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A STUDY ON THE BIOCHEMICAL AND CELLULAR EFFECTS OF ENVIORMENTAL TOBACCO SMOKE ON ADULT AND DEVELOPING RAT BRAIN BIOCHEMISTRY

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

ABC	Ammonium bicarbonate
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
ATP	Adenosine-5'-triphosphate
ATP5	ATP synthase
CCT	Chaperonin containing TCP1 complex
CD	Conduct disorder
CNS	Central nervous system
Dnm1l	Dynamin-like protein 1
Eef	Elongation factor protein family
Ehd	EH domain-containing protein
ETC	Electron transport change
ETS	Environmental tobacco smoke
FADD	Fas-associated protein with death domain
Gap43	Growth associated protein
GFAP	Glial fibrillary acid protein
GL	Granular layer
HK1	Hexokinase 1

HSP70	Heat shock protein 70 kDa
IMAC	Immobilized metal ion affinity chromatography
iNOS	Inhibit inducible nitric oxide synthase
L1cam	Neural cell adhesion molecule L1
MAP	Mitogen-activated protein
Mapt	Microtubule-associated protein Tau
Map2	Microtubule-associated protein 2
Marks	Myristoylated alanine-rich C-kinase substrate
MBP	Myelin basic protein
MH+	Monoisotopic reduced mass
ML	Molecular layer
MS	Mass spectrometry
MT	Microtubules
Ncam 1	Neural cell adhesion molecule 1
NF-kB	Nuclear factor-kappa B
PABPC1	Polyadenylate-binding protein 1
PAHs	Polycyclic aromatic hydrocarbons
PD	Parkinson's disease
PD	Postnatal day
PEA-15	Phosphoprotein enriched in astrocytes
PKC	Protein kinase C
PLGS	Protein Links Global Server

PTM	Post-translational modifications
Rac	Ras-related C3 botulinum toxin substrate
RhoA	Ras homolog gene family member A
ROS	Reactive oxygenated species
RSP	Respirable suspended particulate
RT	Retention time
SHS	Secondhand smoke
SOD 1	Superoxide dismutase 1
TCA	Tricarboxylic acid
2D-LC/MS-MS	Two-dimensional liquid chromatography-tandem mass spectrometry
WM	White matter

Abstract

A STUDY ON THE BIOCHEMICAL AND CELLULAR EFFECTS OF ENVIORMENTAL TOBACCO SMOKE ON ADULT AND DEVELOPING RAT BRAIN BIOCHEMISTRY

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Major Director: Andrew K. Ottens
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Exposure to environmental tobacco smoke (ETS) has been recognized as a significant health risk for adults and children. In adults, ETS exposure has been linked to increased incidences of cardiovascular disease and dementia. In children, exposure has been linked to behavioral and cognitive deficits. Studies on the effects of ETS in the brain have been largely epidemiological, and have lacked a defined explanation of the molecular/biochemical effects of ETS. The present dissertation aims to test whether ETS exposure leads to altered biochemistry in the adult and developing mammalian brain. A rat

ETS exposure model was employed to investigate changes at the molecular and cellular level. In an adult ETS exposure study, we focused on markers of astrogliosis , oxidative stress, and cell death. We observed altered GFAP suggestive of reactive astrogliosis. Yet, markers of oxidative and cell stress were unaffected by ETS exposure in the brain regions examined. Increased degradation of αII-spectrin and dephosphorylation of serine$_{116}$ on PEA-15 indicated greater apoptotic cell death signaling in the brains of ETS exposed animals. β-synuclein was greatly upregulated by ETS, a neuroprotective protein previously reported to exhibit anti-apoptotic and anti-fibrillogenic properties. We next employed a rodent model of postnatal ETS exposure to investigate effects on developing cerebellum using a system biology approach involving mass spectrometry (MS). Proteins at statistically different abundance between groups were correlated with relevant biochemical processes and pathways by bioinformatics. ETS responsive data were enriched in elements associated with all aspects of aerobic respiration. These results were substantiated by orthogonal molecular measures, along with evidence for increase mitochondrial biogenesis/fission. These findings suggest an increase mitochondrial density driven by a demand for ATP. Further exploration of the ETS responsive proteome identified statistically significant associations of the ETS with neuron projections, in particular axon associated proteins and synaptic vesicles. Immunotbloting and microscopy experiments substantiated altered process outgrowth and synaptogenic processes. The presented data depict a striking modulation in cerebellar formation consequent to ETS exposure and the energy source to allow that modulation to occur. Our findings could provide a biochemical and cellular rational for adverse neurological effects observed in ETS exposed children.

Motivation

ETS, also known as secondhand smoke (SHS) and passive smoking, has become a recent global concern with known health risk for both non-smoking adults and children (1, 2). ETS is a combination of sidestream smoke from burning tobacco and mainstream (exhaled) smoke. The chemical composition is remarkably similar between mainstream smoke, sidestream smoke and ETS (3). Over 4,000 chemical compounds have been found in ETS (4). However, many chemical concentrations were observed to be ten-fold greater in sidestream smoke relative to mainstream smoke (5). Of the 4,000 identified chemical compounds an estimated 250 are known to be toxic or carcinogenic (6).

In 2006, the Surgeon General of the United States released a comprehensive study of the effects of ETS on toxicology, exposure, reproduction/development, respiratory effects, cancer and cardiovascular disease. Missing from the report was the under-reported effects of ETS on the adult brain. ETS effect on neuronal development was reported, citing evidence of a potential impact; however the report goes on to state that the available data on the relationship between ETS exposure and CNS function was limited and confounded and more research was needed (7). Their findings echo those of the World Health Organization's earlier report on the effects of ETS on childhood cognition and behavior. Both reports found that current research was either inconclusive or unclear about the relationship between ETS and its adverse effects on cognition and behavior among

children due to significant confounds in experimental procedures and study variations (2), and emphasize the need for further research.

Research into the effects of ETS has been focused on damage to the heart and lungs (8). Findings suggest the ETS exposure induces damage that is predominantly a result of cellular stress caused by increased reactive oxidative species (ROS) and cell death. Few studies have explored possible biochemical effects on the mature brain as a result of ETS exposure. Moreover if effects exist would they be detrimental during neuronal development. The motivation behind my graduate dissertation work is to further explore this gap in the research, elucidating the biochemical changes caused by ETS exposure in the adult and developing rat brain.

Research Objectives

The general hypothesis of my dissertation research is that ETS exposure will induce biochemical and cellular changes within the adult and developing rat brain. Previous research suggests that cellular stress, cell death, and other biochemical processes may be induced by ETS exposure**.** Proposed is a comprehensive study to test the effects of ETS in the adult and developing rat pup brain. The enclosed work focuses on the identification and elucidation of biochemical and cellular domains affected by exposure to ETS. To test my hypothesis, I pursued the following specific aims:

1. Evaluated cell stress and cell death dynamics within the brain using an *in vivo* ETS-exposure paradigm with adult rats. Numerous proteins involved in cellular stress and cell death as a result of ETS exposure were identified in the literature. However, the focus of earlier studies was on non-neuronal tissue such as heart, lungs and cells grown in culture. For this aim I characterized these and other proteins within the brain following ETS exposure in the adult rat (Chapter 2). Experiments involved a combination of immunoblot and mass spectrometry analyses.

2. Evaluated novel biochemical pathway in the developing rat brain affected by ETS exposure during cerebellar development using an *in vivo* ETS-exposure paradigm with rat

pups. Initial epidemiological studies have suggested a direct relationship between ETS exposure, hyperactivity disorders and cognitive/behavioral deficits. The ETS exposure model was adapted for rat pups in order to examine biochemical and cellular effect on cerebellar development. Differential proteomics using a label-free method established in our laboratory provided a novel holistic examination of effects on the developing rat cerebellum. A systems biology approach was employed to interpret and substantiate the mass spectrometry results. Microscopy further extended our results into the cellular domain.

CHAPTER 1

EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE IN ADULT BRAIN

1.1. Epidemiology of environmental tobacco smoke in adult brain.

Environmental tobacco smoke has been well documented for its effect on the pulmonary and cardiovascular system. Non-smokers exposed to ETS have a 25-30% increased risk for heart disease, with about 46,000 deaths annually. The increase in lung cancer risk for non-smokers exposed to ETS is between 20-30% with an estimated 3,400 deaths (1). While the negative effects of ETS have been well defined in the heart and lungs of exposed non-smokers what is less clear is the relationship between ETS and its effect on the brain.

The overall relationship between smoke and neurological disorders can be traced back to the 1950s. In 1959 Dorn published a study reporting an inverse relationship between cigarette smoking and Parkinson's disease (PD) risk (9). Since that time the debate on the effects that smoke has on the brain has been hotly debated. Two types of experimental paradigms have been utilized in studies on the effects of ETS on the brain. The first type relies on self-reported assessment surveys looking at the amount and duration of ETS exposure. The second relies on blood measurements of cotinine to determine the amount of ETS exposure. Cotinine is a metabolite of nicotine that has been widely used as a biomarker of ETS exposure in non-smokers. Both types of studies have

been used to examine the relationship between ETS and various neurological disorders such as dementia and Alzheimer's disease (AD).

A connection between ETS and dementia was first reported in 2007 at the annual meeting of the American Academy of Neurology by a research group at the University of California at San Francisco (10). Recent work has determined that cigarette smokers in older adults can lead to an approximate doubling in the risk of dementia (11). The current thought on the connection between ETS exposure and dementia is an increase in cerebrovascular disease (12). ETS has been well documented in the literature to cause negative vascular changes that can range from platelet aggregation to stroke (13, 14). Barnes et al. used this association as the rational for their 2010 study of ETS and dementia risk in older adults. They hypothesized that lifelong exposure to ETS among older adults who have underlying clinical or subclinical vascular disease could result in an increase risk of dementia. Their findings demonstrated a threefold increase of dementia risk in subjects who had a high level of exposure (self-reported) to ETS, and a 25% increase in carotid artery stenosis. From these finding the authors conclude that ETS, when combined with undiagnosed cardiovascular disease, can lead to an increase risk of dementia (11). Llewellyn et al. published a study exploring a possible link between cotinine measurements and cognitive impairment. Their results demonstrated that high levels of cotinine in non-smoking adults associated with increased odds for cognitive impairments (15). The authors did point out that previous studies in which a non-significant trend was reported between self reported exposure and dementia risk could have been due to the bias of self-reported data. The risk of Alzheimer's disease as a specific form of dementia has

only been examined in smokers (16). Tyas et al. found a association between smokers and AD risk, with a dose dependent relationship between the smoking and AD risk as well as an increased risk for cerebrovascular disease and dementias (16).

1.2. Molecular effects of environmental tobacco smoke exposure.

Environmental tobacco smoke is a combination of 15% mainstream smoke and 85% sidestream smoke each of which contains a large compilation of toxic and biologically-reactive chemicals. Mainstream smoke consists of a particulate and gas phases, both containing a high number of free radicals (17). Heavy metals are found in mainstream smoke as well as toxins such as ammonia, cresol, catechol, carbon monoxide, lead, ketone, nitiric oxide, phenol, styrene, toluene, and butane (18). Sidestream smoke has a similar chemical composition to mainstream smoke, but is made up of a greater concentration of the different components (5). Studies on sidestream smoke revealed that animals exposed to sidestream smoke only had higher concentrations of carboxyhemoglobin, nicotine and cotinine in their blood relative to those exposed to mainstream smoke only. It was also found that sidestream condensates were more tumerogenic than mainstream condensates (19, 20).

Cell Stress

Side stream and mainstream smoke contain reactive, long lived free radicals (21, 22). Gaseous free-radicals and other chemicals will induce production of reactive oxygen species (ROS) in the body; e.g., superoxide anions, reactive aldehyde species, nitric oxides and peroxynitrite (21). Polycyclic aromatic hydrocarbons (PAHs) can generate

ROS, are carcinogenic and are potent atmospheric pollutant, are also in mainstream smoke (23). Cigarette smoke has been shown to promote protein oxidation *in vivo* resulting in oxidative stress along with the reduction of circulating antioxidants (24-26). Further, cigarette smoke contains heavy metals such as cadmium (27) that are detrimental to the nervous system (28), easily crossing the blood brain barrier and catalyze free-radical reactions to promote cell death by apoptotic and necrotic pathways (29).

Oxidative stress can result in tissue damage when pro-oxidants (ROS) become prevalent over anti-oxidants. For example, superoxide anions produced from excess mitochondrial respiration will cause oxidative damage by nitrosylating tyrosine residues on proteins. Another ROS, hydrogen peroxide, is a dangerous byproduct of oxidative metabolism that causes cytotoxic effects in cells if not removed quickly. Hydrogen peroxide has been found to cause increase cell death in an aged mouse strain of cultured astrocytes (30). Free radicals contained within cigarette smoke can activate redox sensitive transcription factors such as nuclear factor-kappa B (NF-kB), which can lead to the transcription of pro-inflammatory genes in the brain (31), to promote glial activation as previously monitored by increased levels of glial fibrillary acidic protein (GFAP) (32, 33). Reactive gliosis has also been suggested as a possible consequence of prenatal and perinatal exposure to ETS, though; this suggestion was based on observed changes in cell size rather than molecular markers (34). Mazzio et al. have found cigarette smoke to inhibit inducible nitric oxide synthase (iNOS) in glia (35). iNOS is an enzyme responsible for the generation of nitric oxide. Nitric oxide is a potent signal in many diverse physiological processes at low concentrations but at high concentrations may cause DNA

damage and cell death (36). iNOS inhibition can be used as another indicator of oxidative damage within examined tissues in response to cigarette smoke exposure. In all, cigarette smoke promotes ROS within the cell which must be counteracted to prevent oxidative damage to cellular structures by anti-oxidants.

The anti-oxidant defense system functions to process ROS and limit the toxicity. For example, ascorbic acid is an anti-oxidant that acts as a free radical scavenger in plasma, and is a frontline defense against ROS. Research has shown that anti-oxidant levels are reduced by ETS exposure, disabling the body's natural defense against ROS damage. Ascorbic acid levels in blood were reduced in children exposed to ETS in the home - even with minimal exposure (37). Another study by Mendez-Alvarez et al. found that cigarette smoke inhibited the activity of the anti-oxidant catalase in the brain (38). Active smoking was also found to affect the anti-oxidant enzymes superoxide dismutase and glutathione peroxidase, which results in a significant increase in oxidative stress (39). These data highlight an important detrimental effect of ETS – not only to induce ROS but to also cripple the body's natural defenses against ROS resulting in oxidative damage.

Cell Death

The two well defined cell death processes are necrosis, a non-programmed, involuntarily event and apoptosis, which is a controlled form of cell death that is triggered voluntarily by activation of discrete molecular pathways (40). In contrast to necrosis, morphologically and biochemically, apoptosis is a programmed form of cell death – the ordered process of turning off and dismantling the cell. Apoptosis can be triggered by the

immune system, signaling cells that have been damaged beyond repair with Fas or TNF ligands. ETS exposure has been shown to induce increased apoptosis in lung tissue due to irreversible cell damage (41), which can be monitored by the activation of caspases. Caspases are a family of calcium-independent cysteine proteases integral to apoptosis pathways. Caspase 8 is activated when an extrinsic "death signal" is received at the cell membrane by Fas or TNF receptors. Conversely, caspase 9 is activated when an intrinsic signal is received. The extrinsic and intrinsic signal cascades both activate caspase 3, which is the primary executioner caspase. Caspase 3 will cleave important cellular proteins leading to the morphological changes observed during apoptosis. Though caspase activation is specific to apoptosis, it is also important to note that calpains are also activated during apoptosis leading to further protein degradation. Kuo et al. made a similar connection between ETS exposure and apoptosis in rat heart muscle cells, observing that adult rats exposed to ETS had increased levels of caspase 3, Fas, and the active form of caspase 9 in their tissue (42). Their work demonstrates a molecular basis for apoptotic cell death and tissue damage as a result of exposure to ETS.

In chapter two we present our first study on the molecular and cellular effect of ETS on the brain, which were begun with adult animals. Given limited literature on the effects of ETS on the brain our initial aim was to demonstrate a measurable effect of ETS exposure relative to control on the mature rat brain. Our hypothesis was based on the literature suggesting potential oxidative stress, gliosis, and apoptotic cell death. Both oxidative stress and cell death were previously identified as possible molecular mechanism for negative effects of ETS on coronary and pulmonary systems. It is also of note that our

lab also explores the molecular effects of traumatic brain injury, from which we built the tools used in our ETS studies on the adult rat brain.

CHAPTER 2

EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON ADULT RAT BRAIN BIOCHEMISTRY

2.1. ABSTRACT

Environmental tobacco smoke (ETS) has been linked to deleterious health effects, particularly pulmonary and cardiac disease; yet, the general public considers ETS benign to brain function in adults. In contrast, epidemiological data has suggested that ETS impacts the brain and potentially modulates neurodegenerative disease. The present study begins to examine yet unknown biochemical effect of ETS on the adult mammalian brain. In the developed animal model, adult male rats were exposed to ETS three hours a day for three weeks. Biochemical data showed altered GFAP levels as a main treatment effect of ETS suggestive of reactive astrogliosis. Yet, markers of oxidative and cell stress were unaffected by ETS exposure in the brain regions examined. Increased proteolytic degradation of αII-spectrin by caspase-3 and the dephosphorylation of serine$_{116}$ on PEA-15 indicated greater apoptotic cell death modulated by the extrinsic pathway in the brains of ETS exposed animals. Further, β-synuclein was upregulated by ETS, a neuroprotective protein previously reported to exhibit anti-apoptotic and anti-fibrillogenic properties. These findings demonstrate that ETS exposure alters the neuroproteome of the adult rat brain, and suggest modulation of inflammatory and cell death processes.

2.2. INTRODUCTION

Exposure to environmental tobacco smoke (ETS) is a known health risk in adults and children. A recent U.S. Surgeon General Report (2007) reviewed the causal relationship of ETS with disease, particularly pulmonary and cardiac. Neurological effects were deemed inconclusive, with the stated need for more research (7). Yet, ETS is a known risk factor for cerebrovascular disease (43-45). Further, epidemiological studies recently indicated ETS as a risk factor for Alzheimer's disease (AD) ((10, 15). At the same time, ETS appears to decrease risk for Parkinson's disease (PD) (46). Despite an apparent clinical impact on the adult brain, the molecular influence of ETS is underexplored, and is largely considered benign.

ETS administered has been shown to alter morphology during primate brain development (34). ETS exposure caused an increase in smaller glial cells suggestive of reactive astrogliosis. Astrogliosis was also observed in the developing brain after prenatal nicotine exposure, with increased glial fibrillary acid protein (GFAP) levels in the cerebellum and hippocampus of offspring (33). Importantly, GFAP levels remained elevated out to postnatal day 60, and deficiencies in basic sensory motor skills were observed (32), a long term, functional effect. Susceptibility to ETS may differ between the developing and mature mammalian brain, but these data raise the potential of an astrocytic response following adult ETS exposure.

Chemicals from ETS have also been found to influence apoptotic processes in cell cultures. Apoptosis is a cell death processed that can be triggered via internal or external cues. Regulation of extrinsic apoptosis involves PEA-15 (phosphoprotein enriched in

astrocytes), an inhibitory protein that binds FADD (Fas-associated protein with death domain) when phosphorylated on Serine 116 (S_{116}) (47). Dephosphorylation leads to caspase 3 activation and subsequent protein cleavage events. One such cleaved protein is αII-spectrin, the cleavage product of which is a known marker selective for apoptosis in neurons (48). ETS chemical extracts induced apoptosis in cardiac cells, with increased Fas and active forms of caspases 3 and 9 (41, 42). In contrast, nicotine administration alone was neuroprotective in spinal cord neurons challenged by apoptosis inducing arachidonic-acid. Activation of caspases 3, 8 and 9 and release of cytochrome c were all reduced with nicotine relative to vehicle (49, 50). These data illustrate how the effects of ETS, with a complex chemical formulation (51), may not be sufficiently modeled by nicotine administration alone. Different components of ETS may induce competing pro- and anti-apoptotic responses.

Given the aforementioned relationships between ETS exposure and the molecular processes astrogliosis and apoptosis in other systems, we present this initial study examining molecular effects in the adult mammalian brain. The glial selective marker GFAP and the neuronal selective caspase 3 proteolytic fragment of αII-spectrin were employed to characterize these processes across multiple brain regions. Mass spectrometry methods were also employed to test for ETS effects on the neuroproteome. This initial assessment focused on the limbic areas frontal cortex and hippocampus as well as cerebellum. Hippocampus and cerebellum were areas shown affected by ETS in developing brain, and limbic regions, such as frontal cortex and hippocampus, are known

to be affected in smokers (52). Further, these limbic areas are affected by increased apoptotic and gliotic pathology in adult onset neurodegenerative disease (53-55).

2.3. EXPERIMENTAL PROCEDURES

Animal Procedures and Tissue Collection- Ten-week old male Sprague Dawley rats (Harlem, Indianapolis, IN) were acclimated to the laboratory for four days prior to exposure. Rats were kept under a controlled environment and housed two to a cage. Food and water were provided *ad libitum* except during exposure when food was removed. No enrichment was provided to either the control or treated groups.

Following acclimation, rats were placed in a Teague TE-10 smoke exposure system (56) for three hours per day over a three week period. The ETS group (n=8) was exposed to a mixture of 15% mainstream (aspirated through filter) and 85% sidestream smoke diluted with air to a concentration of 5 mg/m^3 of respirable suspended particulate (RSP). The control group (n=8) was exposed simultaneously to room air. During each exposure, twenty Kentucky 3R4F reference cigarettes (University of Kentucky, Lexington, KY) were smoked at a rate of one puff per minute, two seconds per puff (35 cm^3) for eight puffs in nine minutes per cigarette. This model provided comparable airborn exposure as experienced in a 50 m^3 household room (0.7 air changes per hour) with a smoker consuming 2 cigarettes per hour over 10 hours (per tables in (57)), which was validated by repeated mass measurements of respirable suspended particles.

A day after the last ETS exposure, rats were decapitated under deep anesthesia (5% isoflurane for 5 minutes). Frontal cortex, hippocampus and cerebellum were dissected, and

snap frozen in liquid nitrogen. All procedures conformed to the United States Public Health Service policy with approval of the Institutional Animal Care and Use Committee.

Statistical analysis- Experiments were performed with n=4 biological replicates per group. Multivariable datasets were analyzed by a two-way repeated measure ANOVA with the Holm-Sidak distribution test and Bonferroni correction. Single variable datasets were analyzed by a t-test with a Kolmogorov-Smirnov distribution test. A Q-test was applied to identify outlier values.

Immunoblotting- Lysates were prepared from the brain tissue as described before (58). Protein concentration was determined via Bio-Rad DC Protein Assay (Hercules, CA). Protein-balanced samples were prepared for SDS-PAGE, 4-20% Tris-glycine gel, in a two-fold Tris-glycine loading buffer (Invitrogen, Carlsbad, CA). Samples were heated for 90 s at 90°C, and centrifuged for 2 min. Following electrophoresis, separated proteins were transferred to polyvinylidene fluoride membranes by the semi-dry method. Membranes were probed with primary antibodies to: GFAP (Millipore, Billerica, MA) at 1:5,000; αII-spectrin caspase-3 breakdown product (University of Florida, Gainesville, FL) at 1:2,000; β-synuclein (BD Biosciences, San Jose, CA) at 1:20,000; α-synuclein (BD Biosciences) at 1:1,000; heat shock protein 70 (Stressgen, Victoria, BC) at 1:2,500; inducible nitric oxide synthase (BD Biosciences) at 1:5000; superoxide dismutase 1 (Millipore) at 1:500; β-actin (Sigma-Aldrich, St. Louis, MO) at 1:2000. The blots were then incubated with a biotinylated-conjugated secondary antibody followed by a streptavidin alkaline

phosphatase conjugate. Bound antibodies were visualized by colorimetric development with the phosphatase substrate BCIP/NBT (KPL, Gaithersburg, MD). Quantitative evaluation of protein levels was performed via densitometric analysis of 16-bit grayscale images using Image J software (National Institute of Health, v 1.6, Bethesda, MD).

Mass spectrometry- Fresh-frozen hippocampus tissues were prepared for IMAC (immobilized metal ion affinity chromatography) analysis as described previously (59). Briefly, Trizol reagent (Invitrogen) was employed for protein extraction per the manufacturer instructions. The protein pellet was resuspended with phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined by DC protein assay. Protein (50 µg) was then reduced and alkylated with DTT and iodoacetamide, respectively, and digested with endo-Lys-C (Roche, Indianapolis, IN) overnight at 37°C. ETS exposed and control group samples were reacted for 2 h with heavy and light methanolic HCl, respectively, as described previously (60). Sample pairs were loaded onto a Poros MC (PerSpective Biosystems, Framingham, MA) packed IMAC column, and separated as described previously (59). The phosphopeptide enriched fractions were separately resolved by reversed-phase gradient separation from 0.7% to 28% acetonitrile/0.2% formic acid in 150 min online with a ThermoElectron (San Jose, CA) LTQ Orbitrap XL with electron transfer dissociation source (61). ETD produced c/z· spectra were searched against a Uniprot Rattus protein database (v14.1) and the reversed image of that database with the OMSSA search engine, and were filtered for a 1% false-detection rate. Integrated peak areas were used to quantify the deuterated and non-deuterated forms of the PEA-15

apoptosis-signaling phosphopeptide.

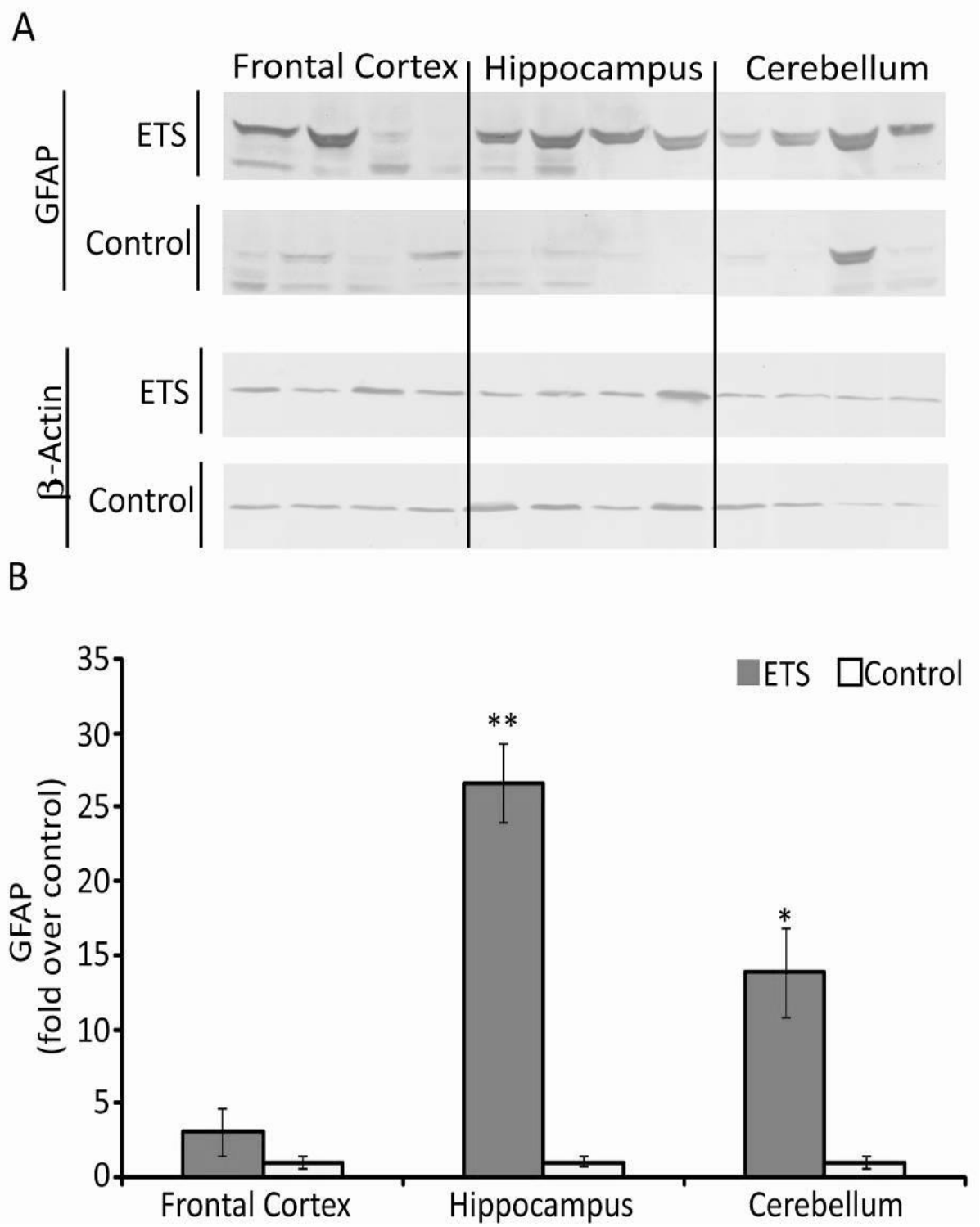
2.4. RESULTS

ETS exposure animal mode- The ETS exposure model developed for this study produce no notable stress in the research animals. Exposed and control animals were handled daily. Normal curiosity to a change in environment was observed for all animals. Animals exposed to ETS displayed less spontaneous movement during exposure than controls, but became immediately alert and explorative whenever the ETS exposure ceased (prior to any other change in environment). The pre-exposure mean body mass for the ETS animal group was 5 g less than the control group, and finished 8 g less 3 weeks later. Overall, no statistical difference in weight gain rate was observed between the two groups.

ETS induces GFAP expression in brain- GFAP was modulated as a main treatment effect of ETS exposure ($p = 0.003$). Multiple comparison analysis also showed an ETS treatment within brain region interaction on GFAP expression (Fig. 2-1) that was statistically significant in hippocampus ($p = 0.001$), and cerebellum ($p = 0.02$). Frontal cortex exhibited a lower, non-significant ($p = 0.09$) interaction, with the data exhibiting a bimodal distribution of individual values, confirmed by replicate assay to rule out experimental error.

ETS affects markers of apoptosis, not cell stress- ETS exposure also had a main treatment effect on increased caspase-3 proteolysis of αII-spectrin in the adult rat brain ($p < 0.001$) in exposed animals. There was also an interaction of ETS treatment within brain

Fig. 2-1: Effect of ETS on the astrogliosis marker GFAP in the adult rat brain. (A) GFAP immunoblot analysis as a marker of astrogliosis in three regions of the adult rat brain after a 3-week ETS or room air exposure. (B) Normalized densitometric quantification of GFAP with β-actin used as a loading control. Symbols indicate significant differences from control (* $p \leq 0.02$, and ** $p < 0.001$) by a repeated measures ANOVA multiple comparison of treatment x brain area.



regions (Fig. 2-2), with a statistically significant increase in frontal cortex ($p = 0.001$). The breakdown product was up for ETS treatment within hippocampus and cerebellum as well, but the observations were not statistically significant ($p = 0.03$ and $p = 0.4$, respectively). Mass spectrometry analysis revealed dephosphorylation of PEA-15 at S_{116} ($p < 0.001$) in the hippocampus of ETS exposed animals. Site specific phosphorylation was confirmed with the selective pattern of c and z· (ETD) fragment ions (Fig. 2-3). Additional immunochemical studies showed that levels of the oxidative and cell stress associated proteins (data not shown), inducible nitric oxide synthase (iNOS), superoxide dismutase 1, and heat shock protein 70 kDa (HSP70) were unaffected by ETS exposure across the three brain regions examined in this study.

ETS induces synuclein protein expression- Mass spectrometry analysis also revealed a large quantitative difference in synuclein protein abundance, though the data lacked isoform specificity. Further immunochemical analysis determined that it was the neuroprotective isoform β-synuclein which increased ($p < 0.001$) in the hippocampus of ETS exposed animals relative to room-air controls (Fig. 2-4). In contrast, aggregate forming α-synuclein expression trended slightly lower among ETS exposed animals, but was not a statistically significant difference.

2.5. DISCUSSION

ETS exposure was verified to influence the adult rat brain neuroproteome in this investigative study. Molecular effects were observed across multiple brain areas while animal growth, as a basic physiological measure, was unaffected. Changes in protein

Fig. 2-2: Effect of ETS exposure on a neuronal apoptosis marker in the adult rat brain. (A) Immunoblot analysis of the neuronal αII-spectrin caspase-3 breakdown product as a marker of apoptosis in three regions of the adult rat brain after a 3-week ETS or room air exposure. (B) Normalized densitometric quantification of the breakdown product with β-actin used as a loading control. Symbols indicate significant differences from control (* $p \leq 0.02$, and ** $p < 0.001$) by a repeated measures ANOVA multiple comparison of treatment x brain area.

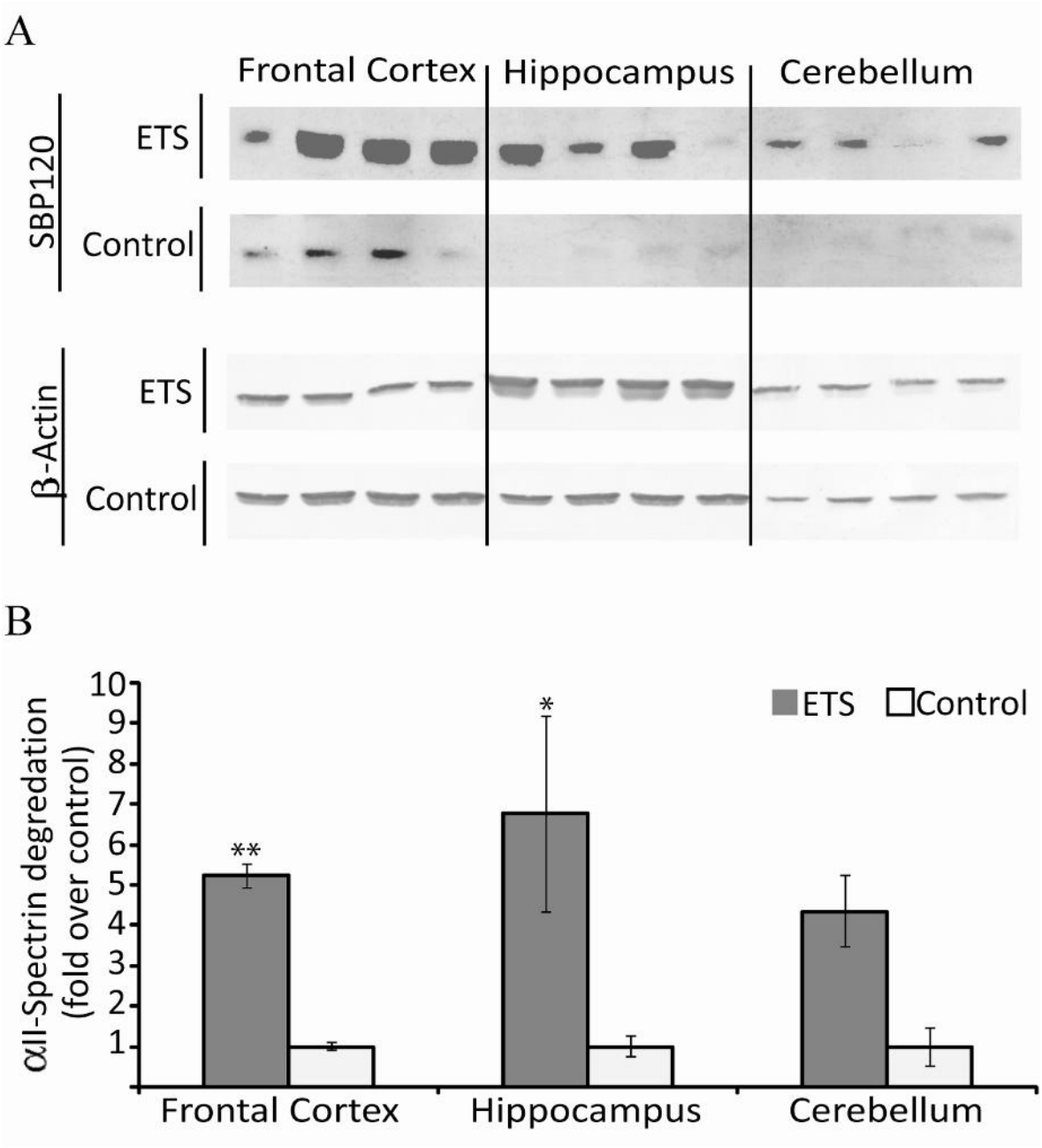


Fig. 2-3: Effect of ETS exposure on DISC inhibiting dephosphorylation of PEA-15. (A) Example electron transfer dissociation spectrum of $c/z^{\cdot}$ fragment ions selectively identifies the phosphorylated S_{116} site, with the phosphorylation state assessed for ETS exposed (B) and control (C) groups by chromatographic peak integration.

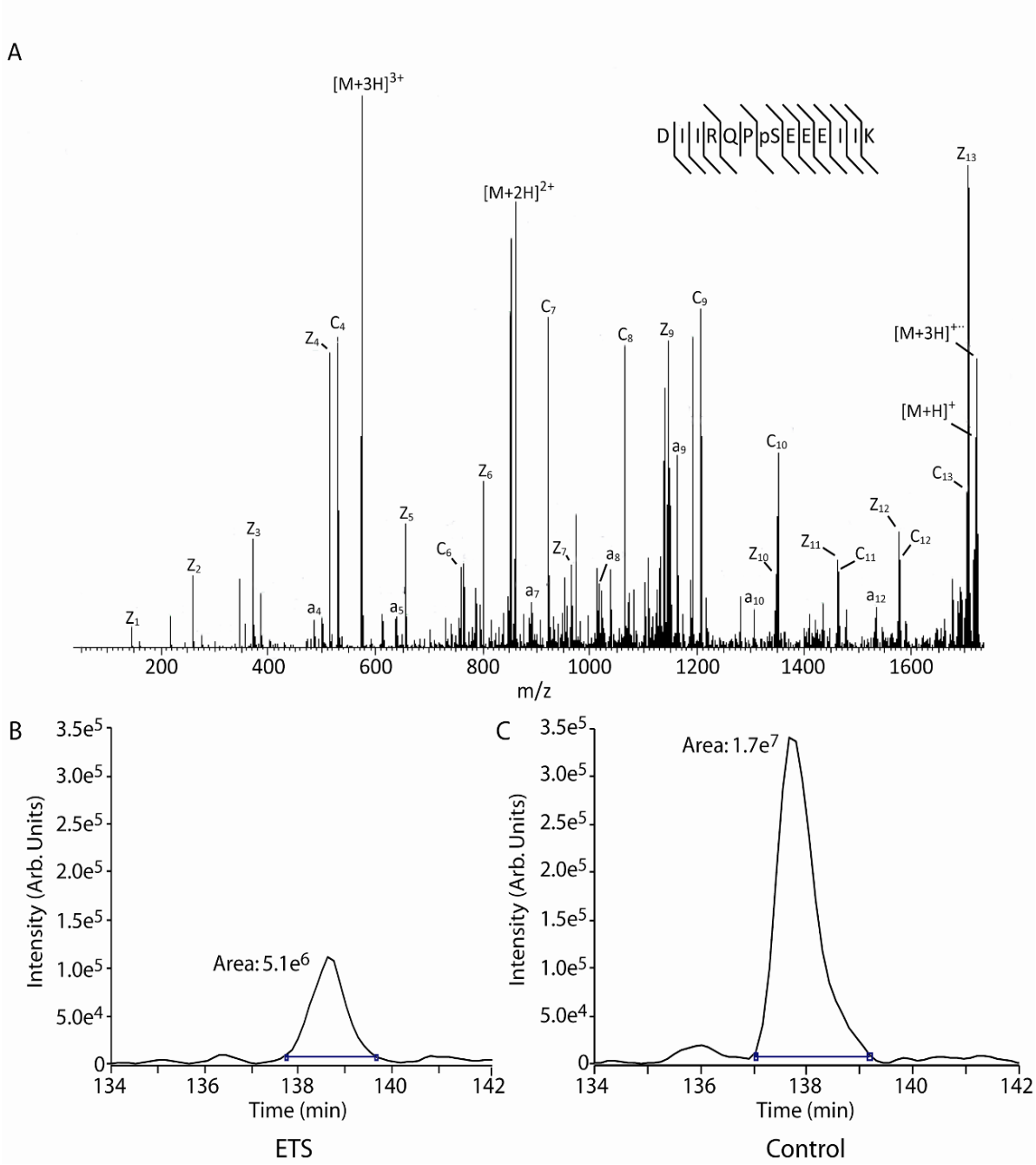
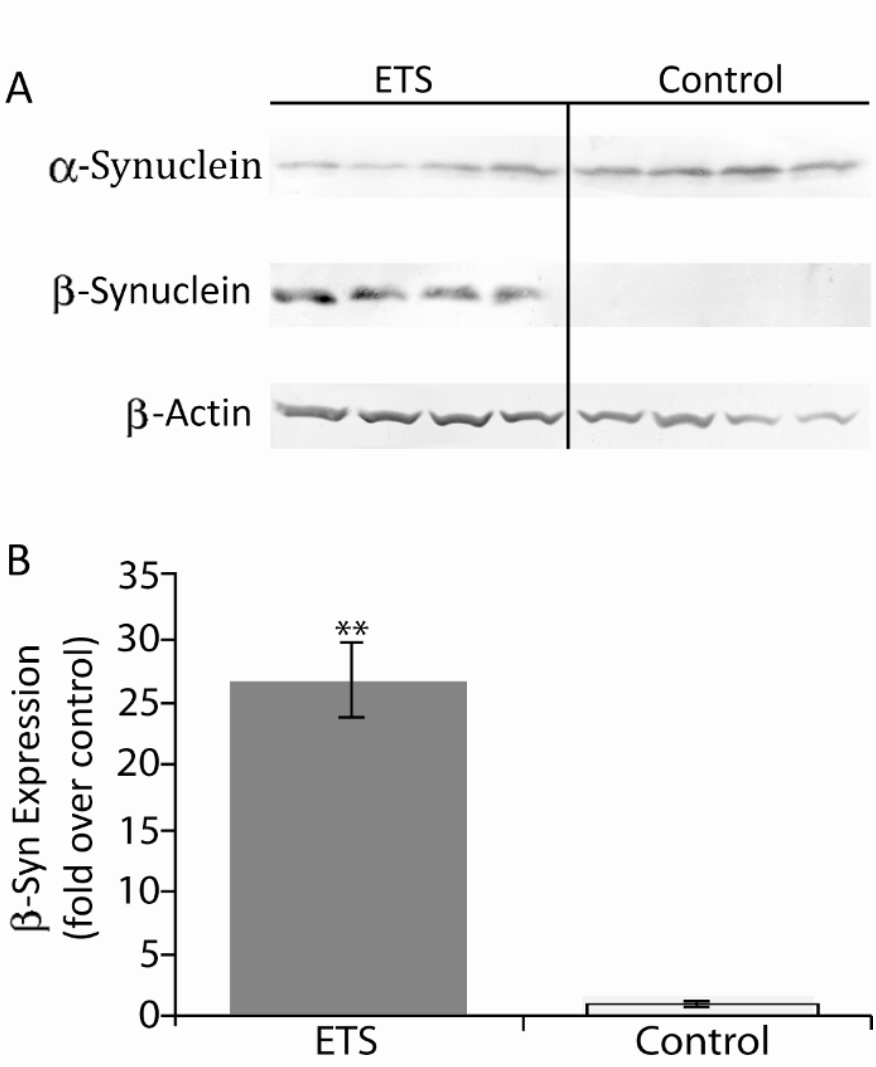


Fig. 2-4: Effect of ETS exposure on synuclein proteins in the adult rat hippocampus. (A) Immunoblot analysis was performed with antibodies against α- and β-synuclein with hippocampal tissue from adult rat brain after a 3-week ETS or room air exposure. (B) Normalized densitometric quantification of synuclein proteins with β-actin used as a loading control. Symbol indicates a significant differences from control (** $p < 0.001$) by a t-test.



markers suggested modulated astrogliosis (GFAP), apoptotic cell death (cytoskeletal degradation of neurons, and DISC complex formation), and the over-expression of β-synuclein as a potential neuroprotective response.

ETS induces astrogliosis in adult rat brain- ETS resulted in modulation of GFAP across the three brain areas examined. GFAP levels are a known marker of astrogliosis in damaged brain (62). The ETS induced difference in GFAP was statistically significant as a main effect and as a regional interactive effect in hippocampus and cerebellum. The adult GFAP results correlate with previous observations of increased GFAP expression consequent to prenatal nicotine exposure (32, 33) and an increase in cell density in the developing brain from ETS (34).

ETS induces apoptosis in adult rat brain- Apoptotic cell death was demarked by greater caspase 3 degradation of neuronal αII-spectrin in the adult rat brain. Increased degradation was a main effect of ETS treatment; though, multiple comparison analysis showed statistically significant treatment within region interactions for frontal cortex. Apoptosis has been reported as a direct result of cigarette smoke induced cell stress in other organs and cultures. ETD tandem mass spectrometry revealed the dephosphorylation of PEA-15 at S_{116} in ETS exposed hippocampus, suggesting the activation of the extrinsic apoptotic pathway, which was previously found activated by ETS in cardiac cells (41, 42).

Neuroinflammation can induce the extrinsic apoptotic pathway through the production of reactive oxygen species (ROS). The ROS nitric oxide is often, though not always (63, 64), induced from increased iNOS expression. In this study, ETS treatment showed no effect on iNOS expression by immunoblot. While ETS may induce astrogliosis, it separately may be suppressing iNOS expression in glia. Cigarette smoke condensates were previously observed to inhibit iNOS in glial cultures (35). Superoxide dismutase 1 (SOD 1), an antioxidant that is also upregulated as a response to brain ROS (65). SOD 1 expression, like iNOS, was unaffected by ETS exposure in this study. Rats exposed to direct smoke (as opposed to ETS) have been shown to express significantly greater levels of HSP70 in brain (66), pulmonary airways (67), and mammalian cell cultures (68). HSP70 is a neuroprotective protein upregulated during times of cell stress; however, in this study, HSP70 expression was also found to be unaffected by ETS exposure in replicate assays. Together, these data suggest that ROS is not induced by ETS exposure in the brain areas studied with this model, and is, therefore, not likely the causal factor promoting apoptosis.

ETS affects β-synuclein protein expression- β-synuclein expression was upregulated in ETS exposed animals. α- and β-synuclein are functionally distinct, with the α isoform prone to Lewy body forming aggregation found in the PD brain (69). In contrast, β-synuclein is immune from aggregation due to its lack of a non-amyloidogenic domain (70). β-synuclein has shown anti-apoptotic properties through down-regulation of p53 expression (71). β-synuclein has also been shown to restore the anti-apoptotic function

of α-synuclein (71). The marked increase in β-synuclein levels observed here may be a neuroprotective response to the observed neuronal apoptosis.

Smoking has long been known to reduce PD incidence (9) in a dose dependent fashion (72, 73). A recent epidemiological study showed ETS as having a similar effect on PD incidence (46). Many studies have explored a connection between smoke exposure and PD neuroprotection. This is the first study to demonstrate increased expression of neuroprotective β-synuclein by ETS, which could be involved in reduced PD incidence. β-synuclein is known to inhibit α-synuclein aggregation in addition to its anti-apoptotic properties (71, 74). A recent study, pointed to nicotine as an active agent that retards the fibrillogenic activity of α-synuclein (75). Future work will examine β-synuclein as an intermediate in PD relevant brain regions.

In conclusion, the results from this study demonstrate a main treatment effect of ETS on adult rat brain biochemistry, which begins to dispel the notion that ETS exposure is benign to the adult mammalian brain. The data point to modulated apoptosis and astrogliosis via increases in markers of these processes, but without the influence of ROS. The data also suggest differences among individual animals that may signify variable susceptibility to ETS effects. β-synuclein expression is significantly increased by ETS exposure, which may be a neuroprotective response with a potential benefit relative to PD. Future work will investigate the induction of apoptosis by ETS and its connection with the increase in β-synuclein in the adult rat brain.

CHAPTER 3

ENVIRONMENTAL TOBACCO SMOKE IN BRAIN DEVELOPMENT

3.1. EPIDEMIOLOGY OF ENVIRONMENTAL TOBACCO SMOKE IN CHILDREN

The National Cancer Institute and the World Health Organization released a 1998 report on the effects of ETS on childhood cognition and behavior. Their report concluded the existing research was insufficient to establish a relationship between ETS and adverse effects on cognitive and behavior domains among children. A lack of clarity resulted from significant confounds in epidemiological experimental procedures and study variations (2). Six years later it was estimated that 11% of all US children age 6 and under were still exposed to daily ETS (57). Reports that focused on cognitive development assessed a wide range of variables - scores for reading and language skills, grade retention and standardized tests that measure specific skills like speech, intelligence and special abilities. A number of reports found varying degrees of association between ETS and cognitive development (76-79). Roeleveld et al. found that exposure to ETS during pregnancy and the first 6 months postpartum led to an increased risk of mental retardation (80). These findings are significant since 50% of mothers who quit smoking during pregnancy resumed within 6 months after giving birth (81). Fried et al. observed that postnatal ETS exposure, led to impaired language abilities (82). Other studies using multiple standardized behavioral and learning aptitude tests have found a statistical difference in approximately 80% of tests

administered. However, there also have been a number of reports that could not demonstrate an association between ETS exposure and increased cognitive deficits (83-85).

Studies linking ETS exposure and behavioral problems in children have been limited. Weitzman *et al*., 1992, reported a direct correlation between heavy maternal smoking and behavioral problems (86). Similar findings were reported by Makin et al. where children exposed to ETS were reported to have more behavioral problems than children who were not exposed to ETS (77). Fergusson et al. also demonstrated a statistically significant association between ETS exposure and childhood disruptive behaviors including conduct problems and attention deficit behaviors (87). These findings appear to correlate with results from Wakschlag et al. who found that maternal smoking lead to an increase in behavioral problems in male offspring (88). Other behavioral disorders such as attention deficit hyperactivity disorder (ADHD) have also been linked to ETS exposure (89, 90).

The epidemiology suggests that ETS may cause cognitive and behavioral problems in children; however, not enough research has been conducted in this vital area to conclusively demonstrate this fact. The need for more research was emphasized by the U.S. Surgeon General's report on ETS exposure (7). It is of particular interest that during the time period when researches were examining a link between ETS and developmental issues, others were beginning to link the cerebellum with some of the same cognitive and behavioral domains.

3.2. CEREBELLUM ARCHITECTURE

The cerebellum is a highly folded brain region that is structurally conserved across mammals, including humans. Distinct layers can be observed within the folded cortex of the cerebellum. The outermost layer of the cerebellum is the molecular layer, which contains the dendritic arbors of Purkinje cells, and parallel fibers, the excitatory granular cell axons. The molecular layer also contains stellate and basket cells, which are inhibitory interneurons that synapse onto Purkinje cells. Below the molecular layer is a single-cell thick layer of Purkinje cells, which receive input from parallel fibers and form the inhibitory output of the cerebellar cortex. Below the Purkinje layer is the granular layer, which is the most densely packed compilation of neurons in the entire brain. Below the granular layer are white matter tracts that contain the myelinated axons entering the cerebellar cortex. Input into cerebellum occurs via climbing fibers and mossy fibers. Climbing fibers originate from the inferior olivary nucleous and synapse onto multiple Purkinje cells, but each Purkinje cell can only receive input from one climbing fiber. Mossy fibers on the other hand terminate onto granular cells. The organization of layers and connections of the cerebellum are relatively homogenous compared with other brain regions. The cerebellum matures later in development, much like the prefrontal cortex. Much of molecular layer formation, the most significant synaptic organization in the cerebellum, occurs after birth in both rats and humans.

The cerebellum has long been recognized for its control of muscular coordination of complex movements and motor learning. However, neurobehavioral, neuroimaging, and clinical studies over the past twenty years have begun to identify the cerebellum as critical

to cognitive functions partially involving language and attention (91). Neuroimaging studies have found a direct relationship between increased activation of the dorsolateral prefrontal cortex and the cerebellum during the completion of a cognitive task. This co-activation has been seen during a wide variety of cognitive assessment task such as the verb generation task (92), verbal fluency task (93), Wisconsin card sorting task (94), and various other non motor, working memory tasks (95-97). The activation of the dorsolateral prefrontal cortex and the cerebellum are critical parts of a neuronal circuit when a cognitive task is difficult for the participant, novel, under new experimental conditions, if a quick response is required, and if the participant must concentrate on the task at hand (98). Similar, findings were found in patients who were injured in the cerebellum. These patients fail typical tests associated with the prefrontal cortex such as verb generation (99), verb frequency (100), or working memory tasks (99). However cerebelluar injury patients have no trouble completing the Wisconsin card sorting test (101). The cerebellum has also been linked to attention and hyperactivity disorders such as ADHA, which incidentally have also been linked to ETS exposure.

Often overlooked aspects of ADHD are motor control issues. Studies have found that many ADHD children also have poor motor control (102, 103). It has also been observed that boys with ADHD have smaller cerebellums relative to normal controls (104, 105). The cerebellum has been shown to be vulnerable to perturbations during development. As early as 1975 Lynch et al. found an association between malnourishment of pregnant rats during gestation and lactation produced prodigy who showed signs of motor control deficiencies (106). Further quantitative stereological procedures at the light

and electron microscope levels found a 31% decrease in the synapse-to-neuron ratios of the granular layer, and a decrease in both the size and amount of Purkinje cells in rats who were undernourished during early postnatal life (107, 108). Exposure to alcohol was also reported to cause a decrease in Purkinje cell population of neonatal rats but not gestating or adult rats (109). The vulnerability in the postnatal state was further explored with x-ray irradiation and under nourishment induced injury in the cerebellum. These experiments discovered a vulnerable period from birth through postnatal day (PD) 21 in rats, where damage to the cerebellum resulted in sustained and functionally significant changes (110). Damage to the cerebellum following PD8 resulted in no significant motor deficits, but instead induced hyperactivity (111). Children with cerebellar hypoplasia exhibited significant impairments, but the most notable area affect was attention (91). Combining all the evidence, we hypothesized that exposure to ETS during the vulnerable period of cerebellar development could result in perturbed formation, which consequently may be involved in cognitive and behavioral deficits potentially impacted by ETS.

3.3. SYSTEMS BIOLOGY

Systems biology is the holistic study of biological systems. Involved is the study of interactions from how a group of proteins act in a metabolic pathway, to how that metabolic pathway interacts with other pathways in a cell, to how that cell interacts with the cells around it, and so on. The key is the identification of the interactions and the elements that comprise them. In 2004, Weston et al. listed and explained five key elements that allowed for the emergence of systems biology as a field of study. The first was the

completion of the human genome project, which led to further discoveries on the mRNA and proteins in individual organisms and cell types. The second was the rise of cross-disciplinary biological science, which has allowed for the combined knowledge of scientist, engineers, mathematicians, chemists and computer sciences. Next was the internet followed by the idea of biology as a science of information. Large amounts of data comprised of gene and protein sequences are now actively being sought out and shared between scientists over the internet. Finally advances in high-throughput platforms for the study of genomics, proteomics, and metabolomics have allowed the creation of massive amounts of data (112). Recently, advances in proteomics have placed it at the forefront of systems biology research (113, 114).

Proteomics is the study of proteins encoded by a genome, their modification, dynamics and cellular function. The application of proteomics to systems biology follows a logical research progression from DNA to mRNA to protein. While they provide a wealth of information, mRNA levels only relate indirectly to protein levels, a balance between synthesis and subsequent processing. Also mRNA levels cannot detect biologically relevant changes at the protein level such as post translational modifications (PTMs), *spacial* information, and interactivity of proteins. However there have been many resources created for analyzing genomic data to discover gene or interactions and provide gene enrichment data. Enrichment analyses use computational methodology to determine if a set of genes or proteins exhibits statistically significant correlation to a specific function, localization or biochemical pathway relative to chance. The ToppGene Suite provides publically available tools for enrichment analysis that can be utilized to for enrichment

analysis, and display significant associations in molecular function, biological process, cellular component and pathways (115). Detailed mapping of relevant protein pathways can be accomplished using the KEGG database tools (Kyoto Encyclopedia of Genes and Genomes) (116, 117). Another valuable tool for understanding protein interactions is protein network analysis. The String database offers a publically accessible collection of tools to mine known and predicted protein-protein interactions (118). At the time of our research the String database contains information from 2,590,259 proteins across 630 organisms. These tools allow a user to examine a proteomics data set that can include hundreds of responsive proteins to visualize interactivity and aide the investigator in deciphering relevance within the biological system.

Chapters four and five represent a paradigm change to a holistic, systems biology experimental design in contrast with the reductionist design in chapter two. Chapters four and five are focused on elucidating the biochemical changes in the developing rat cerebellum initialized by utilizing a peptide-centric mass spectrometry approach. Bioinformatics allowed us to identify 662 responsive peptides to ETS treatment. Further analysis divulged relevant biochemical pathways and cellular structure modulated by ETS.

CHAPTER 4

SYSTEMS BIOLOGY REVEALS ALTERED MITOCHONDRIAL PROLIFERATION AND METABOLISM IN DEVELOPING CEREBELLUM CONSEQUENT TO ENVIRONMENTAL TOBACCO SMOKE EXPOSURE

4.1. ABSTRACT

Postnatal environmental tobacco smoke exposure, ETS, has recently been linked to behavioral and cognitive deficits in children. The functional domains found affected by ETS are symptomatic of attention deficit hyperactivity and conduct disorders, which represent a substantial mental health burden in the United States and abroad. New clinical imaging on such diagnosed children reveals persistent evidence for abnormal morphology and function in the cerebellum. Our study aims to test how postnatal ETS exposure can alter the developing mammalian cerebellum, which may in part underlie the biological bases for functional deficits in exposed children. In this study, ETS was administered during the vulnerable period of postnatal rat cerebellar development. Using a label-free proteomics approach, we identified 662 peptide measures with a statistically significant response to ETS treatment. Bioinformatic tools were exploited to identify relationships within, and interpret the biological relevance of the results. ETS responsive data highlighted altered aerobic respiration, which was confirmed upregualted by orthogonal measures, along with molecular evidence for increase mitochondrial biogenesis mediated by dynamin 1 like protein. Molecular findings correlated with an ETS-induced two-fold

greater density of mitochondria within the cerebellum, observed by immunofluorescence and electron microscopy. A healthy mitochondrial morphology was also confirmed by electron microscopy with no discernible ultrastructural differences between groups. Results were consistent with an ATP-demand driven rationale for the ETS induced upregulation of aerobic respiration within an increased mitochondrial population. In all, this study demonstrates the successful application of a proteomics-based, systems biology approach to divulge novel neurobiology. Abnormal mitochondrial biogenesis in combination with increased ATP demand during the critical time of cerebellar synaptogenesis points to potential irregularity in synapse formation, which in future research may be found to underlie functional deficits associated with mental health issues in children.

4.2. INTRODUCTION

Recent epidemiological studies have reproducibly found a dose-dependent risk for behavioral and associated cognitive problems in children exposed to environmental tobacco smoke (ETS), otherwise known as secondhand smoke (90, 119-124). Importantly, their findings were found to be independent of prenatal exposure (smoking during pregnancy) and in most cases were reported based on unbiased biofluid measurements, alleviating criticism of earlier studies with similar findings (79, 86, 87, 125). Postnatal ETS exposure was found to increase the incidence of inattention, hyperactivity, impulsivity, written and spoken language deficits and conduct problems. The affect of ETS on those domains was often assessed in direct association with diagnosed attention-deficit/hyperactivity disorder (ADHD) and conduct disorder (CD), two comorbid mental

health concerns in children that may share an underlying neurological dysregulation (126, 127). Findings from the epidemiology show a consistent effect with up to a 9-fold increased risk associated with postnatal ETS exposure dependent on the functional domain assessed, level of exposure, gender and race. These studies clearly addressed a call by the U.S. Surgeon General for further research (1), and concluded with the need to explore the biological mechanisms underlying the relationship between ETS exposure during brain development and functional deficits associated with ADHD and CD.

Preclinical research with *in vivo* models is instrumental to providing a foundation of biological mechanisms linking postnatal ETS exposure with neurodevelopmental affects (90, 121, 122, 128). Bias and confounding factors of cross-sectional epidemiological studies can be controlled within the laboratory, with biochemical and cellular measurements made directly on the mammalian brain following modeled ETS exposure. To that end, we modified our *in vivo* ETS model (129) to provide postnatal passive exposure to test an effect on the developing mammalian cerebellum. A growing body of research demonstrates abnormal cerebellar morphology as one of the most prominent and consistent clinical imaging features that distinguishes children with ADHD or ADHD comorbid with CD from other children (104, 105, 130-134, 134, 134). The cerebellum, while commonly associated with motor coordination, is now known to regulate behavioral and cognitive functions through cerebello-thalamo-striatal-prefrontal cortex circuits. Domains with cerebellar involvement include attention, planning, working memory, spoken and written language and inhibition control (98, 100, 134-138), which match deficits found modulated by postnatal ETS exposure and that underlie symptoms

associated with ADHD and CD phenotypes (104, 139). Ultimately, there is a significant population at greater risk for such mental health problems in the United States due to postnatal ETS exposure. Consider that 50% of mothers who ceased smoking during pregnancy resumed within six months of delivery; one in five U.S. children are exposed to ETS in the home, 4.1 million under the age of 5; and that children under the age of 5 have the highest exposure levels due to an increase in respiration rate and little control over their environment (81, 140, 141).

The cerebellum is also distinguished by a late start to development relative to most other brain regions in mammals, to include humans. Research pioneered by Dobbing et al. established the precept of a vulnerable period for neurodevelopment. In humans, the vulnerable period for cerebellar development extends a year or more following birth (142-145), while the corresponding period in the rat extends out to 21 days from birth (146, 147). Animal research demonstrated dramatic cellular and functional affects on the cerebellum if damaged during the vulnerable period that could not be recovered once the period had closed, and that damage caused after this vulnerable period had less of an effect on function (98, 106, 107, 110, 111, 136-138, 148-150). Altman et al. even found distinct functional deficits depending on if the rat cerebellum was damage within the first or second week after birth, which resulted in motor deficits or pronounced hyperactivity, respectively (111). Thus, environmental exposure would likely have the greatest impact on cerebellar development during the vulnerable period with potential long term functional consequences.

In the current study, we applied a systems biology approach to test our hypothesis that exposure to ETS during the rat cerebellar vulnerable period will result in measurable biochemical and cellular alterations that can influence development. The findings herein begin to build a foundation to discern the biological mechanisms by which postnatal ETS exposure may induce childhood deficits with cerebellar involvement that are symptomatic of ADHD and CD. We performed label-free proteomics as the foundation of our study to define the ETS-responsive neuroproteome. A host of available bioinformatic tools were exploited – enrichment analysis, pathway mapping and protein-protein network analysis – to elucidate the biological response to treatment and begin to understand its impact on cerebellar development. Our data interpretation was then substantiated by orthogonal molecular measures and microscopic evaluation at cellular and sub-cellular levels.

4.3. EXPERIMENTAL PROCEDURES

Animal Procedures and Tissue Collection- Timed-pregnant Sprague Dawley rats (n=8) (Harlan Laboratories, Indianapolis, IN) were order to allow five days of acclimation prior to birth. Food and water were provided *ad libitum* except during exposure, when food was removed. Litters were placed within a Teague TE-10 smoke exposure system (56) for 3 h per day until PD 21. The TE-10 system was operated as described previously with the ETS concentration confirmed by daily gravimetric analysis of respirable suspended particulate. Carbon monoxide levels were also affirmed at sub 10 ppm, non-hypoxic levels. Litters were subdivided by treatment n=4 in the ETS exposed and room air only control groups. Brain tissues were collected and snap frozen in liquid nitrogen three days after

completed the ETS exposure, which allowed assessment of a lasting rather than a transient effect of ETS just after the cerebellar vulnerability period would normally end. Animal weight was recorded every 3rd day, with no statistical difference found between groups throughout the study. All procedures conformed to the U.S. Public Health Service policy with approval by the institutional Animal Care and Use Committee.

Sample Preparation- Collected cerebella were bisected at the sagittal midline with one half processed by a three part protein extraction procedure. First, the tissue was homogenized and incubated for 90 min at 4°C in an aqueous buffer absent detergents or reducing agents but containing Complete Protease Inhibitor Cocktail (Roche Biochemicals, Indianapolis, IN) and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma, St. Louis, MO). After centrifuging the homogenate at 15,000 g for 20 min the supernatant was collected (Matrix Extract). Second, after washing the pellet, it was resuspended in an analogous buffer with the addition of DTT (1 mM) and triton X-100 (1% v/v) and incubated for 90 min at 4°C before collecting the supernatant (Membrane Extract 1). The remaining pellet was then washed and resuspended in 60% triton buffer / 40% isopropanol, incubated as before, and then a third supernatant was collected (Membrane Extract 2). Protein concentrations were determined using a Pierce 660 assay kit (Thermo Scientific, Rockford, IL).

Cerebellum lysates, 100 µg each, were exchanged into 25 mM ammonium bicarbonate (ABC), pH 8, containing 0.1% Rapigest surfactant (Waters, Milford, MA) using Millipore Microcon YM-10 centrifugal filters (Billerica, MA) per manufacturer instructions. Fresh DTT was added to a 5 mM final concentration and lysates were heated

at 60°C for 30 min. After cooling, lysates were alkylated with fresh idioacetamide (15 mM final concentration) for 30 min in the dark. Lysates were digested overnight with modified trypsin (Promega , Madison, WI) at an enzyme to protein ratio of 1:100. Digests were then vacuum-dried and reconstituted in 50 µL of 100 mM ammonium formate (pH 10).

Two-Dimensional Liquid Chromatography-Tandem Mass Spectrometry- Protein digests (4 µL each) were sequentially injected in a treatment-interspersed order onto a two-dimensional nanoACQUITY UPLC system (Waters) for tandem reversed-phase (pH 10) / reversed-phase (pH 2) separation online with a Waters Synapt HDMS mass spectrometer. Injected peptides were subdivided into 4 fractions using step-elution off of a BEH130 300 µm x 50 mm C_{18} column and trapped onto a Symmetry 180 µm x 20 mm C_{18} column using 20 mM ammonium formate (pH 10) in water and acetonitrile mobile phases. Trapped peptides were then gradient eluted off of a BEH130 75 µm x 150 mm C_{18} column using 0.1% (v/v) formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: 8% up to 30% B in 45 min, up to 50% B in 15 min, up to 85% B in 1 min, hold for 4 min and then equilibrate back at 8% B for 15 min. Peptides were electrosprayed into the mass spectrometer, which was operated in V-mode with a nominal resolving power of 10,000. An external standard of [Glu1]-fibrinopeptide B (100 nM) sampled every 30 s facilitated accurate mass correction. Data were acquired via data-independent analysis (151, 152), with a precursor ion scan (m/z 400 to 1,500) followed by a product ion scan (m/z 100 to 1,500) each second. The instrument was mass calibrated daily using fragment ions from the external standard with an RMS mass accuracy within 1.5 ppm. A standard Ecoli tryptic digest (1 ug injected) was analyzed using a single 60 min gradient elution

profile following each daily batch of samples for quality control assessment of system performance prior to injecting the next sample batch.

Mass Spectrometry Data Processing- Continuum mass spectrometry data were processed using Waters Protein Links Global Server (PLGS) software version 2.4 as described elsewhere (153). During preprocessing, precursor and product ions above 250 and 100 count thresholds, respectively, were charge-state reduced, isotope deconvoluted and accurate mass corrected. Product ions were time aligned to precursor ions within ± 0.05 min of their chromatographic peak apex and validated based on their chromatographic peak width and the product to precursor peak area proportion. Preprocessed data were summarized by monoisotopic reduced mass (MH+), peak apex retention time (RT) and the combined chromatographic peak area for all isotopes and charge states. Ion tables for each chromatographic fraction were merged combining data within ± 7 ppm mass and ± 0.5 min RT tolerances, producing one ion table per protein extract, per biological sample. The PLGS Ion Accounting protein database search engine parsed each ion table against a UniprotKB Rattus Norvegicus specific fasta format database (2010_06 release) combined with its reversed-sequence decoy entries. Parameters selected for trypsin specificity, one missed cleavage, fixed carbamidomethyl cysteine modification, variable methionine oxidation and neutral loss of ammonia or water. Serial searches were performed to consider no additional post-translational modifications (PTMs), then variable phosphorylation and finally variable glycosylation.

Alignment of Ion Tables Across Biological Replicates- Ion tables for each sample (n=8) were aligned by precursor ion MH+ (± 7 ppm) and RT (± 0.5 min) utilizing

the PLGS Expressions package (154). Aligned results were generated separately for Matrix Extract data and Membrane Extract data, the later comprised of merged ion tables from Membrane Extracts 1 and 2 that shared measures from a common sub-cellular origin. Search engine results were superimposed the Matrix and Membrane ion tables, resolving reproducible peptide identifications across biological replicates. Aligned ion tables were filtered to select peptide measures quantified in ≥ 75% of biological replicates within a group. Peak area values were collapsed together when split by experimental events such as missed-cleavage, methionine oxidation and neutral loss. Afterward, a single biologically-relevant measure was reported under the MH+ and sequence of the most massive collapsed entry. The cleaned Matrix and Membrane Extract ion tables were combined, and all entries were defined by a unique identifier based on peptide sequence, its integer MH+ value and the protein extract of origin. Data were sorted by their peptide score and filtered to an estimated 1% false discovery rate using a decoy database method.

Statistical Analysis of Final Ion Table Data- Data were imported into the quantitative proteomics package DanteR (ver. 1.0.1.0, Pacific Northwest National Laboratory, omics.pnl.gov/software) (155, 156). Chromatographic peak area values were log2 transformed and normalized (linear model), providing median-centered, normal distributed peptide measures across biological replicates. Group-selective missing values were identified; i.e., quantitative measure were absent from all biological replicates in one group but present in replicates of the other group. Those non-random missing values were deemed biologically relevant, symptomatic of a group-specific divide about the analytical detection limit. Imputation values were simulated to reflect a Gaussian distribution about a

mean equal to the minimum measure within the relevant group and having a distributed proportional to the median empirical variance across all variables in that group (157, 158). One-way ANOVA hypothesis testing was performed within DanteR using treatment as the independent variable. The significance level (alpha) was corrected for multiple measures (k=15,197) using an FDR method to control the family-wise error rate to 5% (159, 160).

Interaction Informatics- Measures found statistically responsive to ETS treatment reflected putative modulation of protein or protein family abundance, localization or modification. The ETS-responsive neuroproteome was summarized by Uniprot database-derived protein symbols, which correlate to HGNC stem symbols common across all vertebrate species. Protein enrichment analysis was performed against GO annotation terms (biochemical process and cellular component) and biochemical pathways accessed from eight public databases using a Fisher's inverse chi-square method with Bonferroni correction (initial alpha 0.05) (ToppGene (115, 161)). Detail on enriched pathways was assessed through the KEGG Pathway Database (116, 117). Proteins associated with the GO term Mitochondrion (GO:0005739), the most significant enriched cellular component, were analyzed further using protein-protein network analysis (STRING Database Ver. 8.3, (162)) with the following parameters: a minimum interaction confidence score of 0.5, ≤ 10 interactors and impute ten intervening nodes. Network results were displayed in the evidence view mode applying MCL clustering.

Immunoblot Analysis- Protein-balanced samples (n=4 per group) were resolved using the NuPAGE gel system, 4–12% Bis-Tris gels and MOPS running buffer (Invitrogen, Carlsbad, CA). Separated proteins were transferred to PVDF membrane

(Millipore) via a semi-dry method. Membranes were probed with the following primary antibodies: Hexokinase-1 (Sigma-Aldrich) at 1:400, adenosine triphosphate (ATP) synthase 5A (Abcam, Cambridge, MA) at 1:1,000, Dynamin 1-Like Protein (Origene, Rockville, MD) at 1:500, and calretinin (Abcam) at 1:1,000. IgG HRP-conjugated secondary antibodies and the SuperSignal West Pico chemiluminescence detection kit (Thermo Scientific) were used following manufacture instructions. Blots were imaged on an Image Station 4000MM Pro CCD imager (Carestream Molecular Imaging, Rochester, NY) with net gray-level intensity measured from 16-bit images. Statistical testing was performed using a Student's t-test method with correction for multiple-testing using the Holm-Bonferroni method (initial alpha 0.05).

Immunofluorescence Microscopy- Fresh frozen cerebella were bisected at the midline and mounted for sagittal sectioning starting at a depth of 1 mm from the lateral surface. Cryosections (10 µm) were collected from cerebellar tissue, n=4 per group. Sections were fixed in 3% paraformaldehyde, washed and blocked with 5% milk containing 0.3% triton X-100. The mitofilin primary antibody (MitoSciences, Eugene, OR) was diluted 1:500 (v/v) in blocking buffer and incubated overnight at 4°C. Alexa Flour Dye conjugated secondary antibody (Invitrogen) was applied (1:1000) and incubated at room temperature for 1 h in the dark. Sections were mounted in Vectashield soft set medium containing DAPI stain (Vector Labs, Burlingame, CA). All sections were visualized on a Zeiss AxioImager A1 fluorescence microscope utilizing identical parameters. Images of the molecular layer, granular layer and white matter were captured per section. Grouped by anatomical layer, images were deidentified and then sorted into

two groups by a blind observer for qualitative differentiation. Fractional area ROI analysis was completed in ImageJ (163). Statistical testing was performed using a Student's t-test method with correction for multiple-testing using the Holm-Bonferroni method (initial alpha 0.05).

Transmission Electron Microscopy- Sagittal sections from ETS and control tissues were collected as above, but at a 50 µm thickness. Sections were fixed with 2% glutaraldehyde, 2% paraformaldehyde in Millonig's buffer for 60 min at 4°C and post-fixed in 1% osmium tetraoxide for 2 h. Sections were then dehydrated, transitioned through propylene oxide and then infiltrated overnight in Embed 812 (Electron Microscopy Sciences, Hatfield, PA). Sections were flat-embedded between vinyl slides and viewed via a light microscope to excise from the molecular layer. Thin sections (80 nm) were collected by ultramicrotomy onto copper 300 mesh Gilder thin bar grids (Electron Microscopy Sciences), which were stained in lead citrate and uranyl acetate. Micrographs were collected on a Jeol JEM-1230 transmission electron microscope equipped with a CCD camera. Mitochondria per field were counted and encircled across five micrographs per group using a stereological template within ImageJ (177 mitochondrial in total). Measurements included mean mitochondria area per group (Mito. Area), mean Fractional Area (% of total field area occupied by mitochondrial) and the number of mitochondria per field (Mito. Density). Statistical testing was performed using a Student's t-test method with correction for multiple-testing using the Holm-Bonferroni method (initial alpha 0.05).

4.4. RESULTS

Neuroproteomic Analysis- The research design summarized in Fig. 1 was employed to test the effect of ETS treatment on the developing cerebellar neuroproteome. Proteins soluble within the cytosol, neucleosol and mitochondrial matrix (Matrix Extract) were resolved from membrane tethered or integrated proteins (Membrane Extract) using an optimized multistep extraction protocol (164). The enriched subproteomes exhibited a minimal 18% overlap, with 92% selectivity for known matrix-associated proteins and 81% selectivity for known membrane-associated proteins. Results from label-free, data-independent analysis included 138,212 raw precursor ion scan measures across group replicates (Fig. 2), of which 41,840 were associated with a peptide sequence. Further processing as summarized in Fig. 1*B* was required to remove redundancy and limit the false-positive sequence identification rate.

Raw mass spectrometry results were confounded by experimental events such as neutral losses, methionine oxidation and missed-cleavage; thus, reproducible, but biologically redundant measures were collapsed under a single representative peptide ion with the largest MH+. The split intensity data were summed for relative quantification of biologically distinct measures between groups. Reduced peptide data contained unique entries distinguished by their peptide sequence, MH+ and protein extraction source. These data were then filtered to an estimated 1% false sequence identification rate using a decoy-database method, providing the final list of 15,197 unique measures. Intensity values were log transformed and normalized prior to ANOVA statistical testing. After

Fig. 4-1: Experimental Workflow. *A,* Schematic of sample processing and ion data generation steps. Replicate samples were processed using sequential protein extractions followed by two-dimensional reversed-phase tandem mass spectrometry (2D-RPRP-MSMS) of tryptic digests. Ion data were searched against a rat protein database, aligned across all biological replicates and filtered for within-group reproducible measures. *B,* Schematic of bioinformatics performed. Ion data were post-processed to collapse biologically redundant ion measures, normalize ion area measures, impute for non-random missing values and perform statistical testing. Results were assessed by enrichment, pathway and network analyses, with biological interpretation substantiated by orthogonal molecular and cellular measures.

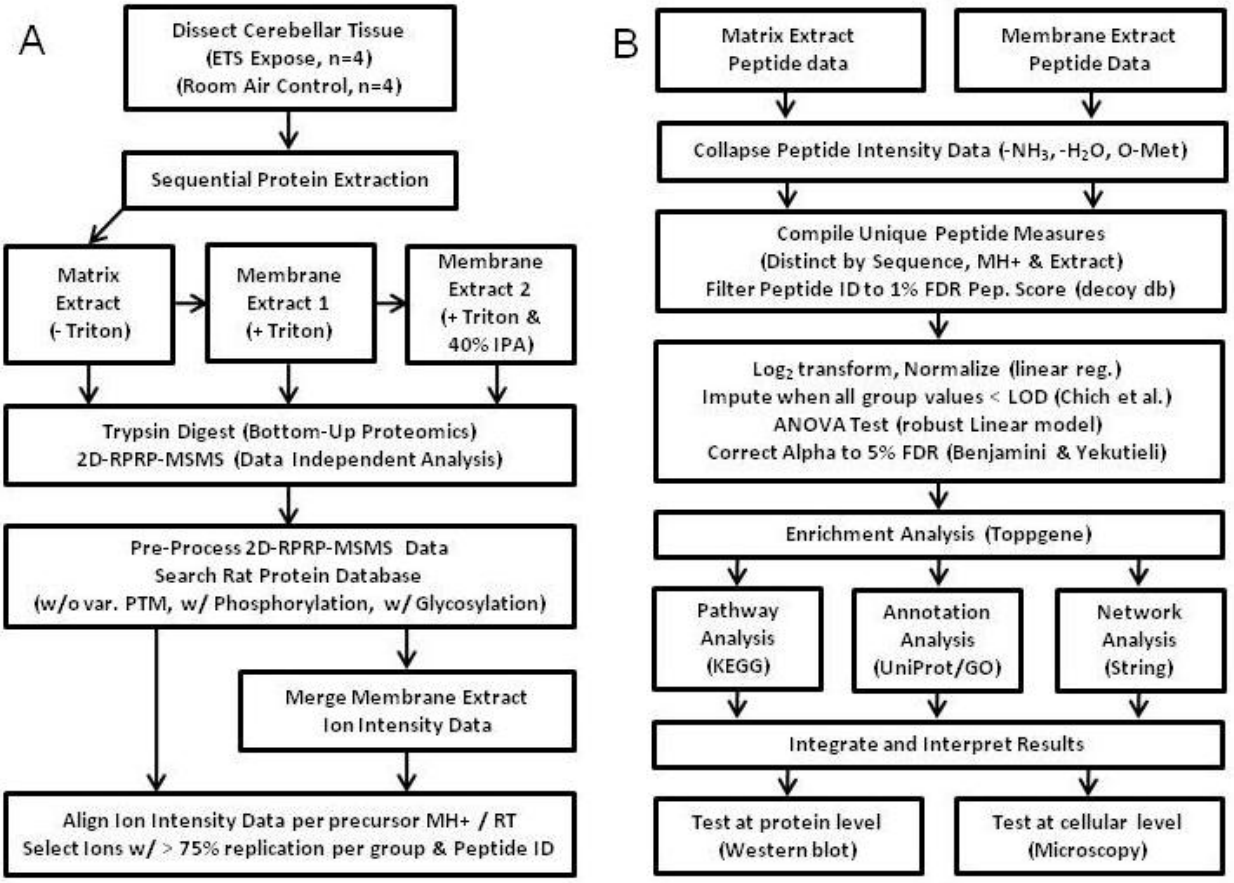
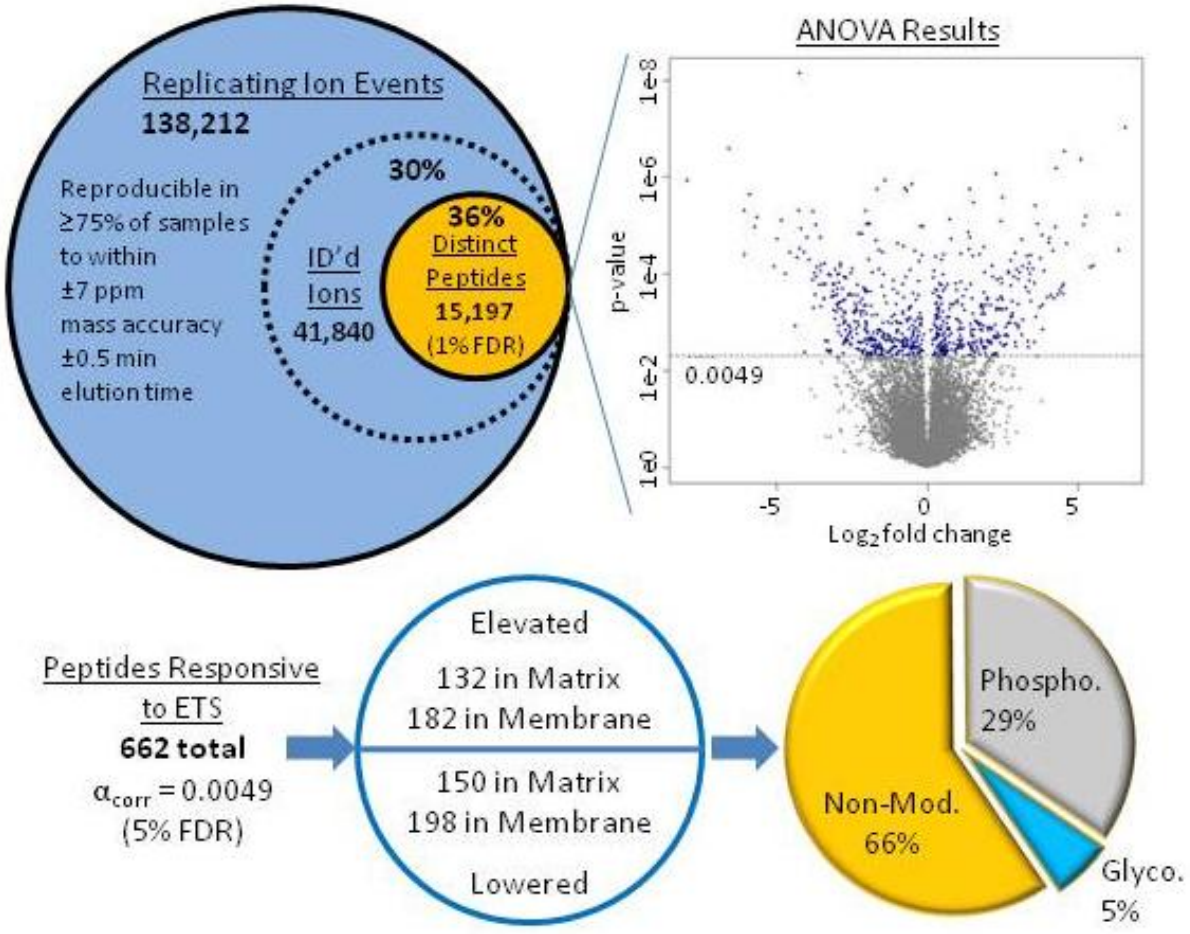


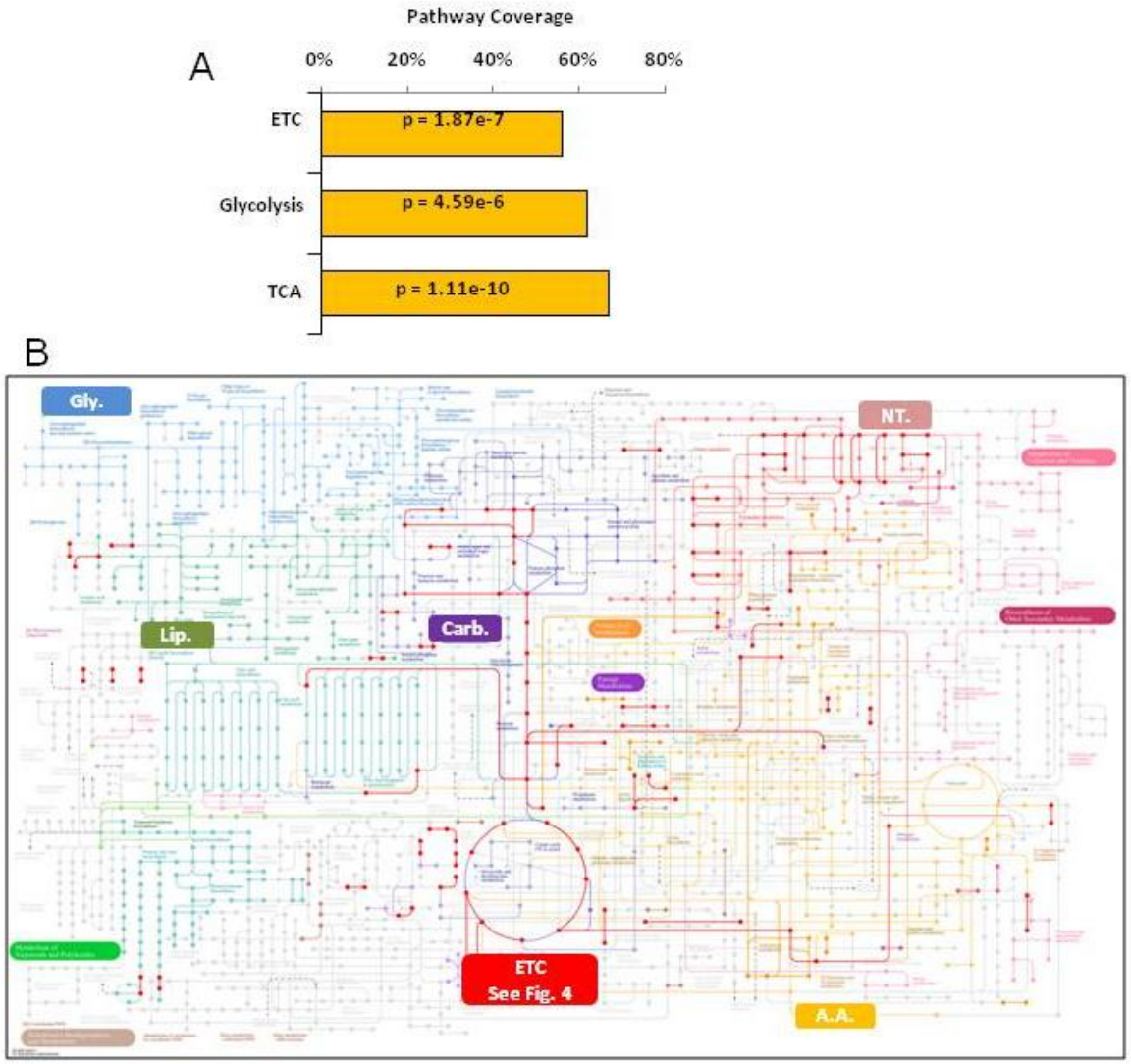
Fig. 4-2: Analysis of reproducible ion data confirms a significant response to ETS-treatment. Based on over 10^5 reproducible ion measures, we were able to discern quantitative data on 15,197 unique peptide measures distinguished by their sequence (limited to an FDR of 1%), their reduced mass (delineates variably modified sequences) and their extract of origin (matrix or membrane). Following ANOVA testing, 662 of the unique peptide measures were discerned as statistically significant in response to ETS treatment at a corrected alpha of 0.0049 using the Benjamini and Yekutieli FDR method to limit the family-wise error rate to 5%. A volcano plot depicts the quantitative response to ETS treatment. The number of responsive peptides was near-equally split between elevated and lowered levels and one-third of responsive peptides were identified with a post-translational modification.



correcting the significance level for performing multiple measures (alpha = 0.0049 for an estimated 5% type 1 error rate), 662 peptides were found as statistically responsive to ETS treatment. The volcano plot in Fig. 2 illustrates the range of responses to ETS treatment. The median group-mean difference among responsive measures was 2.3-fold with a lower quartile cutoff at 65% change and an upper quartile cutoff at 5.2-fold change. The number of increased and decreased ETS-responsive peptides was about even as expected from a normal distribution, with 57% found within the membrane extract. 85% of responsive peptides were found to be isoform specific relative to the target protein database. Of the responsive peptides, 32% were identified as differentially phosporylated and 5% differentially *glycosylated*.

Bioinformatic Analysis- An ETS-induced change in protein function may be represented by one or more responsive peptide due to protein modification (e.g., phosphorylated or glycosylated), altered expression, altered catabolism or translocation between spatial compartments (e.g., between matrix and membrane-tethered states). Further, biologically induced responses will involve protein-protein interaction, whereby significant biochemical events can be elucidated through the interactivity among responsive elements. We thus employed several bioinformatic tools to reveal associations within the ETS-responsive neuroproteome. Enrichment analysis was performed to identify statistically overrepresented annotated biochemical pathways within the responsive data. The results shown in Fig. 3*A* illustrate a concerted response among all protein pathways involved in aerobic respiration, to include statistical overrepresentation

Fig. 4-3: Enrichment and pathway analyses illustrate a prominent affect of ETS treatment on aerobic metabolism. *A*, Protein enrichment analysis performed using the Toppgene suite of bioinformatic tools identified statistically significant associations between ETS responsive data and aerobic respiration pathways of electron transport chain (ETC), glycolysis and the tricarboxylic acid cycle (TCA). Denoted *p*-values were calculated using a Fisher's inverse chi-square method with a Bonferroni correction of the significance factor for multiple testing. The percentage of covered protein elements for each indicated pathway is presented within parentheses. *B*, ETS-responsive proteome superimposed the KEGG global metabolism reference map. Elements identified within the responsive data are shown in bold red, and denote near-complete coverage of aerobic carbohydrate metabolism.



of electron transport chain (ETC), glycolysis and the tricarboxylic acid (TCA) cycle. Superimposed the KEGG global metabolic pathways reference map, the ETS-responsive neuroproteome exhibits a focused, near-complete coverage of aerobic carbohydrate metabolism (Fig. 3*B*).

The ETS-responsive data were also enriched in mitochondrion-associated proteins (28% of the ETS-responsive neuroproteome) tied with the GO term GO:0005739 (p = 5.33e-29). Associations amongst those proteins were further assessed by protein-protein network analysis (Fig. 4), which revealed interactivity between and beyond that annotated in traditional biochemical pathways. As expected from the enrichment analysis, enzymes associated with pyruvate processing (e.g., TCA and associated metabolic side-reactions) and ETC complexes (1, 2, 3 and 5) were well represented protein clusters. Phosphate shuttling, chaperone and proteasome complex proteins represented three other major protein clusters. Hexokinase 1 (Hk1), the loan glycolytic protein present in Fig. 4, is known to traffic to and from the outer mitochondrial membrane to regulate glycolysis.

ETS Amplified Aerobic Respiration Machinery- Aerobic respiration within the brain depends near-exclusively on metabolism of glucose to fuel oxidative phosphorylation within mitochondria. Hk1 is the key regulator in the glycolytic aspect of this process, being the first, rate-limiting enzymatic step of a chain reaction involving 9 other enzymes that were also found responsive to ETS (Fig. 5*A*). Under ATP demand, glycolysis is regulated by increased HK1 binding from the cytosol to the outer-mitochondrial membrane, whereby

Fig. 4-4: Mitochondrion-associated proteins enriched within the ETS-responsive data denote a prominent effect on phosphate dynamics. Protein-protein network analysis using the String Database bioinformatics package revealed distinct clusters associated with the processing, use and shuttling of phosphate. MCL clustering was performed to define protein-protein interactive groups as encircled and labeled. A large array of mitochondrial-associated proteins involved in the downstream processing of pyruvate were found responsive, which provided the necessary input for oxidative phosphorylation via the electron transport chain (ETC). Multiple components of ETC complexes 1, 2, 3 and 5 are defined. Two protein clusters denote elements involved in chaperone and proteasome protein processing that are ATP-dependent. Last, we observed a large group of proteins involved in phosphate shuttling (e.g., phosphate exchanged across the mitochondrial membrane, conversion of AMP to ADP or ATP to NTP).

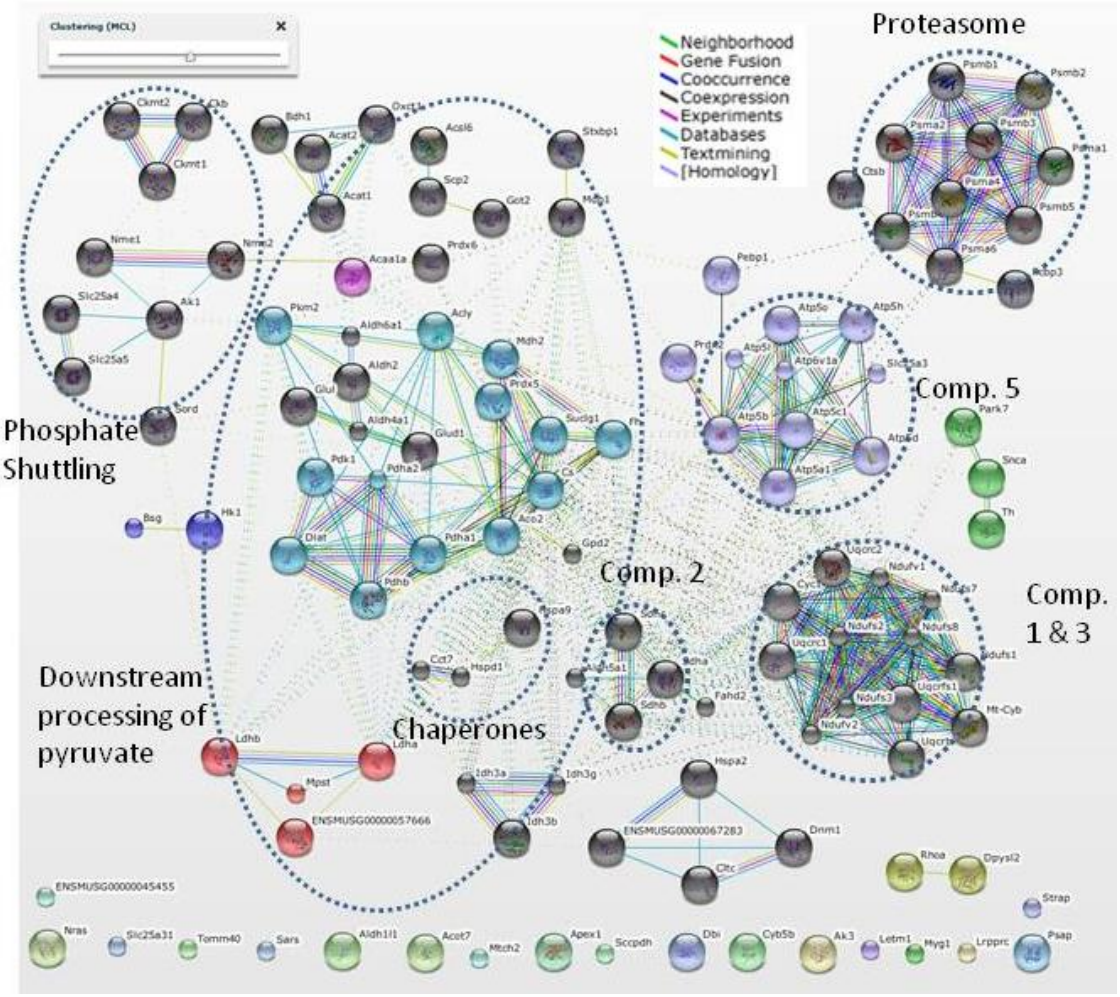
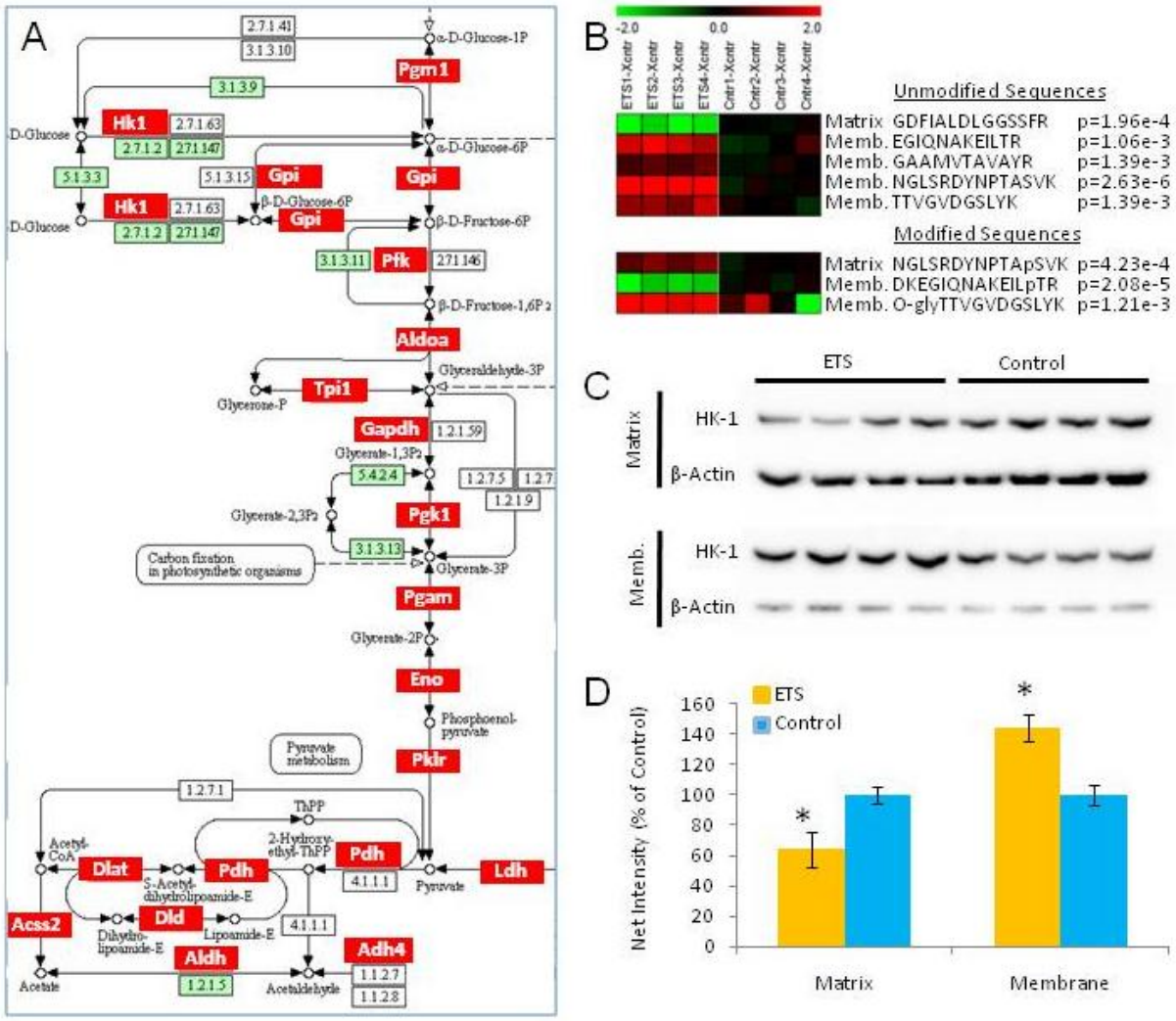


Fig. 4-5: ETS treatment induced an aerobic respiration-dependent increase in glycolysis. *A*, Glycolytic pathway diagram with ETS-responsive elements colored in red. Results were generated via imputing responsive elements into the KEGG pathway database tool, depicting a response in all enzymes associated with the glycolytic pathway. Glucose processing by hexokinase 1 (Hk1) is the rate-limiting step that is regulated by ATP demand on mitochondria. *B*, Heatmap representation of ETS-responsive peptide measures for Hk1, which denote a shift from a matrix to a membrane-tethered localization. *P*-values calculated from ANOVA testing are denoted for each peptide measure. *C*, The translocation of Hk1 was confirmed by orthogonal immunoblot analysis, with *D*, a proportional decrease in the matrix extract and increase in the membrane extract of the ETS group. (Mean ± S.E.M.) Symbols indicate significant differences from control (*$p \leq 0.015$) by a Student's *t*-test method with correction for multiple testing via a Holm-Bonferroni method.

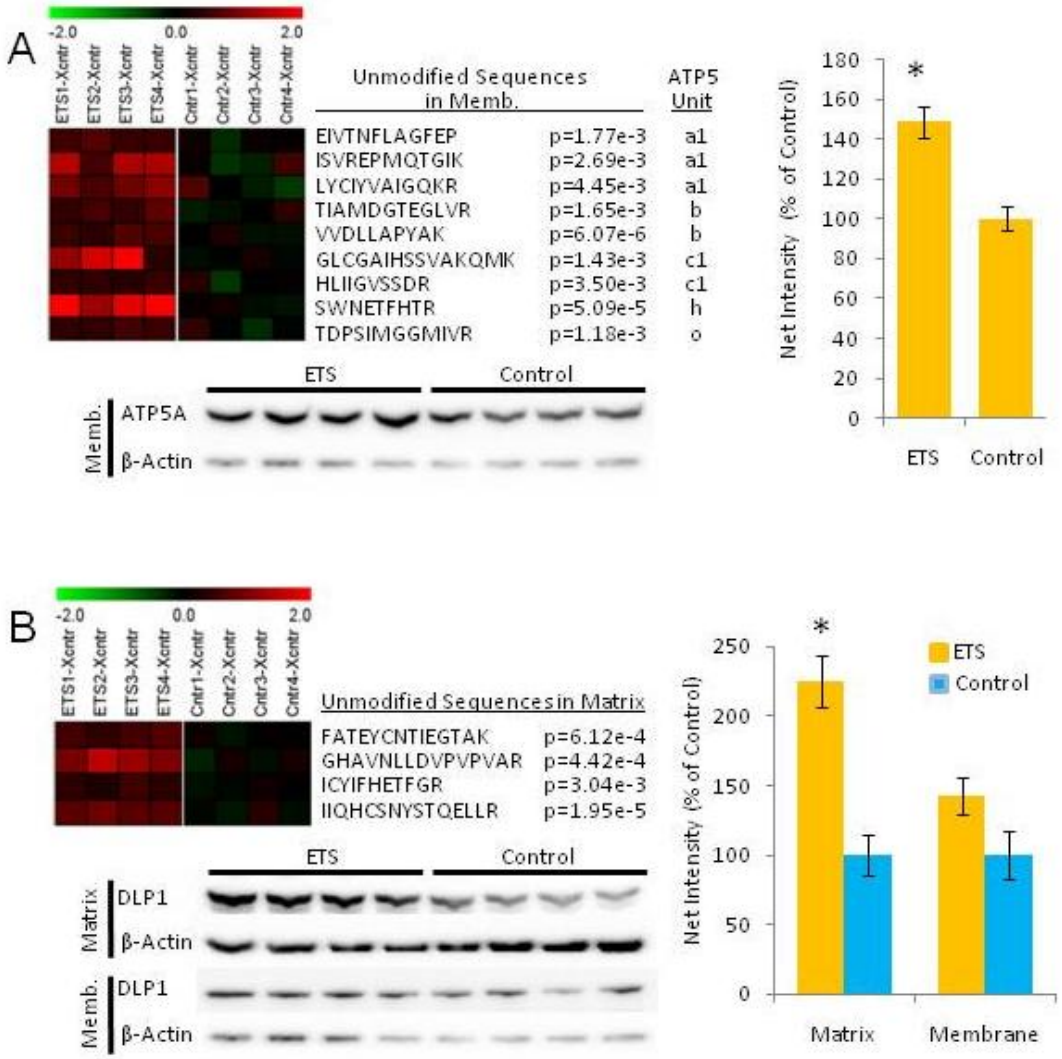


HK1 activity is increased. Such a compartmental shift is suggested from the profiles of Hk1 responsive peptides. As illustrated in Fig. 5*B.*, non-modified HK1 peptides are down in the matrix and up in membrane extracts of the ETS group. Further, phosphorylated and glycosylated HK1 motifs were found to be ETS responsive and may play a regulatory role in this process. HK1 translocation was substantiated by orthogonal immunoblot results, with a proportional decrease of cytosolic HK1 and increase of membrane-tethered HK1 (Fig. 5*C* & *D*).

Mitochondrial function is critical for meeting the ATP demands of the developing brain. Figures 3 and 4 illustrate the sizeable overlap of the ETS-responsive neuroproteome with TCA and ETC mitochondrial processes that culminates in oxidative phosphorylation by ATP synthase (ATP5, ETC complex 5). ATP5 is the multi-protein complex that is directly responsible for ADP to ATP conversion. Measures of non-modified ATP5 responsive peptides all exhibited heightened levels with ETS treatment and span across nine ATP5 protein subunits (Fig. 6*A*). Immunoblot data confirmed a greater amount of ATP5A with ETS treatment.

Taken together, the presented data indicate greater aerobic respiration capacity, from HK1 through ATP5, in response to ETS-treatment. The compelling mitochondrial association warranted further assessment. The rate of mitochondrial biogenesis in the cerebellum is most rapid during the study period as required for initial neuronal process outgrowth and synaptogenesis. ETS-treatment may modulate this biogenesis based on synaptogenesis-induced demand or as an effect of reactive oxygenated species (ROS) induced stress on mitochondrial integrity and function.

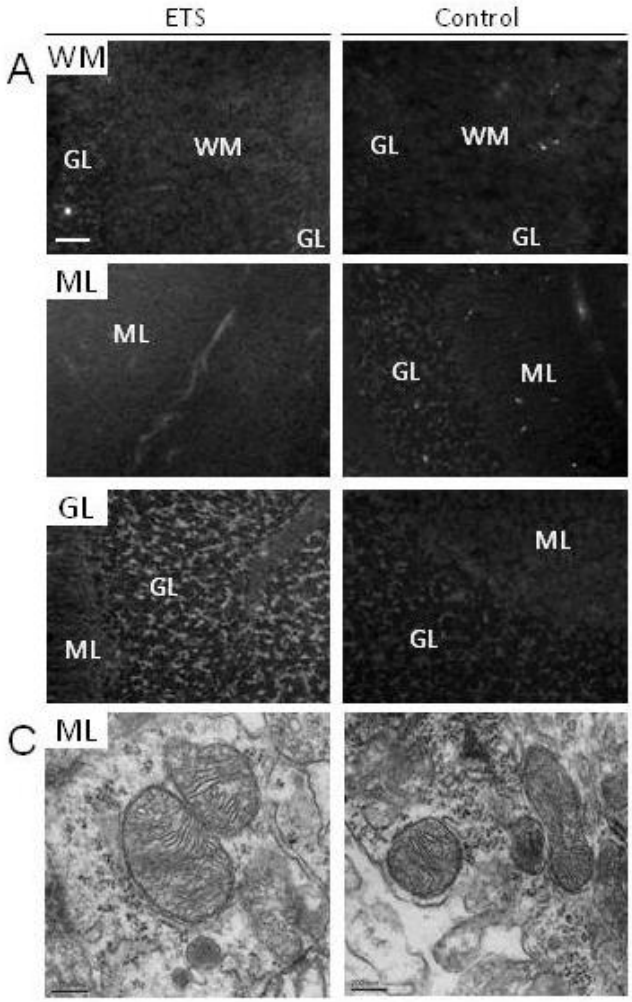
Fig. 4-6: ETS treatment induced an increase in ATP synthase associated with greater mitochondrial biogenesis/fission activity. *A*, Heatmap representation of ETS-responsive peptide measures for ATP synthase (ATP5), which denote a concerted increase in non-modified peptides associated with five different protein components of the complex. *P*-values calculated from ANOVA testing are denoted for each peptide measure. Immunoblot data confirmed a statistically significant increase ATP5A over control. *B*, Heatmap representation of peptide measures for dynamin 1 like protein (Dnm1l). Increased levels of Dnm1l denote greater mitochondrial biogensis/fission activity. *P*-values calculated from ANOVA testing are denoted for each peptide measure. Immunoblot data confirmed an increase in Dnm1l within the matrix and membrane extract. (Mean ± S.E.M.) Symbol indicates significant differences from control (*$p \leq 0.015$) by a Student's *t* test method with correction for multiple testing via a Holm-Bonferroni method.

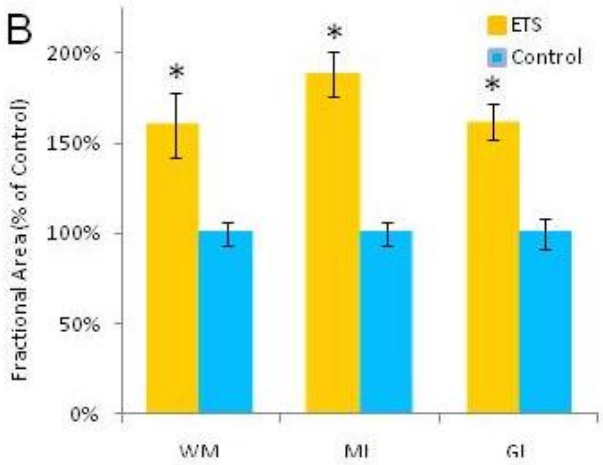


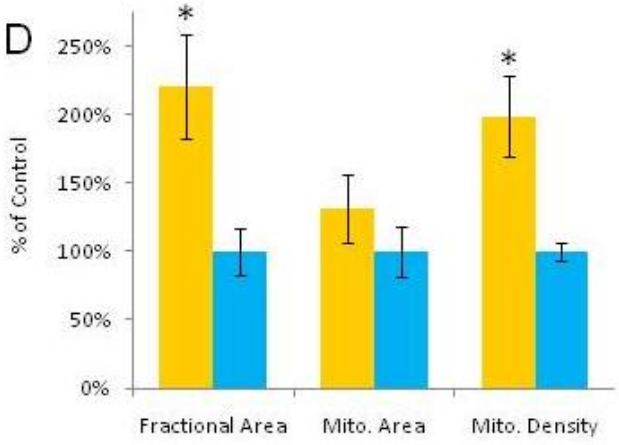
ETS-Altered Mitochondrial Biogenesis/Fission- Dynamin-like protein 1 (Dnm1l), also known as Drp1, is critical to mitochondrial biogenesis/fission and influences trafficking of mitochondria within developing neuronal processes (165, 166). Responsive Dnm1l peptides (non-modified) concertively exhibited a greater intensity within the ETS group (Fig. 6*B*). Dnm1l is primarily found in the cytosol, but a small quantity will bind to the mitochondrial membrane to initiate the fission process. Immunoblot results confirmed a greater Dnm1l abundance in the ETS group, up within matrix and membrane extracts by 125% and 43% over control, respectively. Phosphorylation is known to be involved in signaling Dnm1l translocation. We observed reduced phosphorylation at serine 637 within the ETS group ($p = 0.026$), a phospho motif known to exhibit an inverse correlation with mitochondrial fission activity (167). Further, the ETS-responsive data did not highlight any opposing fusion-associated factors such as mitofusin. Thus, the Dnm1l measures suggest increased signaling and activity of mitochondrial fission in the absence of evidence for an opposing process. Confirmation of an increased mitochondrial system response required cellular observations.

The mitochondrial marker mitofilin was selected for immunohistological assessment of cerebellar sections. Mitofilin was considered ideal in that it provided a uniform staining across inner and outer aspects of the organelle and was known to be uninvolved in mitochondrial fission and fusion dynamics (168). Mitofilin fluorescent images were captured from the cerebellar white matter, the molecular layer and the inner granular layer, which were all in flux during the study period (Fig. 7A). First, qualitative differences between treatment groups were assessed in all three areas by a blinded

Fig. 4-7: ETS exposure induced a 2-fold increase in mitochondrial density with no aberrant morphology. *A*, Representative micrographs of mitofilin immunofluorescence as observed in the white matter (WM), molecular layer (ML) and granular layer (GL) of cerebellum sections from ETS exposed and control groups. Scale bar: (in WM-ETS) 100 µm. *B*, Average fractional area measures normalized to control were calculated for the three regions of interest from n=4 animals per group. *C*, Representative electron micrographs of mitochondria within the ML for ETS and control groups. Outer mitochondrial membrane and cristae morphology are consistant between groups and appear healthy. Scale bar: 200 nm. *D*, Measures of fractional area, mitochondrial area and mitochondrial density measured by counting and encircling individual mitochondrial observed over n=5 replicate electron micrographs per group taken from within the ML. Measurements were made on mitochondria within or bordering the upper and right-hand edge of the stereological template. (Mean ± S.E.M.) Symbols indicate significant differences from control (*$p \leq 0.01$) by a Student's *t*-test method with correction for multiple testing via a Holm-Bonferroni method.







observer, in particular for the granular layer. Subsequent fractional area measures substantiated statistically significant increases in mitofilin occupied area within the three regions of interest (Fig. 7B), which was most prominent in the molecular layer at 2-fold over control. Also, increased mitofilin staining was measured in the soma of Purkinje cells ($p = 0.007$) with ETS treatment (data not shown). These data suggest an ETS-induced increase in mitochondrial biogenesis/fission; although, an increase in mitochondrial size would also result in greater mitofilin area measures.

Observations on individual mitochondria were resolved by electron microscopy. Stereological measurements of mitochondrial counts and area were recorded within the molecular layer. Relative to the total frame area, the fractional area occupied by mitochondria was 2-fold greater in the ETS group (Fig. 7D), which was consistent with the immunofluorescence data. Average mitochondrial size showed no statistically significant difference between groups ($n = 177$ mitochondrial measures); whereas, the number of mitochondrial observations within a given field (density) was significantly greater within the ETS group. Morphology within both groups was consistent with a healthy appearance. Analysis of mitochondrial ultrastructure revealed no discernible difference between groups as confirmed by blinded experts (Fig. 7C). With no evidence for an association with apoptosis in the neuroproteomic or microscopy findings, the greater mitochondrial biogenesis/fission activity observed within the ETS group, evidenced by the presented molecular and cellular results, is most consistent with an increased demand for ATP as met by an assessed upregulation of aerobic respiration pathways.

4.5. DISCUSSION

The experimental design outlined in Fig. 1 permits us to account for a range of protein regulatory events in response to ETS treatment. Inherent to bottom-up proteomics is the direct measurement of peptides, which provides information on post-translational dynamics imperceptible to genomic studies. Altered expression, while important, is but one of many proteomic responses to a stimulus. Post-translational modification and protein translocation are two other important proteomic events considered in this study that underlie altered function. Indeed, a full third of our ETS-responsive measures were identified as phosphorylated or glycosylated (Fig. 2), which independently can represent an important change in the regulation of a protein's function. Results for Dnm1l are a good illustration of the multi-faceted responsiveness to ETS treatment. The non-modified Dnm1l peptides (Fig. 6) all exhibited an increase within the ETS group, while the serine 637 containing phosphopeptide was decreased. The former data suggested greater Dnm1l abundance while the later is known to signify increased signaling for fission activity (169). Taken together, these differential measures support one another in terms of their biological relevance, despite the inverse quantitative response to ETS treatment.

Our experimental design also afforded rudimentary assessment of ETS-induced spatial dynamics. Increased separation is well known to extend proteome coverage; however, it is advantageous to consider fractionation steps that also retain information pertinent to the biology, such as protein localization. Hk1 results aptly illustrate the insight attained with even the most basic spatial discrimination. Data in Fig. 5 demonstrate the altered partitioning of HK1 from a cytosolic to a membrane associated state in response to

ETS treatment that would otherwise have been concealed without spatial separation. Hk1 translocation to the outer mitochondrial membrane governs the rate of glycolysis under aerobic respiration (170).

Reproducibility and statistical stringencies were employed to control for random and systematic experimental error. All measures were observed in 75% of biological replicates, and samples were processed and analyzed in an experimental treatment-interspersed order. Type I error was controlled such that of 662 ETS-responsive measures, an estimated 33 were false detections and 7 had falsely indentified sequences. The responsive data exhibited enrichment in isoform-specific peptides portending relevance to the biology. A differential response of one individual member or an opposing response by multiple members of a larger protein family could be diluted when assessed by common peptide measures. Independent quantitative analysis of isoform-specific peptides provided a more straightforward measure of dynamics among individual translated products (171, 172). We also recognized that some of the non-modified peptide measures were likely confounded by uncharacterized, but potentially biologically relevant modifications. Aware of the above, all responsive measures were considered as only putative indications of a reaction by their parent proteins to ETS treatment. Relevance to the biology was based on a concerted response elucidated through protein-protein interactivity.

Enrichment analysis revealed significant overrepresentation of aerobic metabolic processes (Fig. 3). Further analysis of the ETS-responsive neuroproteome displayed near-complete coverage of all enzymes involved in aerobic respiration pathways (Figs. 3 & 4). The initial regulator of those pathways is HK1, which was found to translocate from a

matrix to a membrane associated state in response to ETS (Fig. 5). Heightened oxidative phosphorylation activity is known to be regulated through recruitment of more HK1 to the outer mitochondrial membrane, where it will utilize ATP derived directly from the intramitochondrial compartment (170). The involved mechanism entails a change in the conformation of the C-terminal binding sites of HK1, which is known to be affected by ATP5 activity (173). In this way, the rate of glycolysis is physically coordinated with the rate of oxidative phosphorylation at ATP5, providing for an ATP-demand dependent regulatory link between the two under aerobic respiration. Results in Fig. 6 support this link by illustrating increased ATP5 with ETS-treatment. ATP-demand driven HK1 activity may also be regulated by post-translational modification (174, 175). A host of kinases and phosphotases have been shown to act on HK1, altering its activity. To our knowledge, Fig. 5 presents two novel phosphorylation motifs and one O-glycosylated that may be involved in regulating HK1 under aerobic respiration.

High ATP-demand is normal during neuronal process outgrowth and synaptogenesis. Manufacture and vesicular transport of building elements to the neuronal growth cone is ATP intensive. Chaperone and proteasome activities found responsive to ETS treatment (Fig. 4) are two examples of ATP-dependent processes involved in neurite extension. An upsurge vesicular transport activity happens as the axon approaches a target and synaptic building blocks are focused. Synaptogenesis activity is proportional to the rate of aerobic respiration. Hence, neurite outgrowth and synaptogenesis will affect the rate of mitochondrial biogenesis (176, 177). Enrichment analysis showed the mitochondrion to be the most ETS-responsive subcellular component. As neurites grow, there is a need to

generate more, smaller mitochondria that can be easily transported. Then as synapses form there is a surge in mitochondrial biogenesis to populate the pre- and post-synaptic densities. Mitochondrial biogenesis/fission activity is controlled via Dnm1l, a GTPase protein that forms a spiral structure that constricts and pinches a mitochondrion in two (178). Upon examination of the neuroproteomic results, Dnm1l was the only mitochondrial fission or fusion associated protein found to be responsive to ETS-treatment. Results indicate ETS-induced calcineurin-mediated modification of and an increase in Dnm1l, which are consistent with greater mitochondrial biogenesis activity (167).

There are at least two possible explanations for increased Dnm1l mediated mitochondrial fission after ETS-treatment, both with potential functional implications for the developing cerebellum. First, mitochondrial fission activity may be induced consequent to increased biogenic demand, particularly by an ETS modulation of synaptogenesis that is the most prominent cellular activity during the exposure period. Were this true, our data would indirectly suggest that postnatal ETS treatment results in abnormal synapse formation in the cerebellum or at least the timing thereof, which could contribute to functional deficits. Li et al. recently demonstrated that overexpression of Bcl-X_L increased synaptogenesis and mitochondrial biogenesis in tandem through a Dnm1l mediated process, further tying these two events together in development (179). Pharmacological nicotine administration has also been shown to modulate synaptic plasticity, suggesting that it may be at least one of the responsible components in ETS (180). Prenatal administration of nicotine has long been shown to influence attention, activity and cognitive functions associated with ADHD and CD as recently reviewed elsewhere (181);

however, findings have been confounded by the dose, route and timing of administration, limiting a clear clinical correlation with postnatal ETS exposure. The present study addressed those limitations, administering a clinically relevant dose of nicotine as well as the other active compounds contained in actual ETS via passive breaking, and during a postnatal period of vulnerability for a brain region known to be involved in the pathobiology of ADHD. We acknowledge that no model is a perfect correlate. The exact timing cannot be exactly correlated between species and the rodent brain is well known to differ in some respects from the human.

In the second scenario, stress-induced apoptotic cell death may underlie ETS increased Dnm1l triggered mitochondrial fission as described in detail elsewhere (182-184). Tobacco smoke condensate has been shown to induce mitochondrial dysfunction *in vitro* accompanied by a significant perturbation to mitochondrial morphology with increased ROS (21, 185, 186). Excessive mitochondrial fission with degraded morphology and function is typical within a stress-induced pro-apoptotic environment as mitochondria are broken down as part of controlled cell-death (169, 184, 187). Indeed, mitochondrial dysfunction and excessive fission has been associated to a wide range of neurodegenerative disorders, including Parkinson's and Alzheimer's diseases (166, 169, 187-189). Ultimately, either scenario required examination at the cellular level to substantiate an ETS effect on the mitochondrial population.

Mitofilin-stained mitochondria were assessed by immunofluorescence microscopy (Fig. 7*A* & *B*). Qualitative analysis by a blinded observer revealed distinct differences in intensity and distribution of the fluorescence between treatment groups. Quantification of

the fractional area occupied by mitochondrial showed as much as a 2-fold increase that was statistically significant in ETS over control within the white matter, molecular layer, granular layer and Purkinje cell body. Immunofluorescence results, thus, substantiated a pronounced affect of ETS treatment on mitochondria across all layers of the cerebellum, implying an effect on granular and Purkinje cell types. However, additional data were needed to confirm that the fractional area was increase due to a greater population of mitochondria (i.e., fission activity) rather than a size increase (i.e., fusion activity).

Electron microscopy provided the decisive evidence for greater mitochondrial biogenesis/fission activity induced by ETS treatment (Fig 7*C* & *D*). Measurements were collected on individual mitochondria within the molecular layer. The results substantiated a 2-fold increase in the number of discrete mitochondrial observations per area (Mito. Density) within the ETS group, while average size remained unchanged. The quantification was consistent with the immunofluorescence data, supporting that mitochondrial biogenesis was increased across the cerebellum. Further, observations of mitochondrial ultrastructure made by blinded experts were consistent with a healthy appearance in both experimental groups. Absent evidence for abnormal mitochondrial morphology or enrichment of apoptotic pathways within the ETS-responsive neuroproteome, the first scenario was determined most applicable – that greater Dnm1l mediated mitochondrial biogenesis along with upregulated aerobic respiration as determined in this study were a likely consequence of ETs induced abnormalities in cerebellar synaptogensis.

In conclusion, results from this systems biology study demonstrate that ETS exposure during the vulnerable period of postnatal cerebellar development resulted in as much as a 2-fold increase in mitochondrial biogenesis. The mitochondrial response was mediated by an ETS-induced increase in Dnm1l, triggered at least in part through calcineurin dephosphorylation of serine 637. Increased Dnm1l activity is known to be associated with synaptogenesis, an ATP intensive process that is the likely driving force behind the abnormal increase in mitochondrial biogenesis. Such a scenario is further support by the increased regulation of aerobic respiration as evidenced through a strong enrichment of associated pathways within the ETS-responsive neuroproteome and discrete HK1 and ATP5 regulatory measures. Future research may find that maladaptive synaptic plasticity within the cerebellum may indeed underlie a causal relationship between ETS exposure and functional deficits symptomatic of ADHD and CD.

CHAPTER 5

SYSTEM BIOLOGY REVEALS ALTERED NEURONAL PROCESS OUTGROWTH IN DEVELOPING BRAIN CONSEQUENT TO ENVIRONMENTAL TOBACCO SMOKE EXPOSURE

5.1. ABSTRACT

A growing number of epidemiological studies suggest a causal association between environmental tobacco smoke (ETS) exposure and behavioral and cognitive deficits in children. The functional domains explored in these studies- behavioral and attentional as well as language disorders are strikingly similar to those caused by perturbations of the cerebellum during development. Using a systems biology approach our study aims to test whether postnatal ETS exposure leads to altered biochemistry in the developing rat cerebellum. Employing a rodent model of postnatal ETS exposure, we investigated differences in the cerebellum neuroproteome of exposed animals relative to controls using a label-free proteomic approach. From these data, we deduced 662 statistically significant peptide measures responsive to ETS treatment. The corresponding ETS responsive proteome was assessed with protein-protein network analysis. Clusters related to protein processing, pre-synaptic transport vesicle activity, cytoskeletal structure dynamics, signal transduction pathways and neurite growth and remodeling factors were discerned. Protein enrichment analysis identified statistically significant associations of the ETS responsive proteome with neuron projections, in particular axon associated proteins and synaptic vesicles. Finally, immunotbloting and microscopy experiments substantiated altered process outgrowth and synaptogenic processes. Taken together, the data depict a striking

modulation in cerebellar formation consequent to ETS exposure that may, in part, be a biochemical and cellular basis for behavioral and cognitive deficits observed in ETS exposed children.

5.2. INTRODUCTION

Epidemiological studies have linked ETS exposure in children with increased incidence of behavioral problems (87, 88, 190) and cognitive deficits to include language disorders and attention issues (51, 79, 119, 191, 192). Most recently, the attention has prominently focused on an association between ETS exposure in children and attention-deficit and hyperactivity disorders e.g. ADHD (89, 90). Separate studies found that 50% of ADHD children have poor motor control, which could be linked to a perturbation of the cerebellum (102, 103). More recently MRI studies demonstrated that children with ADHD have a reduced cerebellar volume relative to controls (104, 105, 133).The cerebellum plays a critical role in coordinated activities beyond locomotion, into such cognitive domains such as language disorders, dyslexia, inhibition of behaviors and attention deficits (98, 193, 194), which are functional domains previously suggested to be impacted by childhood ETS exposure. Exposures during the neonatal period, such as to alcohol, have been shown to perturb cerebellar development (91)(109). Further, insults to the cerebellum when the molecular layer is forming, such as at PD10 or PD15 in rat, have induced hyperactivity, which only resolves later in adulthood (104, 139). The overlap between functional domains impacted by childhood ETS exposure and those with cerebellar involvement suggests a

correlation, whereby ETS may alter cerebellar development that can result in disorders, such as ADHD (134, 195).

The cerebellum stands out as a brain area with rapid post-term development, and thus may have heightened susceptibility to post natal ETS exposure. Work by John Dobbing et al. and others has established a critical time extending from the third trimester as far out as 1.5 years after birth in humans when the cerebellum undergoes rapid development, which has been defined as a vulnerable period (142, 144, 145). An analogous vulnerable period in rats extends from birth to PD21, where damage to the cerebellum resulted in sustained and functionally significant changes (110). During the later 2/3^{rds} of this vulnerable period molecular layer formation is taking place through PD21. During maturation of the molecular layer, granular cells axons extend into the molecular layer where they bifurcate to form parallel fibers. Synaptogenisis then occurs between the parallel fibers dendrites of the Purkinje cells, which send out inhibitory axonal projections as the major regulatory output of the cerebellar cortex.

In this chapter we further asses mass spectrometry data initially described in Chapter 4. Further integration via bioinformatics tools has revealed responsive peptides associated with altered neuronal process outgrowth, in particular axons, and associated synaptogenisis. Presented are the new results along with additional supportive measures.

5.3. EXPERIMENTAL PROCEDURES

ETS exposure, tissue collection, lysate preparation and mass spectrometry procedures are described in Chapter 4. Analysis of the mass spectrometry data utilized

bioinformatics tools for protein enrichment (115) and protein-protein network analyses (118) to identify interactions within our ETS responsive data.

Immunoblotting and immunohistochemsity procedures are described in detail within Chapter 4. Membranes were probed with the following primary antibodies, neurofilament-M (Invitrogen, Carlsbad, CA) at 1:20,000, CRMP2 (Abcam, Cambridge, MA) at 1:20,000, N-Cadherin (BD Biosciences, San Jose, CA) at 1:1,000, MAP2 (Millipore, Billerica, MA) at 1:2,000 and calretinin (Abcam, Cambridge, MA) at 1:20,000. Secondary antibodies utilized were stabilized peroxidase conjugated goat anti mouse and anti rabbit (Thermo Scientific, Rockford, IL) at 1:1500. Bound antibodies were visualized by *chemiluminescence* with either the SuperSignal West Pico or Dura kits (Thermo Scientific) following manufacture instructions. Images were captured on an Image Station 4000MM Pro molecular imager (Carestream Molecular Imaging, Rochester, NY). Quantitative evaluation of protein levels was performed via densitometric analysis of 16-bit grayscale images using Carestream Molecular Imaging Software. Immunohistorchemistry was performed for MAP2 (Millipore, Billerica, MA) at 1:1000 with AlexaFlour 588 fluorescence conjugated secondary antibody (Invitrogen) at 1:1000. See Methods, Chapter 4 for further detail.

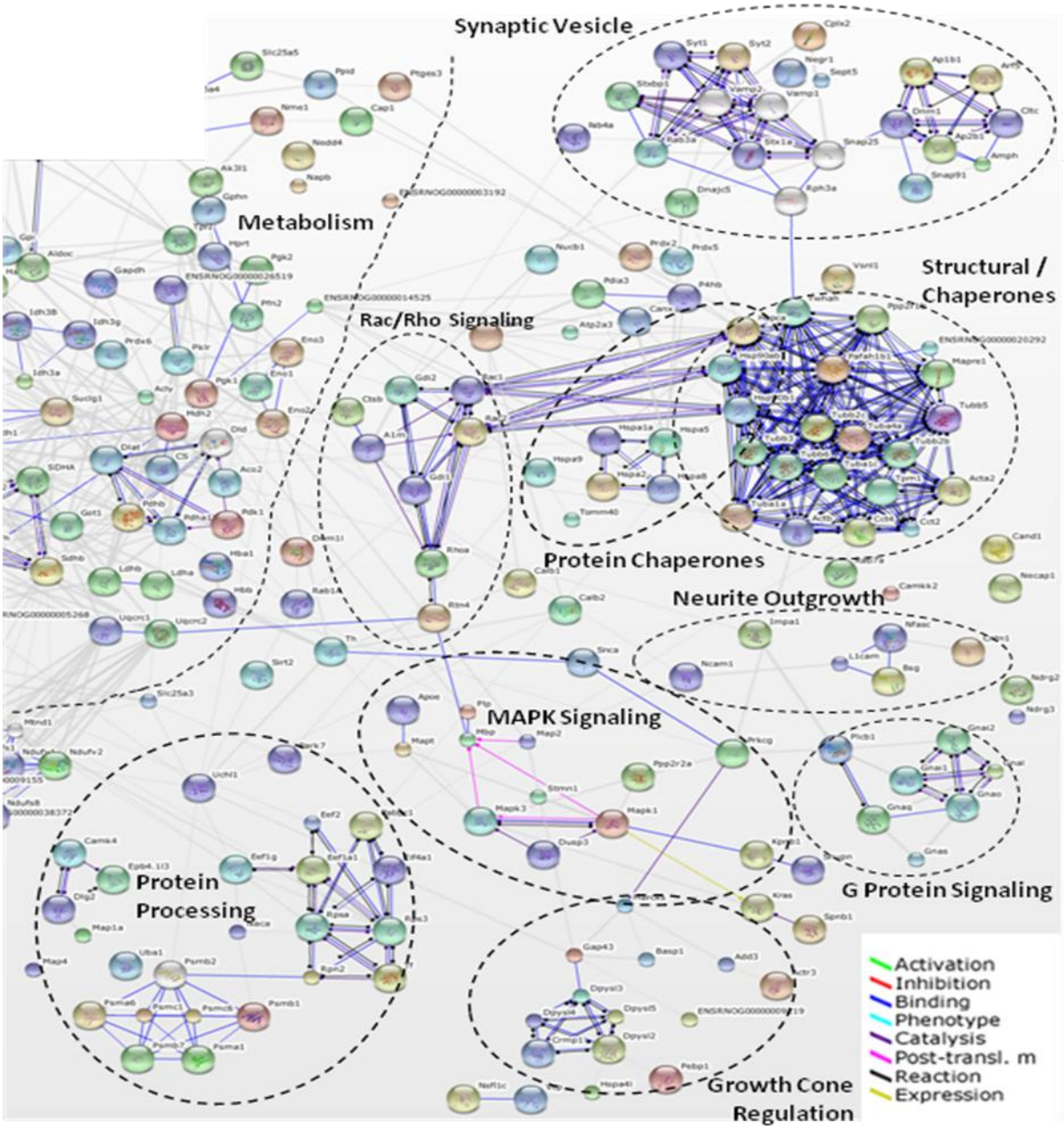
5.4. RESULTS

The associated proteins to all 662 responsive peptides (see Chapter 4) were analyzed by protein-protein network analysis (Fig. 5-1), in Chapter 4 our earlier assessment focused on mitochondrial associated elements. We visualized the results based

on known action-based interactivity between responsive elements (e.g., reaction, binding, modifying activity). The large cluster of peptides associated with metabolism was again present, as explained in Chapter 4. However, we identified nine additional interactive protein clusters.

Four of the identified clusters are involved with protein processing: (1) a group involved in protein elongation includes multiple members of the elongation factor protein family (Eef) and polyadenylate-binding protein 1 **(**PABPC1); (2) 40S ribosomal proteins Rpsa and Rps3 that are involved in protein synthesis as well as six additional (data not shown) responsive ribosomal proteins involved in mRNA processing; (3) a large group of responsive chaperone proteins to include multiple heat-shock 70 (Hspa) and 90 (Hsp90) family members; (4) lastly, multiple proteins associated with *ubiquitin*-proteasome activity were responsive, to include UCHL1, UCHL3, Uba1 and seven components of the proteasome (PsmX). Another cluster highlighted 20 pre-synaptic transport vesicle associated proteins, involved in trafficking the building blocks required for axonal elongation and guidance. In addition, we observed a larger cluster of actin (Act) and

Figure 5-1: Protein-protein network analysis of the ETS responsive proteome. Interaction networks of ETS exposure responsive proteins within the rat cerebellum. Results were generated using the protein-protein interaction algorithm of the String Database (162). Proteins associated with 662 responsive peptide measures were imputed into the software. An action-based display was selected with a minimum correlation factor of 0.5. The white nodes were added for enhanced interactivities. Biologically-relevant protein clusters are highlighted and labeled, respectively.



tubulin (Tubb) elements along with associated chaperones such as HSP90 and chaperonin containing TCP1 complex (CCT) proteins that fold and stabilize actins and tubulins.

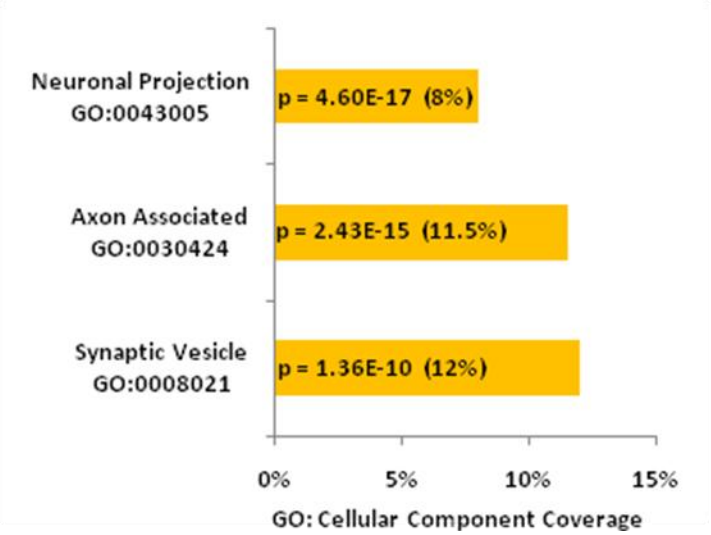
Proteins associated with signal transduction pathways were discerned. Rho family GTPases, to include Ras-related C3 botulinum toxin substrate 1 and 2 (Rac1, Rac2), were observed as well as Ras homolog gene family member A (RhoA). Rac1 and Rac2 play important roles in signaling events required for axon growth (196, 197). In Fig. 5-1, RhoA and one of its binding partners reticulon 4 (Rtn4), also known as nogo are observed together. The binding of Rtn4 with its receptor Rtn4R will induce the RhoA-dependant pathway resulting in neurite outgrowth inhibition (198, 199). Another signaling cluster included mitogen-activated protein (MAP) kinases, specifically Mapk3 and Mapk1. Mapk3 can phosphorylate Mapk1 that in turn can regulated microtubule-associated protein 2 (Map2) via phosphorylation. Map2 is involved in microtubule assembly and plays a important role in determining and stabilizing dentritic shape during development. Other notable elements linked with the MAPK signaling cluster were microtubule-associated protein Tau (Mapt) that is involved in microtubule organization within axons, and myelin basic protein (MBP), which is known to exhibit developmental-specific isoform dynamics during *synaptogenesis* and myelin formation.

The network analysis cumulated with a group of clusters related prominently to various aspects of neurite growth. The first of these clusters contained proteins necessary for process outgrowth. Notable elements included neural cell adhesion molecule 1 (Ncam1) and neural cell adhesion molecule L1 (L1cam), two proteins involved in cell-cell adhesion that play an integral role in process development. Also seen in the neurite

outgrowth cluster were two other adhesion proteins neurofascin (Nfasc) and contactin 1 (Ctn1), which play a role in process outgrowth and synaptic dynamics. A cluster of five collapsin response mediator protein members (Dpysl/Crmp) was also observed. Dpysl/Crmps are phosphoproteins expressed exclusively in the nervous system that are integral to growth cone dynamics regulating extension and collapse. Also observed were proteins associated with the Dpysl/Crmps. Growth associated protein 43 (Gap43) is predominantly found in growth cones during development. The myristoylated alanine-rich C-kinase substrate (Marks) proteins were found to interact with Gap43 and are regulated by protein kinase C (PKC) observed in Fig. 5-1 as Prkcg. Lastly another protein regulated by PKC was observed, Gamma-adducin (Add3), that is involved in the assembly of spectrin-actin networks.

Subsequent to network analysis we further mined for related information by enrichment analysis. We specifically inspected these results for enrichment association with process outgrowth and synaptogenisis. Statistically significant associations were identified with the following Gene Ontology cellular components: neuron projections, axon associated proteins and synaptic vesicles (Fig. 5-2). While the overall coverage was lower than enrichment observed for metabolism in Chapter 4, p-values indicated high significance. The GO term Neuron projections contained elements of both dendrites and axons. However, it was the specific GO term for axons was significantly enriched.

Fig. 5-2: Protein enrichment analysis. Enrichment analysis was performed utilizing the Toppgene suite of tools (161).From the results, we identified an enrichment of three GO Cellular Components terms, which contained a statistically significant number of elements relative to a random assortment. P-values from Fishers exact testing are included for each term, and the percentage of term-associate elements found in our ETS-responsive data are shown in parenthesis.



Exposure to ETS affects Neurite Outgrowth in Cerebellum- Immunotbloting results were generated to substantiate observations from the mass spectrometry data (Fig. 5-3). Neurofilament-M, an axonal marker expressed during development, was two-fold greater in the matrix (soluble) fraction of the ETS group. N-Cadherin (Ncad) is an extra-cellular adhesion molecule that modulates synapse formation. We observed a six-fold greater amount of Ncad in the matrix fraction of the ETS group, relative to the control group. The pro form of Ncad is delivered to the growth cone via presynaptic transport vesicles and is presented extracellularly where it can be cleaved and released during synapse maturation (200). We also examined CRMP2 via immunoblot analysis, which if phosphorylated by Rho kinase will inhibit CRMP-2 from associating with microtubules resulting in growth cone collapse. In both the matrix and membrane fractions more p-CRMP-2 was present in the ETS group, though the measurements did not reach significance after multiple measure correction. Immunoblot analysis also revealed a greater amount of Map2 in the ETS membrane fraction. Map2 is known to be elevated within dendrites synaptogenisis (201) . We further examined Map2 via immunohistochemistry (Fig. 5-4), which revealed an increase in Map2 staining specifically in the molecular layer of ETS exposed cerebellum, corroborating our molecular results and localizing our observation to the molecular layer.

Fig. 5-3: Proteins related to neurite outgrowth as shown responsive by orthogonal immunoblot analysis. (A) Immunoblots for proteins involved in neurite outgrowth in the matrix and membrane fraction. (B) Normalized densitometric quantification of immunoblot results with calretinin used as a loading control in both the membrane and matrix fractions. Symbols indicate significant differences from control (*$p \leq 0.02$ and **$p \leq 0.001$) by a Student's *t*-test with a Holm-Bonferroni method correction for multiple-testing.

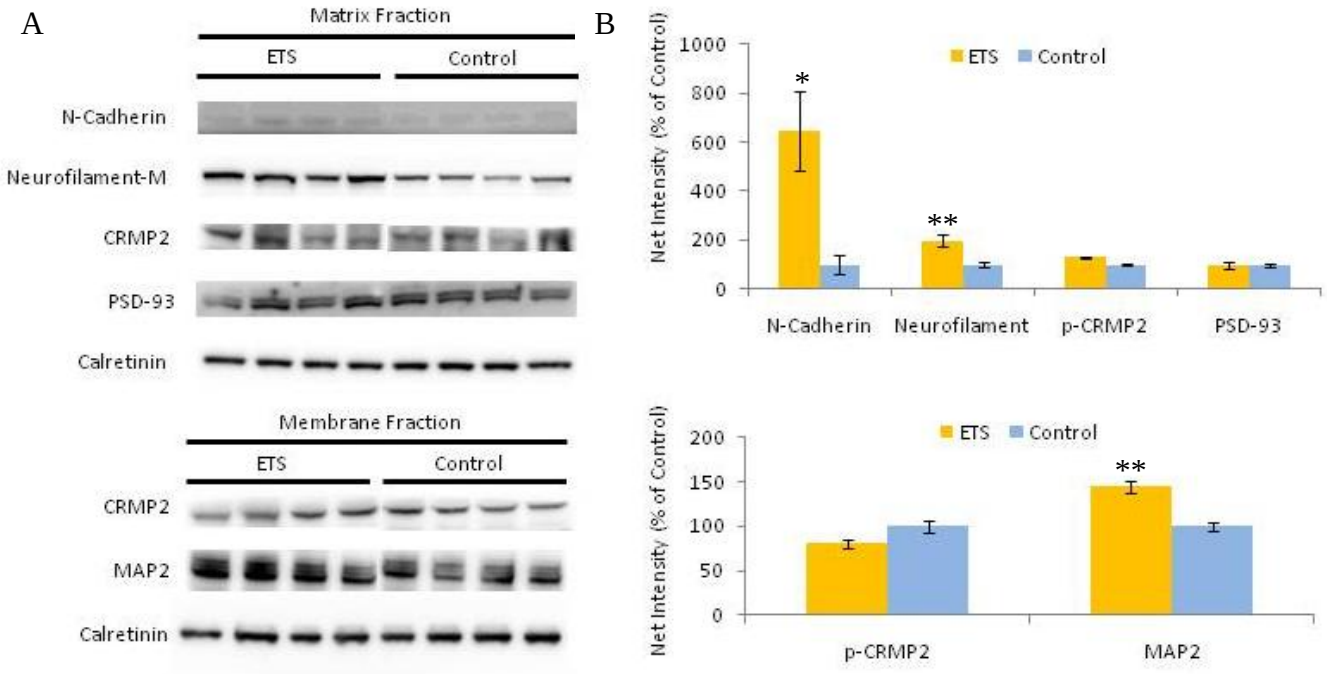
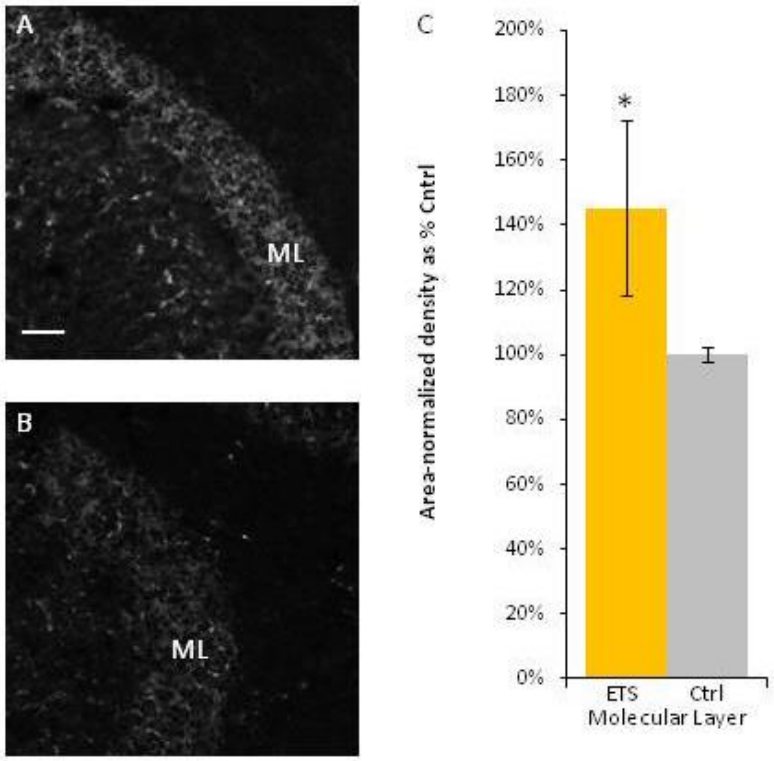


Fig. 5-4: Greater dendritic plasticity within the cerebellar molecular layer following ETS exposure. An increase in Map2 density normalized to area was observed in the molecular layer (ML) of cerebellar sections in the ETS (A) group relative to control (B). Scale bar: (in A) A-B, 200 µm. Area normalized Map2 density was quantified from matched sections of molecular layer (Mean ± S.D., n=3). Symbol indicates a significant difference from control (* p=0.02) by Student's t-test.



5.5. DISCUSSION

At the peptide level, we observed a statistically significant decrease in isoform A of Rtn4 within the membrane fraction, which suggest a reducing in the membrane tethered state (202, 203). Recent evidence suggests that Rtn4-A will operate as a potent outgrowth inhibitor and initiate growth cone collapse via a RhoA-associated signalsome in neurons (204). In this process, extracellular Rtn4-A anchors the growth cone and is rapidly taken up by neurons via a clatherin-independent process mitigated by EH domain-containing proteins (Ehd's). Indeed, we observed an increased level of Ehd1 within the matrix fraction of the ETS treatment group. Disruption of the Ehd endocytosis mechanism will lead to elevated membrane tethered levels of Rtn4-A and disrupts the outgrowth inhibition function of Rtn4-A. Modulation of the Rtn4/RhoA signalsome may therefore result in altered levels of membrane-tethered Rtn4 as indicated by our proteomic data, where the lower level of membrane-tethered Rtn4 suggests greater activation of the inhibitory function of the signalsome within the ETS group. Further confirmation of this was the strong increase in RhoA at the peptide level, which is well known for its involvement in outgrowth arrest and growth cone collapse (198, 205). Activated RhoA will result in Rho Kinase (ROCK) activation that then can acts upon a range of substrates.

A novel, mammalian brain specific substrate of activated ROCK is profilin II (Pfn2), which was found to be greater in the matrix fraction of the ETS group. Pfn2 is now known to be a negative regulator of process outgrowth (206). Indeed Da Silva et al demonstrated that Pfn2 is recruited to the processes via a RhoA/ROCK activation-

dependent pathway. Following RhoA activation, ROCK complexes with Pfn2, which then increases polymerized actin stability that inhibits neurite outgrowth and synaptogenesis. Another relevant RhoA/ROCK substrate is CRMP-2, a member of the dihydropyrimidinase-related protein family that as a whole (all five family members) was responsive to ETS treatment. CRMP-2 phosphorylation by ROCK has been shown to inhibit its association with tubulin and the recruitment to the growth cone as necessary for microtubual elongation and axon growth (207). We further assess CRMP-2 by immunoblot analysis (Fig. 5-3) and observed an increase in the phosphorylated form of the protein in both the matrix and membrane fractions of the ETS group, though the measures did not reach statistical significance after correcting for multiple immunoblot measures performed with these samples. Elevated p-CRMP-2 is a further sign of inhibited axonal outgrowth and growth cone collapse (208).

ETS responsive proteomic data also suggest that protein kinase C (Prkcg or Pkc) may be more active and influencing a number of downstream effectors to further growth cone collapse. The peptide data indicates a reduction in the soluble levels of Pkc, which indirectly suggests that more Pkc maybe be membrane tethered where it is activated. In particular, MARCKS protein was modulated by ETS treatment. We observed increase phosphorylation of MARKS, which would dissociated it from the membrane, also observed as a decreased level in the membrane fraction. We observed a similar trend for the Pkc substrate fascin (Fscn1), which like MARCKS had increase phosphorylation and a decreased amount in the membrane fraction of the ETS group relative to control levels. While a differential phosphopeptide was not observed for GAP43, it was again lower in the

membrane extract of the ETS group and is also a Pkc substrate. Taken together, these data further the case for an increase in growth cone collapse involving Pkc phosphorylation of multiple growth cone elements (209).

Map2 proteins are known to increase following neurite growth initiation and remain elevated through dendritic outgrowth (210, 211). The function of Map2 is to stabilize microtubules (MT) by cross linking MT with intermediate filaments and other MT. Our data showed a greater level of MAP2 in the membrane fraction of the ETS group (Fig. 5-3), which we confirmed to be localized to the molecular layer of the ETS group by immunohistochemistry (Fig. 5-4). Elevated Map2 in the molecular layer suggest increase plasticity in Purkinje cell dendrites. Given that Map2 levels subsequently fall during synaptic maturation after PD 21 in the rat, our Map2 results suggest an immature dendritic phenotype or an abnormal plasticity within the molecular layer. We further assessed PSD-93 by immunoblot analysis, a post-synaptic density marker enriched in cerebellar Purkinje cells that should associated with mature synapses. Results for the ETS group show a dramatic range of biological variability not present within the control group. In two of four smoke animals we saw a decrease in PSD-93 levels across two different bands relative to control supporting a reduction in synapse maturation. However, the other two ETS samples show a prominent increase in the lower of two observed bands suggesting a difference in the modification state of PSD-93, which is known to be regulated by phosphorylation and palmitoylation (212). While it remains unclear as to the significance between the PSD-93 band-pattern within the ETS group, what is clear is that the effect of ETS treatment on PSD-93 is highly variable between individual animals.

Results discussed thus far have suggested ETS induced growth cone collapse and an inhibitory state to axonal outgrowth. However, there was a separate cluster of responsive proteins that supported an increase in axonal guidance and adhesion functionality after ETS treatment. A large number of L1 family adhesion molecules were found responsive to ETS treatment. Ncam1, the most prominent was found increase in a membrane tethered state as was L1cam and contactin 1 (Cntn1). The latter two L1 members were also found with reduced phosphorylation, supporting an extracellular presentation, since phosphorylation by Map kinase 1 (Mapk1) would result in the dissociation of these L1 molecules from actin and destabilization of their extracellular presentation at the growth cone. Mapk1 was also found responsive, with less phosphorylation and a lower amount recruited to the membrane suggesting less activity. Neurofascin (Nfasc) was the only L1 family member found lower in abundance in the membrane fraction of the ETS group, which may be a consequence of isoform dynamics not resolved in our data. Indeed, Nfasc is unique in the L1 family by having many splicing variants (179). Ncam1 and L1 Cam are both well known signal coreceptors in neuronal migration and process outgrowth, and provide cytoskeletal linkage necessary for cell growth cone motility (213). Thus, it remains unclear why their levels as well as that of Cntn1 indicate an increase in guidance and adhesion at odds with other results suggesting inhibited axon outgrowth and growth cone collapse. Another adhesion molecule not directly related to the L1 family found elevated in the ETS group is N-Cadherin (Ncad). A six-fold greater level of Ncad was measured in the matrix fraction of the ETS exposed group indicating a greater abundance in the cytosol (Fig. 5-3). N-cadherin, while primarily

known as a substrate-associated adhesion molecule, is also known to stimulate neurite outgrowth as a soluble molecule in cerebellar neurons (214). N-cadherin is particularly associated with axon guidance (215). Together with the L1 family dynamics, it seems likely that there are two different processes at play within the cerebellum in response to ETS treatment. One that is inhibiting axonal outgrowth with growth cone collapse that is an abnormal effect of the exposure, while a second processes is trying to compensate by maintaining high levels of guidance cues to attempt to complete synaptogenesis inhibited by the first processes. Future studies will examine structural aspects of axons and synapses. For example, neurofilament-M staining may allow us to visualize parallel fibers within the molecular layer. We have already performed immunoblot analysis of NF-M, which indicated an elevated level within the matrix fraction of the ETS group. The carboxy-terminal tail domain of neurofilament-M is essential for the size and cytoskeletal architecture of axons (216).

The results from this study depict a prominent affect of ETS treatment on a cerebellum neural architecture. We have presented evidence of growth cone collapse and inhibited axonal outgrowth induced by ETS treatment. We have provided evidence for a concomitant immature dendritic phenotype. Other data suggests that the cerebellum may be attempting to compensate by maintaining adhesion/guidance cues at a time point when synaptogensis should be largely completed. Other biological processes involved in synaptic development to include protein processing, cytoskepetal dynamics and presynaptic transport vesicle activity are also found responsive to ETS treatment. Those processes are all largely dependent on high-levels of ATP supported by increase aerobic

respiration functionality. Future experiments will look to expand cellular characterization of axonal and synaptic structures within the cerebellum; thus, further substantiating a miss wiring in association with ETS exposure. Indeed recent studies are beginning to identify dysfunction in synaptic plasticity within the cerebellum (217) and how cerebellar deficits may be at least in part responsible for ADHD and other behavioral disorders in children (134).

CHAPTER 6

CONCLUSIONS

6.1. THE EFFECTS OF ETS ON THE DEVELOPING RAT CEREBELLUM

In Chapter 4 we demonstrated an increase in mitochondrial proliferation as a result of ETS exposure in the developing rat cerebellum. Absent of any abnormal morphology, the increase in mitochondria is likely driven by an increased demand on aerobic respiration. Further evidence in Chapter 4 also pointed to an increase in aerobic respiration through increasing of glycolysis, the TCA and the ETC. Most telling was the translocation of Hexokinase-1 from an unbound cytosolic state to a mitochondrial membrane bound state, which happens only under increased utilization of adenosine triphosphate (ATP). In Chapter 5 results depicted altered neuronal process outgrowth, specifically in axon formation. Protein-protein network analysis of the 662 responsive peptides identified a series of interactive protein clusters. A commonality between these clusters is their need for ATP. For example, in protein synthesis ATP is required for aminoacyl tRNA synthetase activity, which is essential for elongation of a growing peptide chain. On the other hand, if proteins are being degraded rather than formed then ATP would also be required. Ubiquitin-proteasome activity is highly dependent on ATP for ubiquitination, recognition by the 19S regulatory particle and unfolding of the protein to be degraded. It is fitting that we also observed responsiveness among various protein chaperones. Chaperones function in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation. Heat shock proteins 70, 90 and chaperonin containing TCP1 complex (CCT) proteins all utilize ATP as a regulator of their binding

ability. No matter if a new protein is being synthesized or targeted for degradation, ATP is required. ATP is also required for the formation of actin filaments and cytokinesis. Presynaptic transport vesicles are dependent on ATP to fuel V-ATPases that in turn create a proton gradient to allow for active transport. These results frame the question- what can this increase in ATP demand in the ETS exposed group tell us about its possible phenotype relative to the control group?

The development of the cerebellum begins after birth in rats and lasts until about PD21. Chapter 3 detailed the vulnerable period in which the cerebellum is particularly vulnerable to perturbations that can result in sustained and functionally significant changes. From a metabolic standpoint this is also a time of increased utilization of aerobic rather than anaerobic respiration. During this time of increased aerobic respiration, the brain is rapidly increasing its synaptic density, utilizing an increased amount of ATP for the formation of neurites, their refinement, and finally maturation of synapses. The developmental increase in aerobic respiration eventually declines, leveling off during adulthood. In Chapter 5 we presented evidence for a concomitant immature dendritic phenotype and data suggesting that the cerebellum may be attempting to compensate by maintaining adhesion/guidance cues at a time point when synaptogenisis should be largely completed. Other biological processes involved in synaptic development, to include protein processing, cytoskeletal dynamics and presynaptic transport vesicle activity, were also found responsive to ETS treatment. Very telling is that those processes are all largely dependent on high-levels of ATP supported by increased aerobic respiration functionality.

Taken together Chapters 4 and 5 present strong evidence for an immature developmental phenotype as a result of ETS exposure, with Chapter 5 describing the immature phenotype and Chapter 4 presenting evidence for the greater energy demand of an immature phenotype.

6.2. IMPLICATIONS OF ETS EXPOSURE IN ADULTS AND CHILDREN

Epidemiological data cited and Chapters 1 and 3 highlighted two populations which appear most vulnerable to ETS exposure, children and the elderly. In both cases there have been numerous studies which have linked exposure to ETS with neurological disorders, namely dementia in the elderly, and ADHD/CD in children. Lacking in these studies are defined biochemical/molecular/cellular pathways which are affected as a result of ETS exposure. While epidemiological studies can determine the detrimental effects of ETS exposure it us unable to elucidate the mechanisms by with ETS is harmful to these vulnerable populations. A better understanding of the basic biology of ETS exposure in the brain is paramount to developing possible treatment options, especially in children with ADHD/CD. The overall scope of this dissertation research was to begin to elucidate the biochemical and molecular effects of ETS on the adult and developing mammalian brain. In this section I will speak to the implications of our research data as it applies to both our adult and developing brain stories.

In Chapter 2 we presented evidence to apoptosis, gliosis and a possible neuroprotective effect. Apoptosis has been previously observed in other tissues and in culture as a result of ETS exposure. In the brain, increased apoptosis has been observed to

be a hallmark of Alzheimer's pathobiology (218, 219). Similarly we also observed an increase in GFAP, a marker of gliosis, which is also common in Alzheimer's pathobiology (220-222). Alternatively ETS induced dementia could be as a result from downstream cardiovascular effects which were discussed in detail in Chapter 1. Finally our adult study observed an increase in β-synuclein, a protein known for its neuroprotective effects. β-synuclein has shown anti-apoptotic properties and has also been shown to restore the anti-apoptotic function of α-synuclein, a key protein in Parkinson's pathobiology. Smoking has long been known to reduce PD incidence in a dose dependent fashion and the marked increase in β-synuclein levels observed here may be a neuroprotective response to the observed neuronal apoptosis. Our experimental findings in Chapter 2 begin to lay a framework for a possible biochemical connection between ETS exposure and an occurrence of dementia in an elderly population.

Chapters 4 and 5 presented data for the effects of ETS on the cerebellum during a vulnerable developmental period. Epidemiological evidence has pointed to a reproducible dose-dependent risk for behavioral and associated cognitive problems in children exposed to ETS. The effect of ETS on those domains was often assessed in direct association with diagnosed ADHD/CD. However, like dementia in the elderly population, the biochemical mechanism has remained unclear. In Chapter 4 we demonstrated altered aerobic respiration, and a greater density of mitochondria within the cerebellum. Normal mitochondrial morphology in the ETS exposed tissue lead to the theory that the increase mitochondrial proliferation was not as a result of a stress response but instead appeared consistent with an altered synaptogenic phenotype. Further data in Chapter 5 provided

further evidence for an immature developmental phenotype as a result of ETS exposure. The immature phenotype was characterized by axons and dendrites that appeared to be in a plastic state and a lack of mature synapses. The current data shows signs of an underdeveloped cerebellum as a result of ETS exposure during the vulnerable period. This could then result in the ADHD/CD like phenotype, which if true, would potential mean that we have taken the first step in understanding the biological mechanism by which postnatal ETS exposure may induce childhood deficits associated with ADHD and CD

6.3. PROTEOMICS AND THE SCIENTIFIC METHOD

In order to evaluate the effectiveness of proteomics in my dissertation research, it was useful to compare and contrast the experimental designs used to study the adult vs. the developing rat brain. For both research projects the defined question was similar, testing whether exposure to ETS results in a measurable change at the molecular and cellular levels. The next step for both projects was to gather supporting information to create a targeted hypothesis. The central hypothesis for the adult study was crafted around previous findings form work associating tobacco smoke exposure with the central nervous system (CNS). To that end, there was very little information relating ETS directly to the CNS. However, I was able to obtain literature on ETS exposure for cultured cells and the cardio and pulmonary systems. From the literature, I crafted the hypothesis that ETS exposure in the adult brain would lead to altered biochemical effects due to gliosis, apoptosis and damage caused as a result of an increase in reactive oxygen species. Literature pertaining to the effects of ETS on the developing brain was also limited. I discovered a wealth of

literature on epidemiological studies that helped provide evidence to the possibility that ETS exposure during the critical neonatal phase causes functional perturbations in cognitive and behavioral domains later in life. Without any *a priori* knowledge of any biochemical or molecular effects, other than our adult study findings, we opted to adopt a holistic, systems biology experimental design. In this approach, data from a large-scale proteomic experiment would be mined to refine a working hypothesis for subsequent targeted analysis. For example, analysis of the proteomic data framed the working hypothesis that ETS exposure during a vulnerable period of cerebellar development would result in excessive mitochondrial biogenesis, increased aerobic respiration demand and modulation of cerebellar circuit formation. From here, projects reported in Chapters 4 and 5 followed similar paths as we tested our hypothesis via orthogonal methodologies such as, immunoblotting and microscopy analyses.

Some points need to be discussed in comparing between the holistic, systems biology experimental design of Chapters 4 and 5 in contrast with the reductionist design in Chapter 2. In the adult study we were able to find changes in representative biomarkers for apoptosis and gliosis. However we tried multiple markers of ROS and none of them had a statistically significant difference between treatment groups. In the developing rat brain we were able to repeatedly discover supporting evidence of our hypothesis via orthogonal methodology. The application of proteomics for systems biology experimentation allowed us to better define a specific hypothesis not solely based on the literature but from novel data.

While a very powerful tool, proteomics is not without its limitations. Many of the computational resources used in the completion of this study in the developing brain were originally designed for application to microarray experiments. Recent studies have demonstrated a poor correlation between changes in mRNA levels and changes in the protein level. Moreover, data collected at the peptide level can be more telling in terms of PTMs, isoform specificity, and spacial locality. Quantification from proteomics data is the collection of replicated measurements of the protein pieces themselves, the peptides. However, a main limitation to mass spectrometry is the inability to detect all peptides from a given protein of a complex protein mixture. This can be attributed to various technical aspects of mass spectrometry experimentation. First is the limitation of detection on the mass spectrometer. The detection capability has increased greatly over the years; however, the ability to capture low abundance peptides remains difficult. Next is the effect of the enzyme used to digest the complex protein sample. In our experiment trypsin was used to digest the proteins into peptides by hydrolyzing the lysine and arginine residues. By only using a single enzyme it is certain that some peptides would have been created that were either too large or too small to be properly ionized and detected. The last limitation is that all peptide identifications are based on matches from user defined peptide databases. As noted in Chapter 4, the numbers of reproducible ions detected among our replicates was large, but only a small percentage of those measured ions were identified. The unidentified ions could have been a result of PTMs not selected in our search algorithm. Future processing of the data, with target searches for PTMs beyond phosphorylation and glycosylation could very well uncover additional peptide information. Also possible is that

the corresponding peptide was not present in the database we used. The upside of the database issue is that it is fluid, with new proteins and peptide sequences being added daily. The successful application of a holistic, systems biology experimental design provided a wealth of biochemical and cellular information for the effects of ETS on the developing cerebellum. Furthermore, the effects seen were not predicted by the literature, and therefore, might have gone unseen using a reductionist design.

6.5. INDIVIDUAL EFFECTS OBSERVED IN ETS EXPOSED RATS

One consistent observation was made during the course of both the adult and developing brain studies. This observation was an increase in the variability in signal intensity among the ETS exposed group relative to the control group during immunoblot experiments. Initially the variability was associated with possible experimental error, with the most likely culprit being unbalanced samples. However as can be observed from the loading controls this did not seem likely. Also, this phenomenon was observed consistently with various different antibodies (Figure 2-1, Figure 2-2). The observed variability led to a non-significant difference between the ETS and exposed groups for those affected proteins. We did not observe a similar increase in variability in our control groups. Furthermore, we also observed this variation, albeit to a lesser extent in our developing rat studies, in particular the immunoblot of PSD-93 a marker of mature synapses (Figure 5-3). In our ETS group two animals showed a noticeable decrease in PSD-93 relative to the control group. The variation can also be observed in the literature which has been discussed in Chapters 1 and 3. In ADHD studies only 50% of those afflicted also showed motor

deficiencies, and a large variance was observed among the behavioral studies which were discussed in Chapter 3. We detected a slightly larger (0.08) increase in variance among the quantitative values for the peptides derived from the ETS exposed cerebellum sample. However, the variance did not have the same statistical effect on the data as found among the above mentioned immunoblot experiments. This leads to the question of what could be responsible for the observed variation. I believe the best evidence can be found in the literature linking ETS to increase dementia risk, detailed in Chapter 1. These studies found that in a population with cardiovascular vulnerability, ETS exposure can increase dementia risk. I hypothesize that one cause for the increased variation in the western blot data is an unknown, possibly cardiovascular related issue in the ETS exposed rats used in the study. Future studies will most likely require a larger test population in order to determine the extent of, and the further elucidation of the underlying cause of the observed variability.

6.5. FUTURE EXPERIMENTS

The studies in the adult and developing brain have showed that there is a measurable difference between the ETS and controlled brains at the protein level. Now that we have established that a difference can be measured we can now outline future experimentation to further study the effects of ETS on the developing rat brain. One concern that arose during our mass spectrometry study was statistical power. Due to the fact that we were utilizing a new mass spectrometer we were unable to do a proper power analysis to determine the number of animals needed to reduce the number of false negatives. Our mass spectrometry results during the developing brain study showed a large

variability between the two groups. In order to correct for this we chose a stringent alpha to reduce our false positive detection rate. However due to the size of our population we also increased the number of false negatives. During the statistical analysis our dilemma was whether to decrease our alpha in order to increase the number of measurements or stay with the corrected alpha which would lead to less measurements overall. We decided to stay with our stringent alpha value which reduced our type I error to ~5% but provided us with enough measurement to tell a biologically relevant story. Moving forward with the ability to perform a proper Power Analysis we have found that we need to increase the sample size from 4 to 6 animals per group. In regards to instrument sensitivity we were quite impressed with the limit of detection of the mass spectrometer. Out of the 662 responsive peptides measured, 119 (18%) had a fold change of 7% or less. Our range of detection ranged from as little as 7% to as high as 256% demonstrating the ability of the mass spectrometer to accurately measure both large and small fold changes between the groups.

Future experiments will also use a different exposure timeline to more accurately match with human exposure. In rats the cerebellum develops completely after birth, but in humans the cerebellum starts to develop prior to birth. In order to adjust our rat model to match a post natal developmental time point we would move our exposure start time to PD8. The vulnerable period in cerebellum development in humans closes when the molecular layer matures which between eight months to one year. In rats this happens around PD21. The new exposure timeline would better match a post natal exposure paradigm in humans. Furthermore since our work has led us towards the idea of a

developmental effect it would be of great interest to have more data from multiple time points rather than a single snapshot at the end of the exposure period, this would allow us to gain a better perspective of the development changes as they are happening.

Much of the literature cited in my dissertation has looked for, but has not been able to determine a direct association between ETS and cognitive development. The same can also be said for behavioral issues caused as a result of ETS exposure during neuronal development. A strong possibility for this lack of a solid connection could be related to the use of surveys for obtaining information. Clinical datasets may not always be reported accurately and/or honestly. In order to eliminate bias, it would be valuable to utilize our rat model for behavioral testing. Our animal model could be used to study both cognitive and behavioral difficulties as a result of ETS exposure. Behavioral deficits such as irritability and anxiety could be assessed by the determination of emotion changes in the test subjects as a result of ETS exposure. Cognitive and behavioral difficulties associated with ETS exposure will be measured using widely utilized and accepted paradigms within the field of psychiatry. In particular, simple measures of movement and exploration could be utilized to measure deficits in attention and or hyperactivity, which could provide valuable data towards our ADHD hypothesis. It would also be beneficial to follow some rat pups into adulthood for long term observation of exposure during the vulnerable period until adulthood which would be ~PD58. Allowing some of the rats to mature into adulthood could provide valuable information on long term changes in behavior, cerebellar structure and the potential to witness a reverse of the hyperactivity deficit as discussed in Chapter 3.

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