High-Throughput Analysis of Age-Dependent Protein Changes in Layer II/III of the Human Orbitofrontal Cortex

By

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Abstract

Studies on the orbitofrontal cortex (OFC) during normal aging have shown a decline in cognitive functions, a loss of spines/synapses in layer III and gene expression changes related to neural communication. Biological changes during the course of normal aging are summarized into 9 hallmarks based on aging in peripheral tissue. Whether these hallmarks apply to non-dividing brain tissue is not known. Therefore, we opted to perform large-scale proteomic profiling of the OFC layer II/III during normal aging from 15 young and 18 old male subjects. MaxQuant was utilized for label-free quantification and statistical analysis by the Random Intercept Model (RIM) identified 118 differentially expressed (DE) age-related proteins. Altered neural communication was the most represented hallmark of aging (54% of DE proteins), highlighting the importance of communication in the brain. Functional analysis showed enrichment in GABA/glutamate signaling and pro-inflammatory responses. The former may contribute to alterations in excitation/inhibition, leading to cognitive decline during aging.
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List of Abbreviations

GABA - gamma-Aminobutyric acid

GO – Gene Ontology

IPA – Ingenuity Pathway Analysis

LCM – Laser Capture Microdissection

LC-MS/MS - Liquid chromatography Tandem Mass Spectrometry

MALDI MS - Matrix-assisted laser desorption/ionization Mass Spectrometry

OFC - Orbitofrontal Cortex

PFC – Prefrontal Cortex

ROS – Reactive Oxygen Species

SDC – Sodium Deoxycholate

SDS- Sodium dodecyl sulfate
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Chapter 1: Introduction

1.1 Context

The world population is estimated to reach 9.7 billion in 2050, of which those over 60 years of age will comprise 2.1 billion. In North America alone, the aging population above 65 years will account for 41% of the population by 2030 (United Nations, 2015). The rise in the world’s aging population increases the incidence of age-related diseases, such as heart disease, diabetes and brain disorders (United Nations, 2015). Therefore it is essential to understand the biological processes of normal aging in order to design effective therapeutic interventions to delay or prevent age-related illnesses.

1.2 Theories of Aging

Multiple theories have been proposed to explain the biological basis or molecular mechanisms/pathways of aging over the past seven decades. Previously, proposed theories are based on DNA damage and programed-based hypothesis, which are further classified into several postulates. DNA damage based theories such as Orgel’s hypothesis of aging define aging to be based on molecular mechanisms that cause damage to DNA which transcends to errors at the protein level that accumulates over time, leading to autophagy and cell death. (Orgel, 1963). Other popular DNA damage-based theories were based on changes that occur in energy metabolism and accumulation of free radicals through time and age, which ultimately has detrimental effects on cellular and organ systems overall (Ames et al., 1993; Duffy et al., 1990; Gerschman et al., 1954; Harman, 1956; McCarter and Palmer, 1992). In parallel, other theories have also been proposed based on the idea that aging is a pre-determined programmed process within the human genome. These theories highlight mechanisms associated with progressive changes in the programmed endocrine signaling system (Breese et al., 1991; de Magalhaes and Sandberg, 2005; Hammerman, 1987; Ho et al., 1987; Klass and Hirsh, 1976; Sapolsky et al., 1986). These theories provide insight into specific molecular mechanisms that mediate aging, however do not provide a holistic view of the aging process.
1.3 Hallmarks of Aging

In a recent review by Lopez-Otin et al. 2013, the authors summarized the aging phenotype in the form of a combination of nine molecular processes, termed as “Hallmarks of Aging”. These hallmarks which are based on evidence observed in peripheral somatic tissue, attempt to comprehensively explain the underlying biological processes that mediate the aged phenotype in cells and tissues (Lopez-Otin et al., 2013). The term ‘aged phenotype’ is defined here as a decline in functionality of a cell or tissue which can hinder the ability to adapt to changing environment, and cause loss of homeostasis or cellular/organism death (Fedarko, 2011). According to Lopez-Otin et al (2013), the nine hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and intercellular communication, may be classified into three categories, i.e. primary, antagonistic and integrative hallmarks.

Genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis, are termed as primary hallmarks. For example, the integrity of DNA is regularly challenged by various threats (e.g. reactive oxygen species, DNA replication errors, chemical alterations, mutations) which can contribute to genomic instability, telomere attrition and epigenetic alterations (epigenetic drift caused by DNA sequence variation and mutations)(Lu et al., 2004; Teschendorff et al., 2013). Changes and damage to DNA can alter gene expression patterns and consequently the functionality of a cell. Simultaneously, there is an accumulation of misfolded proteins during aging that can lead to deterioration of mechanisms that regulate protein folding, stability, functionality and ubiquitination and eventual loss of proteostasis (Lopez-Otin et al., 2013; Taylor and Dillin, 2011). Thus, these hallmarks can cause intracellular damage to the cell during aging, leading to loss of homeostasis and/or cell death, thus eventually contributing to an aged phenotype.

Other hallmarks of aging such as deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence, are termed antagonistic hallmarks. For example, molecular mechanisms involved in nutrient sensing are in place to regulate and promote cell survival and growth, however at chronic levels of activity, deregulated nutrient sensing can promote overgrowth and aging of tissue (Efeyan et al., 2015; Katewa and Kapahi, 2011). In contrast, cellular senescence normally protects tissue from overgrowth of cells, however at chronic levels, old senescent cells
accumulate in a tissue, inducing inflammatory responses, which can lead to aged damaged tissue over time (Collado et al., 2007; Kapahi et al., 2010; Rodier and Campisi, 2011; Wang et al., 2009). Lastly, mitochondria are normally involved in energy production and the production of reactive oxygen species (ROS, an unstable byproduct of energy production). Cells rely on the ATP produced by mitochondria and downstream signaling produced by ROS molecules for cellular defense, cell proliferation, inflammation (Liochev, 2013; Murphy, 2009; Turrens, 2003). However, at high levels of activity and dysfunction, excessive ROS become toxic to the cell, damaging intracellular mechanisms and compartments (Edgar et al., 2009; Zhang et al., 2009).

The phenotypic results of primary and antagonistic hallmarks of aging are termed integrative hallmarks. Overall function of a tissue greatly depends on the interactions and communication between its constituents and therefore the aging of individual cells can cause altered intercellular communication, leading to defects in function and homeostasis of the tissue. (Lopez-Otin et al., 2013). These changes include alterations and deficits in neuroendocrine function and inflammation signaling (Nelson et al., 1995; Shaw et al., 2010; Tosato et al., 2007). In addition to intercellular communication, tissue homeostasis also relies on the ability to maintain its cellular population. Cellular damage caused by primary and antagonistic hallmarks to a tissue’s stem cell population can significantly decrease the regenerative ability of tissue. Decreases in stem cell division, stem cell death or even over proliferation of stem cells can cause stem cell exhaustion in a tissue, yielding an aged phenotype (Cheng et al., 2000; Conboy and Rando, 2012; Lopez-Otin et al., 2013).

In summary, the hallmarks of aging attempt to provide a holistic overview of biological processes that mediate aging at a molecular level. However, the relevance of these peripherally-defined hallmarks of aging to brain aging remains to be determined.
Figure 1: Summary of Hallmarks of Aging (Adapted from Lopez-Otin et al. Cell 2013)

Stem cell exhaustion and altered intercellular communication are categorized as integrative hallmark. Deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence are classified as antagonistic hallmarks. Genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis are classified as primary hallmarks of aging.
1.4 Brain Aging

The brain is particularly vulnerable to the deleterious effects of aging. This is because neuronal cells have a higher metabolic rate in comparison to peripheral cells types and therefore are more exposed to several metabolic byproducts such as reactive oxygen species (ROS) that cause intracellular damage (Boumezbeur et al., 2010; Murphy, 2009; Sibille, 2013). Intracellular damage poses a threat to the cell’s physiology as it causes a whole range of downstream effects including modifications to cellular macromolecules and induction of inflammatory process (Liochev, 2013; Skaper, 2007; Temple et al., 2005). Normally, this form of oxidative toxicity in peripheral somatic tissue can activate pathways to induce apoptosis and eliminate the damaged cell (Circu and Aw, 2010; Ott et al., 2007). However, in the brain where most of the cells are non-dividing, the deleterious consequences of aging are usually progressive and lasting.

Unlike intercellular communication which relies mostly on signaling via endocrine secreted hormones and paracrine chemical signals, neuronal communication involves the transmission and storage of information via fast acting electric impulses mitigated by Na⁺/K⁺ current (Agnati et al., 1995; Blalock, 1994; Yamada and Katagiri, 2007). Neuronal communication includes specific neuronal structures (e.g. synapses, dendrites, axons) and molecules (e.g. neuropeptides, neurotransmitters such as GABA, glutamate and Ca²⁺) that propagate and code signals between neurons (Kandel et al., 2000; Quan and Banks, 2007; Squire et al., 2012; Yamada and Katagiri, 2007). These differences in the type of communication between the brain and peripheral tissue can yield different age-related outcomes. Age-related changes in neuronal communication maybe attributed to differential gene expression of signaling/signal transduction molecules (e.g. neural peptides, neurotransmitters, receptors etc.) either locally or across different brain regions (Mattson et al., 2002; Rugg, 2013; Yuste, 2015). The gene expression changes in signaling cascades may alter efficacy of information processing, as well as the transmission and storage of information in the brain, yielding specific age-related cognitive deficits (Burke and Barnes, 2006; Park and Bischof, 2013; Yu et al., 2006).

Aging can alter the delicate balance of a neuron’s intracellular homeostasis and its ability to communicate effectively with other neurons. This can increase the risk for the development of various neuropsychiatric and neurodegenerative disorders (He et al., 2007; Winkelmann et al., 2014). Alterations in neural communication due to loss of functionality in specific neurons has
previously been shown in various neuropsychiatric disorders, e.g. in the case of depression and schizophrenia, loss of function of interneurons in the Prefrontal Cortex (PFC) has been reported (Canetta et al., 2016; Inan et al., 2013; Warner-Schmidt et al., 2012). Studies investigating neuropsychiatric (bipolar disorder and schizophrenia) and neurodegenerative diseases (Alzheimer’s, Parkinson’s), have shown an increase in gene expression of oxidative stress related responses (Berk et al., 2011; Niranjani, 2014; Okusaga, 2014; Venkateshappa et al., 2012). These observations suggest an overlap of biological mechanisms that may mediate aging and neuropsychiatric-neurodegenerative disorders. However, it remains to be investigated if age is causal to or a promoter for these neurological disorders. In order to better understand the relationship between brain aging and the progression of neuropsychiatric and neurodegenerative disorders, we need to understand the complex process of brain aging at the various levels.

Normal brain aging is a complex process that involves progressive and persistent changes occurring at the functional, neural network, morphological and molecular levels (Glòrisoso and Sibille, 2011; Yankner et al., 2008).

At the functional level, aging affects different aspects of cognitive function (e.g. vocabulary, general knowledge, and occupational expertise do not show age-related decline) (Christensen, 2001; Park and Reuter-Lorenz, 2009). However, normal brain aging is associated with a decline in working, long-term and spatial memory, motor function (reaction time, speed, difficulties with balance and gait) and mood as well as inability to maintain focus and perform high-level cognitive problem solving (Grady, 2012; Leal and Yassa, 2015; McQuail et al., 2015) (Fiske et al., 2009; Koenig and Blazer, 1992; Mahncke et al., 2006; Rosso et al., 2013).

It is at this level where the symptoms of neuropsychiatric and neurodegenerative disorders manifest, in the form of dementia, anxiety, hallucinations and other cognitive deficits (Levenson et al., 2014). There is variability in how individuals experience age-related changes, ranging from rapid to no decline in cognitive functions (Li et al., 2001; Ylikoski et al., 1999). This can potentially be due to differences in genetic (e.g. APOE allele) and environmental factors (e.g. smoking, body mass index, cardiovascular health, nutrition) (Anstey and Christensen, 2000; Fillit et al., 2002; Morris et al., 2010; Sibille, 2013).

Age-related functional changes in the brain are paralleled at the neural network and morphological levels, where changes in neuronal communication within/between various brain
regions occur. Disruptions in connectivity among brain regions and a global loss of integrative function, specifically in regions responsible for higher-order cognitive functions e.g., PFC, have been associated with increase in age (Andrews-Hanna et al., 2007; Cabeza et al., 1997; Ferreira et al., 2015; Geerligs et al., 2015). Age-related loss of gray and white matter volume have been shown to be brain area specific, where decline in specific regions of the frontal lobe (e.g. PFC) have been reported, as opposed to areas such as the amygdala and hippocampus (Crivello et al., 2014; Ge et al., 2002; Good et al., 2001; Resnick et al., 2003; Tisserand et al., 2002). A study investigating normal age-related changes in the human brain using magnetic resonance imaging in healthy subjects (20-86 years), demonstrated that gray matter volume loss is continuous, following a linear trajectory, while white matter volume starts after “middle adult life” (40-50 years) (Ge et al., 2002). The reason for gray/white matter volume loss was thought to be neuronal loss/death. However, emerging evidence suggest that the volume loss in normal brain aging is the result of plausible shrinkage of dendritic spines and subsequent loss of synapses during aging. This loss of synaptic structures is thought to be the underlying cause of cognitive decline during aging (Benavides-Piccione et al., 2013; Dickstein et al., 2007; Duan et al., 2003; Morrison and Hof, 1997).

Aging in the brain at a molecular level is a dynamic process involving changes in DNA, RNA and proteins. Several endogenous and exogenous factors contribute to the changing molecular environment during aging in the brain, which ultimately affects the viability and functionality of neural cells and brain tissue. In neural cells, decline in DNA integrity is associated with an increase in mutation rates and DNA damage both of which are correlated with oxidative stress (Best, 2009; Floyd and Carney, 1992). It is postulated that genes involved in neuronal cell survival and function are selectively vulnerable to age-related genomic changes in DNA (Lu et al., 2004; Oberdoerffer et al., 2008). Such changes in DNA and other age-related cellular factors can an impact gene expression. Transcriptomic changes in the aging brain have been shown to be associated with biological pathways implicated in inflammatory processes, mitochondrial disruption, oxidative stress, calcium deregulation, and reduced neurotropic support (Berchtold et al., 2008; Liang et al., 2007). Age-related gene expression changes have also been shown to be cell type-specific between neuronal and glial populations (Erraji-Benchekroun et al., 2005). These biological processes are associated with changes in neuronal structure (volume) and function (Sibille, 2013). Some of the age-related gene expression changes observed in the
transcriptome have been validated in age-related protein changes. Protein alterations in pathways of oxidative stress and mitochondrial dysfunction have been reported (Poon et al., 2005).

Multiple studies have shown the link between age-related changes in pro-inflammatory pathways and its vulnerability in developing neurodegenerative disorders like Alzheimer’s, and Parkinson’s diseases (De Felice and Ferreira, 2014; Hirsch et al., 2012; Lema Tome et al., 2013; Michaud et al., 2013; Niranjan, 2014; Norris et al., 2005; Orre et al., 2014). Moreover, susceptibility to developing neuropsychiatric disorders such as depression, schizophrenia and bipolar disorder has also been reported (Baune et al., 2012; Berk et al., 2011; Drexhage et al., 2010; Fung et al., 2010; Na et al., 2014; Okusaga, 2014; Oxenkrug, 2011; Patterson, 2009; Rao et al., 2010).

An “age-by-disease” model, which suggests an interaction between aging and neurological disorders at a molecular level, was recently proposed. The theory hypothesizes that biological processes, which mediate normal brain aging, occur in parallel with those implicated in several neurological disorders (e.g. depression) and overall interact with each other. The same processes follow an age-related life-long continuous trajectory which is proposed to create an intrinsic vulnerability in biological processes associated with pathologies of the brain (Sibille, 2013). Genetic and environmental factors augment (at risk) or reduce (protect) this vulnerability in the early or late onset development of neurological disorders (Sibille, 2013).
1.5 Previous Studies

Previous studies have focused on specific regions of the brain, namely limbic regions (e.g. hippocampus) and the PFC. Based on previous evidence from our group, we know that certain regions of the brain are highly vulnerable to age-related changes that might lead to certain neurological disorders, therefore in this study we focused on the OFC sub-region of the PFC.

1.5.1 Aging in the Prefrontal Cortex

The PFC (PFC) represents approximately 30% of the frontal cortex (Fuster, 2015; West, 1996). Anatomically, the PFC is divided into various sub-regions such as dorsolateral prefrontal cortex (DLPFC), ventral medial prefrontal cortex (VMPC), anterior cingulate cortex (ACC) and the OFC (OFC) (Ongur and Price, 2000; Teffer and Semendeferi, 2012). It is characterized as a multimodal association area and is the central hub where various sensory modalities are integrated to form and regulate memory, mood, and high level cognitive functions (Fuster, 2015; Siddiqui et al., 2008; Teffer and Semendeferi, 2012).

Rabbitt and Rogers (1965) were among the first researchers to show age-related cognitive decline in older individuals. Compared to younger subjects, older subjects showed poor differentiation abilities in tasks related to the frontal cortex such as, response times to insignificant tasks, and age-related cognitive impairment in memory (Rabbitt and Rogers, 1965). Subsequent neuropsychological tests found the frontal lobe, more specifically the PFC, as the primary region vulnerable for age-related cognitive impairment and memory (McDowd and Oseas-Kreger, 1991; Shimamura et al., 1994; Stuss et al., 1982). The emergence of PET/MRI imaging based studies provided further evidence for decreased action of the PFC in blood flow during memory, face recognition tasks and shrinkage of the PFC in older patients when compared with younger counterparts (Grady et al., 1995; Raz et al., 1998). These studies were complemented by additional evidence showing decreases in gray matter volume, spine densities and white matter myelination in the PFC in the aging brain in humans and monkeys (Peters et al., 1994; Struble et al., 1985). Early studies attempting to characterize the molecular and cellular underpinnings of age-related cognitive decline in the PFC showed decline of neurotransmitter signaling in the primate brain (Goldman-Rakic and Brown, 1981). More recent studies showed
age-related deregulation of GABA/glutamate receptor signaling between excitatory pyramidal neurons and diverse populations of interneurons (e.g. somatostatin, parvalbumin, neuropeptide Y), in the PFC, which is attributed to low working memory and cognitive impairment (Banuelos et al., 2014; Gonzalez-Burgos, 2010; Gonzalez-Burgos et al., 2009; McQuail et al., 2012; Pinard et al., 2010). Age-related decline in concentrations of GABA and glutamate in the PFC has also been reported (Grachev and Apkarian, 2001).

The PFC is also vulnerable to degradation and deregulated function in neurodegenerative and neuropsychiatric disorders, respectively. Patients diagnosed with mood disorders such as depression, schizophrenia, and bipolar disorder, have been shown to have structural and functional changes in the PFC (Lyoo et al., 2004; Rajkowska and Stockmeier, 2013), decreased gray matter volume, alterations of excitatory and inhibitory signaling within the local circuitry of gray matter, and changes in the morphology (loss of density and size of GABAergic interneurons) of gray matter structure in the PFC (Benes et al., 1991; Crespo-Facorro et al., 2000; DeKosky and Scheff, 1990; Hastings et al., 2004; Hirayasu et al., 2001; Konopaske et al., 2014; Lewis et al., 2012; Lyoo et al., 2004; Nakazawa et al., 2012; Rajkowska and Stockmeier, 2013; Salat et al., 2001; Selvaraj et al., 2012; Sotrel et al., 1991; Webb et al., 2014). Alterations in interneurons, more specifically SST and NPY expressing interneurons in the PFC have been reported in Schizophrenia (Gonzalez-Maeso et al., 2002). Lastly, Alzheimer’s and Huntington’s disease patients have been shown to experience selective degradation of the PFC leading to symptoms of dementia and cognitive decline (DeKosky and Scheff, 1990; Gray et al., 2013; Kulijewicz-Nawrot et al., 2013; Salat et al., 2001; Sotrel et al., 1991; Unschuld et al., 2013; Wolf et al., 2007; Wong et al., 2014). Although research until now has demonstrated normal and pathological age-related changes in the PFC, it remains unclear of how age affects different sub-regions within the PFC.

1.5.2 Gray Matter Layers in the Prefrontal Cortex

Gray matter of the human PFC is organized into six layers: pia/molecular Layer (I), external granular layer (II), external pyramidal layer (III), internal granular layer (IV), internal pyramidal layer (V) and the multiform layer (VI) (Figure 2). Categorization of these layers is primarily based on cellular architecture and organization of neuronal cell types and their projections to cortico-cortical, subcortical and intra-laminar connections, which can vary
between brain regions (Fuster, 2015; Shipp, 2007). Each layer consists of a complex network of neural cell populations (pyramidal neurons and interneurons), as well as non-neuronal cell types (astrocytes, microglia and oligodendrocytes), which maintain the balance of neuro-chemical (e.g. neurotransmitters, GABA, glutamate) and electrical (e.g. Na and K) signaling that mediate overall brain function (Nowak and Bullier, 1998b).

Distribution of the six layer of gray matter within the PFC is relatively uniform (Semendeferi et al., 2001). Layer I primarily consists a matrix of apical dendrite extensions of sub-laminar pyramidal cells and horizontal axonal extensions from other gray matter layers and regions of the brain (e.g., thalamus) (Cauller, 1995; Shipp, 2007). Inputs received in layer I are vital connections for facilitating associative learning and cognitive function (Shipp, 2007). Layer II mostly includes pyramidal neurons and interneurons from which pyramidal neurons receive inputs from other laminar layers (Shipp, 2007). Layer III contains pyramidal neurons flanked by a diverse population of interneurons, and non-neuronal cell types (e.g. astrocytes, microglia, and oligodendrocytes). Dendritic projections of layer III pyramidal neurons extend to layer I, receiving cortico-cortical afferent and efferent signals (Shipp, 2007; Teo et al., 1997). Layer III also contains spine/synapse extensions from layer V pyramidal neurons that are flanked by a diverse group of inhibitory interneurons, which tightly regulates the coding of excitation output signaling of both layer III and V pyramidal neurons. This controls the excitation inhibition balance which mitigates cognitive function in the PFC (Peters and Sethares, 1997).

Layer IV and V contain pyramidal neurons that receives inputs from subcortical regions of the brain (e.g. thalamus, basal ganglia) (Jones, 1998; Peters and Sethares, 1997; Shipp, 2007). Lastly, layer VI consists a mixture of pyramidal and spindle shaped neurons whose axons extend to various other regions of the brain (Jones, 1998; Lam and Sherman, 2010; Shipp, 2007).
Figure 2: Graphical depiction of local circuitry of pyramidal neurons and interneurons in layers I-VI of the PFC (Adapted from Sibille. 2013)
1.5.3 Aging Layer II/III

Layer II/III contains pyramidal cells, as well as a complex network of interneurons, maintaining excitatory (pyramidal) and inhibitory (interneurons) balance. Maintenance of this balance is thought to underlie brain region, neural network for high-order cognitive function in the human PFC. Layer II/III has been shown to be particularly vulnerable to age-dependent decrease in spine/synapse architecture. When testing firing action potentials of neurons in layer II/III of aged rhesus monkeys, age-related PFC dysfunction was related to significant changes with increasing and decreasing firing rates, indicating an alteration of excitation/inhibition of neural circuit in layer II/III may be responsible for age-related cognitive decline (Dickstein et al., 2013; Luebke et al., 2004; Luebke et al., 2015; Peters et al., 2008b; Yadav et al., 2012). In addition, electron microscopic analysis of neurons of the PFC cortex showed a significant 30% decrease in synaptic distribution of layer II/III in aged rhesus monkeys, which was highly correlated with cognitive impairment (Peters 2008). This has also been shown in human post-mortem brain tissue where a loss of spine and synapse structure in layer III has been observed (Benavides-Piccione et al., 2013; Sala and Segal, 2014).

Layer II/III is a crucial area for high-level cognitive function in the brain which has shown to be selectively vulnerable to functional deregulation (Mostany et al., 2013; Nowak and Bullier, 1998a; Peters et al., 2008a). Little research on age-dependent molecular changes in layer II/III exist to account for the observed synaptic/dendritic loss and altered excitation/inhibition mentioned above. Hence, we selected layer II/III for our proteomic analysis of age related changes in post-mortem OFC in young and old subjects.

1.5.4 The Orbitofrontal Cortex

It is well known that the PFC is selectively vulnerable to age-related decline in structure and function (Resnick et al., 2000), however whether are uniform across all the sub-regions of the PFC is unknown. (Raz et al., 1997). Recent emerging evidence from longitudinal and cross-sectional human studies have shown the OFC to be selectively vulnerable to structural change with age within the PFC (Convit et al., 2001; Resnick et al., 2000).
The OFC (BA11/47) has been shown to play an important role in functional tasks including exteroceptive and interoceptive information processing, learning and decision making related to emotion and reward stimuli (Ongur and Price, 2000). More specifically, studies in humans and monkeys show the OFC to be involved in primary reinforcements (touch and taste sensory input) and controlling reward/punishment related behavior, thereby controlling emotion (O'Doherty et al., 2001; Tremblay and Schultz, 1999).

MRI imaging studies in healthy aged human adults, confirm a pronounced grey matter volume loss in the OFC in comparison to other brain regions (Convit et al., 2001; Raz et al., 1997; Resnick et al., 2003). Subsequent fMRI studies have highlighted significant functional decline of OFC-related cognitive task to be correlated with structural deficits in the OFC in healthy aged adults (Lamar and Resnick, 2004; Lamar et al., 2004; Resnick et al., 2007; Weiler et al., 2008). In terms of brain network connectivity, the OFC has been reported to experience age-related compensatory changes where it is thought a weaker cortical efficacy causes an increase in neural communication of top-down cortical signaling (e.g. with the primary visual cortex) (Kaufman et al., 2016). At the molecular level, age-related changes in the OFC transcriptome have been shown to be highly correlative with changes in the DLPFC (Erraji-Benchekroun et al., 2005). Further investigation is required to understand the exact changes occurring in the OFC during aging and the molecular pathways involved.

1.6 High throughput methods for studying Brain Aging

Over the last 15 years, several high-throughput methods such as microarrays (for the transcriptome) and mass spectrometry (for the proteome) have been employed to investigate brain aging. A benefit of the high throughput approaches is the ability for discovery transcriptomics or proteomics towards probing molecular mechanisms associated with brain aging.
1.6.1 Microarray Assays and Large-scale Transcriptomic Studies of Aging in the PFC.

Microarrays are DNA based assays that contain dense arrays of complementary cDNA sequences/oligonucleotides (probes), where each probe represents a known gene. These probes (10-20,000 genes) are organized in specific locations on a solid surface. The assay involves isolating RNA from samples (e.g. tissues, cell cultures, disease state samples), and transforming the RNA to cDNA (Canales et al., 2006; Pongrac et al., 2002). Subsequently, the cDNA samples are fluorescently tagged and loaded on the predesigned microarray with associated controls (e.g. control versus disease; old versus young). Hybridization of sample cDNA to probes enables the identification and quantification of altered genes within the sample (Jaluria et al., 2007). Automatic software’s are available (e.g. Illumina, Affymetrix) to scan and analyze the florescent intensity and probes induced by sample-probe hybridization to determine which genes are altered and the represented intensity to which they are altered (Harr and Schlotterer, 2006; Lin et al., 2008). In the end, the assay provides a list of altered genes between experimental groups. Subsequent inferences of altered genes can be done by functional analysis.

Microarray assays have enabled large-scale high-throughput analysis of altered mRNA express levels, giving insight to basic molecular mechanisms at a genomic level in a single assay (Quackenbush, 2002; Schena et al., 1995). The advantages of current microarray assays include their ability to provide quick and easy data at low cost. The identification and quantification of genes are highly accurate, precise and reproducible (Russo et al., 2003; t Hoen et al., 2008). However, microarrays are limited by the number of genes available on the microarray. Therefore, relatively new genes or gene variants may not be analyzed. This also creates a bias in the large-scale analysis, as the data is only limited to the age of the microarray chip (Somorjai et al., 2003). In addition, although microarray assays provide large-scale information on the molecular biology of a sample, it only provides data on gene expression changes, which is not always correlative to the proteomic changes. Most research on the molecular biology of aging in the brain has been microarray-based (Fu et al., 2009; Malone and Oliver, 2011).

To understand molecular underpinnings that mediate aging of the human PFC, several large-scale microarray studies have characterized changes at the transcriptomic level. Firstly, genome wide mRNA studies in the PFC have shown age-related gene expression changes to be limited to about 10% of the entire transcriptome, suggesting at the transcriptomic level, age-
related changes remain relatively unchanged (Erraji-Benchekroun et al., 2005; Tang et al., 2009). Gene expression changes were also shown to be persistent and progressive across the lifespan in the PFC. Life-long expression trajectories of these genes were shown to be highly robust and correlated with chronological age \((r=0.73)\). Therefore, it was concluded that the “molecular age” of an individual (defined by the trajectories of genes that change with age) could be a predictor for the chronological age of an individual (Erraji-Benchekroun et al., 2005; Loerch et al., 2008b).

Microarray analysis of age-related gene expression changes in the PFC indicated that the alterations in molecular pathways are cell type specific. Genes involved in glial dystrophy (GFAP, NFkB) were enriched and shown to be upregulated as a function of age (Berchtold et al., 2008; Erraji-Benchekroun et al., 2005). Conversely, there was a decrease in neuronal based genes such as: neurotransmitter receptors (5-Hydroxytryptamine Receptor 2A (HTR2A), Dopamine receptor D2 (DRD2)), calcium signaling proteins (Calbindin 1 (CALB1), Calcium/Calmodulin-Dependent Protein Kinase IV (CAMK4)) and neurotropic factors (Brain-Derived Neurotrophic Factor (BDNF), Insulin like Growth Factor 1 (IGF-1)) (Berchtold et al., 2008; Erraji-Benchekroun et al., 2005; Loerch et al., 2008b; Primiani et al., 2014; Toescu et al., 2004). More specifically, genes associated with synaptic function and GABA/glutamatergic neurotransmission were downregulated (Loerch et al., 2008b; Yu et al., 2006). Mitochondrial dysfunction and neuro-inflammation are two other molecular pathways reported to be altered in the PFC during aging (Berchtold et al., 2008; Erraji-Benchekroun et al., 2005; Primiani et al., 2014). Changes in the latter have specifically been attributed to glial cell populations (Erraji-Benchekroun et al., 2005; von Bernhardi et al., 2015; Wu et al., 2016).

Taken together, these studies show that aging in the PFC at the transcriptomic level is highly robust and associated with changes in pathways involved in loss of synaptic function, mitochondrial dysfunction, and neuro-inflammation. Although transcriptomic studies provide insights into alterations in gene expression of biological pathways, they do not address the physiological state of the aging PFC, which may be better understood at the proteome level.
1.6.2 MS-based Proteomic Analysis of the Aging Brain

Mass spectrometry is a technique for qualitative and quantitative analysis of molecules based on their mass to charge ratios. The convergence of multiple technologies over the last decade has allowed for high-throughput identification and quantification of proteins by mass spectrometry. To date, mass spectrometry remains the most comprehensive way for large-scale proteomic analysis (Domon and Aebersold, 2006; Law and Lim, 2013). Complex mixtures of proteins from samples extracted from biological sources can be analyzed, identified and quantified, with high accuracy. The shotgun proteomic analysis strategy involves extracting protein homogenates, reducing and alkylating them, then sequentially digesting them with known enzymes (e.g. trypsin, LysC) to obtain peptides, which can be detected by the mass spectrometer and identified using bioinformatics platforms (Levin and Bahn, 2010; Wisniewski et al., 2009b). Firstly, the peptides are separated by liquid chromatography and then introduced to MS instrument by electro spray ionization (ESI), which gives peptides a charge (Van Riper et al., 2013; Yates, 2000). Ionized peptides can then ‘fly’ into the mass analyzer, where they are separated according to their mass to charge ratios as they move to the detector. Protein identifications are based on bioinformatics platforms which analyze peptide sequences in database that match them to the peptides identified in a given samples (Gallien et al., 2013; Hu et al., 2005; Olsen et al., 2005; Yates, 2000).

By necessity, data gathered for the identification of peptides can also be used for quantification of the resulting proteins using labeled or label free quantification. Labeled quantification involves labeling the peptides with a mass tag before LC-MS/MS analysis and comparing the sample run data to a reference mass tag run. Labeled quantification may be incorporated into the LC-MS/MS work flow, by labelling amino acids with a stable isotope in cell culture (SILAC), Isotope Coded Affinity Tags (ICAT), isobaric Tag for Relative and Absolute Quantification (iTRAQ) or Tandem Mass Tags (TMT), to name a but a few (Gygi et al., 1999; Ong et al., 2002; Ross et al., 2004). This form of analysis yields precision in protein quantification, however, introduces a bias on how the tag binds to certain peptides which can limit protein identification. (Antoniewicz, 2013)

Label-free quantification on the other hand is based on measurement of ion abundance (e.g MaxQuant) or spectral counts (e.g. SAINT) obtained from MS run data (Cox and Mann,
Although this method has previously lacked in the precision of quantification, advances in bioinformatics analysis tools, e.g. MaxQuant, has greatly improved the reliability of label-free quantification. Moreover, it provides an unbiased (avoids differences in labeling efficiency of peptides) large-sale overview of proteomic changes in a sample at low cost (Choi et al., 2012; Cox and Mann, 2008a).

Several studies have investigated age-related proteomic changes in the brain, although no one study has specifically performed large-scale proteomic analysis in the PFC (Chen et al., 2003; Sato et al., 2005; Xu et al., 2016a). Early MS-based proteomics studies of aging in the brain were primarily based on 2DE gel electrophoresis coupled to Matrix-assisted laser desorption/ionization (MALDI) MS to identify differentially expressed proteins between young and old brain samples (Tsugita et al., 2000). Studies in different regions of the mouse brain highlighted age-dependent downregulation of proteins involved in neuronal function and disturbance in ion signaling/synaptic transport, which are consistent with findings in transcriptomic studies (Erraji-Benchekroun et al., 2005; Glorioso et al., 2011; Loerch et al., 2008b). Moreover, they also found downregulation in proteins enriched in protein quality control, a cellular process not robustly shown to be altered in transcriptomic studies (Poon et al., 2006a; Sato et al., 2005; Yang et al., 2008). A human post-mortem brain study also used MALDI MS to identify four differentially expressed proteins one of which was apolipoprotein A-1 precursor (Chen et al., 2003).

With the latest LC-MS/MS instruments, MS analysis of aging in the human hippocampus tissue was performed. The study investigated age-related changes in 16 subjects (22-88 age range) using TMT 4-plex tagged LC-MS/MS analysis. A total of 4582 proteins were identified, of which 35 were differentially altered as a function of age. Altered pathways included 25 downregulated proteins enriched in synaptic vesicle formation, electron transport chain (Xu et al., 2016b).

Thus far large-scale proteomic studies have also investigated specific protein alterations during aging. For example, oxidative stress in the brain can lead to various protein alterations such as carbonylation, a risk factor for the onset of age-related neuro-pathologies (Cabisco et al., 2014; Perluigi et al., 2014; Uttara et al., 2009). A study by Soreghan et al (2003) performed large-scale proteomic analysis (LC-MS/MS) on young and aged mice, subjecting the proteome to
hydrazide biotin-streptavidin to identify altered age-related proteins modified by carbonylation. Their results revealed enrichment in mitochondrial proteins, which regulate glucose and energy metabolism as well as signaling proteins involving in insulin and insulin-like growth factors (Soreghan et al., 2003).

Emerging transcriptomic and proteomic evidence has suggested, deregulation of mitochondria as a key drive for age-related changes in the brain (Green et al., 2011; Ott et al., 2007; Yang et al., 2008). Recently, LC-MS/MS analysis of mitochondrial fractions was performed to test for age-related changes between 5 month old, 12 month old and 24 month old male mice. A total of 1,233 proteins were identified and quantified among the aged groups. Their investigation showed age-related changes in pathways of glycolysis, and oxidative phosphorylation, suggesting little change in mitochondrial function at the protein level (Stauch et al., 2014; Stauch et al., 2015).

In summary, proteomic analysis by mass spectrometry gives insight into the physiological age-related changes that occur in the brain. Several molecular key players, such as proteins involved in oxidative stress, glucose metabolism, protein quality control, neuronal function, and neural signaling have previously been reported by MS analysis of aging in the brain. It is important to note that, no proteomic study has yet examined normal age-related changes in the human PFC.

1.7 Functional Analysis Overview

High-throughput experiments typically generate large datasets with a list of genes or proteins from which meaningful biological information has to be extracted. The challenge is to identify biologically relevant information that provides insight on functional modules that are over (enriched) or under (depleted) represented within a dataset. Such analysis requires a priori knowledge of individual relationships between genes in the literature (Ashburner et al., 2000; Huang da et al., 2009a). Bioinformatics based tools has allowed for the collection and curation of this knowledge in the form of databases such as Gene Ontology (GO).

GO, is a bioinformatics-based set of network databases that classifies and annotates all known knowledge of genes across multiple species and genomes. It attempts to classify their biological processes, molecular interactions, and cellular compartments. GO-based analysis
exploits this vast network of functional annotations to statistically compare gene lists obtained from large-scale data analyses (Dutkowski et al., 2013; Gene Ontology, 2015; Gene Ontology et al., 2013). A multitude of these software’s include, but are not limited to, gProfiler, Gorilla, ClueGO, DAVID, Panther etc. (Bindea et al., 2009; Eden et al., 2009; Huang da et al., 2009a, b; Mi et al., 2013). Typically, lists of proteins and genes of interest are compared to a background list of all known genes (or customized set of background genes) in the GO database. From which, the type of annotation of GO can be selected (e.g. biological processes). Enrichment, depletion or enrichment/depletion of functional annotations (modules) can be determined using right sided, left sided, or two-sided hypergeometric tests, respectively. Significance of these associations is determined by a p-value (<0.05; fisher’s extract p-value) threshold, which is usually subjected to Benjamini-Hochberg or Bonferroni correction (Balakrishnan et al., 2013; Draghici et al., 2007; Grossmann et al., 2007). Each gene annotation is supported by various forms of evidence, which can include experimental data, computational data (in silico analysis of gene sequences), author statements (annotation based on statement by an author of a reference), or electronic annotations (evidence assigned by automated methods) (Gene Ontology et al., 2013).

Other independent tools such as Ingenuity Pathway Analysis (IPA), which are not solely based on GO, but also contain their own hand curated independent gene database based on experimental literature surveys. IPA is a widely used platform that provides various analytical tools, ranging from functional annotation to enrichment of specific altered pathways with a dataset(Jiménez-Marín et al., 2009; Werner, 2008). IPA also contains designed tools to extrapolate drug interactions and targets within an inputted dataset. Network analysis in IPA integrates plausible interactions between the genes of the dataset, highlighting key node genes within a particular gene interaction network. The advantage is that it also inputs genes that have not been identified within the dataset but are important interactors within the network. Other tools, such as the enrichment of diseases and cellular within a dataset are also available in IPA (Kramer et al., 2014).

In the end, the goal of functional analysis to provide insights in causal networks and relationships of altered biological processes and disease states from a simple list of altered genes. In this study, we opted to use gProfiler for functional annotations as well as IPA for network analysis of our large scale proteomics dataset.
1.8 Rational and Goals of the Study

Brain aging is a complex process that occurs at multiple levels (functional, neural network, morphological, molecular). To understand the source for functional, neural network, and morphological changes, a holistic view is needed to understand its basic underlying molecular changes. Transcriptomic based studies provides sufficient but incomplete information on the molecular changes that occur in the aging brain as it limits the understanding of age-related changes at the physiological level, which is the proteome. Large-scale proteomic analysis can provide a comprehensive and complementary view of molecular aging of the brain.

We hypothesize that aging in the brain at the proteomic level, is associated with molecular alterations in specific hallmarks of aging (e.g. altered neural communication), more precisely, affecting glutamate/GABA signaling, whereas other peripherally-defined hallmarks, such as telomere attrition, may be less relevant to brain aging. To test these hypotheses, we performed large-scale proteomic profiling of age-altered changes in layer II/III of OFC (Brodmann Areas BA11/47) of healthy aging subjects using LC-MS/MS analysis, as outlined in Figure 1. In the long-term, gaining insights on age-associated proteomic changes in the OFC in a temporal and layer resolution may provide leads and strategies for delaying onset of several neurological disorders.

Goals

The overall goal is to explore age-related proteomic changes in layer II/III of OFC. Specific goals are:

- To develop methods to optimize and obtain LC-MS/MS qualitative and quantitative proteomic data from 15 young and 18 old human post-mortem, OFC layer II/III samples.
- To perform functional analysis of differently expressed proteomic changes OFC layer II/III to identify altered biological processes during aging.
- To summarize age-related proteomic changes in the form of hallmarks of aging in the brain.
- To determine which hallmarks are specific to the brain versus peripheral somatic tissue.
1.9 Study Workflow

Overall this study involved the collection of OFC layer II/III by laser capture microdissection from young and old human post-mortem brains. Subsequent protein extraction, denaturation, alkylation, reduction and digestion and sample preparation by Filter Aided Sample Preparation (FASP, refer to materials and methods for further details) was done prior to LC-MS/MS analysis (Figure 3)

Ideal conditions for optimal staining of OFC gray matter were required for accurate identification and collection of layer II/III. In addition, due to our very limited access to tissue of layer II/III, optimization of tissue extraction had to be done to create an ideal protocol for subsequent LC-MS/MS analysis. Optimization steps are highlighted in orange boxes below (Figure 3)

Micro-dissected layer II/III of OFC from 15 young and 18 old human post-mortem brains was processed for LC-MS/MS analysis. Label-free quantification of raw mass spectrometry data by MaxQuant software followed by statistical analysis using the random intercept model resulted in a list of differentially-expressed proteins between young and old subject groups. Subsequent functional analysis by gprofiler and IPA softwares was performed to investigate altered age-related biological processes (See summary Figure 3).
Figure 3: Experimental workflow of proteomic profiling of layer II/III during aging of the OFC in human post-mortem brain samples.
Chapter 2

2 Materials and Methods

2.1 Human Post-Mortem Brain Samples

Samples of the Orbitofrontal cortex (BA 11/27) post-mortem human brains were obtained from the Douglas-Bell Canada Brain Bank, Canada. 33 male samples were collected and divided into two groups, 15 young (age 15 – 43) and 18 old (62-88). The collection procedures were approved by the Douglas Hospital Research Ethics Board, the Douglas Brain Bank Scientific Review Committee and the CAMH Research Ethics Board. All subjects were reported to be free of psychiatric illness based on a standardized psychological autopsy obtained from a family member in accordance to Diagnostic and Statistical Manual of Mental Disorders I (DSMV) criteria. Physiological autopsies of individual’s were done by a trained medical professional for all subjects, concluding all subjects as free of neuropathological diseases of brain with no signs of neurotoxicity. Death of all subjects was either deemed as an accident or cardiovascular disease events, with no prolonged agonal period before death.

Samples were chosen based on availability of post-mortem samples, selected age range, sex of individual, matching for postmortem interval (PMI) and brain pH. The age ranges of young and old subjects were based on previous reports showing age-related gene expression changes are relativity unchanged within those two ranges and overall negatively correlated between the ages 42-73 (Lu et al., 2004). Lastly, fewer female samples were available, therefore only male subjects were chosen to maintain consistency among the subjects, as sex differences in male and female aging in the brain have been reported (Coffey et al., 1998).

Statistical analysis of the effect of PMI and brain pH within subject groups was concluded to be non-significant (Table 1). Tissue blocks were stored at -80°C until further use.
Table 1: Significance of PMI, pH and Age of Cohort subjects in young and old groups.

PMI and pH shows no significance among subject groups. The effect of age is shown to be significant among subject groups.

<table>
<thead>
<tr>
<th></th>
<th>Avg</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤45</td>
<td>≥60</td>
</tr>
<tr>
<td>PMI</td>
<td>23.3</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>6.3</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>30.7</td>
<td>71.1</td>
</tr>
<tr>
<td></td>
<td>9.6</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>2.89E-15</td>
<td></td>
</tr>
</tbody>
</table>

2.2 Method Development

2.2.1 Identification of Layers / Sample Collection

A combination of multiple staining and fixing conditions were performed to optimize the identification of layer II/III.

Table 2: Summary of conditions tested to stain 20um of human post-mortem brain tissue to optimize the identification of layer II/III

<table>
<thead>
<tr>
<th>Fixing Condition</th>
<th>Fixing Time</th>
<th>Concentration of Thionin stain (in 66% Acetone)</th>
<th>Staining Time</th>
<th>Staining protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% formalin</td>
<td>5min and 10 min</td>
<td>0.2%</td>
<td>10min</td>
<td>Fixing solution for 5 min or 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 min PBS wash (for 10% fixing only)</td>
</tr>
<tr>
<td>100% Methanol</td>
<td>5min and 10 min</td>
<td>0.5%</td>
<td>5min</td>
<td>1 min 50% ethanol</td>
</tr>
<tr>
<td>75% ethanol</td>
<td>5min and 10 min</td>
<td>2%</td>
<td>1min</td>
<td>Thionin stain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1min MilliQ water</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50% ethanol 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75% ethanol 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>95% ethanol 30 sec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100% ethanol 2x for 30 sec each</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Xylene for 3 min.</td>
</tr>
</tbody>
</table>
2.2.2 Optimization of Extraction buffers components for LC/MS-MS Analysis

Multiple extraction buffers were tested to optimize extraction and compatibility of peptide solution for LC-MS/MS analysis

Table 3: Summary of extraction buffers tested to optimize extraction and sample preparation for LC-MS/MS analysis

<table>
<thead>
<tr>
<th>Standard Components of Extraction Buffer</th>
<th>Altered detergents and detergent concentrations of extraction buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM HEPES, (pH:8), 150mM NaCl, 1mM EDTA, 1mM DTT, 1X Protease Cocktail Inhibitor (Roche, Basel, Switzerland) and 1X PhosSTOP (Roche)</td>
<td>5% SDC</td>
</tr>
<tr>
<td>3% SDS (wt/vol)</td>
<td>1% (vol/vol) NP-40, 3.5% SDS (wt/vol)</td>
</tr>
<tr>
<td>1.5% SDS (wt/vol)</td>
<td>No Detergent</td>
</tr>
<tr>
<td>8M Urea, 0.1M Tris pH 8</td>
<td></td>
</tr>
</tbody>
</table>

2.3 Tissue Collection

Tissue blocks were equilibrated at -15°C for 45 min in the Leica CM1950 cryostat chamber (Wetzlar, Germany) for sectioning. Fresh frozen samples were collected on PEN membrane glass slides (Thermoscientific Fisher Scientific, MA, USA), with 20 μm thickness. To identify layers, collected sections were stained with the following optimized thionin staining protocol; fixing in -20°C in 75% and 50% ethanol for 5 min and 1 min, respectively. Slides were then stained in 0.2% thionin (composed of 66% acetone) (Sigma Aldrich, MI, USA) for 10 min. The following washing and dehydrating steps were performed; 1 min MilliQ water, 50% ethanol, 75% ethanol, 95% ethanol and 100% ethanol for 30 sec each, and Xylene for 3 min. Samples were kept at -20°C until subsequent laser capture microdissection for layer II/III collection.

Layer II/III gray matter of OFC was collected using the ArcturusXT™ Laser capture microdissection system (Thermoscientific Fisher Scientific, MA, USA.). Layer II/III was identified based on its distinct cellular architecture of cells, which was highlighted by the thionin stain. A total of 30-40 mm² of gray matter layer II/III was collected per sample. Tissue sample collection was randomized within pairs of young and old subject groups.
2.4 Sample Preparation

The collected tissue from all samples were added in the optimized buffer (50mM HEPES, pH 8, 150mM NaCl, 1.5% SDS (wt/vol), 1mM EDTA, 1mM DTT, 1X Protease Cocktail Inhibitor (Roche, Basel, Switzerland) and 1X PhosSTOP (Roche). The mixture was then vigorously pipetted, vortexed briefly, and sonicated for 6 cycles of 1 min each, with 2 min incubations on ice in between. For denaturation, the samples were heated at 99°C for 5 mins. To collect the resulting extracted protein, samples were spun down at maximum speed (21000g) for 20 min and the supernatant (protein homogenate) was retained for each sample. Sample quality and protein concentration were estimated by running 2.5% (vol/vol) of each sample on a 10% SDS-PAGE gel, with a range of BSA standards (0.05ug – 0.5ug) for comparison.

2.5 Filter Aided Sample Preparation (FASP)

An estimated 20ug of protein homogenate was collected and reduced by adding 10mM Dithiothreitol (DTT) (Sigma Aldrich) with incubation at 60°C for 15 min. The sample was then alkylated with 50mM Iodoacetamide (IAA) (Sigma Aldrich) and 5mM TCEP in the dark at room temperature for 30 minutes. The reduced and alkylated protein homogenates were added to prewashed/equilibrated Amicon Ultra-Millipore Protein 0.5ml Filters with 10kDa cut-off (Millipore, MA, USA) for Filter Aided Sample Preparation (FASP) as described below (Scifo et al. 2015)

Amicon Ultra-Millipore Protein 0.5ml Filters were washed/equilibrated with 300ul of the following wash buffers and steps at maximum speed for 15min. two times with MilliQ water, two times with 0.1M NaOH, three times with MilliQ water, and once in Urea Buffer (8M Urea, 0.1M Tris pH 8.0).

20ug of reduced and alkylated protein homogenate was mixed along with 300ul of Urea Buffer (8M Urea, 100mM Tris pH:8) and added to Amicon Ultra-Millipore Protein 0.5ml Filters. The resulting mixture in the filter was spun down for 40min at maximum speed. This step denatured the proteins and introduced the protein samples to the filter membrane. The samples were then washed and denatured with five additional washes with 300ul of urea buffer washes, again spun down at 30min each at maximum speed. The process of lysate preparation and LC-
MS/MS analysis from all subjects were performed in four batches with 15 pairs (young and old) and three separate samples for ensuring reproducibility between runs.

The resulting denatured proteins were first subjected to enzyme digestion (1:10, enzyme to protein) by LysC endoproteinase (CAT:90051 Wako/ThermoScientific) by incubation with shaking at 25°C overnight. The resulting cleaved LysC peptides were then collected by centrifugation at max speed for 20min and collecting the resulting flow through. The flow through was then stored at 4°C. Remaining protein on the Millipore Filter was then subject to enzymatic digestion by Modified Sequence Grade Trypsin (CAT: V511a Pomega) in a 1:10 enzyme to protein ratio, incubated at 25°C overnight. Once digested, the tryptic peptides were collected by spinning the columns at maximum speed at 20 minutes and pooled with the Lys-C digested peptides. To ensure all the peptides were collected 100ul of 0.5M NaCl was added to the column and spun down at maximum speed for 20 min. The resulting flow through was re-applied to the filter and spun again to collect a maximum yield of peptides.

For detergent depletion, an equal volume of 2M KCL was added to the collected peptide samples for SDS precipitation. After an incubation of 30 minutes at room temperature, the samples were set to spin at maximum speed for 5 minutes. The supernatant was collected as a detergent depleted peptide sample. The volumes of the peptides were reduced by SpeedVac 100. Desalting of peptide samples was done using C18 ZipTips (200ul volume) (EMD Millipore: CAT: ZTC18S096, Darmstadt, Germany). The following solutions were prepared for desalting ZipTip protocol:

Table 4: Solutions of ZipTip desalting protocol

<table>
<thead>
<tr>
<th>Solution</th>
<th>Solution Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50% Acetonitrile: MilliQ H2O</td>
</tr>
<tr>
<td>B</td>
<td>1% TFA</td>
</tr>
<tr>
<td>C</td>
<td>0.1% TFA: 5% Acetonitrile</td>
</tr>
<tr>
<td>D</td>
<td>0.1% TFA, 0.05% Formic Acid in 70% Acetonitrile</td>
</tr>
</tbody>
</table>
The following Ziptip procedure was used:

- Wetting Step: A 100ul of solution A was aspirated with the ZipTip 8 – 10 times.
- Washing/Equilibration: 100ul of solution B was aspirated 8-10 times.
- Peptide Binding: The sample peptide solution was then aspirated 10-15 times.
- Washing Step: Bound peptides were then washed in solution C by aspiration 8-10 times.
- Peptide elution: Peptides were then eluted aspirating 40ul of solution D and saved. Elution was repeated 5x to total 200ul. The final desalted peptides samples were dehydrated in SpeedVac.

2.6 LC-MS/MS Mass Spectrometry and Label-Free Quantification

The final peptide samples were submitted to the SickKids SPARC BioCentre Facility for LC-MS/MS analysis. Samples were run on an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific) as previously described in (Zhang et al., 2015). Briefly, peptides were separated on a 50-cm column (75 μm inner diameter) packed with PepMap®RSLC C18 resin at 60 °C. Samples were run for 3 hr with a flow rate of 250 nl/min using a 5% - 30% acetonitrile in 0.1% formic acid gradient. Columns were washed between samples using a 30% to 90% acetonitrile in 0.1% formic acid gradient for 2 min, followed by a wash of 90% acetonitrile in 0.1% formic acid for 12 min. Peptides were ionized with a nano-electrospray ion source (EASY-SPRAY, Thermo Fisher Scientific) prior to introduction into the mass spectrometer. Mass spectra were acquired in a range of (400–1200 m/z) with a resolution of 240,000 at m/z 400 in the Orbitrap, followed by 10 data-dependent MS/MS scans.

Raw MS data from age cohort samples was input into MaxQuant v1.5.3.8, a freely available statistical platform for proteome wide quantification of MS data (Cox and Mann 2008). MaxQuant utilizes correlation analysis and graph theory to detect peaks as three-dimensional objects in m/z, elution time and signal intensity space (Cox and Mann 2008). Label-free quantification (LFQ) of a protein in a given sample is based on peak intensity values of individual peptides. LFQ intensity values of individual peptides for a given protein were averaged and the fold ratio of each protein was calculated for both young and old subjects. Differentially expressed proteins were determined after statistical analysis of the LFQ data using the random intercept model. Label free quantitation of peptides was performed by searching
against the human Uniprot database (released November, 2015; 20,193 entries; http://www.uniprot.org). The match bin feature of MaxQuant was employed. Other key parameters included: fragment ion mass tolerance of 20 ppm, maximum two missed cleavages (Trypsin and Lys-C), fixed modification as carbamidomethylation of cysteine, and variable modification as oxidation of methionine and acetylation of protein N-terminal. False discovery rate was set to 1% for both peptide and protein levels in target/decoy to minimize false positives. Label-free quantification resulted in the identification of 4,193 proteins among young and old subject groups.

2.7 Data imputation and statistical analysis

**Note: The following statistical analysis was done by our collaborators in George Tseng’s research group at the University of Pittsburgh.**

Label-free quantification can lead to missing values of data among samples due to technical limitations of MS instrument (Matzke et al., 2013; Nahnsen et al., 2013; Webb-Robertson et al., 2015). Therefore to further assess the reliability of 4,193 identified proteins, we classified them into five categories based on the percentage of samples with non-missing values, molecular weights and the mean intensity in all 33 samples. The categories were as follows:

- category I: consists of complete data with no missing values in any group;
- category II: non-missing proportion >=70% in both old and young groups;
- category III: non-missing proportion >=70% in one group but <70% in the other group;
- category IVA: non-missing proportion <70% in both groups, both their mean intensity and molecular weight above 33th percentile;
- category IVB: Non-missing proportion <70% in both groups, either their mean intensity or molecular weight below 33th percentile

Data imputation was performed using k-nearest neighbor regression (k-NN) with k=10 to impute for zeros only for category II. Proteins in categories I and II were considered high-confidence data. To account for potential co-variants (PMI, pH), effecting the significance and fold change of each protein, random intercept model (RIM) with parameter selection using the smallest Bayesian Information Criterion was performed. (Ding et al., 2015) To correct the potential bias of the variable selection procedure, we performed a permutation analysis that
randomly shuffled the disease labels within each pair to generate a null distribution for p-value assessment (B=500).

The differentially expressed protein list was obtained with the following thresholds; proteins only in category I and II (only proteins identified in >70% of samples), p-value ≤0.05 and a minimum of 20% effect size (-log2 fold change = ±0.263).

2.8 Functional Analysis

2.8.1 gProfiler Enrichment Analysis

Differentially expressed proteins (n=118, p-value<0.05, >20% effect size) were subjected to GO based functional analysis using g:Profiler (Reimand et al., 2011). Enrichment analysis was limited to biological pathways, searching in GO and Reactome databases. The significance of enrichment between differentially expressed proteins and GO database was corrected by the Benjamini-Hochberg method of controlling the false discovery rate (FDR) (Haynes, 2013; Thissen et al., 2002). The range of functional category size was set between 3 and 500 with a minimum of 3 proteins from the data list per GO annotation. No electronic annotations were included in the analysis (only experimental based annotations). Resulting enriched GO annotations were downloaded in the form of Generic Enrichment Map (TAB), which was then subsequently uploaded to Enrichment map app (v.2.1.0) in Cytoscape (v. 3.2.4) (Mericó et al., 2010). Parameter for enrichment map visualization was set to p-value and FDR cut-off at 0.05.

2.8.2 Ingenuity Pathway Analysis

Differentially expressed proteins and associated statistics (p-value and effect size) were uploaded onto IPA interface. IPA Core analysis was performed with the following parameters; comparison of differentially expressed protein list with Ingenuity Knowledge Base (Genes only) for background, experimentally observed data only, the species set to humans, including data from all tissues. Subsequent investigation of canonical pathways and diseases was done, performing statistical analysis of significance by Benjamini-Hochberg. The IPA tools canonical pathway analysis, network analysis and disease analysis were used in the investigation of our differentially expressed proteins.
2.8.3 Hallmarks of Aging

To categorize our differentially expressed proteins into hallmarks of aging, we identified the GO biological processes of individual proteins by GeneCards. We then proceeded to manually categorize proteins based on specific key words (e.g. inflammation, apoptosis) associated with our hallmarks of aging (e.g. cellular senescence).

To validate the proteomic findings obtained from LC-MS/MS, we opted to test the expression of proteins of interest within our dataset by Western Blot analysis. However, due to limitations in protein quantity from the extracted layer II/III, the Western blot analysis proved unsuccessful. Based on our pilot experiments, the extracted proteins from layer II/III were below the detection limit of the Western blot analysis. For instance, low abundant proteins are relatively difficult to identify by immunoblotting analysis in comparison to more abundant ones. Dot blot experiments are an alternative to validation by Western blot and are currently ongoing. They allow for concentration of more dilute samples, which is ideal for the low protein quantities of our layer 2/3 postmortem brain tissue samples.
3 Results

3.1 Method Development

3.1.1 Optimization of Layer II/III Identification and Collection

Thionin staining is one of the most effective methods to identify cellular architecture under the microscope and the most predominant technique used to visualize neurons. It stains cell bodies, highlighting unique triangular soma structures of neurons (Pearse, 1949; Schmued, 1990; Windle et al., 1943). Although other tissue stains are available (e.g. Nissl, Golgi stains), thionin stains have been used in many studies for the staining of gray matter to identify its laminar distribution for laser capture microdissection collection (Byne et al., 2008; Kohler and Chan-Palay, 1983; Lin and Sibille, 2015; Seney et al., 2015; Tolbert and Morest, 1982).

Here we used thionin staining to identify gray matter layers under the microscope and capture them by laser capture microdissection (LCM). The use of LCM precludes application of mounting media and glass slides to tissue slices thereby affecting visualization of cellular architecture. In order to optimize identification of layer II/III gray matter from our postmortem OFC tissue under these less-than-optimal conditions, we tested several tissue staining and preservation techniques. Samples of 20um thickness were collected by cryosection at -15°C (see materials and methods for details). Different Nissl (thionin) based staining protocols (which stains Nissl bodies within cell bodies) were tested, ranging in concentration of thionin from 0.2%-1%) to determine the most suitable procedure for visualization of cellular architecture. A multitude of tissue preservatives, ranging from 75% ethanol, to 100% methanol and 10% formalin, 4% PFA fixing were also attempted to optimize tissue conditions for layer II/III laser capture microdissection collection. Fixation by alcohols (e.g. methanol, ethanol) dehydrates the denaturing proteins and exposes hydrophobic residues. Other fixing agents such as formalin and PFA are involved in crosslinking reactions among proteins, preserving protein architecture. Both types of preservatives are easy to use and are compatible with MS analysis (Ahram et al., 2003; Cruickshank-Quinn et al., 2014; Paulo et al., 2012). Based on our pilot experiments, we concluded that optimal visualization of all six layers was obtained by fixation with 75% ethanol and staining in 0.2% Thionin  (See Materials and Methods for details).
Figure 4: Optimized Staining and Collection of Gray Matter of the OFC, Layer II/III

(A) Optimized staining of human OFC gray matter using 0.2% Thionin stain, showing six gray matter layers (I-VI). Tissue is shown at 10x magnification (B) Laser capture microdissection (LCM) of layer II/III with optimized Thionin staining protocol. Left panel (before LCM), middle panel (after dissection of tissue) and right panel (capture tissue on a cap).
3.1.2 Optimization of Extraction for LC-MS/MS Analysis

Although LC-MS/MS is a common technique for proteomic analysis, most neurodegenerative and psychiatric studies involve using large amounts of starting tissue material (5-10mg) for sample preparation (Andreev et al., 2012; Martins-de-Souza et al., 2009; Polisetty et al., 2012). However, here we opted for analysis of a small section of human gray matter OFC, requiring microdissection precision technology to capture layer II/III. We also performed several pilot experiments to test various extraction buffers and determine the minimum tissue amount required for comprehensive LC-MS/MS analysis. We aimed to optimize: (1) protein extraction protocols (2) tissue amounts to be collected, detergents type and concentration in the extraction buffer. The goal was to maximize total protein extraction from the LCM dissected OFC layer II/III, while minimizing detergent concentration in the digested peptides since most detergents are incompatible with LC-MS/MS analysis and would therefore limit peptide/protein identification (Chen et al., 2007b). Secondly, due to limitations in availability of postmortem brain samples we also sought to establish the minimum tissue requirements for our MS-based workflow (Figure 3).

The tested extraction buffers were similar in composition (see materials and methods), except for their SDS detergent concentrations (0, 1.5% and 3%). Following protein extraction, 2% of the total protein yield was run on a 10% SDS-PAGE and analyzed by silver staining to estimate protein yield based on known BSA standards.
Figure 5: Silver stained 10% SDS-PAGE gel showing 2% (vol/vol) of extracted protein homogenates from 35-40mm² gray matter tissue testing different extraction buffers.

The silver stain contains a dual color marker, BSA standards (0.05ug-0.5ug) and 2% (vol/vol) of protein homogenate obtained from extractions with extraction buffers containing different detergent concentrations; 3% SDS, 1%NP-40, 1.5% SDS, and No SDS. No detectable protein was observed in the conditions with no SDS added in the extraction buffer. Sub-optimal protein levels (<0.4ug) were detected in samples extracted with 3% SDS, 1%NP-40. Optimal protein levels (>0.5ug) were obtained in samples extracted with 1.5% SDS.
Based on silver staining of 10% SDS-PAGE loaded with protein homogenates extracted using the various protein extraction protocols, we observed that the extraction buffers with 1.5% and 3% SDS yielded more total protein than the buffer supplemented with 6M Urea (Figure 5). After sample preparation using a modified FASP protocol (see Materials and Methods), the resulting peptides from the samples extracted with SDS were treated with 2M KCL for detergent depletion. All peptide samples were concentrated and desalted on C\textsubscript{18} stage tip columns prior to MS analysis (Table 4, see Materials and Methods). We evaluated the total ion chromatogram profiles and number of identified proteins from all tested protein extraction protocols. The total ion chromatogram is the sum of signal intensities obtained from all mass spectral peaks scanned from a given sample during the MS run. Peptides from a standard sample lysate were used as a reference which contained no contaminants and yielded high intensity peaks for peptide identification. Based on this analysis, the sample processed with a 1.5% SDS extraction protocol showed a similar total ion chromatogram as the reference sample and yielded the most protein identifications (1392) with minimal contaminants (eluting at the end of the run) in a 2 hour MS run in comparison to samples obtained using 3% SDS (1115) or 6M Urea (286) which had lower total ion chromatograms (Figure 6).
Figure 6: Chromatogram showing time verses peak intensities of LC-MS/MS comparing peptide samples, from samples extracted with different concentrations of SDS

Tissue starting material for all samples was 35mm$^2$ of 20um thickness. Green indicates an MS run profile of an optimal control sample of a reference lysate which represents a clean protein sample that yields high intensity peaks for peptide identification and minimal contaminants eluted at the end of a MS run. The blue line represents a sample extracted with 1.5% SDS. Pink line represents samples extracted from 3.0% SDS concentration. SDS detergent removal from these samples was done with 2M KCL precipitation. Orange line represents samples extracted with urea buffer, no SDS added.
3.2 Proteomic Profiling of Layer II/III of OFC in human post-mortem subjects

Given the role of layer II/III in information processing (described in section 1.6) we were interested in MS-based proteomic analysis of the same laminar boundaries in the OFC from 33 healthy male subjects (including, 15 young and 18 old) (Table 5). Laser capture micro-dissected layer II/III samples were subjected to 1.5% SDS extraction buffer protocols to obtain total protein. Following sample preparation as described above, ~1-2.5ug peptide solution was utilized for LC-MS/MS analysis on a 3h gradient.

Table 5: Summary table of subjects.

Table of young group (A) subjects and old group (B) subjects showing subject age, PMI, pH, and cause of death (natural or accidental). Natural death corresponds to death by cardiovascular disease or old age with no chronic medical conditions. Accidental death corresponds to quick death with no prolonged pre-agonal phase.

<table>
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<th>PMI</th>
<th>pH</th>
<th>Cause of Death</th>
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</table>
To determine the proteomic changes occurring during aging in the OFC layer II/III, we performed label-free LC-MS/MS analysis on LCM captured layer II/III Figure 3) from 33 male subjects, from 15 young and 18 old (Table 5). Label-free quantification of the raw MS data by MaxQuant resulted in the identification of 4,193 proteins from which 2,317 proteins were confidently identified in at least 70% of subjects (Categories I and II, Figure 7). Additional, statistical analysis, by the random intercept model (RIM), adjusting for cofactors (e.g. PMI and pH) resulted in a total of 118 differentially expressed proteins, with a p≤0.05 and minimum 20% effect size (RIM effect size ≥ ±0.263; -log2 fold change. From the 118 differentially expressed proteins and equal number of proteins (59) were upregulated and downregulated. (See Appendix Table 1)

The most significantly upregulated proteins as a function of age included, AHNAK, PPT1, GFAP, ASAH1, APOD. The most significantly age-related downregulated proteins were, SERPINB6, BRK1, CXADR, THY-1, CALB1 (See Summary figure 4, table 6, appendix table 1). Proteins such as GFAP and APOD have been previously observed to be significantly upregulated, while CALB1 was shown to be downregulated in human brain aging. (Dassati et al., 2014; Erraji-Benchekroun et al., 2005; Rodriguez et al., 2014), providing internal validation controls for our experiment. A summary of top 30 most affected differentially expressed proteins shows 20 out of 30 proteins are upregulated. (Table 6).
Figure 7: Classification of 4,193 proteins into categories based on missing protein values per subject.

Category I, consists of proteins identified in all samples. Category II contains proteins found in $\geq 70\%$ of all samples. Category III contains proteins found $\geq 70\%$ in one group but $\leq 70\%$ in the other group. Category IVA contains proteins found $\leq 70\%$ in both groups, both their mean intensity and molecular weight above 33th percentile. Category IVB contains proteins $\leq 70\%$ in both groups, either their mean intensity or molecular weight below 33th percentile.
Figure 8: Volcano Plot of 2,317 proteins confidently identified in at least 70% of subjects.

Dashed red line represents $p=0.05$ with points above represent significant proteins. The dashed orange lines represent +/-0.263 effect size (-log2fold change) cutoff based on label-free quantification using MaxQuant. Downregulated significant proteins are represented as green points and upregulated proteins are represented as red points. Top five most significantly downregulated (SERPINB6, BRK1, CXADR, THY-1, CALB1) and upregulated proteins (AHNAK, PPIT, GFAP, ASAH1, APOD) are highlighted.
Table 6: Summary of top 30 most affected differentially expressed proteins during aging of the OFC layer II/III

Table shows the gene symbol, protein name and effect size of each differentially expressed proteins between young and old subjects. Effect size highlighted in red show upregulated proteins during aging, while downregulated proteins that increase in age are highlighted in green.

<table>
<thead>
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<th>Gene Symbol</th>
<th>Protein Name</th>
<th>P-Value</th>
<th>Effect Size</th>
</tr>
</thead>
<tbody>
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<td>AHNK</td>
<td>Neuroblast differentiation-associated protein AHNK</td>
<td>0.009886159</td>
<td>1.22</td>
</tr>
<tr>
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<td>Palmitoyl-protein thioesterase 1</td>
<td>0.000402201</td>
<td>1.10</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>0.000112456</td>
<td>1.10</td>
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<tr>
<td>ASAH1</td>
<td>Acid ceramidase</td>
<td>0.000683544</td>
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<td>APOD</td>
<td>Apolipoprotein D</td>
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<td>Cathepsin D</td>
<td>0.018497266</td>
<td>0.50</td>
</tr>
</tbody>
</table>
3.3 Functional Analysis

3.3.1 gProfiler Enrichment Analysis

GProfiler (http://biit.cs.ut.ee/gprofiler/) is a web based platform for analysis of enriched functional modules within a dataset and when coupled to Cytoscape, allows for improved visualization of networks associated with the modules of interest. We therefore performed gProfiler enrichment analysis to functionally characterize the 118 differentially expressed proteins obtained from postmortem OFC layer II/III from young and old subjects (Figure 8). Cytoscape (v3.4) was used to visualize the interconnections of these pathways with the significance threshold cut-off p-value<0.05 and FDR<0.05. We obtained a network map consisting of 7 disconnected, but distinct modules which were organized into larger categories such as altered neuro-cognitive signaling and metabolism (Figure 9).

Biological processes were involved in altered neuro-cognitive signaling in three main modules. These modules included learning and memory (e.g. NRXN3, CALB1, PPP3CB, CAMK4, and PLCB1), glutamate/GABA signaling (e.g. CACNG3, HOMER1, GRM2, PLCB1, GRM3) and divalent ion transport (e.g. CACNG3, TF, TPT1, HOMER1, THY1, CACNB4), which were significantly downregulated with age within our 118 differently expressed proteins. (Figure 9) Glutamate/GABA receptor signaling pathway (p-value= 9.02e10) and learning and memory (p-value= 1.7e10) was shown to be most significantly enriched within our dataset. More specifically, glutamate/GABA related signaling proteins included, Glutamate Metabotropic Receptor 2 and Glutamate Metabotropic Receptor 3 which have been shown to be downregulated during aging (Lindenberger et al., 2008; Michaelis, 1998).

In addition, biological processes involved in metabolism, included modules for carbohydrate (e.g. PYGB, GPI, PFKL, AGL, CRYL1, and APOD) and small molecule metabolism (e.g. GPI, ALDH6A1, NAGK, PFKL, ALDH9A1, ALDH7A1, CRYL1, ECI2), which were demonstrated to be upregulated and enriched during aging.

Pro-inflammatory response was also shown to be upregulated which included proteins involved in lymphocyte migration (e.g. SLC7A5, PTK2B, MSN, SCG2, APOD, PPIA). More specifically Apolipoprotein D and secretogranin II have been known to be upregulated during aging (Gomar et al., 2014; Kalman et al., 2000; Lassmann et al., 1992; Loerch et al., 2008a).
Central nervous system development was also shown to be upregulated during aging within our dataset. See Appendix figure 1 for full gProfiler enrichment map with node labels.

Figure 9: gProfiler enrichment map of GO gene-set enrichment results of 118 differentially expressed proteins between young and old subjects.

Nodes represent protein sets and edges indicate their GO relationships. Nodes are colored based on enrichment results, where red and blue represents upregulation and downregulation, respectively of protein sets with increase in age. Color intensity correlates to enrichment significance. Significance of enrichment was corrected by Benjamini-Hochberg FDR. The range of functional category size was set between 3 and 500 with a minimum of 3 proteins from the data list per GO annotation. No electronic annotations or background dataset were included in the analysis. Manual classification of modules in general biological processes are shown encircled (blue line), highlighting commonalities among modules. The p-values and associated genes of the top three most, significant nodes are highlighted.
3.3.2 Ingenuity Pathway Analysis

IPA is a tool for comprehensive analysis of large-datasets in the form of complex biological systems. It is a peer reviewed database, regularly updated with multiple analysis tools including canonical pathway analysis, network and interactome analysis of genes, disease enrichment and drug interactions. Several studies have used IPA in the analysis of microarray, microRNA, and largescale proteomics data, including in our own lab (Currie et al., 2016; Erraji-Benchekroun et al., 2005; He et al., 2014; Ishida et al., 2015; Wang et al., 2015). We used canonical pathway analysis tool to identify enriched canonical pathways, networks and diseases within our 118 differentially expressed proteins between young and old subjects, in layer II/III of the OFC.

3.3.2.1 Canonical Pathway Analysis

Canonical pathway analysis by IPA at a p-value threshold of 0.05 was performed to investigate the association of proteins within our aging differentially expressed dataset. A total of seven significant canonical pathways were enriched from our dataset, including: glutamate receptor signaling, synaptic long term potentiation, aryl hydrocarbon receptor signaling, xenobiotic metabolism signaling, PI3Ks signaling in B lymphocytes LPS/IL-1 mediated inhibition of RXR function, and FXR/RXR activation(Figure 10).

This analysis also showed enrichment in neuro-cognitive signaling which included pathways involved in glutamate signaling and synaptic long term potentiation(Table 7). The two pathways showed overlap of GRM2, CAMK4, GRM3, which have been shown to be involved in glutamate/GABA and calcium signalling. These pathways have previously been observed to decline with age in the brain (Lindenberger et al., 2008; Loerch et al., 2008a; Michaelis, 1998).

Pathways involved in age-related pro-inflammatory responses consisted of which PI3K signaling in B lymphocytes and LPS- LPS/IL-1 mediated inhibition of RXR function were enriched in our 118 differentially expressed proteins. In addition, aryl hydrocarbon receptor signaling and xenobiotic metabolism were also altered within our dataset. This finding has not been previously described in the context for aging in the brain. LPS- LPS/IL-1 mediated inhibition of RXR function, aryl hydrocarbon receptor signaling and xenobiotic metabolism
show overlap of ALDH1L1, GSTM2, ALDH6A1, ALDH9A1, ALDH7A1 upregulated proteins, suggesting functional homology in the pathways (Table 7).

A

![Aryl Hydrocarbon Receptor Signaling](image1)

B

![Downregulated Upregulated](image2)
Figure 10: IPA top canonical pathways of differentially expressed proteins during aging in layer II/III of the OFC

(A) X-axis shows the significant canonical pathways (after Benjamini-Hochberg multiple testing correction with a threshold p-value of 0.05 [-log(B-H p-value) =1.3]). The left y-axis shows the –log10 p-value for each canonical pathway. The right y-axis corresponds to the line graph showing the percentage of proteins within a given pathway that are represented in our dataset. Z-score analysis by IPA is represented by the color coding, where red indicates pathway activation within the dataset. Blue represents inhibition of the canonical pathway within the dataset. Gray represents insufficient data (number of genes) to make a z-score prediction. White represents z-score prediction with no change (no activation or inhibition) within the dataset (B) X-axis represents the number of proteins per canonical pathway where red shows upregulated proteins and green shows downregulated proteins. Red boxes highlight canonical pathways synaptic long-term potentiation and glutamate receptor signaling which show significant decrease as a result of age.

Table 7: Upregulated and downregulated proteins in altered-canonical pathways during aging in the OFC layer II/II

<table>
<thead>
<tr>
<th>Canonical Pathway</th>
<th>Upregulated</th>
<th>Downregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl Hydrocarbon Receptor Signaling</td>
<td>TGM2, CTSD, ALDH1L1, NCOA7,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM2, ALDH6A1, ALDH9A1,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALDH7A1</td>
<td></td>
</tr>
<tr>
<td>Glutamate Receptor Signaling</td>
<td></td>
<td>GRM2, CAMK4, GRM3, GLUL,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HOMER1</td>
</tr>
<tr>
<td>Xenobiotic Metabolism Signaling</td>
<td>ALDH1L1, GSTM2, ALDH6A1,</td>
<td>MAP2K4, CAMK4, PPP2R5C</td>
</tr>
<tr>
<td></td>
<td>ALDH9A1, ALDH7A1</td>
<td></td>
</tr>
<tr>
<td>LPS/IL-1 Mediated Inhibition of RXR Function</td>
<td>ALDH1L1, GSTM2, ALDH6A1,</td>
<td>MAP2K4, FABP3</td>
</tr>
<tr>
<td></td>
<td>ALDH9A1, ALDH7A1</td>
<td></td>
</tr>
<tr>
<td>Synaptic Long Term Potentiation</td>
<td></td>
<td>GRM2, CAMK4, PPP3CB,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GRM3, PLCB1</td>
</tr>
<tr>
<td>PI3K Signaling in B Lymphocytes</td>
<td>PDPK1</td>
<td>CAMK4, C3, PPP3CB, PLCB1</td>
</tr>
<tr>
<td>FXR/RXR Activation</td>
<td>C4A/C4B, APOD</td>
<td>MAP2K4, C3, TF</td>
</tr>
</tbody>
</table>
3.3.3 Network Analysis

Network analysis by IPA was performed to explore the biological interactions that are most significantly interconnected between the proteins of that network. The most significant network contained 25 proteins from our dataset, with a significance p-value of $10^{-46}$ (determined by Fisher’s exact test, testing the probability of the assembly of proteins to happen by chance) is shown in figure 10. All colored proteins within the network (red/pink are upregulated and green are downregulated) represent proteins found within the dataset. Interconnections of the proteins are defined by solid (direct interaction) and dashed (indirect interaction) between the proteins. It is to note, that all interconnections between proteins are based on a minimum of one literature reference/canonical pathway information in the Ingenuity Pathway Knowledge Base. This network shows these 25 proteins belong to an interconnected network, and maybe involved in related biological activities. We hoped to identify key nodes (focus genes) for which our differently expressed proteins interact. This analysis revealed key nodes/protein complexes such as NF-kB, IgG, PI3K, AKT, ERK1/2, CD3, 26S proteasome, estrogen receptor and CREB, involved in neurological disease, cell-to-cell signaling and interaction, nervous system development and function and inflammation. (Figure 10). Out of the 10 nodes identified within the network three (NFkB, CREB, estrogen receptor) are localized to the nucleus, indicating age-related gene regulation within the network. Five identified nodes (CD3, ERK1/2, AKT, PI3K, 26S Proteasome) show to be localization in the cytoplasm/cell membrane indicating age-related changes in signal transduction within a cell.
Figure 11: Top network identified by IPA for 118 differentially expressed proteins during aging of the OFC layer II/III.

IPA network analysis tool was used to identify the biological relationships among 118 differentially expressed proteins. The most significant network (determined by Fisher’s exact test) was associated with neurological disease, cell-to-cell signaling and interaction, nervous system development and function. Pink/Red and green nodes represent proteins that are upregulated and downregulated during aging, respectively where color intensity is represented by the degree of fold change within the dataset. Proteins are arranged based on their subcellular local. Solid and dotted edges between the nodes represent direct and association between the two genes, respectively. Key nodes identified within the network include NF-kB, IgG, PI3K, AKT, ERK, and CREB. It is to note these proteins were not a part of 118 differentially expressed proteins. The molecule shapes represent the functional group each protein belongs to; double lined circle represents a protein complex, a vertically elongated diamond represents an enzyme, a horizontally elongated diamond represents a peptidase, an equilateral trail represents a phosphatase, an inverted equilateral triangle represents a kinase and a circle shows the protein is involved in another category.
3.3.3.1 Disease Analysis

The disease analysis feature in IPA maybe utilized to probe for diseases associated with proteins within a given dataset. We analyzed diseases associated with the differentially expressed proteins using BH multiple testing with a threshold set to p-value 0.05. This analysis yielded 8 disease associations, of which the top two categories enriched within our dataset were shown to be neurological and psychological diseases. The top eight most significant diseases within these categories are shown in Figure 12. A collection of neuropsychiatric and neurodegenerative disorders was shown to be enriched, including Huntington’s disease, dementia, disorders of the basal ganglia, Alzheimer’s disease, schizophrenia, Parkinson’s, and major depression. It is important to note that five out of the eight most significant diseases enriched in our age-related differentially expressed dataset are related to disorders of the brain including neurodegenerative disorders (e.g. Alzheimer’s) and neuropsychiatric disorders (e.g. dementia, schizophrenia, mood disorders, and major depression). (Figure 12) As mentioned in the introduction, these findings are consistent with the recently proposed age by disease model proposed by Sibille et al. 2011 which suggested an underlying molecular interaction between aging and the development of neuropsychiatric and neurodegenerative disorders.
Figure 12: Diseases most significantly associated with differentially expressed (DE) proteins during aging in layer II/III of the OFC.

X-axis shows top associated diseases within DE dataset. The left y-axis corresponds to significance p value of the associated diseases. Benjamini-Hochberg multiple testing correction with a threshold p-value of 0.05 was used to calculate the p-value. The right y-axis (line graph) corresponds to the number of proteins associated with each disease with the DE protein dataset. Red boxes highlight significant neuropsychiatric and neurodegenerative diseases enriched within the proteomic dataset.
Table 8: Summary table of most significant diseases and their respective enriched proteins within our 118 differentially expressed dataset.

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huntington's Disease</td>
<td>ALDH6A1, AQP4, C3, C4A/C4B, CALB1, CAMKK2, CRYAB, EPHB2, GFAP, GPI, HOMER1, HPCA, MAP2K4, PLCB1, PTK2B, SCARB2, SCG2, TGM2, VCAN</td>
</tr>
<tr>
<td>Dementia</td>
<td>ALDH11L1, APOD, ATP8A1, C3, C4A/C4B, CACNB4, CALB1, CTSD, GFAP, GRM2, GRM3, HOMER1, IGSF8, MAPT, NPTX1, PRKDC, PSAP, SERPINE2, TF, TGM2, THY1</td>
</tr>
<tr>
<td>Disorder of Basal ganglia</td>
<td>ALDH6A1, APOD, AQP4, C3, C4A/C4B, CALB1, CAMKK2, CRYAB, EPHB2, GFAP, GPI, HOMER1, HPCA, MAP2K4, MAPT, PLCB1, PTK2B, SCARB2, SCG2, TGM2, THY1, TPT1, VCAN</td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>ALDH11L1, APOD, ATP8A1, C3, C4A/C4B, CALB1, CTSD, GFAP, GRM2, GRM3, HOMER1, IGSF8, MAPT, NPTX1, PRKDC, PSAP, SERPINE2, TF, TGM2, THY1</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>APOD, C3, C4A/C4B, CALB1, CPLX1, GFAP, GRM3, HSPA2, KIAA1549L, MLC1, NCS1, NPTX1, PPP1R9B, PPP3CB, SCG2, TF</td>
</tr>
<tr>
<td>Parkinson's Disease</td>
<td>APOD, AQP4, CRYAB, GFAP, MAPT, SCARB2, TGM2, THY1, TPT1</td>
</tr>
<tr>
<td>Mood Disorders</td>
<td>APOD, AQP4, CACNB4, CPLX1, CRYAB, GFAP, GRM3, MAPT, NCS1, NDUFS8, PPP1R9B</td>
</tr>
<tr>
<td>Major Depression</td>
<td>AQP4, CPLX1, CRYAB, GFAP, GRM3, MAPT</td>
</tr>
</tbody>
</table>
3.4 Categorization of Differentially Expressed Proteins into Hallmarks of Aging

Although the hallmarks of aging have been described for peripheral tissue, it is not yet known how they recapitulate aging in the brain (Lopez-Otin et al., 2013). Therefore to explore how our age-related differentially expressed proteins are involved in the hallmarks of aging, we manually categorized 118 differentially expressed proteins based on each protein’s biological functions, into the hallmarks of aging of peripheral somatic tissue outlined by (Lopez-Otin et al., 2013) (Refer to materials and methods for details). Within our dataset, most proteins that change as a function of age were shown to categorize under altered neural communication, with a total of 64 proteins (54%), from which 23 were upregulated and 41 were downregulated. A total of 46 proteins (39%) were involved in deregulated nutrient sensing, of which 28 were upregulated and 18 were downregulated. Loss of proteostasis and cellular senescence constituted of 41 (35%) and 36 (31%) proteins, respectively. Hallmarks of aging affected less by aging in the brain were stem cell exhaustion, mitochondrial dysfunction, epigenetic alterations and genomic instability contained 16 (14%), 15 (13%), 8 (7%), and 3 (3%) proteins, respectively. Lastly, no proteins within our dataset were involved in telomere attrition. It is to note, several proteins were categorized into multiple hallmarks, due to the functional heterogeneity or multi-functionality of proteins.
Figure 13: Functional categorization of differentially expressed proteins that change with increase in age, in layer II/III of the OFC, into nine hallmarks of aging.

Based on the biological function, each protein was categorized into hallmarks of aging. Red represents upregulated and green represents downregulated proteins within each hallmark. Compared to other hallmarks, altered neural communication is shown to be the most affected hallmark of aging, with 54% coverage of differentially expressed proteins.
Chapter 3

4 Discussion

4.1 Project Summary

In an effort to understand molecular mechanisms associated with normal brain aging, we undertook a large scale unbiased study to generate a proteomic profile of layer II/III OFC of young and old individuals, using LCM and LC-MS/MS analysis.

The experimental strategy used, presented several challenges: (1) optimal identification of layer II/III was required prior to LCM, (2) maximum protein extraction from limited tissue had to be done by optimizing detergent concentration and (3) depletion of detergents from peptides to ensure compatibility with LC-MS/MS analysis. We determined the optimum staining for identification of layer II/III (with 0.2% thionin for 20 min). We also identified that 1.5% SDS was the ideal concentration for protein extraction and the condition required for removal of the detergents to ensure compatibility with LC-MS/MS analysis.

We found a total of 4,193 proteins from which 2,317 proteins were reliability identified and quantified in >70% of subjects among 33 young and old subjects. Further statistical analysis revealed a total of 118 differentially expressed proteins with an equal number (59 each) of upregulated and downregulated proteins.

Functional analysis by gProfiler highlighted age-related downregulation of glutamate/GABA and Ca$^{2+}$ signaling, learning and memory, as well as upregulation of lymphocyte migration. IPA canonical pathway analysis showed enrichment in synaptic long-term potentiation and glutamate signaling as well as pathways involved in pro-inflammatory responses such as lymphocyte migration, PI3K signaling of B-lymphocytes, LPS/IL-1 mediated inhibition of RXR function, aryl hydrocarbon signaling and xenobiotic signaling. All gProfiler IPA canonical pathway analysis and IPA network analysis showed analogous enrichment of decreased neuro-cognitive signaling and increased pro-inflammatory response. Disease analysis by IPA showed enrichment of neuropsychiatric (e.g. dementia, major depression, schizophrenia, anxiety disorders) and neurodegenerative (e.g. Alzheimer’s).
Investigation of hallmarks of aging within our dataset showed 8 out of 9 hallmarks of aging were represented in the brain. The most represented hallmark to be altered neural communication, while genomic instability and telomere attrition were not highly represented processes in the brain aging.

4.2 LCM and Proteomic analysis by LC-MS/MS

Age-related changes in the brain at the molecular level include alterations in both the transcriptome and proteome. Although microarray studies have highlighted age-related changes in the transcriptome of the PFC, the physiological context of a cell is best understood at the proteome level. Changes in the transcriptome may not necessarily correlate with the proteome (Chin et al., 2008; Zhu et al., 2012). For instance, Ghazalpour et al. (2011) compared transcriptomic (microarray analysis, 22,000 transcripts) and proteomic datasets (LC-MS/MS labeled quantification, 5000 proteins identified) derived from mouse liver tissue experiments. Correlation analysis of 7,185 and 486 significantly quantified transcripts and identified proteins, respectively, showed poor correlation (r=0.27) between the two datasets (Ghazalpour et al., 2011).

Undertaking a proteomic study of this scope using human post-mortem tissues posed its own set of challenges and required us to establish our own new methods and procedures. It is critical to note that post mortem tissues are notoriously challenging because of the limited amounts of starting material and also the biological viability of the samples (e.g. PMI, pH, cause of death, etc.)

In this study, we performed LC-MS/MS analysis which is the most effective high-throughput assay for the analysis of the proteome. We utilized LCM to collect and capture layer II/III of the OFC from young and old subjects. LCM is well suited for precise, contact-free collection and capture of specific areas of tissue with minimum contamination under a microscope. This technique has been applied to investigations of various tissues and cell types in cancer, cardiovascular diseases, and neurological disorders (Brady et al., 2016; Kandathil et al., 2013; MacDonald et al., 2014; Siniard et al., 2015; Vedantham et al., 2015). The use of LCM required us to optimize the visualization of layer II/III of OFC to accurately dissect this brain region for a systematic, precise collection, suitable for subsequent proteomic analysis.
Another critical step in method development was to optimize protein extraction from the limited micro-dissected layer II/III OFC samples in order to maximize protein yield. Typically, protein extraction involves the use of detergents (e.g. SDS, NP-40, SDC, TritonX) in the extraction buffer to maximize protein retrieval from cellular compartments (Cox and Emili, 2006; Wisniewski et al., 2009a). However, it is important to balance the need for efficient protein extraction which is achieved by using strong detergents with compatibility of detergents for MS analysis. The use of strong detergents which are not readily depleted from protein extracts often hinder LC-MS/MS analysis and should therefore be minimized. (Annesley, 2003; Chen et al., 2007a; Yates et al., 2009) We optimized the ideal SDS concentration to ensure efficient protein extraction, yield and compatibility with MS analysis.

Label free quantitative MS analysis is an unbiased and economical technique for large scale identification and quantification of proteomic changes in complex protein mixtures. Although labeled MS analysis has traditionally provided greater reliability in protein quantification, label-free MS has a higher dynamic range for peptide quantification and has greatly benefited from new statistical platforms like MaxQuant to improve quantitation and accuracy (Cox and Emili, 2006). The sensitivity and resolution of peptide identification is dependent on the type of mass analyzer in the mass spectrometer. Early mass analyzers yielded low resolution in peptide detection, limiting protein identification. The latest generation of mass analyzers (e.g. high Resolution Linear Ion Trap in Orbitrap Elite Mass Spectrometer) allows for high-throughput, high-resolution of peptide identification of up to 4000-5000 proteins in a single MS run (Holcapek et al., 2012; Hu et al., 2005; Michalski et al., 2012; Olsen et al., 2005). MS-based proteomic approaches have successfully been applied to studies of neurodegenerative and cardiovascular diseases and therefore have great potential in the investigation of age-related changes in the brain (Andreev et al., 2012; Arab et al., 2006; Castegna et al., 2002; Castegna et al., 2003; Taylor et al., 2003; Tilleman et al., 2002).

4.3 GABA/glutamate signaling alterations in cortical layer II/III in older human subjects

Functional analysis by gProfiler showed significant enrichment and downregulation of proteins involved in neuro-cognitive signaling (e.g. learning and memory, glutamate receptor
signaling, and divalent molecule transport (e.g. Ca\textsuperscript{2+}). Canonical pathway analysis by IPA further validated this by showing enrichment in glutamate signaling and synaptic long term potentiation. The balance of GABA/glutamate signaling mediates cognition (e.g. learning, memory, and synaptic long term potentiation) in the PFC where synapses/dendrites of excitatory (glutamate) pyramidal neurons receive inputs from GABAergic inhibitory interneurons, that code for excitatory output signals which mediate cognitive signaling. The greatest density of GABAergic interneurons in the frontal cortex is located in layer II/III therefore the greatest control of excitatory signaling occurs at these layers. (Bartolini et al., 2013b; Cauli et al., 1997; Gupta et al., 2003; Hoffmann et al., 2015; Kepecs and Fishell, 2014; Somogyi et al., 1998).

Downregulation of specific proteins involved in glutamate/GABA, Ca\textsuperscript{2+} signaling during aging, is a novel finding of this study and may contribute to the observed age-related decline in cognition. This decrease is in agreement with previous reports (by magnetic resonance spectroscopy) showing age-related decrease in GABA and glutamate concentration and alterations in excitation/inhibition in brain (Grachev and Apkarian, 2001). It implicates dysfunction of the GABA/glutamate regulatory system in normal brain aging. Previous studies have also suggested that age-related cognitive decline may be due to loss of function of GABAergic interneurons in layer II/III in the PFC and hippocampus (Beas et al., 2016; Kishimoto et al., 1998; McQuail et al., 2012; Vela et al., 2003). Progressive alteration in GABA/glutamate signaling has been involved in the dysfunction of pyramidal neurons of layer II/III of the PFC as a function of age (Banuelos et al., 2014). This effect on pyramidal neuron activity and decline in synaptic/spine structure was shown to involve GABA clearance impairment and to contribute to age-related working memory impairment (Banuelos et al., 2014).

Regarding glutamatergic signaling deficiency, we report here changes in glutamate receptor subunits (e.g. GRM2, GRM3), and binding proteins of glutamatergic receptor HOMER1 as well as calcium signaling cascades and secondary signal transductor (e.g. PCLB1) (Galván et al., 2015; Hoffmann et al., 2015; Otsu et al., 2014). Together this supports an overall alteration in the excitability of the aging layer II/III neuron involved in cognitive decline.
4.4 Increase in pro-inflammatory responses in layer II/III of OFC

Aging in layer II/III of the OFC at a proteomic level revealed significant enrichment in pro-inflammatory signaling pathways, including: lymphocyte migration (gProfiler), PI3K signaling of B-lymphocytes, and LPS/IL-1 mediated inhibition of RXR function (IPA). Surprisingly, Ingenuity pathway analysis of DE proteins associated with aging yielded enrichment in aryl hydrocarbon signaling (AHS) and xenobiotic receptor signaling. Classically, AHS/xenobiotic signaling has been associated with response to exogenous chemicals, inducing pro-inflammatory responses and anti-toxin pathways in the body (Denison and Nagy, 2003; Vogel et al., 2014).

Within our aging cohort, the induction of AHS/xenobiotic signaling during aging may be a left-over effect of long-term environmental exposure to exogenous environmental toxins (e.g. Dioxins) in the older population compared to the younger subjects. The molecular response to dioxins primarily mediated the Aryl Hydrocarbon Receptor (AhR). Epidemiological studies have shown that these toxins remain in the body across a life-time with limited removal from the body (White and Birnbaum, 2009). Studies have yet to investigate the effect of chronic AHS/xenobiotic signaling as function of age in the brain. However, a study testing the effect of AHS in the context of aging in the cardiovascular system showed a significant vascular stiffness with an increase in AHS signaling persistent in the context of age in humans and mice. These results imply that chronic dioxin-mediated AHS signaling may promote an aged phenotype (Eckers et al., 2016).

In addition, chronic AHS signaling as result of exposure to toxins, such as dioxins, has been shown to disrupt dendritic growth and maturation of the brain (ref?). Studies of mothers exposed to dioxins during pregnancy showed elevated AHS signaling in the offspring, with abnormal brain function in later life (Endo et al., 2012; Kakeyama et al., 2014; Kimura et al., 2016; Kimura et al., 2015; Schantz et al., 1996). This shows that exposure to dioxins has profound effects on the brain and it developments. Although the effect of AHS/xenobiotic signaling in the brain in the context of aging remains to be investigated, the appearance of these pathways within our cohort maybe due to the residual effect of toxins in the brains of older subjects. Together, this suggests that this particular molecular phenotype observed in the older subjects may represent a cohort effect, rather than an age-effect per se. Recent evidence has also
shown activation of AHS signaling/xenobiotic signaling to be much more diverse, involving endogenous ligands to stimulate pro-inflammatory response (Denison and Nagy, 2003; Vogel et al., 2014). More specifically, AHS in the brain has been shown to be involved in microglial and astroglial mediated inflammatory response (Lee et al., 2015; Rothhammer et al., 2016). Therefore, enrichment of aryl hydrocarbon/xenobiotic signaling suggests the induction of age-related chronic pro-inflammatory response. These findings were further supported by the IPA network analysis where NF-kB was enriched as a central signaling node. NF-kB has been previously shown to be involved in inflammatory response in the brain (Capiralla et al., 2012; Gabuzda and Yankner, 2013). Whether the induction of AHS signaling/Xenobiotic signaling within our age-cohort is due to exogenous toxins or an endogenous response due to the effect of age remains to be investigated.

Multiple transcriptomic and proteomic studies reported increased pro-inflammatory responses in the aging brain (Bigagli et al., 2015; Chung et al., 2001; Korolainen et al., 2005; Lynch et al., 2014; Poon et al., 2006b; Primiani et al., 2014). Neuro-inflammation is a response to injury or stress releasing cytokines, reactive oxygen species (ROS) and nitric oxide (NO), processes intended to repair damaged tissue (Lee et al., 2012). Acutely, pro-inflammatory response is beneficial for neuronal survival, however chronically, it induces downstream responses which can result in neurotoxicity of the cell and cellular senescence during aging, exacerbating the aging phenotype of brain tissue (Blasko et al., 2004; Joseph et al., 2005; Perry, 2004; Salminen et al., 2011). Although the exact cause for chronic inflammation in the aging brain is not yet well understood, deregulation and/or exacerbation of microglial and astroglial function has been implicated (Blasko et al., 2004; Joseph et al., 2005; Perry, 2004).

Within layer II/III, an increase in inflammation maybe correlated with the loss of function of interneurons. In mice, it was reported that IL-6 pro-inflammatory signaling facilitates loss of PV-expressing GABAergic interneurons during aging in the PFC (Dugan et al., 2009a; Dugan et al., 2009b). Moreover, increases in the expression of other pro-inflammatory factors including IL-1β and TNF-α have been shown during aging in the hippocampus. This was correlated with a reduced density in SST expressing GABAergic interneurons; however, no change was observed in PV-expressing interneurons (Gavilán et al., 2007; Lin and Sibille, 2015). Our findings of decreased proteins involved in GABA/glutamate signaling and increased pro-inflammatory response in layer II/III of the OFC are consistent with previously mentioned reports linking loss
of GABAergic interneuron function to increased inflammatory signaling in the aging brain. Based on our proteomic findings these processes are potentially related and since PFC layers II/III contain the most abundant population of GABAergic interneurons within the gray matter (Bartolini et al., 2013a; Darmanis et al., 2015; Markram et al., 2004; Sugino et al., 2006) this suggests an association between interneuron dysfunction and pro-inflammatory factors. However the exact molecular pathways that connect GABA/glutamate signaling to inflammation and the direction of causality (if any) remain to be investigated.

4.5 Hallmarks of aging, as defined in peripheral somatic tissue, partly recapitulates aging in the brain, showing predominance of altered neural communication.

We also evaluated whether the observed age-related proteomic changes in the OFC layer II/III of young and old subjects recapitulated biological processes outlined in the hallmarks of aging, described for peripheral somatic tissue. We manually categorized 118 differentially expressed proteins into the nine hallmarks of aging. Based on our annotations, 8 out of the 9 hallmarks of aging was shown to be represented, with certain hallmarks being more represented than others. More specifically, altered neural communication was observed to be the most affected hallmark. This was expected since age-related changes in the brain are primarily due to alterations in signaling of/between neural cells. Adaptation of neural communication may occur in response to the changing environment of the cell and brain overall during aging. Also, we have found altered neural communication to encompass intercellular signaling such as neurotransmission, neuron-glial signaling (e.g. calcium voltage-gated channel auxiliary subunit beta 3 (CACNG3)), intracellular signaling including cellular signaling cascades (e.g. Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2)) and structural signaling involving morphological, synapse/spine changes (e.g. doublecortin like kinase 1 (DCLK1)) signaling. Such forms of communication are unique to the brain, and therefore merit further investigation to characterize the age-related changes associated with in altered neural communication.

Other hallmarks such as deregulated nutrient sensing, loss of proteostasis and cellular senescence were also significantly represented. Many of the age-related differentially expressed proteins categorized to deregulated nutrient sensing, loss of proteostasis and cellular senescence, which were also classified in the “altered neural communication” hallmark. This may be because
the molecular machineries and pathways that mediate deregulated nutrient sensing, loss of proteostasis and cellular senescence involve intracellular signaling, which is also part of altered neural communication. In addition, it has been implied that loss of proteostasis is one of the key processes of intracellular damage to a neuron during aging, leading to loss of function, neuronal death and vulnerability to brain disorders (Cuanalo-Contreras et al., 2013). Our results also highlight the contribution of cellular senescence during aging, which is in correlation to what was expected as the incidences of cellular senescence is a key marker for the induction of inflammation and cellular stress.

Furthermore, our analysis showed less representation of hallmarks such as stem cell exhaustion, mitochondrial dysfunction, epigenetic alterations, genomic instability and telomere attrition. The hallmark of aging of epigenetic alterations included genes involved in genomic instability and inflammation and stem cell exhaustion. More specifically, since cell proliferation in the human brain is restricted to specific cell types (e.g. glial) and regions (e.g. hippocampus, supraventricular zone) (Eriksson et al., 1998) it is understandable that we found proteomic contribution to stem cell exhaustion in the OFC layer II/III in aged individual.

Interestingly, there were very few proteins involved in mitochondrial dysfunction. Many transcriptomic studies have reported robust enrichment of age-related gene expression changes involved in mitochondrial dysfunction in the brain. Mitochondrial dysfunction can lead to leakage of otherwise regulated reactive oxygen species and inflammation, whose long term exposure is shown to contribute to age-related cellular dysfunction in the brain (Salminen et al., 2012; Wang et al., 2013). However, age-related mitochondrial dysfunction in the brain has not been replicated at the proteomic level and is therefore probably limited to age-related changes at the transcriptomic level. For instance previous proteomic studies showed limited proteomic changes in the function of mitochondria and more changes in glycolytic, carbohydrate metabolism pathways (Stauch et al., 2015). Little to no proteins were shown to be involved in genomic instability and telomere attrition which is in agreement with what was expected. There is less cellular division in the brain to introduce mutations and genomic alteration by DNA replication.

Overall, we propose that the biological processes that mediate aging in the brain (at the proteomic level) can be recapitulated by hallmarks of aging. More precisely, we show altered
neural communication to be a predominant hallmark of aging in the brain while hallmarks such as genomic instability and telomere attrition show less significance possibly due to limited cell division in the brain. In further studies we propose to subdivide altered neural communication into intercellular, intracellular and structural signaling to provide an in depth characterization of aging in the brain.

4.6 Enrichment of protein changes associated with neuropsychiatric and neurodegenerative disorders in age-related proteomic changes in layer II/III of the OFC

The top five enriched pathways based on IPA disease pathway analysis were neurological disorders including neuropsychiatric diseases such as dementia, schizophrenia, mood disorders (e.g. pathological anxiety), and major depression and neurodegenerative disorders such as Alzheimer. These results support the theory of age-by-disease interaction at a proteomic level (Sibille, 2013). Although many studies have investigated aging and its relation to neurological disorders (Sibille, 2013), no unbiased study showed an association of age-related proteomic changes in multiple neuropsychiatric and neurodegenerative disorders. Interestingly the IPA disease analysis also showed enrichment for proteins in disorders of the basal ganglia including Huntington’s and Parkinson’s disease, all diseases of the thalamus. This can be due the thalamic neural projections that project to pyramidal neurons of layer III (Middleton and Strick, 2002). This may also indicate that the biological processes that occur during aging in the OFC are similar to those occurring in the thalamus.

This is particularly interesting, as it is contrast to transcriptomic studies whose changes are specific to the region analyzed, where mRNAs are synthesized. However, the origin of the collected proteins is dynamic and proteins not necessarily have to be synthesized in the region analyzed. Layer II/III of the OFC afferences/inputs from various regions (e.g. thalamus) (Altar et al., 1997; Benowitz et al., 1988; Conner et al., 1997). This is one method that provides validation of potential proteomic communication between the OFC and the basal ganglia in human post-mortem brain tissue.

In addition, specific proteins within our dataset have robustly been shown to change as a function of age and implicated in neurological disorders. We confirm the astroglial hypertrophy and altered function (e.g. Glial Fibrillary Acidic Protein (GFAP), Aquaporin 4 (AQP4),
Glutamine Synthetase (GLUL)), robustly shown altered during aging (Bondy, 2014; Lynch et al., 2014; Nichols et al., 1993; Rodríguez et al., 2014). Indeed, we found an increase in astroglial reactivity (e.g. increase GFAP) in human brain samples with aging. This process is classically associated with increased neuronal cell death and synaptic losses described in neurodegenerative and neuropsychiatric disorders such as Alzheimer’s and late life depression and schizophrenia (Miguel-Hidalgo et al., 2000; Rial et al., 2015). GFAP is a cell specific protein involved in structural and morphological cellular architecture in astroglial populations. The underlying cause of upregulation of GFAP has been correlated with neuronal loss, as astrocyte populations attempt to compensate and remediate the decreased cellular density by increasing volume and number (Rial et al., 2015). Associated with GFAP increase, we found an increase in AQP4 membrane protein which confirms the increase in volume and/or number of astrocytes. However, these volumetric changes are associated with loss of function since we found a decrease in GLUL (Magistretti, 2009; Miguel-Hidalgo et al., 2000).

4.7 Limitations

The inference of proteins from peptides identified by LC-MS/MS analysis creates a problem of how to deal with non-unique or “shared” peptides (Neilson et al., 2011). Shared peptides refer to peptide sequences that match to two or more proteins in a database and therefore present ambiguity during protein identification. In MS-based proteomics, protein assignment is typically based on identification of at least two unique peptides (Carr et al., 2004). Several strategies may be utilized to deal with this experimental limitation. For instance, they can be ignored and thus counted several times; removed and only rely on unique peptides or distributed across homologous proteins (Choi et al., 2008; Usaite et al., 2008). The chosen strategy to deal with shared peptides will affect label free quantitation (Podwojski et al., 2010). Protein inference based on only unique peptides leads to underestimation of true protein quantities (Usaite et al., 2008). In the MaxQuant platform that was utilized for label free quantitation of raw MS data in our study, both unique and shared peptides are used in protein quantitation (Cox and Mann, 2008b).

Most mass spectrometers including the Orbitrap Elite used in this study utilize a data dependent acquisition (DDA) strategy to select peptide ions for MS/MS (Kalli et al., 2013). Technically, the DDA strategy is limited by sequencing speed, sensitivity and dynamic range,
which leads to poor identification and quantitation of low abundant proteins (Michalski et al., 2011). The most abundant ions are selected for fragmentation scans which may create a bias against lower abundant peptides, in event they co-elute with more abundant peptides (Venable et al., 2004). As such, a subset of low abundant proteins may remain effectively unseen due to the mentioned peptide sampling limitations (Ghaemmaghami et al., 2003). Ionization efficiency and ion-trapping capacity of an MS instrument may also limit the MS signal to more abundant proteins. Targeted MS workflows may improve the dynamic range for detection and quantitation of a selected subset of proteins (Picotti et al., 2009; Savitski et al., 2010). Data independent acquisition (DIA) workflows have recently been demonstrated to improve identification of low-abundance peptides (Schmidt et al., 2011). In comparison to RNA-seq studies which can identify 10-12,000 genes or transcripts, most MS-based proteomics are still limited to identification of 4-5000 proteins in a 3 hour MS run (Lundberg 2010 and Zubarev 2013).

In addition, validation of LC-MS/MS results is also limited by the sensitivity of other proteomic techniques. For example, Western blot analysis can lack the dynamic range to observe relatively low abundant changes observed by LC-MS/MS. Western blot validation requires significantly greater amounts of starting protein material than LC-MS/MS analysis, therefore, proof of LC-MS/MS proteomic change, must be correlated with relatively large amounts of protein, an option that may not be available, and a challenge we were confronted with using human brain samples.

Lastly, the observed proteomic changes are a “snap-shot” of actual dynamic molecular processes that occur during aging and therefore some proteins associated with biological processes involved in aging processes may not be identified.

4.8 Future Directions

Additional analysis investigating cellular specificity of age-related proteomic changes within layer II/III could be done to provide more precise resolution at a molecular level. Layer II/III contains a diverse population of neural cell populations including pyramidal neurons, interneurons, astrocytes, microglia, and oligodendrocytes (Bartolini et al., 2013a; Darmanis et al., 2015; Markram et al., 2004; McQuail et al., 2015; Sugino et al., 2006) Our proteomics data showed significant changes in GABA/glutamate signaling and pro-inflammatory response in layer II/III. However, it remains to be investigated if the observed age-related proteomic changes
are specific to particular interneurons, or glial populations. If so, this may provide insight on affected cellular populations during aging.

Although for this study we opted for the analysis of layer II/III as literature showed the region to be selectively involved in age-related alterations in signaling and spine degeneration, layer V has also been greatly implicated to experience age-related changes (Mostany et al., 2013; Peters et al., 2008a; Young et al., 2014). Layer V contains somas of excitatory pyramidal neurons that mediate cognition of the PFC. Age-related changes in these pyramidal neurons have also been shown, therefore proteomic analysis of this layer would provide insight on altered on how aging effects these neurons at a proteomic level (Kasper et al., 1994; Nowak and Bullier, 1998a). In addition to layer V, comparative proteomic analysis of all layers could be performed, to investigate, the dynamic laminar changes that occur during aging. This analysis would highlight layer specific molecular pathways as well as pathways common to gray matter as a whole during aging. Such analysis can answer questions such as the decline GABA/glutamate specific for layer II/III at the proteomic level, and to what degree these changes are layer specific.

In terms of mass spectrometry analysis, targeted labeled quantitative MS workflows e.g. selected reaction monitoring (SRM) or parallel reaction monitoring (PRM) would provide more precise quantitation of a subset of proteins from our dataset. For instance we would limit the targeted MS analysis to synaptic function and GABA/glutamate related proteins (Gallien et al., 2012; Peterson et al., 2012; Podwojski et al., 2010).
4.9 Conclusions

- In conclusion we were able to conclusively optimize LCM mediated identification and extraction of proteins from postmortem human brain samples, and more specifically from layer II/III of the OFC for LC-MS/MS analysis.

- Functional analysis using gProfiler and IPA revealed significant downregulation in GABA/glutamate receptor signaling and upregulation pro-inflammatory responses. This indicated an interplay between altered interneuron function in layer II/III (shown by GABA/glutamate signaling) being affected by pro-inflammatory responses during aging at a proteomic level. In addition, our data suggest that aging of the brain can be recapitulated by proposed Hallmarks of Aging, showing specific profile compared to the periphery, highlighting alterations in neural communication.

- Finally, proteins involved normal brain aging in layer II/III of the OFC was shown to be significantly enriched in neurological disorders (e.g. dementia, major depression, and Alzheimer) showing an age-by-disease interaction at a proteomic level.
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## Appendices

### Appendix 1: Table of 118 differentially expressed proteins

<table>
<thead>
<tr>
<th>Uniprot Accession</th>
<th>Gene Symbol</th>
<th>Protein Name</th>
<th>Effect Size</th>
<th>P-value</th>
<th>Potential Co-Factors</th>
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<td>O15075</td>
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<td>6.84E-04</td>
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<tr>
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<tr>
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<td>Versican core protein</td>
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See full table for additional information on each protein, including Uniprot Accession, Gene Symbol, Protein Name, Effect Size, P-value, and Potential Co-Factors.
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<th>Uniprot Accession</th>
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<th>Protein Name</th>
<th>Effect Size</th>
<th>P-value</th>
<th>Potential Co-Factors</th>
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Appendix 2: Full gProfiler enrichment map of GO gene-set enrichment results of 118 differentially expressed proteins between young and old subjects.

Nodes represent protein sets and edges indicate their GO relationships. Nodes are colored based on enrichment results, where red and blue represents upregulation and downregulation, respectively of protein sets with increase in age. Color intensity correlates to enrichment significance.
Appendix 3: Summary table of age-related differentially expressed proteins categorized into hallmarks of aging.

DE proteins were manually classified into hallmarks of aging based on their biological processes outlined by GO. Red proteins show downregulation, while green proteins show upregulation.

<table>
<thead>
<tr>
<th>Hallmarks of Aging</th>
<th>Number of Proteins</th>
<th>Percent of Dataset</th>
<th>Categorized Proteins</th>
</tr>
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<tbody>
<tr>
<td>Altered Neuronal Communication</td>
<td>64</td>
<td>54%</td>
<td>ABI12, ACTN4, AHNK1, APOD, AQP4, ARHGFE2, ATP8A1, BRK1, C3, CACNB4, CACNG3, CALB1, CAMK1, CAMK2, CPE, CPLX1, CXADR, DCLK1, DLGAP1, DYNC1L1, EPHB2, GLUL, GPN1, GPM2, GRM3, GSTM2, HBB, HOMER1, HPCA, HSPA5, IKK2, KIAA1545, LASTIN, MAGI1, MAP2K4, MLC1, MSN, NCKIPSD, NCSTN, NEXN, NPAPP, PCBP1, PP1R10B, PP2R5C, PPP5CB, PRKDC, PSAP, PTK2B, PVRL1, RANBP1, SCA1, SCAR2, SERPINB6, SERPINE2, SGF1, SLC7A5, STIN1, SYT12, TUG2, THY1, TIP1, TRAPP1, VCAN</td>
</tr>
<tr>
<td>Deregulated Nutrient Sensing</td>
<td>46</td>
<td>39%</td>
<td>ABI12, AG1R, ALDH1A1, ALDH7A1, ALDH1A4, APOD, AQP4, ARHGFE2, ASAH1, BLM, BSN, C3, CACNB4, CALB1, CRL1, DCLK1, DYNC1L1, ECL2, FABP3, GLUL, GPN1, GPP1, GSTM2, HBB, ISCU, IPK1, KIAA1545, MAGI1, NAGK, NPTX1, PIP5K1B1, PIP5K2A, PIP5K3, PIP5K4, PIP5K1, PIP5K2B, PIP5K3, PIP5K4, PLCB1, PP1R10B, PPP5CB, PPT1, PRKDC, PSAP, PTGFR, SDC2, SH3T, TF, TIGAR, UCHL5</td>
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<tr>
<td>Loss of Proteostasis</td>
<td>39</td>
<td>33%</td>
<td>ADRM1, AHNK1, APOD, ATG7, ATP8A1, BRK1, CAMK4, C3, C5, CEP58, CPE, CRYAB, CTSD, FABP3, GSTM2, HPCA, HSPA5, LAMP2, MLC1, MSN, NCKIPSD, NCSTN, PDK1, PP1A, PP2R5C, PPT1, PRKDC, PTK2B, PQS1, SCAR2, SERPINB6, SERPINE2, SGF1, SLC7A5, TIGAR, TIP1, TRAPP1, TUG2, UCHL5, VTA1, VT1</td>
</tr>
<tr>
<td>Cellular Senescence</td>
<td>36</td>
<td>31%</td>
<td>APOD, ARHGFE2, C3, C4, CACNB4, CRYAB, CXADR, DCLK1, DYNC1L1, EPHB2, GFAP, GPR1, HEPACAM, IGSF8, KIAA1217, KIF1B, MAGI1, MAP1, MLC1, NCKIPSD, NPTX1, PAF1B2, PPP5CB, PPT1, PRKDC, PTK2B, PVRL1, SDC2, SERPINE2, STEN, TGF2, THY1, TIP1</td>
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<tr>
<td>Stem Cell Exhaustion</td>
<td>16</td>
<td>14%</td>
<td>ACTN4, APOD, ARHGFE2, DCLK1, GFAP, HEPACAM, IGSF3, PCDHB, PHGDH, PPT1, PVRL1, SLC7A5, TIP1, TPT1, UCHL5, VCAN</td>
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<td>Mitochondrial Dysfunction</td>
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<td>ALDH1L1, CCKAP4, CRYAB, EC12, FABP3, GSTM2, HBB, KIAA1545, MAP2K12, NDUF5, NPTX1, PSAP, PTGFR, SDC2, SH3T, TF, TIGAR, UCHL5</td>
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<td>Epigenetic Alterations</td>
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<td>Genomic Instability</td>
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<td>Telomere Attrition</td>
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