) and stored at -20^{o}C until analysis.

GFAP protein quantitation

Quantitation of GFAP protein was performed as previously described (Jany et al. 2013). CSF samples were diluted directly in ELISA buffer and evaluated using a sandwich ELISA. Results were expressed as ng/L for CSF using purified bovine GFAP as a standard. For room temperature incubations, samples were thawed in room temperature water for 2 minutes then allowed to sit undisturbed for 24 hours before quantification of GFAP by ELISA. Under these conditions the lower limit of detection was 11 ng/L, and the biological limit of detection (BLD) (after accounting for the minimum 1:1 dilution with reaction buffer) was 46 ng/L. For analysis of plasma effects on GFAP, plasma from *Gfap*-null mice was spiked into the GFAP standard at a ratio of 1:1 (1 part plasma + 1 part GFAP standard) (Fig. 7.2).

Methanol/chloroform protein precipitation and western blot

For methanol/chloroform protein precipitation, CSF samples were thawed at room temperature then pooled. Four volumes of methanol were added to one volume of CSF sample

and mixed. One volume chloroform was then added before vortexing the solution. Three volumes of double distilled water were added and the total mixture was vortexed. The solution was then centrifuged at 14,000 *x g* for 2 minutes at room temperature. The aqueous layer was removed and discarded. To the remaining solution, four volumes of methanol were added, vortexed, and then centrifuged for 14,000 *x g* for 15 minutes. Supernatant was removed leaving the protein pellet, which was air dried and resuspended in 20 µL 1x Laemmli buffer, 0.1% 2-mercaptoethanol (BioRad).

After the sample is resuspended, samples were incubated at 95°C for 15 minutes. Standard curves were generated using bovine GFAP (Fitzgerald Industries International, # RDI-PRO62007) diluted in Laemmli buffer. Samples and Precision Plus protein Dual Color Standard (BioRad) was loaded (15 and 7µL respectively) into a 10% TGX gel (BioRad) submerged in 1x Tris/Gly/SDS Running buffer (BioRad) and ran at 125 V for 1.5 hours. Proteins were transferred from the gel to an Immobilon FL membrane overnight at 4°C in 1x Tris/Gly (BioRad) with 20% methanol at 30 V. After removing the gel from the membrane, the membrane was blocked for 1 hour in LiCor Odyssey (LI-COR) block then rinse in Tris buffered saline with 0.05% Tween 20 (TBS-Tw20). After washing, a rabbit polyclonal antibody (Dako, Z334) was used to detect GFAP followed by a Dylight 800 conjugated anti-rabbit IgG secondary antibody (Thermo Scientific, #35571) diluted in TBS-Tw20. After washing again with TBS-Tw20, fluorescence was detected and analyzed using the Odyssey Imaging System (LI-COR).

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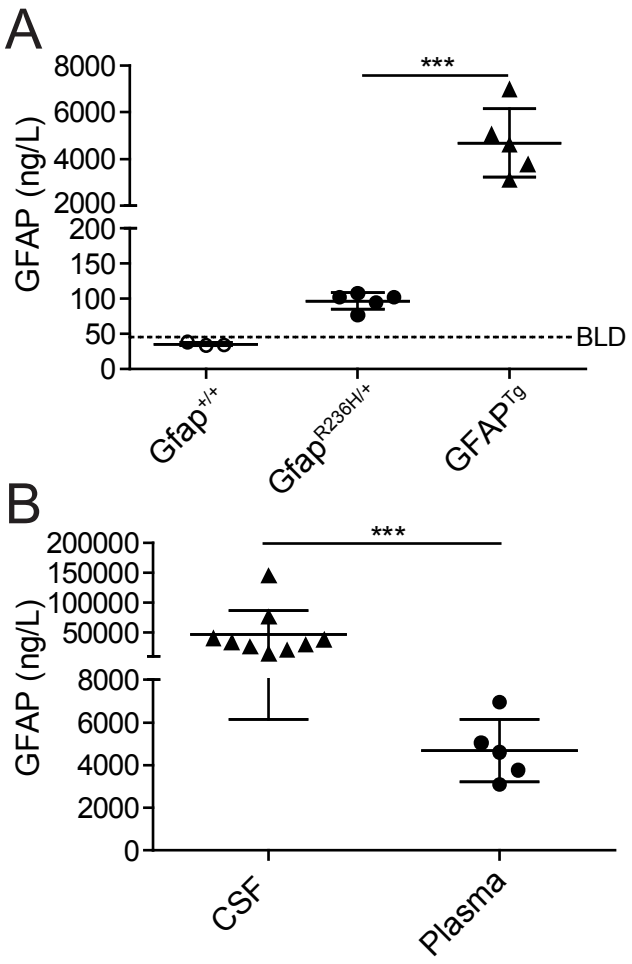
A
8000
6000
4000
2000
200
150
100
50
0
GFAP (ng/L)
***
BLD
Gfap+/+
GfapR236H/+
GFAPTg
B
200000
150000
100000
50000
8000
6000
4000
2000
0
GFAP (ng/L)
***
CSF
Plasma

Figure 7.1: GFAP in plasma and CSF samples from mouse models of AxD. (A) GFAP concentrations in plasma from wild-type mice (*Gfap*$^{+/+}$) was undetectable (n=3). GFAP in plasma was detectable in *Gfap*$^{R236H/+}$ mice and significantly elevated in *GFAP*^{Tg} mice (n=6 per genotype). (One-way ANOVA with post-hoc Bonferroni t-test; *** p<0.001). (B) In plasma, GFAP concentrations were significantly decreased when compared with GFAP in CSF (n=5 plasma and n=9 CSF). (Student's t-test; *** p<0.001). Presented as mean ± 1 standard deviation (SD). BLD, biological limit of detection.

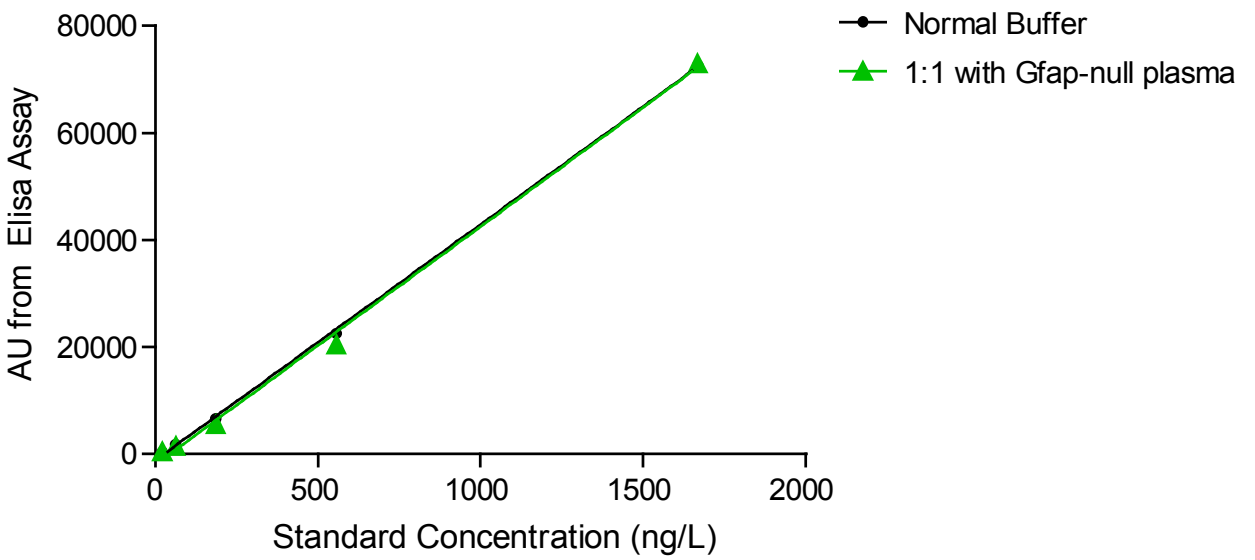
80000
60000
40000
20000
0
AU from Elisa Assay
0
500
1000
1500
2000
Standard Concentration (ng/L)
Normal Buffer
1:1 with Gfap-null plasma

Figure 7.2: Plasma effects on GFAP quantification using an ELISA. Spiking plasma (1:1) from *Gfap*-null mice into the GFAP standard did not change GFAP quantification when compared with the GFAP standard diluted with the normal ELISA buffer. Representative of replicate two experiments.

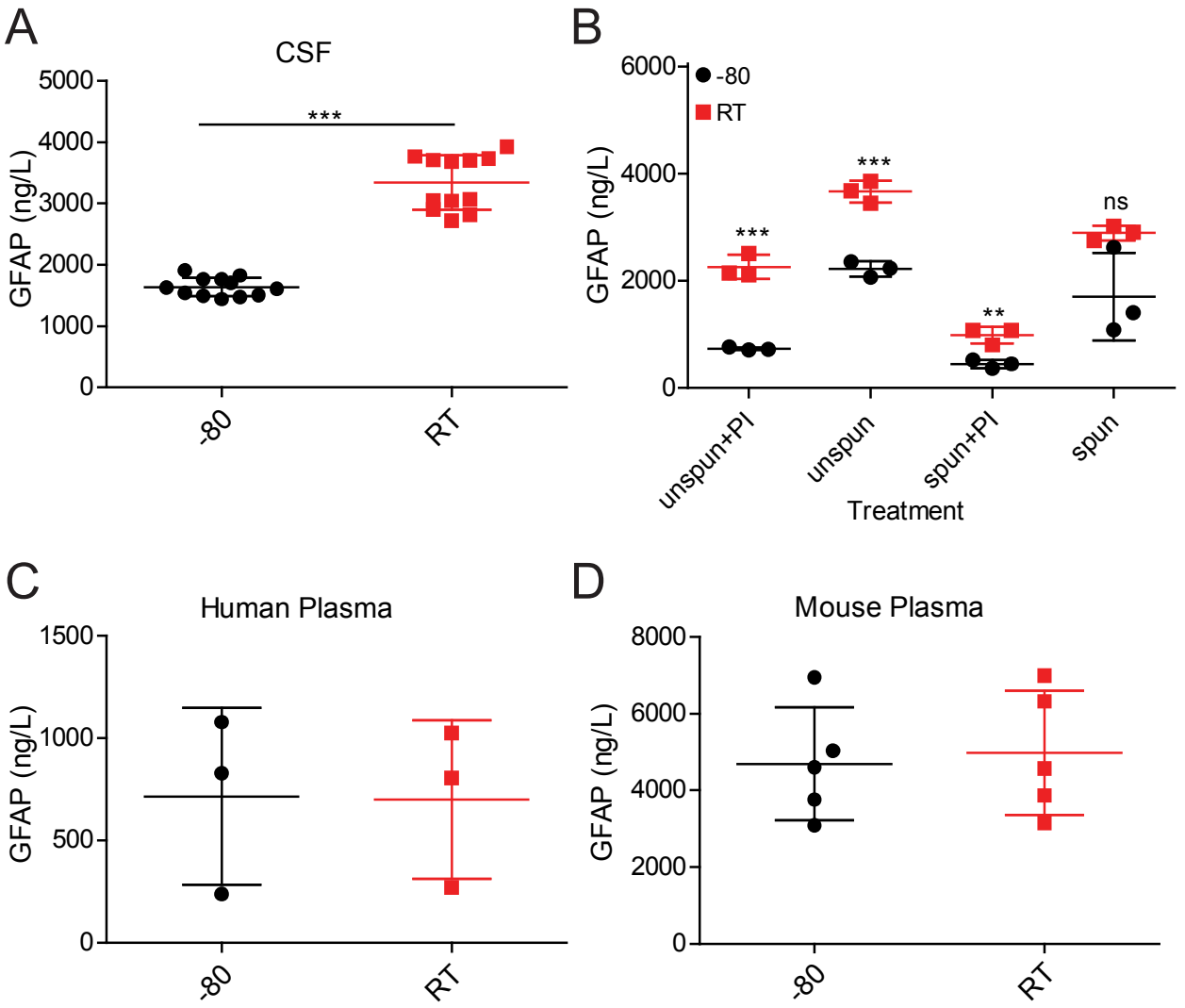
A
CSF
GFAP (ng/L)
5000
4000
3000
2000
1000
0
***
-80
RT
B
GFAP (ng/L)
6000
4000
2000
0
-80
RT
***
***
**
ns
unspun+PI
unspun
spun+PI
spun
Treatment
C
Human Plasma
GFAP (ng/L)
1500
1000
500
0
-80
RT
D
Mouse Plasma
GFAP (ng/L)
8000
6000
4000
2000
0
-80
RT

Figure 7.3: Room temperature incubation effects on the stability of GFAP in mouse CSF and mouse and human plasma. (A) After a 24 hour room temperature (RT) incubation, GFAP concentrations in CSF significantly increased compared with a -80^{o}C incubation (n=12 per incubation condition). (Student's t-test; *** p<0.001). (B) To determine if GFAP is increasing due to degradation, cells which may release degradation enzymes were removed by spinning the CSF (spun) and treating the CSF with protease inhibitors (PI). After the RT incubation, GFAP still increased in all CSF samples (n=3 per condition). (One-way ANOVA with post-hoc Bonferroni t-test; ns, not significant; ** p<0.01; *** p<0.001 compared with -80^{o}C incubations). (C and D) In plasma, RT incubations did not alter GFAP concentrations in both human (C) and mouse (D) plasma samples (n=3-5 per incubation condition). (Student's t-test). Presented as mean ± 1 SD. RT, room temperature; PI, protease inhibitors.

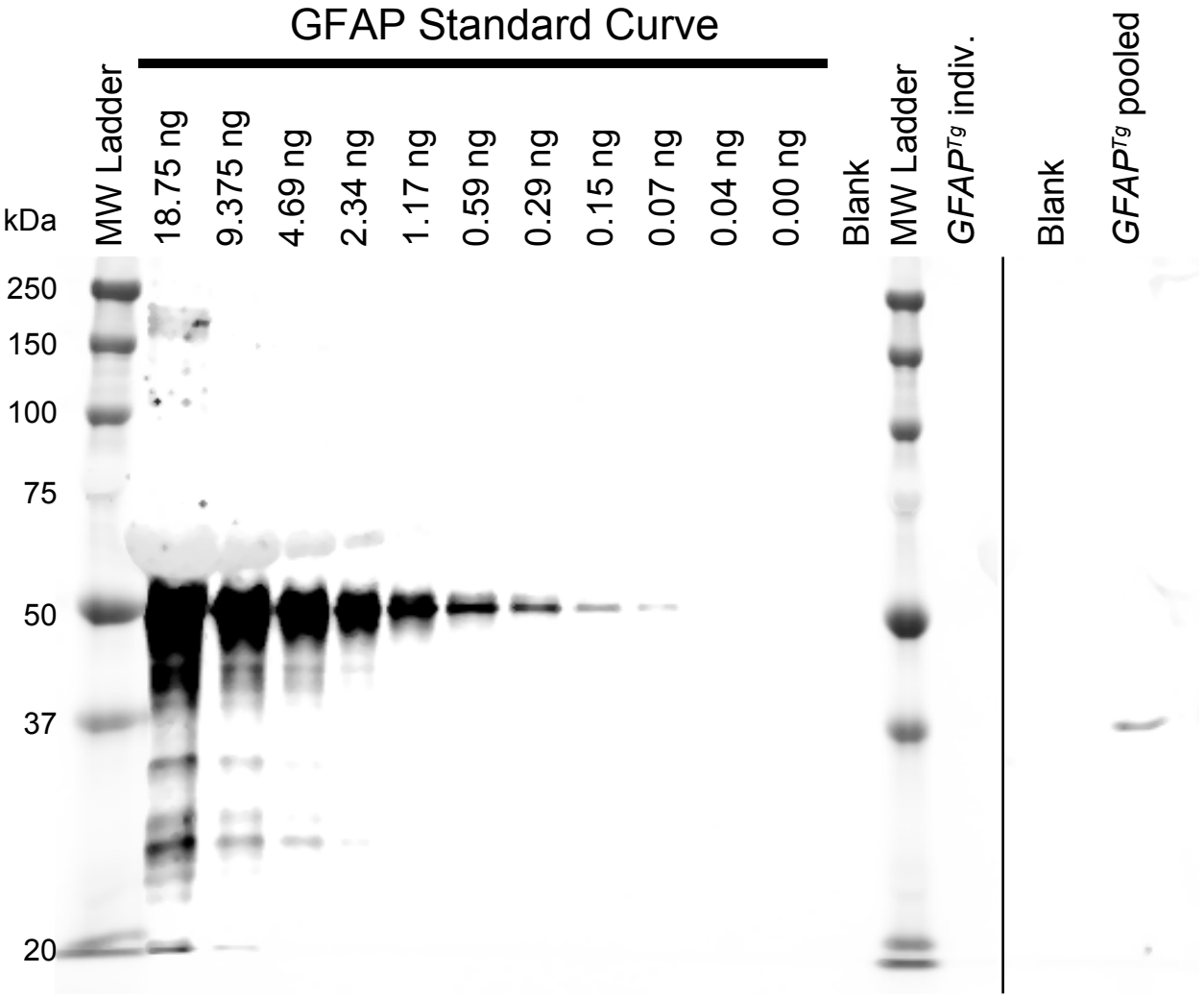
GFAP Standard Curve
kDa
MW Ladder
18.75 ng
9.375 ng
4.69 ng
2.34 ng
1.17 ng
0.59 ng
0.29 ng
0.15 ng
0.07 ng
0.04 ng
0.00 ng
Blank
MW Ladder
GFAPTg indiv.
Blank
GFAPTg pooled
250
150
100
75
50
37
20

Figure 7.4: 37 kDa GFAP degradation product in pooled *GFAP^{Tg}* CSF sample. CSF from an individual *GFAP^{Tg}* mouse and pooled *GFAP^{Tg}* mice was subjected to methanol/cholorform precipitation followed by GFAP analysis by western blot. GFAP was detectable (0.11 ng) in pooled, precipitated *GFAP^{Tg}* CSF samples, but undetectable in the individual *GFAP^{Tg}* CSF sample. Purified bovine GFAP dilutions were used for quantification. kDa, kilodalton; MW, molecular weight.

REFERENCE

Acarin, L., S. Villapol, M. Faiz, T. T. Rohn, B. Castellano and B. Gonzalez (2007). "Caspase-3 activation in astrocytes following postnatal excitotoxic damage correlates with cytoskeletal remodeling but not with cell death or proliferation." Glia **55**(9): 954-965.

Biniossek, M. L., D. K. Nagler, C. Becker-Pauly and O. Schilling (2011). "Proteomic identification of protease cleavage sites characterizes prime and non-prime specificity of cysteine cathepsins B, L, and S." J Proteome Res **10**(12): 5363-5373.

Chen, Y. S., S. C. Lim, M. H. Chen, R. A. Quinlan and M. D. Perng (2011). "Alexander disease causing mutations in the C-terminal domain of GFAP are deleterious both to assembly and network formation with the potential to both activate caspase 3 and decrease cell viability." Exp Cell Res **317**(16): 2252-2266.

Cho, W. and A. Messing (2009). "Properties of astrocytes cultured from GFAP over-expressing and GFAP mutant mice." Exp Cell Res **315**(7): 1260-1272.

Cirman, T., K. Oresic, G. D. Mazovec, V. Turk, J. C. Reed, R. M. Myers, . . . B. Turk (2004). "Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins." J Biol Chem **279**(5): 3578-3587.

Cunningham, R., P. Jany, A. Messing and L. Li (2013). "Protein changes in immunodepleted cerebrospinal fluid from a transgenic mouse model of alexander disease detected using mass spectrometry." J Proteome Res **12**(2): 719-728.

DeArmond, S. J., M. Fajardo, S. A. Naughton and L. F. Eng (1983). "Degradation of glial fibrillary acidic protein by a calcium dependent proteinase: an electroblot study." Brain Res **262**(2): 275-282.

DeMattos, R. B., K. R. Bales, M. Parsadanian, M. A. O'Dell, E. M. Foss, S. M. Paul and D. M. Holtzman (2002). "Plaque-associated disruption of CSF and plasma amyloid-beta (Abeta) equilibrium in a mouse model of Alzheimer's disease." J Neurochem **81**(2): 229-236.

Hagemann, T. L., S. A. Gaeta, M. A. Smith, D. A. Johnson, J. A. Johnson and A. Messing (2005). "Gene expression analysis in mice with elevated glial fibrillary acidic protein and Rosenthal fibers reveals a stress response followed by glial activation and neuronal dysfunction." Hum Mol Genet **14**(16): 2443-2458.

Jany, P. L., T. L. Hagemann and A. Messing (2013). "GFAP expression as an indicator of disease severity in mouse models of Alexander disease." ASN Neuro **5**(1): e00109.

Kwon, B. K., A. M. Stammers, L. M. Belanger, A. Bernardo, D. Chan, C. M. Bishop, . . . M. F. Dvorak (2010). "Cerebrospinal fluid inflammatory cytokines and biomarkers of injury severity in acute human spinal cord injury." J Neurotrauma **27**(4): 669-682.

Lamparska-Przybysz, M., B. Gajkowska and T. Motyl (2005). "Cathepsins and BID are involved in the molecular switch between apoptosis and autophagy in breast cancer MCF-7 cells exposed to camptothecin." J Physiol Pharmacol **56 Suppl 3**: 159-179.

Liu, Z., J. Cao, X. Gao, Q. Ma, J. Ren and Y. Xue (2011). "GPS-CCD: a novel computational program for the prediction of calpain cleavage sites." PLoS One **6**(4): e19001.

Messing, A., M. W. Head, K. Galles, E. J. Galbreath, J. E. Goldman and M. Brenner (1998). "Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice." Am J Pathol **152**(2): 391-398.

Messing, A., C. M. LaPash Daniels and T. L. Hagemann (2010). "Strategies for treatment in Alexander disease." Neurotherapeutics **7**(4): 507-515.

Mouser, P. E., E. Head, K. H. Ha and T. T. Rohn (2006). "Caspase-mediated cleavage of glial fibrillary acidic protein within degenerating astrocytes of the Alzheimer's disease brain." Am J Pathol **168**(3): 936-946.

Papa, L., L. M. Lewis, J. L. Falk, Z. Zhang, S. Silvestri, P. Giordano, . . . K. K. Wang (2012). "Elevated levels of serum glial fibrillary acidic protein breakdown products in mild and moderate traumatic brain injury are associated with intracranial lesions and neurosurgical intervention." Ann Emerg Med **59**(6): 471-483.

Petzold, A., G. Keir, A. J. Green, G. Giovannoni and E. J. Thompson (2004). "An ELISA for glial fibrillary acidic protein." J Immunol Methods **287**(1-2): 169-177.

Seiffert, D. (1996). "Hydrolysis of platelet vitronectin by calpain." J Biol Chem **271**(19): 11170-11176.

Turk, B., D. Turk and V. Turk (2000). "Lysosomal cysteine proteases: more than scavengers." Biochim Biophys Acta **1477**(1-2): 98-111.

Wei, J., C. H. Lin, H. Wu, Y. Jin, Y. H. Lee and J. Y. Wu (2006). "Activity-dependent cleavage of brain glutamic acid decarboxylase 65 by calpain." J Neurochem **98**(5): 1688-1695.

Zatz, M. and A. Starling (2005). "Calpains and disease." N Engl J Med **352**(23): 2413-2423.

Zoltewicz, J. S., D. Scharf, B. Yang, A. Chawla, K. J. Newsom and L. Fang (2012). "Characterization of Antibodies that Detect Human GFAP after Traumatic Brain Injury." Biomark Insights **7**: 71-79.