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PROTEOMICS AND PHOSPHOPROTEOMICS OF BRAIN: (1) ANALYSES OF THE PINK1 KNOCKOUT MODEL OF PARKINSON DISEASE; (2) INSIGHTS INTO THE PROGRESSION OF ALZHEIMER DISEASE; AND (3) THE NAKED MOLE-RAT MODEL OF SALUBRIous AGING

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PROTEOMICS AND PHOSPHOPROTEOMICS OF BRAIN: (1) ANALYSES OF THE PINK1 KNOCKOUT MODEL OF PARKINSON DISEASE; (2) INSIGHTS INTO THE PROGRESSION OF ALZHEIMER DISEASE; AND (3) THE NAKED MOLE-RAT MODEL OF SALUBRIOUS AGING

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By

Judy Carol Triplett
Lexington, Kentucky

Director: Dr. D. Allan Butterfield, Professor of Chemistry
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2015

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PROTEOMICS AND PHOSPHOPROTEOMICS OF BRAIN: (1) ANALYSES OF THE PINK1 KNOCKOUT MODEL OF PARKINSON DISEASE; (2) INSIGHTS INTO THE PROGRESSION OF ALZHEIMER DISEASE; AND (3) THE NAKED MOLE RAT MODEL OF SALUBRIOUS AGING

Proteomics is the field of science in which proteins produced by an organism (the proteome) are identified. The level of a particular protein can vary with time and the influence of cellular stressors. The study of phosphoproteomics is vital because tyrosine, serine and threonine phosphorylation modulate protein structure and function and is a crucial regulator of cellular signaling pathways. Dysregulation of protein and phosphorylation levels has been reported in multiple neurodegenerative disorders including Parkinson disease (PD) and Alzheimer disease (AD).

PINK1 is a mitochondrial serine/threonine kinase that polices mitochondrial integrity. Mutations in this protein are associated with familial early-onset PD. PD is characterized by accumulated Lewy bodies, largely composed of aggregated alpha-synuclein, and progressive dopaminergic neuronal degeneration in the substantia nigra. In this dissertation study, proteomics identified differences in protein expression and protein phosphorylation levels in the brains of PINK1 knockout mice. The observed changes suggest that perturbed metabolism, diminished proteostasis, decreased neuronal plasticity, and aberrant cellular signaling are implicated in familial PD pathogenesis.

AD is characterized by senile plaques, neurofibrillary tangles and synapse loss. Previously, multiple AD brain proteins were reported from our laboratory as abnormally phosphorylated, indicating that deregulated phosphorylation may play a key role in AD pathogenesis. In this dissertation study, quantitative phosphoproteomic analyses were conducted on the inferior parietal lobule from three different clinical stages of AD, i.e., late-stage AD, amnestic mild cognitive impairment (MCI) and preclinical AD (PCAD). The differential phosphoproteins identified provide insights into underlying mechanisms promoting the
preservation of memory in PCAD with expansive AD pathology, while uncovering early aberrant phosphorylation events in MCI that conceivably may be involved in the progressive cognitive decline leading to dementia.

Aging is a primary risk factor for development of neurodegenerative disorders, including AD and PD. The naked mole-rat (NMR), which can live for 32 years, is currently under investigation to gain insights into extending human lifespan and healthspan. In this dissertation research, age-related alterations of the brain proteome and phosphoproteome of the NMR were identified, revealing key proteins involved in neuronal plasticity, energy metabolism, autophagy, and the ubiquitin-proteasome system that may contribute to salubrious longevity.

KEYWORDS: Proteomics, Phosphoproteomics, Parkinson Disease, Alzheimer Disease, Naked Mole-Rat

Judy Carol Triplett
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April 30, 2015
Date
PROTEOMICS AND PHOSPHOPROTEOMICS OF BRAIN: (1) ANALYSES OF THE PINK1 KNOCKOUT MODEL OF PARKINSON DISEASE; (2) INSIGHTS INTO THE PROGRESSION OF ALZHEIMER DISEASE; AND (3) THE NAKED MOLE RAT MODEL OF SALUBRIOUS AGING

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To my family and friends,

I could not have accomplished so much

without your love and support.
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LIST OF ABBREVIATIONS

AAT – aspartate aminotransferase
Aβ – amyloid-β-peptide
ACN – acetonitrile
AD – Alzheimer disease
ADF – actin depolymerizing factor
AKT – protein kinase B
ALDOA – aldolase A
ALP – autophagy lysosomal pathway
APT1 – acyl-protein thioesterase 1
ARPC2 – actin-related protein 2/3 complex subunit 2
ATP5A1 – ATP synthase subunit alpha
BACH – brain acyl-CoA hydrolase
BCA – bichinchoninic acid
BSA – bovine serum albumin
BVR – biliverdin reductase
CaM – calmodulin
CBR3 – carbonyl reductase 3
ChT-L – chymotrypsin-like
CNP – 2',3'-cyclic nucleotide-3'-phosphodiesterase
CRMP2 – collapsin response mediator protein 2
CS – citrate synthase
DA – dopaminergic
Dnm – dynamin
DI – deionized
DRP2 – dihydropyrimidinase-related protein 2
DS – Down syndrome
DTT – dithiothreitol
EDTA – ethylenediaminetetraacetic acid
EFhd1 – EF-hand domain-containing protein D1
EF2 – elongation factor 2
EGTA – ethylene glycol tetraacetic acid
ENO – enolase
ETC – electron transport chain
ERAD – endoplasmic reticulum-associated degradation
FA – formic acid
FR – flavin reductase
GLUT – glucose transporter
GNAO1 – guanine nucleotide-binding protein G(o) subunit alpha
GPI – glucose-6-phosphate isomerase
GSH – glutathione
GSN – gelsolin
GSNO – S-nitrosoglutathione
G6PD – glucose-6-phosphate dehydrogenase
HIF – hypoxia-inducible factor
HNE – 4-hydroxy-2-nonenal
HPRT – hypoxanthine-guanine phosphoribosyltransferase
HRP – horseradish peroxidase
HSP – heat shock protein
IA – iodoacetamide
IDH1 – isocitrate dehydrogenase, cytosolic
IEF – isoelectric focusing
IP – immunoprecipitation
IPL – inferior parietal lobule
KO – knockout
LC3 – light chain 3
LDHB – lactate dehydrogenase B chain
MAPK1 – mitogen-activated protein kinase 1
MAPKK1 – mitogen-activated protein kinase kinase 1
MCI – mild cognitive impairment
MDH1 – malate dehydrogenase, cytosolic
MDH2 – malate dehydrogenase, mitochondrial
MMSE – mini-mental state examination
MOPS – 3-(N-morpholino) propanesulfonic acid
MS – mass spectrometry
mTOR – mammalian target of rapamycin
NFL – neurofilament light
NFM – neurofilament medium
NFT – neurofibrillary tangles
NMR – naked mole-rat
NSF – vesicle-fusing ATPase
PARL – presenilins-associated rhomboid-like protein
PCAD – preclinical AD
PD – Parkinson disease
PGPH – post-glutamyl peptide hydrolyzing
PHB – prohibitin

PINK1 – PTEN-induced kinase 1

PI3K – phosphoinositide 3-kinase

PK – pyruvate kinase

PMSF - phenylmethanesulfonylfluoride

PPlaseA – peptidyl-prolyl cis-trans isomerase A

PPP – pentose phosphate pathway

PSA2 – proteasome subunit alpha type-2

PSβ1 – proteasome subunit beta 1

PRDX1 – peroxiredoxin 1

PTEN – phosphatase and tensin homologue

RT – room temperature

SDS – sodium dodecyl sulfate

SMP-30 – senescence marker protein 30

SOD – superoxide dismutase

SP – senile plaques

Stxbp1 – syntaxin-binding protein 1

TAGLN3 – transgelin-3

TBS-T – tris-buffered saline and tween

TCP1 – T-complex protein 1

TGS – Tris/Glycine/SDS

TK – transketolase

TNF – tumor necrosis factor

UCH – UB carboxy-terminal hydrolase

UPS – ubiquitin proteasome system
VCP – valosin-containing protein
VDAC – voltage-dependent anion channel
VTAF – V-type proton ATPase subunit F
WT – wild type
3-NT – 3-nitrotyrosine
3-PGDH – 3-phosphoglycerate dehydrogenase
CHAPTER 1
Introduction

The studies described in this work utilize proteomics and phosphoproteomics techniques to gain a greater understanding of underlying biochemical mechanisms that contribute to pathology and clinical presentation of Alzheimer and Parkinson diseases. Additionally, changes in the brain proteome and phosphoproteome that occur with age in the naked mole-rat (NMR) are probed to examine mechanisms that may be central in maintaining an exceptionally long, healthy lifespan. Further, proteomic insights into key proteins involved in promoting/hindering neurodegenerative disease mechanisms may provide biomarkers to aid in early detection and development of effective, targeted therapeutics.

Many neurodegenerative diseases share common characteristics including: changes in energy metabolism, diminished proteostasis networks, increased oxidative stress, altered cellular signaling, and reductions in neuroplasticity, neurotransmission and cellular structure (Beal 1992, Butterfield and Kanski 2001, Palop, Chin et al. 2006, Rubinsztein 2006). Parkinson disease (PD) and Alzheimer disease (AD) are the two most common age-related neurodegenerative diseases. Although these disorders usually appear later in life, mutations in key genes can induce early onset, inheritable forms of the disease. In this dissertation research, we examined the proteome and phosphoproteome of the PINK1 (phosphatase and tensin homologue induced kinase1) knockout mouse model of PD at the earliest inception of disease.
presentation (6 months-old). Further, we analyzed changes in the phosphoproteome in the inferior parietal lobule from three different pathological stages of AD: preclinical AD (PCAD), amnestic mild cognitive impairment (MCI), and AD to evaluate mechanisms involved in disease progression from MCI to AD as well as understanding underlying mechanisms that preserve memory and cognitive function despite the presence of expansive senile plaque (SP) and neurofibrillary tangle (NFT) pathology typical to AD brains.

Even though the greatest single risk factor for neurodegeneration is aging (Lin and Beal 2006), most neurodegenerative research is conducted on short-lived species. However, by examining the age-related changes in the proteome and phosphoproteome of the longest-lived rodent, the NMR, may provide superior insights into proteins that may play critical roles in mechanisms that resist neurodegeneration and facilitate cellular proteostasis for healthy cognitive aging and improved quality of life.

The overall hypothesis of this dissertation research is that the underlying biochemical changes, as evident by evaluation the proteome and phosphoproteome of neurodegenerative brain, contribute to disease progression, pathogenesis, and clinical presentation and how proteomic investigations of the NMR could possibly identify key proteins that contribute to mechanisms promoting salubrious longevity. To support the overall hypothesis, this dissertation addresses the following:
1. What changes occur in the proteome and phosphoproteome of brain from PINK1-KO mice at the earliest stage of PD-like pathology?

2. Which proteins exhibit altered phosphorylation states in the inferior parietal lobule (IPL) of amnestic MCI and are they harbingers of AD?

3. What are the differences in the phosphoproteome of IPL of PCAD and AD subjects and how does a brain heavy with SPs and NFTs negate cognitive impairment?

4. What are the underlying mechanisms promoting a long healthspan and lifespan in the NMR?
   a. What are the biochemical changes in brain metabolism as these animals age?
   b. Which proteins are implicated in maintaining robust proteostasis networks throughout their long lives?
   c. Are there alterations in levels or activity of proteins associated with neuroplasticity, neurotransmission, or structure which may contribute to the extraordinary traits of the NMR?
CHAPTER 2

BACKGROUND

2.1 Neurodegeneration overview

Neurodegenerative diseases are a collection of disorders characterized by selective, progressive loss of neuronal structure and function. Oftentimes, these diseases share certain common characteristics, including: alterations in energy metabolism, mitochondrial dysfunction, increased oxidative stress, failing proteostasis networks, aggregation of misfolded proteins, and neuronal cell death (Beal 1992, Butterfield and Kanski 2001, Lin and Beal 2006, Palop, Chin et al. 2006). Since aging is the greatest risk factor for developing a neurodegenerative disease, a potential global therapy may conceivably lie in targeting key proteins in one or more of these common neurodegenerative pathways to ameliorate disease severity and promote a healthy lifespan.

2.1.1 Parkinson disease (PD)

2.1.1.1 PD overview

PD is the most common neurodegenerative movement disorder that affects approximately 1-2% of the population over the age of 65 and 3-5% of the population over 85 years-old (Fahn 2003). Additionally, 5-10% of PD patients may have an early-onset form of the disease with symptom onset occurring before the age of 50 (Alves, Forsaa et al. 2008). Except for incidences of PD caused by inheritable DNA mutations, the etiology of PD remains unclear, though
a number of contributing factors (in addition to age) have been implicated: exposure to environmental toxic metals or pesticides (Priyadarshi, Khuder et al. 2001), having a family member with PD (Kurz, Alves et al. 2003), mitochondrial dysfunction and oxidative stress (Henchcliffe and Beal 2008, Bueler 2009), and failure of proteostasis networks (McNaught, Olanow et al. 2001, Lynch-Day, Mao et al. 2012).

2.1.1.2 PD Pathology and physiology

PD is characterized by the aggregation of phosphorylated α-synuclein (Ser-129) within Lewy bodies and by the catastrophic death of the majority of dopaminergic (DA) neurons in the pars compacta region of the substantia nigra, although other neuronal types have been reportedly depleted as well (Braak, Del Tredici et al. 2003, Anderson, Walker et al. 2006). Significant DA neuron loss can result in one or more of the four cardinal motor symptoms of PD: resting tremor, bradykinesia, muscular rigidity, and postural instability (Jankovic 2008). The vulnerability of DA neurons has been linked to their reportedly reduced mitochondrial reserve as compared to that of other neuronal types (Matsuda, Kitagishi et al. 2013). Without an ample reserve of mitochondria, DA neurons are more susceptible to apoptosis during periods of acute energy crisis, mitochondrial dysfunction and oxidative stress (Hauser and Hastings 2013, Van Laar and Berman 2013). Other typical non-motor PD symptoms, which include anosmia, constipation, depression, hallucinations, REM sleep disorder, and
cognitive decline (Chaudhuri, Healy et al. 2006) may appear many years before the onset of motor-related symptoms.

2.1.1.3 PINK1

2.1.1.3.1 PINK1 function

Mutations in PTEN induced kinase 1 (PINK1), a mitochondrial serine/threonine kinase, are associated with autosomal recessive-inherited early onset PD (Kawajiri, Saiki et al. 2011). PINK1 is short-lived neuroprotective protein that polices mitochondrial integrity (Narendra, Jin et al. 2010). When mitochondria are functioning in a healthy steady-state, PINK1 is imported to the inner mitochondrial membrane where it is immediately cleaved by proteases, including PARL (presenillins-associated rhomboid-like protein), and retro-translocated to the cytosol for proteasomal degradation (Figure 2.1) (Deas, Plun-Favreau et al. 2011).
Figure 2.1 PINK1 in healthy mitochondria. Cleavage of PINK1 at the inner mitochondrial membrane in healthy mitochondria.

However, when mitochondria become dysfunctional and depolarize (Figure 2.2), PINK1 import into the mitochondria is blocked and PINK1 accumulates on the outer mitochondrial membrane where it recruits and phosphorylates Parkin, an E3 ligase, to initiate mitophagy for disposal of the damaged organelle (Matsuda, Sato et al. 2010).
**Figure 2.2 Pink1 in damaged mitochondria.** PINK1 accumulating on the outer membrane of damaged mitochondria, recruiting Parkin for mitophagy.

With malfunctioned PINK1, damaged mitochondria accumulate and disrupt energy and calcium homeostasis (Heeman, Van den Haute et al. 2011).

**2.1.1.3.2 PINK1 knockout (KO) PD mouse model**

PINK1 KO mice do not develop α-synuclein-containing Lewy bodies, nigrostraital neurodegeneration or manifestation of the four cardinal motor symptoms; however, pre-motor symptoms are present, including anosmia and gait disturbances (Glasl, Kloos et al. 2012). Consequently, PINK1 KO mice are a good model to study early PD development and early biomarkers (Glasl, Kloos et al. 2012). Further, PINK1 KO mice reportedly have decreased dopamine release, reduced synaptic plasticity (Kitada, Pisani et al. 2007), progressive weight loss and motor disturbances that correlate to reduced dopamine (Gispert, Ricciardi et al. 2009). Further, in the brain of PINK1 KO mice, mitochondria exhibit functional
defects as they have decreased ATP production (Gispert, Ricciardi et al. 2009), reduced capacity for Ca\textsuperscript{2+} storage (Akundi, Huang et al. 2011) and increased sensitivity to oxidative stress (Gautier, Kitada et al. 2008).

### 2.1.1.4 Mitochondria and superoxide

#### 2.1.1.4.1 Mitochondria

The mitochondrial respiratory chain, located on the inner mitochondrial membrane, is composed of five multisubunit protein complexes, which generate ATP by electron (e-) mediated coupling of H\textsuperscript{+} and O\textsubscript{2} to form H\textsubscript{2}O. The majority of energy generated by oxidative phosphorylation is spent maintaining ion gradients, propagating action potentials, releasing and recycling neurotransmitters; therefore, mitochondrial dysregulation can be detrimental to neuronal survival (Attwell and Laughlin 2001).

#### 2.1.1.4.2 Superoxide

Up to 2\% of oxygen consumed in this process is converted to superoxide (O\textsubscript{2}\textsuperscript{-}) due to e- leakage from the ETC (primarily from complex I and II) and transference to O\textsubscript{2} (Chouchani, Pell et al. 2014). Superoxide can lead to oxidative damage in a number of ways: (i) by direct damage by free radical chain reactions; (ii) by reaction with nitric oxide to form peroxynitrite leading to protein nitration; and (iii) indirectly by conversion to H\textsubscript{2}O\textsubscript{2} by superoxide dismutase (SOD), which though Fenton reactions can oxidize H\textsubscript{2}O\textsubscript{2} producing a hydroxide anion and a hydroxyl radical leading to lipid peroxidation and protein oxidation
(Figure 2.3). The hydroxyl radical is considered to be one of the most toxic free radical reactive oxygen species due to high reactivity and irreversible lipid, protein and DNA modifications (Halliwell 2007).

![Figure 2.3 Consequences of superoxide production. Pathways to protein nitration, lipid peroxidation and protein oxidation.]

2.1.2 Alzheimer disease (AD)

2.1.2.1 AD overview

AD, the most common neurodegenerative disease, afflicts 11% of the population over the age of 65 and 32% of people over 75 (Hebert, Weuve et al. 2013). Interestingly, about two-thirds of the 5.2 million Americans with AD are women (Hebert, Weuve et al. 2013). Since the greatest risk factor for developing AD is age; and to date, no significant difference has been found between men and women who develop AD (Hebert, Scherr et al. 2001), except for the longer average lifespan of women. Other proposed AD contributing factors include: Aβ

2.1.2.2 AD pathology and physiology

Neuropathological hallmarks of AD include the extensive distribution of extracellular senile plaques (SPs), intracellular neurofibrillary tangles (NFTs), amyloid β-peptide (Aβ) oligomers, and loss of synapses, which results in increased neuronal death. SPs are insoluble, fibrous Aβ-containing aggregations that are often surrounded by dystrophic neurites. NFTs are insoluble aggregates containing hyperphosphorylated tau proteins that have uncoupled from microtubules, leading to disassembly of the microtubules and decimation of neuronal infrastructure and axonal transport. Severity of AD clinical symptoms tends to correlate with the quantity and distribution of SPs, NFTs and lost synapses (Braak and Braak 1995, Thal, Rub et al. 2002). The pathology and underlying biochemical mechanisms lead to escalating memory loss and cognitive dysfunction that culminates in severe dementia.

2.1.2.3 AD progression

2.1.2.3.1 Amnestic mild cognitive impairment (MCI)

MCI is considered to be a transition between normal cognition and AD as the MCI brain generally displays levels of SPs, NFTs, neuronal loss, and oxidative stress that are intermediate to levels in AD (Morris, Storandt et al. 2001); however, SP density and abundance can vary widely (Villain, Chetelat et
A more reliable indicator that correlates with increased cognitive impairment and synapse loss is the measurement of Aβ oligomers, of which Aβ(1-42) is most toxic (Butterfield and Kanski 2002, Pham, Crews et al. 2010). Additionally, MCI subjects have non-age-related cognitive deficits but exhibit no signs of dementia or hindrance in performing every-day tasks (Petersen 2004). MCI is divided into two specific categories: amnestic MCI, which involves memory loss, and non-amnestic MCI, which does not affect memory (Portet, Ousset et al. 2006). Moreover, amnestic MCI is considered to be a preferential harbinger of AD (Portet, Ousset et al. 2006) and the conversion rate to AD is approximately 10-15% per annum (Maioli, Coveri et al. 2007). Proteomic research into this disease stage may conceivably uncover biomarkers to facilitate early AD detection and increase understanding of early mechanisms involved in disease progression.

2.1.2.3.2 Preclinical AD (PCAD)

Individuals with PCAD display no cognitive deficits, however, at postmortem, they exhibit extensive distribution of SP and NFT pathology (Erten-Lyons, Woltjer et al. 2009). PCAD brains are not only uncommon but also possess some remarkable traits including: minimal or no loss of neurons (Price, Ko et al. 2001), increased synaptic plasticity (O'Brien, Resnick et al. 2009), levels of oxidative stress similar to control brain (Aluise, Robinson et al. 2010, Aluise, Robinson et al. 2011), increased neuronal hypertrophy (Iacono, O'Brien et al. 2008), and alterations to proteins involved in anion transport (Lyubartseva, Smith et al. 2012).
et al. 2010). Identifying neurochemical mechanisms that inhibit cognitive decline despite heavy SP and NFT burden may provide critical clues towards therapeutic interventions stopping progressive memory loss.

2.1.3. Oxidative stress

Oxidative stress is implicated in multiple neurodegenerative disorders (Uttara, Singh et al. 2009). Oxidative stress occurs in a system when levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) overwhelm antioxidant defenses (Dasuri, Zhang et al. 2013). An oxidatively-stressed environment leads to damaged biomolecules, such as lipid membranes and proteins, and can induce post translational modifications that may result in altered protein structure and function, protein unfolding and aggregation, among others (Butterfield and Lauderback 2002).

2.1.3.1 Reactive oxygen species (ROS) and reactive nitrogen species (RNS)

ROS and RNS are toxic species and free radicals that include: superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide, and peroxynitrite, among others. ROS and RNS are tightly controlled by antioxidant enzymes, e.g., superoxide dismutase (SOD), biliverdin reductase, glutathione peroxidase, peroxiredoxins, catalase, and many heat shock proteins, and molecules, such as glutathione, vitamins C, D and E, and lipoic acid, to maintain the cellular redox state (Gilgun-Sherki, Melamed et al. 2001). An imbalance in this system can lead to oxidative damage to lipids, proteins and DNA (Halliwell 2007).
2.1.3.3 Lipid peroxidation and 4-hydroxy-2-nonenal (HNE)

Lipid peroxidation occurs in three stages: initiation, propagation and termination. Initiation occurs when an allylic hydrogen is removed from the lipid leaving a carbon-centered radical that can react with O$_2$ to form a peroxyl radical. Propagation occurs as a radical-radical chain reaction leads to further allylic hydrogen abstraction, which can cause production of reactive alkenals, loss of lipid asymmetry and apoptosis (Castegna, Lauderback et al. 2004, Butterfield, Bader Lange et al. 2010). Lipid peroxidation of arachidonic acid leads to the formation of 4-hydroxy-2-nonenal (HNE). HNE can render proteins dysfunctional by binding to His, Lys and Cys residues via a Michael’s addition reaction, thereby altering the protein’s structure and function (Butterfield, Bader Lange et al. 2010).

2.1.3.4 Tyrosine nitration

Tyrosine nitration occurs when a nitro group is added to tyrosine at the 3-position. The presence of this nitrite group sterically blocks tyrosine kinase-mediated phosphorylation, causing altered protein activity (Tangpong, Cole et al. 2007). Further, increased levels of tyrosine nitration elevate the likelihood of proteasomal degradation of the protein and can lead to neuronal death (Gow, Duran et al. 1996, Mattson, Goodman et al. 1997).

2.1.3.5 Protein carbonyl (PC)

PCs are the most wildly measured marker of oxidative stress (Dalle-Donne, Scaloni et al. 2005). PCs can be formed on proteins through a variety of
mechanisms, including: Michael addition reactions with lipid peroxidation products, direct oxidation of amino acid side chains, peptide backbone scission, and glycooxidation (Aksenov, Aksenova et al. 2001).

2.2 The naked mole-rat (NMR)

The NMR is arguably best known due to their unusually long healthspans and hold the world record as the longest lived rodent (Buffenstein 2005, Grimes, Reddy et al. 2014). Though being the size of a mouse, NMRs can live up to 32 years while maintaining normal body function and activity for 75-80% of their lifespan (Buffenstein 2008, Edrey, Park et al. 2011). These remarkable NMRs are not only resistant to cellular senescence, but they do not develop cancers, as there are no indications of spontaneous neoplasia and only one route to tumor development with experimentally-induced tumorigenesis (Buffenstein 2008, Liang, Mele et al. 2010, Tian, Azpurua et al. 2013), while other typical lab rodents develop cancers. It was recently discovered that malignant transformation of cells could occur in NMR cell culture if high-molecular-mass hyaluronan (HMM-HA) was removed (Tian, Azpurua et al. 2013). The mass of NMR HMM-HA is over 5 times that of a mouse or human analog and is implicated in mechanisms of early contact inhibition (Tian, Azpurua et al. 2013). The role that HMM-HA plays in mediating NMR cancer resistance is currently under investigation. Because of these extraordinary traits, the NMR genome was sequenced and released in 2011 to help decipher underlying mechanisms that promote a long and healthy lifespan.
2.2.1 NMR: Habitat and behavior

The naked mole-rat (NMR; *Heterocephalus glaber*; sand puppy) is a subterranean burrowing rodent indigenous to the hot and arid sub-Saharan region of North East Africa. NMRs live in large colonies that average 75-80 individuals, but can be as large as 300 NMRs (Brett 1991).

NMRs are the only known eusocial mammals, a social structure akin to that of bees or termites. Each colony has one dominate, breeding female (the queen) that consorts with up to 3 breeding males (Lacey and Sherman 1991). All other members of the community are somewhat smaller in size and reproductively repressed; however, these subordinates can become reproductive if removed from the colony (Holmes, Goldman et al. 2008). Interestingly, subordinate males and females exhibit a remarkable lack of differences in anatomy and behavior (Edrey, Park et al. 2011). Furthermore, a NMR colony has a strict division of labor of communal tasks, including: gathering of food, defense against predation, juvenile caretaking, and excavating of tunnel systems (Jarvis 1981).

Living in a deep and thermally stable ecological niche, NMRs have evolved several remarkable traits. This sealed underground environment is highly hypoxic, yet NMRs manage to cope with low oxygen levels, where in many vertebrates, only a few minutes of oxygen deprivation leads to irreparable neurological injury (Larson, Drew et al. 2014). Notably, as with most subterranean endothermic species, NMRs have low basal metabolism rates and
NMRs can greatly reduce their metabolism during periods of starvation (O’Connor, Lee et al. 2002). Additionally, NMRs are thermoconformers and therefore expend no energy in regulating their body temperature. Instead, NMRs huddle together or retreat deeper into burrows for warmth.

2.2.2 NMR and oxidative stress

Surprisingly, NMRs have been shown to produce high levels of ROS compared to mice (Lambert, Boysen et al. 2007). Even at an early age, NMRs reportedly have high levels of oxidative damage; however, these global levels do not increase and oxidized proteins do not accrue with age (Andziak and Buffenstein 2006, Lewis, Andziak et al. 2013). High levels of oxidative damage typically affect multiple cellular processes and contribute to age-related decline (Morimoto and Cuervo 2009). But clearly, NMRs have seemingly evolved mechanisms to repair or eradicate oxidative damage accumulation, since even young NMRs are more resistant to resultant oxidative stress damage than mice (Labinskyy, Csiszar et al. 2006). Antioxidants are a key ROS combatant; however, when levels of selected antioxidants (SOD 1 & 2, catalase and glutathione peroxidase) were measured in the NMR, they were not elevated compared to shorter-lived species (Andziak, O’Connor et al. 2005), suggesting that a superior antioxidant defense system is not the primary contributing factor for NMR resistance to oxidative stress.
2.2.3 Proteostasis in the NMR

NMRs are extremely resilient to cellular stress as their proteins are resistant to unfolding (Perez, Buffenstein et al. 2009). In most species, damaged proteins accumulate with age and is one of the hallmarks of aging (Zwickl, Seemuller et al. 2001). These proteins can aggregate and cause damage, such as the clogging proteasomes, disrupting cellular homeostasis. NMRs are an exception to this age-related accumulation of damaged proteins (Perez, Buffenstein et al. 2009). This resistance to oxidative stress in the NMR is hypothesized to be a result of their ability to maintain proteostasis through mechanisms assisting in protein repair or elimination (Rodriguez, Wywial et al. 2011).
CHAPTER 3

METHODS

3.1 Tissue homogenization

For all studies in this dissertation, selected brain tissues were prepared using a Wheaton glass homogenizer (~40 passes) with ice-cold isolation buffer [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 0.2 µg/ml PMSF, 4 µg/ml leupeptin, 4 µg/ml pepstatin, 5 µg/ml aprotinin and 10 µg/ml phosphatase inhibitor cocktail 2]. Homogenates were vortexed and then sonicated on ice for 10 s at 20% power, two times resting the sample for 20 s in-between, using a Fisher 550 Sonic Dismembrator (Pittsburgh, PA, USA). Protein concentrations of the homogenates were determined by the Pierce BCA method (Rockford, IL, USA) (Smith, Krohn et al. 1985).

3.2 Bicinchoninic acid (BCA) protein assay

Global protein concentrations of all samples in this dissertation research were determined using the bicinchoninic acid (BCA) protein assay. The first step of this assay utilizes the Biuret reaction for the reduction Cu^{2+} to Cu^{+} by the chelation of nearby peptide bonds. The second step is the formation of a chelating complex containing 2 BCA molecules in a 4-coordination complex with one Cu^{+} (Figure 3.1). A greater amount of protein will result in a darker purple solution (absorbing at 562nm) as there would be a greater number of chromophoric BCA complexes in solution. Bovine serum albumin (BSA) was used as the protein standard and Beer’s law was used for calculations of protein concentration.
3.3 Measurement of global oxidative stress markers

The slot blot method was used to determine levels of global makers of oxidative stress: protein carbonyls, 3-nitrotyrosine (3NT) and protein-bound 4-hydroxy-2-nonenal (HNE). The slot blot process involves preparation of samples (described below), loading samples in duplicate onto a nitrocellulose membrane utilizing the slot blot apparatus, blocking the membrane, detecting the desired modification with a primary antibody, labeling the primary antibody with a secondary antibody for detection and the quantification of the data using BioRad Image Lab software.

3.4 Detection of oxidative stress markers

3.4.1 Quantification of protein carbonyls

Global levels of protein carbonyls (PC) were determined in naked mole-rat brain. First, 5 µL of homogenized sample, combined with 5 µL of a 12% sodium
Dodecyl sulfate (SDS) solution was derivatized with 10 μL of a 2,4-dinitrophenylhydrazine (DNPH) solution [1:10 dilution with a 1x PBS, a phosphate buffer solution with sodium chloride and mono and dibasic sodium phosphates] to form dinitrophenylhydrazone Schiff bases. The solution was incubated for 20 min at 22°C. Next, 7.5 μL of a neutralization buffer [2 M Tris, pH 6.8, in 30% (v/v) glycerol] was added to react with the remainder of the DNPH solution. Utilizing the BCA data, the solution is further diluted with phosphate saline buffer (PBS) solution (10 mM, pH 8 with 0.88% NaCl) for a final protein concentration of 1 ng/µL. The protein-bound hydrazone-adducts were transferred to a nitrocellulose membrane using the slot blot apparatus and then detected immunochemically.

### 3.4.2 Determination of 3-nitrotyrosine and protein-bound HNE

To measure global levels of 3-NT and protein-bound HNE, 5 μL of the sample is added to 5 μL of the 12% SDS solution and 10 μL of a Laemmle buffer [0.125 M Tris base, pH 6.8, 4% (v/v) SDS, and 20% glycerol] for 20 min, then diluted to 1 ng/µL with PBS, transferred to a nitrocellulose membrane and detected immunochemically.

### 3.4.3 Immunochemical development of nitrocellulose membrane

Nitrocellulose membranes were blocked in 3% BSA in Wash Blot solution [150 mM sodium chloride, 3 mM monobasic sodium phosphate, 17 mM dibasic sodium phosphate and 0.04% (v/v) Tween 20], with gentle rocking for 1.5 h at 22°C. To this solution, the appropriate antibody was added (derivatized-PC
(1:300 dilution), 3-NT (1:5000) and HNE (1:5000)) for 2 h. The membrane was then rinsed and rocked in Wash Blot three times for 5, 5 and 10 min. Next, 2.5 µL of an anti-rabbit IgG alkaline phosphatase conjugated secondary antibody in 20 mL of wash blot was added for exactly one hour. The membrane was rinsed and rocked in Wash Blot again three times for 5, 10 and 10 min. The secondary antibody was detected by adding 30 mL of an alkaline phosphatase (ALP) buffer [0.5 M Tris, 0.1 M sodium chloride, 5 mM magnesium chloride hexahydrate, pH 9.5] with 99 µL of 5-bromo-4-chloro-3-indolyl phosphate dipotassium (BCIP) and 198 µL of nitrotetrazolium blue chloride (NBT). BCIP is hydrolyzed by ALP to form 5-bromo-4-chloro-3-indole and inorganic phosphate and NBT is added as an oxidizer to form an insoluble dark blue precipitate after being reduced for colorimetric detection. Membranes were rocked in this developing solution until colored slots appear. Relative color intensities are compared between the samples, relating relative oxidation levels.

3.5 Proteomics

For all studies in this dissertation, significantly altered protein levels and phosphorylation states were detected using a 2D-PAGE approach in conjunction with protein-gel staining and protein identification by MS/MS.

3.5.1 Isoelectric focusing

Isoelectric focusing (IEF) is a technique for separation of proteins based on their isoelectric point (pI). First, 200 µg of brain homogenate proteins were shaken for 2 h at 22°C in 200 µl of rehydration buffer [8 M urea, 2.0% (w/v)
CHAPS, 2 M thiourea, 50 mM DTT, 0.2% Biolytes, 0.01% Bromophenol Blue].
Samples were sonicated for 10 s and applied to 11 cm ReadyStrip IPG strips, pH 3-10 (Bio-Rad, Hercules, CA, USA). The IPG strips were actively rehydrated at 20 °C for 18 h at 50 V and then isoelectrically focused at a constant temperature of 20 °C beginning at 300 V for 2 h linearly, 500 V for 2 h linearly, 1000 V for 2 h linearly, 8000 V for 8 h linearly, and finishing at 8000 V for 10 h rapidly, using a Protean IEF cell (Bio-Rad). IPG strips were stored at -80 °C until second dimension separation.

3.5.2 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

2-D-Page separates proteins based on their molecular weight and shape. First, IPG strips were thawed for 10 min and equilibrated in buffer A [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 0.5% dithiothreitol (DTT)] for 10 min and then in buffer B [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 4.5% iodoacetamide (IA)] for 10 min. IPG strips were next placed into Linear Gradient (8-16%) Precast Criterion Tris–HCl polyacrylamide gels, 11 cm (Bio-Rad). Precision Plus Protein All Blue (Bio-Rad) or PeppermintStick (Invitrogen, Grand Island, NY, USA) molecular weight standards were run with samples at a constant voltage of 200 V for approximately 65 min in Tris-Glycine SDS running buffer.

3.5.3 Pro-Q Diamond phosphoprotein staining

Pro-Q Diamond phosphoprotein gel stain allows for the detection of phosphoserine, phosphothreonine and phosphotyrosine protein residues; and the
intensity of the resultant protein spot is correlated with the number of phosphate
groups attached. After completion of 2D-PAGE, gels were removed from their
casings and fixed in 50mL solution [10% (v/v) acetic acid, 50% (v/v) methanol] for
30 min, two times, at 22˚C on a gently rocking platform. Gels were washed in
100mL of deionized (DI) water (3 times; 30 min each). Next, 60 mL of Pro-Q
Diamond stain was added to the gel in the dark and covered with aluminum foil
and rocked for 90 min. Gels were destained in 100 mL of destaining solution
[20% acetonitrile (ACN), 50 mM sodium acetate, pH 4] in the dark (4 times; 30
min each). The gels were then washed 3 times for 30 min each in 100 mL of DI
water in the dark. The gels, transported wrapped in aluminum foil, were scanned
at 580 nm using either a GE Healthcare Life Sciences Typhoon FLA 9500
scanner (PINK1 knockout project) or a BioRad ChemiDoc MP System Imager
(Alzheimer disease and naked mole-rat projects).

3.5.4 Sypro Ruby protein staining

Sypro Ruby gel protein stain allows for the detection and quantification of
total protein content. This staining method is also used in conjunction with Pro-Q
Diamond phosphoprotein stain to allow for normalization of the phosphorylated
proteins so that a highly abundant but lightly phosphorylated protein can be
distinguished from a lowly expressed but highly phosphorylated protein.

After scanning Pro-Q Diamond-stained gels, the gels were fixed in 50 mL
solution [7% (v/v) acetic acid, 10% (v/v) methanol] for 45 min. After removing the
fixing solution, 50 mL of Sypro Ruby stain was added for overnight staining (15
hr) at 22°C with gentle rocking. Gels were rinsed and transferred to 50 mL DI water and scanned at 450 nm using the afore-mentioned scanners. Gels were stored in DI water at 4 °C until protein spot excision.

3.5.5 Image analyses

For all proteomic and phosphoproteomic studies in this dissertation, spot intensities from the Pro-Q Diamond and Sypro Ruby-stained 2D-gel images were quantified densitometrically according to the total spot density using PDQuest 2-D analysis software (Bio-Rad).

3.5.5.1 Differential protein expression

Spot intensities from Sypro Ruby-stained 2D-gel images of were quantified by densitometry. Intensities of gel spot were normalized to the total gel density. Protein spots of gels from a particular study were first manually matched together and then matched by the PDQuest program using powerful automatching algorithms for accurate spot-matching. Resultant data of normalized intensity of each protein spot were compared between groups using the appropriate statistical analysis. Figure 3.2 shows the method used to determine differential protein levels in samples and provides an illustration of a PDQuest gel map comparison.
Figure 3.2 Proteomic methods. Flow chart depicting analysis sequence in 2-D gel-based detection of differential protein levels.

3.5.5.2 Differential phosphorylation levels

Protein spots from Pro-Q Diamond-stained 2D-gel images were quantified and matched together in the same manner as the Sypro Ruby-stained gels. Next, two master gels, one from the Pro-Q Diamond images and one from the Sypro Ruby stained images, were matched together in the same manner as described above. Figure 3.3 shows the phosphoproteomics method for determining the identity of proteins with differential protein phosphorylation levels. The PDQuest software provided numerical data corresponding to the intensity of the protein spot and the intensity of the phosphoprotein spot. The phosphoprotein spot densities were then normalized to the Sypro Ruby spot densities and the resultant normalized phosphoprotein spot densities were compared. Spots with a
statistically significant difference based on appropriate statistical testing were considered for in-gel trypsin digestion and subsequent identification.

![Figure 3.3 Phosphoproteomic methods. Flow chart depicting analysis sequence in 2-D gel-based detection of differential protein phosphorylation.](image)

**3.5.6 In-gel trypsin digestion**

Significant protein spots were excised from 2D-gels with a clean, sterilized razor blade or pipette tip and individually transferred to Eppendorf microcentrifuge tubes. Gel plugs were incubated with 20 µL of 0.1 M ammonium bicarbonate (NH₄HCO₃) for 15 min, and with 30 µL of ACN for 15 min. Gel plugs were dried under a flow hood at RT for 30 min. Next, 30 µL of 20 mM DTT in 0.1 M NH₄HCO₃ was added at 56°C for 45 min. The DTT/NH₄HCO₃ solution was then removed and replaced with 30 µL of 0.05 M IA in 0.1 M NH₄HCO₃ and
incubated at 22°C for 15 min. Next, the IA solution was removed and plugs incubated for 15 min with 150 µL of 0.05 M NH₄HCO₃. Then, 200 µL ACN was added to this solution and incubated for 15 min. Solvent was removed and gel plugs were allowed to dry for 30 min under a flow hood. Plugs were rehydrated with 10 µL modified trypsin solution (Promega, Madison, WI, USA) in 0.05 M NH₄HCO₃ (enough to completely cover the gel plugs) and incubated with shaking overnight at 37 °C.

3.5.7 Gel-peptide extraction and purification

The next day, the digest solution was transferred into a new Eppendorf microcentrifuge tube. Next, approximately 20 µL of a 5% ACN, 0.1% formic acid (FA) solution was added to the old tube containing the gel plug (twice the volume necessary to submerge the gel), and sonicated in a bath for 15 min. To this, 30 µL of a solution containing 95% ACN, 0.1% FA and 0.001 M NH₄CO₃ was added and sonicated for 15 min. This resulting solution was combined with the supernatant digest solution in the new Eppendorf tube. Using a Speed Vac, the samples were concentrated to a volume of 10 µl. Using C18 ZipTips (Sigma-Aldrich, St. Louis, MO, USA), salts and contaminants were removed from the tryptic peptide solutions. To prepare the column in the ZipTip, 10 µL of 100% ACN was aspirated into the tip and then expelled 5 times. Next, 10 µL of a 50% ACN solution containing 0.1% FA was aspirated and expelled 5 times for column equilibration. The trypsin digested solution was then slowly drawn up and pushed gently out of the column 10 times for peptide adherence. Unwanted contaminants
were removed from the peptides in the ZipTip by washing with 10 µL of a 5% ACN and 0.1% FA solution 3 times. To elute the peptides from the column, 10 µL of a 50% ACN and 0.1% FA solution was drawn up and then expelled into a new Eppendorf tube. The eluant was drawn up and expelled 5 times to ensure complete peptide removal from the column. Samples were stored at -80°C until MS/MS analysis.

### 3.5.8 NanoLC-MS

Samples desalted with C₁₈ Zip Tips and reconstituted in 10 µL 5% ACN/0.1% FA were analyzed by a nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) system in data dependent scan mode. An in-house packed capillary column (0.1 x 130 mm column packed with 3.6 µm, 200Å XB-C18) and a gradient with 0.1% FA and ACN/0.1% FA at 200 nL/min were used for separation. The MS spectra were acquired by the orbitrap at 30,000 resolution and MS/MS spectra of the six most intense ions in MS scan were obtained by the orbitrap at 7,500 resolution.

### 3.5.9 Database interrogation and protein identification

Data files from all samples in this dissertation research were searched against the most current version of the Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4, Thermo Scientific). At least two high-confidence peptide matches were required for protein identification (false discovery rate <1%). Proteins matched with the same peptides are reported as one protein group. Exact peptide masses of digested proteins from the MS experiments were
compared with theoretical peptide masses in the database to produce a list of potential peptide matches. Proteins receive a confidence score based on the number of peptides identified and the percentage of the protein’s amino acid sequence covered. The data accompanying the identified protein includes the expected molecular weight and isoelectric point. Preliminary verification of the identified protein is made by comparing the position of the excised gel plug with the expected position of the identified protein.

### 3.5.10 Immunoprecipitation

Brain proteins (250 µg) were suspended in 500 µL IP buffer [0.05% NP-40, leupeptin 4 µg/mL, pepstatin 4 µg/mL, aproprotin 5 µg/mL, phosphatase inhibitor cocktail 10 µg/mL in a phosphate buffer solution, pH 8 (8 M NaCl, 0.2 M KCl, 1.44 M Na₂HPO₄, 0.24 M KH₂PO₄, pH adjusted with NaOH)] and allowed to shake for 30 min at 4˚C. Next, 50 µL of Protein A/G beads (per sample) were washed and centrifuged at 2500 RPM (5 min; 4˚C). The washed beads were added to pre-clear the samples of non-specific protein A/G artifacts (90 min; 4˚C). Samples were centrifuged at 2500 RPM (5 min; 4˚C) and the supernatant was transferred to new Eppendorf tubes with an antibody (1:50 dilution) and shaken overnight at 4˚C. The next morning, 50 µL of protein A/G beads were washed as and incubated with the protein samples (90 min; 4˚C). The supernatant was removed and the beads were washed in IP buffer with shaking (5 times; 10 min each). Beads were preserved for 1D-PAGE experiments.
3.5.11 One-dimensional polyacrylamide gel electrophoresis (1-D PAGE)

Whole brain homogenates (50 µg) were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] diluted to 1X with distilled water, heated at 95 °C for 5 min and cooled on ice. Samples and Precision Plus Protein All Blue Standards were loaded into a Criterion precast (4-12%) Bis-Tris polyacrylamide 12 well gels and run at RT in a Criterion Cell vertical electrophoresis buffer tank (Bio-Rad) filled with a 1X dilution of XT MOPS running buffer at 80 V for 15 min to ensure proper protein stacking. The voltage was then increased to 120 V for approximately 100 min at RT for the duration of the electrophoretic run.

3.5.12 Western blotting

1D-gels were directly transferred to nitrocellulose membranes (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad) at 25 V for 30 min. After the transfer, membranes were blocked with 3% bovine serum albumin (BSA) in Wash Blot [150 mM NaCl, 3 mM NaH₂PO₄, 17 mM NaH₂PO₄ and 0.04% (v/v) Tween 20] or TBS-T [8 M NaCl, 2.4 M Tris, and 0.1% (v/v) Tween 20] for 1.5 h. Primary antibodies were added to blocking solution for 2 h. Blots were rinsed two times for 5 min each and one time for 10 min in either Wash Blot or TBS-T, followed by a 1 h incubation with a secondary antibody. Blots were rinsed three times for 5, 10 and 10 min each in Wash Blot or TBS-T and developed chemiluminescently using Clarity Western ECL Substrate (Bio-Rad). After developing for 5 min at RT in the dark, blots were scanned using a Bio-Rad
ChemiDoc XRS+ imaging system and quantified using Image Lab software (Bio-Rad). Blots were then typically rinsed with Wash Blot or TBS-T (5 min, two times) and then stripped with Re-Blot Plus Strong solution (GE Healthcare, Pittsburgh, PA, USA) for 10 min at RT, followed by two 5 min rinses with Wash Blot or TBS-T for re-probing with additional antibodies.
CHAPTER 4

QUANTITATIVE EXPRESSION PROTEOMICS AND PHOSPHOPROTEOMICS PROFILE OF BRAIN FROM PINK1 KNOCKOUT MICE: INSIGHTS INTO MECHANISMS OF FAMILIAL PARKINSON DISEASE

4.1 Overview

Parkinson disease (PD) is an age-related, neurodegenerative motor disorder characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta and presence of α-synuclein-containing protein aggregates. Mutations in the mitochondrial Ser/Thr kinase PTEN-induced kinase 1 (PINK1) are associated with an autosomal recessive familial form of early-onset PD. Recent studies have suggested that PINK1 plays important neuroprotective roles against mitochondrial dysfunction by phosphorylating and recruiting Parkin, a cytosolic E3 ubiquitin ligase, to facilitate elimination of damaged mitochondria via autophagy-lysosomal pathways. Loss of PINK1 in cells and animals leads to various mitochondrial impairments and oxidative stress, culminating in dopaminergic neuronal death in humans. Using a 2-D PAGE proteomics approach, the differences in expressed brain proteome and phosphoproteome between six month-old PINK1-deficient mice and wild-type mice were identified. The observed changes in the brain proteome and phosphoproteome of mice lacking PINK1 suggest that defects in signaling networks, energy metabolism, cellular proteostasis and neuronal structure and plasticity are involved in the pathogenesis of familial PD.
4.2 Introduction

Parkinson disease (PD) is the most common neurodegenerative movement disorder. The relative risk of developing PD increases with age and approximately 1-2% of the population above 65 years is affected (Twelves, Perkins et al. 2003, Pilsl and Winklhofer 2012). PD is characterized by the accumulation of Serine-129 phosphorylated α-synuclein aggregates (Anderson, Walker et al. 2006) within Lewy bodies and the loss of the majority of dopaminergic (DA) neurons in the substantia nigra pars compacta, causing drastically diminished dopamine release in the striatum (Braak, Tredici et al. 2003). In addition to the cardinal motor symptoms (resting tremor, bradykinesia, rigidity, postural instability), PD patients frequently suffer from a variety of non-motor symptoms that may occur decades earlier and include cognitive decline, depression, hallucinations, REM sleep disorder and anosmia (Chaudhuri, Healy et al. 2006, Jankovic 2008). Although the precise cause of PD remains elusive, significant contributing factors are believed to include exposure to environmental toxins, oxidative stress, inflammation, mitochondrial dysfunction, and failure of proteostasis networks associated with protein aggregation (Henchcliffe and Beal 2008, Bueler 2009, Tansey and Goldberg 2010).

While the majority of PD cases are sporadic, an estimated 5-10% of patients develop PD as a result of inheritable mutations in one of several genes (Gasser, Hardy et al. 2011). Mutations in \textit{Pink1} are the second most common cause of autosomal recessive familial early onset PD (Valente, Abou-Sleiman et
al. 2004, Bonifati, Rohe et al. 2005, Bonifati 2012). PINK1 is a mitochondrial kinase consisting of 581 amino acids that encode for a mitochondrial targeting sequence, a transmembrane domain and a Ser/Thr kinase domain. PINK1 is believed to confer neuroprotection by policing mitochondrial integrity (Narendra, Jin et al. 2010, Jin and Youle 2012), and a growing amount of research links dysfunction of mitochondrial dynamics with PD (Heeman, Van den Haute et al. 2011, Hauser and Hastings 2013, Van Laar and Berman 2013). DA neurons may be particularly vulnerable to mitochondrial dysfunction and oxidative stress due to reduced mitochondrial reserve compared to other types of neurons and the reliance on calcium influx for pace-making (Liang, Wang et al. 2007, Surmeier, Guzman et al. 2011). In normal cells with healthy mitochondria, PINK1 is imported to the inner mitochondrial membrane where the N-terminal mitochondrial targeting sequence is cleaved by the mitochondrial protease, PARL (Deas, Plun-Favreau et al. 2011, Meissner, Lorenz et al. 2011). Subsequently, PINK1 is retro-translocated to the cytosol where it is degraded by the proteasome (Yamano and Youle 2013). However, a proportion of PINK1 remains in the cytoplasm (Haque, Thomas et al. 2008, Lin and Kang 2008) and cytosolic PINK1 has been implicated in the regulation of various pathways, including AKT signal transduction and cell survival, synaptic plasticity, DA synthesis and mitophagy (Haque, Thomas et al. 2008, Murata, Sakaguchi et al. 2011, Akundi, Zhi et al. 2012, Fedorowicz, de Vries-Schneider et al. 2014, Zhou, Refai et al. 2014). Import of PINK1 to the inner mitochondrial membrane is blocked when the mitochondrial electron transport chain becomes dysfunctional.

In this study, we utilized a 2-D PAGE proteomics approach to identify proteins with differential expression levels and phosphorylation states in the brains of PINK1 knockout (KO) mice versus wild-type controls to further study the molecular mechanisms of PINK1-related PD.

4.3 Materials and Methods

4.3.1 Materials

All chemicals used in these studies were purchased from Sigma-Aldrich (St. Louis, MO, USA) with noted exceptions: Criterion precast polyacrylamide gels, ReadyStrip IPG strips, TGS and XT MOPS electrophoresis running buffers, 0.2 nm nitrocellulose membrane, Precision Plus Protein All Blue Standards,
Sypro Ruby Protein Stain, mineral oil, dithiothreitol, iodoacetamide, biolytes, and urea were purchased from Bio-Rad (Hercules, CA, USA). Pro-Q Diamond phosphoprotein stain, PeppermintStick phosphoprotein molecular weight marker, and anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies raised in rabbits were purchased from Invitrogen (Grand Island, NY, USA). Anti-HSP70 and anti-aldolase A antibodies raised in rabbits were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MAPK1/2 polyclonal antibody raised in rabbits, C\textsubscript{18} ZipTips, and Re-Blot Plus Strong stripping solution were purchased from Millipore (Billerica, MA, USA). Calmodulin polyclonal antibody raised in rabbits was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Amersham ECL rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody, protein A/G beads and ECL-Plus Western blotting detection reagents were purchased from GE Healthcare (Pittsburgh, PA, USA). Modified trypsin solution was obtained from Promega (Madison, WI, USA).

4.3.2 Animals and brain tissue

The generation and characterization of the PINK1-deficient mice used in this study has been described previously (Akundi, Huang et al. 2011). The animal housing facility regularly underwent serological testing to certify a healthy, virus and pathogen free facility. Subjects were fed Richland Laboratory Rodent Diet 5001 and water ad libitum. The animal rooms were maintained with a 12 hour light:12 hour dark cycle (lights on at 0600) at 20-22°C. For the experimental
groups of animals, n=6 per group, whole brains were isolated from mice rendered unconscious by CO\textsubscript{2} inhalation and euthanized by cervical dislocation using procedures approved by the UK Institutional Animal Care and Use Committee. Dissected brains were flash-frozen in liquid nitrogen and stored at -80˚C until use.

4.3.3 Sample preparation

Mouse brains were thawed and individual homogenates were prepared using a Wheaton glass homogenizer (~40 passes) with ice-cold isolation buffer [0.32M sucrose, 2mM EDTA, 2mM EGTA, 20mM HEPES, 0.2µg/ml PMSF, 4µg/ml leupeptin, 4 µg/ml pepstatin, 5 µg/ml aprotinin and 5 µg/ml phosphatase inhibitor cocktail 2]. Homogenates were vortexed on ice and then sonicated on ice for 10 s at 20% power, two times, with a Fisher 550 Sonic Dismembrator (Pittsburgh, PA, USA). Protein concentrations of the homogenates were determined by the Pierce BCA method (Rockford, IL, USA) (Smith, Krohn et al. 1985).

4.3.4 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Isoelectric focusing (IEF). IEF was performed as described previously (Sultana, Boyd-Kimball et al. 2007). Briefly, brain homogenate proteins (200 µg) were shaken for 2 h at RT in 200 µl of rehydration buffer [8 M urea, 2.0% (w/v) CHAPS, 2 M thiourea, 50 mM DTT, 0.2% Biolytes, 0.01% Bromophenol Blue]. Samples (200 µl) were applied to 11 cm pH 3-10 ReadyStrip IPG strips (linear gradient). Strips were actively rehydrated at 20 °C for 18 h at 50 V. Next, the
samples were isoelectrically focused at a constant temperature of 20 °C beginning at 300 V for 2 h, 500 V for 2 h, 1000 V for 2 h, 8000 V for 8 h, and finishing at 8000 V for 10 h. IPG strips were stored at -80 °C until second dimension separation (SDS PAGE).

**SDS PAGE.** IEF strips were thawed and equilibrated with buffer A and buffer B (buffer A [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 0.5% DTT], buffer B [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 4.5% iodoacetamide]). IPG strips were next placed into Criterion precast linear gradient (8–16%) Tris–HCl polyacrylamide gels, 1 IPG + 1 Well Comb, 11 cm. Invitrogen PeppermintStick molecular weight marker and samples were run at a constant voltage of 200 V for approximately 65 min in Tris-Glycine SDS running buffer.

### 4.3.5 Gel staining

Proteins and phosphoproteins were detected using SYPRO Ruby and Pro-Q Diamond phosphoprotein gel stain according to manufacturer’s directions and as previously described (Di Domenico, Sultana et al. 2011). Briefly, gels were fixed in solution [10% (v/v) acetic acid, 50% (v/v) methanol] (50 mL) and washed in deionized water. Next, 60 mL of Pro-Q Diamond stain was added with gentle rocking for 90 min. Gels were then destained 4 times for 30 min each in 100 mL of destaining solution [20% acetonitrile, 50 mM sodium acetate, pH 4]. The gels were washed in deionized water and scanned at 580 nm using a GE Healthcare Life Sciences Typhoon FLA 9500 scanner. The gels were then
stained overnight with 50 mL of Sypro Ruby gel stain. Gels were rinsed and placed in deionized water. Gels were scanned at 450 nm and then stored at 4 °C until protein spot excision.

4.3.6 Image analysis

Expression Proteomics. Spot intensities from SYPRO Ruby-stained 2D-gel images of wild-type (WT) and PINK1 KO samples were quantified by densitometry using PDQuest 2-D Analysis Software (Hercules, CA, USA). Intensities of each gel were normalized to the total density of each gel. Protein spots were manually and automatically matched with the PDQuest program. Spot densities in KO and WT samples were compared and spots with a statistically significant difference based on a Student’s two-tailed t-test (p<0.05) were considered for in-gel trypsin digestion and subsequent identification.

Phosphoproteomics. Protein spots from Pro-Q Diamond-stained 2D-gel images of PINK1−/− and WT samples were quantified and matched together in the same manner as the SYPRO Ruby-stained gels. Next, two master gels were chosen, one from the Pro-Q Diamond images and one from the Sypro Ruby stained images. The phosphoprotein master image was then matched to the master Sypro Ruby stained image in the same manner as described above. The PDQuest software provided numerical data corresponding to the intensity of the protein spot. The phosphoprotein spot densities were then normalized to the Sypro Ruby spot densities and the resultant normalized phosphoprotein spot densities in KO and WT samples were compared and spots with a statistically
significant difference based on a Student’s two-tailed t-test (p<0.05) were considered for in-gel trypsin digestion and subsequent identification.

4.3.7 In-gel trypsin digestion/peptide extraction

Protein spots were excised from 2D-gels with a clean, sterilized razor blade and individually transferred to Eppendorf microcentrifuge tubes for trypsin digestion as previously described (Thongboonkerd, McLeish et al. 2002). Gel plugs were incubated with 20 µL of 0.1 M ammonium bicarbonate (NH₄HCO₃) for 15 min, and with 30 µL of acetonitrile for 15 min. Gel plugs were dried under a flow hood at RT for 30 min. Next, 30 µl of 20 mM DTT in 0.1 M NH₄HCO₃ was added at 56°C for 45 min. The DTT/NH₄HCO₃ solution was then removed and replaced with 30 µl of 0.05 M iodoacetamide (IA) in 0.1 M NH₄HCO₃ and incubated at RT for 15 min. Next, the IA solution was removed and plugs incubated for 15 min with 150 µl of 0.05 M NH₄HCO₃ at RT. Then, 200 µl acetonitrile was added to this solution and incubated for 15 min at RT. Solvent was removed and gel plugs were allowed to dry for 30 min at RT under a flow hood. Plugs were rehydrated with modified trypsin solution in 0.05 M NH₄HCO₃ (enough to completely cover the gel plugs) and incubated with shaking overnight at 37 °C. The next day, the salts and contaminants were removed from the tryptic peptide solutions using C₁₈ ZipTips in accordance with manufacturer’s directions. Samples were stored at -80°C until MS/MS analysis.
4.3.8 NanoLC-MS with Data Dependent Scan

Samples desalted with C₁₈ Zip Tips were reconstituted in 10 µL 5% ACN/0.1% FA and analyzed by a nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) system in data dependent scan mode. An in-house packed capillary column (0.1 x 130 mm column packed with 3.6 µm, 200Å XB-C18) and a gradient with 0.1% FA and ACN/0.1% FA at 200 nL/min were used for separation. The MS spectra were acquired by the orbitrap at 30,000 resolution and MS/MS spectra of the six most intense ions in MS scan were obtained by the orbitrap at 7,500 resolution. Data files from each sample were searched against the most current version of the Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4, Thermo Scientific). At least two high-confidence peptide matches were required for protein identification (false discovery rate <1%). Proteins matched with the same peptides are reported as one protein group.

4.3.9 Immunoprecipitation and Western blotting validations

Immunoprecipitation (IP). Whole brain homogenized protein extracts (250 µg) were suspended in 500 µL IP buffer [0.05% NP-40, leupeptin 4 µg/mL, pepstatin 4 µg/mL, aproprotin 5 µg/mL, phosphatase inhibitor cocktail 10 µg/mL in a phosphate buffer solution, pH 8 (8 M NaCl, 0.2 M KCl, 1.44 M Na₂HPO₄, 0.24 M KH₂PO₄, pH adjusted with NaOH)] and allowed to shake for 30 min at 4°C. Next, 50 µL of Protein A/G beads (per sample) were washed and centrifuged at 2500 RPM for 5 min at 4°C. The washed beads were added to the
individual protein samples to pre-clear of non-specific protein A/G artifacts for 90 min at 4°C. Samples were then centrifuged at 2500 RPM for 5 min at 4°C and the supernatant was transferred to new Eppendorf tubes with either anti-MAPK1 antibody (1:50 dilution) or anti-calmodulin antibody (1:50) and shaken overnight at 4°C. The next morning, 50 µL of protein A/G beads were washed as and incubated with the protein samples for 90 min at 4°C. After removing the supernatant, the beads were again washed 5 times with IP buffer with 10 min of shaking in IP buffer between centrifugations. The beads were preserved for a 1D-PAGE experiment.

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE). Whole brain homogenates (50 µg) or beads from immunoprecipitation experiments were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] diluted to 1X with distilled water, heated at 95 °C for 5 min and cooled on ice. Samples and Precision Plus Protein All Blue Standards were loaded into a Criterion precast (4-12%) Bis-Tris polyacrylamide 12 or 18 well gels and run at RT in XT MOPS running buffer at 80 V for 15 min. The voltage was then increased to 120 V for approximately 100 min at RT for the duration of the electrophoretic run.

1D-Western blotting. 1D-gels were directly transferred to nitrocellulose membranes (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA, USA). After the transfer, membranes were incubated in a blocking solution of 3% bovine serum albumin (BSA) in Wash Blot [150 mM NaCl, 3 mM
NaH$_2$PO$_4$, 17 mM NaHPO$_4$ and 0.04% (v/v) Tween 20] at RT for 1.5 h. Each protein of interest was detected by incubation with a primary antibody (1:8000 dilution), in blocking solution at RT with gentle rocking for 2 h. Blots were rinsed two times for 5 min each and one time for 10 min in Wash Blot, followed by a 1 h incubation with a horseradish peroxidase (1:3000) secondary antibody at RT with gentle rocking. Blots were rinsed three times for 5, 10 and 10 min each in Wash Blot and developed chemiluminescently using Clarity Western ECL substrate. After developing for 5 min at RT in the dark, blots were scanned using a Bio-Rad ChemiDoc XRS+ imaging system and quantified using Image Lab software (Hercules, CA, USA). Blots rinsed and then stripped with Re-Blot Plus Strong solution for exactly 10 min at RT, followed by three 5 min rinses with Wash Blot. The membranes were then blocked once again in 3% BSA for 1.5 h. Next, an anti-tubulin antibody (1:20000) or anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies (1:10000) were added to the Wash Blot solution with 2 h more of gentle rocking at RT. Membranes were then washed, incubated with a secondary HRP-conjugated antibody, washed, developed chemiluminescently and scanned.

**4.3.10 Statistical analysis**

All statistical analyses were performed using a two-tailed Student’s t-test, in which p<0.05 was considered statistically significant for Western blot and PDQuest analysis. Fold-change values of easily discernible protein spots were determined by dividing the average, normalized spot intensities in the knockout
gels by the average, normalized spot intensities of the wild-type gels. Only spots with a 1.25 fold change or greater in normalized spot density were considered for MS/MS analysis. Protein and peptide identifications obtained with the SEQUEST search algorithm with \( p<0.01 \) were considered to be statistically significant.

4.4 Results

4.4.1 PDQuest and MS/MS analysis

Proteomic analyses of the isolated brain proteins were conducted using a 2D-PAGE approach with Sypro Ruby and Pro-Q Diamond staining in conjunction with MS/MS analysis. Protein spot intensities were compared between \( PINK1^{(-/-)} \) and \( PINK1^{(+/-)} \) control mice to determine differentially expressed and phosphorylated proteins. Figure 4.1 shows representative examples obtained from SYPRO Ruby-stained 2-D gel images of the isolated proteins from 6 month old \( PINK1^{(-/-)} \) and \( PINK1^{(+/-)} \) mice with significant differentially expressed proteins labeled. Figure 4.2 shows typical images of Pro-Q Diamond-stained gels with protein spots showing altered phosphorylation states labeled in the images. Graphs displaying normalized phosphorylation intensity of each sample are also provided.
Figure 4.1 Sypro Ruby-stained gels of PINK1 KO and WT mice.

Representative 2-D gel images of isolated proteins from the brains of 6 month-old WT mice and PINK1 KO mice (n=6). Proteins whose expression was significantly altered are labeled in the images and NFL spot is enlarged to demonstrate differential levels (p<0.05). Used by permission of John Wiley and Sons.
Figure 4.2 Pro-Q Diamod-stained PINK1 KO and WT gels. Representative images of Pro-Q Diamond-stained 2-D gels with protein spots showing altered phosphorylation states labeled in the images. Bar graphs displaying normalized phosphorylation intensity (Pro-Q Diamond spot density divided by SYPRO Ruby
spot density) of each of n=6 WT (blue) and n=6 KO (red) samples [total of 12 individual gels] are also provided (p-value<0.05). Used by permission of John Wiley and Sons.

PDQuest analysis of all of the 2-D images found 29 protein spots suitable for extraction and whose expression or phosphorylation state was significantly altered in the brains of \( PINK1^{(-/-)} \) mice as compared to the WT controls. After in-gel trypsin digestion and peptide extraction, MS/MS analysis coupled to interrogation of protein databases was utilized to determine the identity of the proteins. Tables 4.1 and 4.2 list the proteins in the brains of \( PINK1^{(-/-)} \) mice that showed significantly altered expression or phosphorylation states. Other information listed in the tables include: the spot number as labeled by the PDQuest program, the SwissProt accession number, percentage of the protein sequence covered by matching peptides, the number of peptide sequences identified by the MS/MS analysis, the protein confidence score, the expected molecular weight and isoelectric point of the identified protein, as well as the fold-change levels and p-values obtained from the PDQuest analysis. Regarding fold-change values, a ↑1.33-fold means the protein expression in the KO brain is 33% more than in WT brain. A ↓0.0885-fold means protein expression in the KO brain is 91.15% less than in WT brain. All proteins were identified by more than one peptide sequence. Further, identified protein spots were visually compared against the theoretical molecular weights and isoelectric points from the mass spectrometry analysis.
Table 4.1 Proteins with altered expression in brain of PINK1 KO mice. PDQuest and MS/MS results of brain proteins with significantly altered expression in the PINK1 KO mice versus WT mice. Fold calculated by dividing average KO protein spot intensity by WT protein spot intensity.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Identified</th>
<th>Accession #</th>
<th>Coverage (%)</th>
<th>Number of identified peptides</th>
<th>Score</th>
<th>MW (kDa)</th>
<th>pl</th>
<th>P value</th>
<th>Fold Increase/Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1701</td>
<td>Neurofilament light peptide (NFL)</td>
<td>P08351</td>
<td>31.12</td>
<td>13</td>
<td>104.31</td>
<td>61.5</td>
<td>4.64</td>
<td>0.012</td>
<td>0.580</td>
</tr>
<tr>
<td>2008</td>
<td>V-type proton ATPase subunit F (VTAF)</td>
<td>Q8D1K2</td>
<td>41.18</td>
<td>4</td>
<td>20.46</td>
<td>13.4</td>
<td>5.82</td>
<td>0.041</td>
<td>0.725</td>
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<tr>
<td>3202</td>
<td>Prohibitin (ProB)</td>
<td>Q77778</td>
<td>44.12</td>
<td>10</td>
<td>53.45</td>
<td>29.8</td>
<td>5.76</td>
<td>0.0057</td>
<td>0.464</td>
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<tr>
<td>3603</td>
<td>Dihydropyrimidinase-related protein 2 (DRP2)</td>
<td>Q08553</td>
<td>15.21</td>
<td>7</td>
<td>30.03</td>
<td>62.2</td>
<td>6.38</td>
<td>0.046</td>
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<td>3703</td>
<td>Heat shock-related 70 kDa protein 2 (HSP70.2)</td>
<td>P17156</td>
<td>23.70</td>
<td>13</td>
<td>68.18</td>
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<td>6.57</td>
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<td>Asp-proline thioesterase 1 (APIT1)</td>
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<td>5102</td>
<td>Prostate-specific antigen-alpha type-2 (PSA2)</td>
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<td>14.96</td>
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<td>5109</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)</td>
<td>P04993</td>
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<td>6.58</td>
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<td>Malate dehydrogenase, cytoplasmic (MDHc)</td>
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<td>6.58</td>
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<td>34.1</td>
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<td>82.6</td>
<td>6.95</td>
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<td>6802</td>
<td>Elongation factor 2 (EF2)</td>
<td>P82152</td>
<td>3.33</td>
<td>3</td>
<td>19.70</td>
<td>95.3</td>
<td>6.83</td>
<td>0.042</td>
<td>0.411</td>
</tr>
<tr>
<td>7001</td>
<td>Peptidyl-prolyl cis-trans isomerase A (PPIaseA)</td>
<td>F7G4J</td>
<td>10.98</td>
<td>2</td>
<td>8.65</td>
<td>18.0</td>
<td>7.90</td>
<td>0.028</td>
<td>0.181</td>
</tr>
<tr>
<td>3007</td>
<td>Gamma-enolase (ENO2)</td>
<td>D3V0D3</td>
<td>25.41</td>
<td>2</td>
<td>3.81</td>
<td>13.4</td>
<td>5.15</td>
<td>0.01</td>
<td>0.124</td>
</tr>
<tr>
<td>3614</td>
<td>Tubulin epsilon 1 (TUB1)</td>
<td>P80316</td>
<td>17.19</td>
<td>9</td>
<td>30.59</td>
<td>59.6</td>
<td>6.02</td>
<td>0.012</td>
<td>0.133</td>
</tr>
<tr>
<td>4617</td>
<td>D3-phosphoglycerate dehydrogenase (PFDH)</td>
<td>Q85723</td>
<td>12.76</td>
<td>6</td>
<td>38.81</td>
<td>56.5</td>
<td>6.54</td>
<td>0.0096</td>
<td>3.69</td>
</tr>
<tr>
<td>5314</td>
<td>Isotrophic 3 of dynamin-1 (Dnm1.3)</td>
<td>P80535</td>
<td>23.62</td>
<td>18</td>
<td>73.81</td>
<td>93.9</td>
<td>6.76</td>
<td>0.033</td>
<td>1.21</td>
</tr>
<tr>
<td>6104</td>
<td>Transketo-lase (TASGL)</td>
<td>08P08</td>
<td>18.59</td>
<td>9</td>
<td>9.05</td>
<td>22.5</td>
<td>7.33</td>
<td>0.0081</td>
<td>1.47</td>
</tr>
<tr>
<td>7412</td>
<td>Fructose-biphosphate aldolase A (FABAA)</td>
<td>P00564</td>
<td>16.76</td>
<td>4</td>
<td>30.29</td>
<td>39.3</td>
<td>8.09</td>
<td>0.024</td>
<td>2.99</td>
</tr>
</tbody>
</table>

The 23 brain proteins with significantly altered expression were identified as: neurofilament light peptide (↓0.580-fold), V-type proton ATPase subunit F (↓0.725-fold), prohibitin (↓0.464-fold), dihydropyrimidinase-related protein 2 (DRP2, also called collapsing response mediated protein 2, CRMP2) (↓0.0186-
fold), heat shock-related 70 kDa protein 2 (↓0.213-fold), acyl-protein thioesterase 1 (↓0.0130-fold), carbonyl reductase [NADPH] 3 (↓0.0244-fold), proteasome subunit alpha type-2 (↓0.0898-fold), hypoxanthine-guanine phosphoribosyltransferase (↓0.0885-fold), malate dehydrogenase, cytoplasmic (↓0.0121-fold), actin-related protein 2/3 complex subunit 2 (↓0.0110-fold), voltage-dependent anion-selective channel protein 2 (↓0.607-fold), isocitrate dehydrogenase [NADPH] cytoplasmic (↓0.226-fold), vesicle-fusing ATPase (↓0.0396-fold), elongation factor 2 (↓0.411-fold) and peptidyl-prolyl cis-trans isomerase A, also known as cyclophilin A (↓0.181-fold), gamma-enolase (↑1.24-fold), T-complex protein 1 subunit epsilon (↑1.33-fold), D-3-phosphoglycerate dehydrogenase (↑3.69-fold), isoform 3 of dynamin-1 (↑1.41-fold), transgelin-3 (↑1.47-fold), fructose-bisphosphate aldolase A (↑2.99-fold) and transketolase (↑1.52-fold).

The six proteins found to have differential phosphorylation states were: calmodulin (↓0.418-fold), 14-3-3 protein epsilon (↓0.480-fold), neurofilament medium polypeptide (↓0.275-fold), V-type proton ATPase catalytic subunit A (↓0.313-fold), dual specificity mitogen-activated protein kinase kinase 1 (↑2.34-fold), and mitogen-activated protein kinase 1 (↑3.39-fold).
Table 4.2 Proteins with altered phosphorylation levels in brain of PINK1 KO brain. PDQuest and MS/MS results of proteins with significantly altered phosphorylation states in the brain of PINK1 KO mice versus WT mice.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Identified</th>
<th>Accession #</th>
<th>Coverage</th>
<th>Number of identified peptides</th>
<th>Score</th>
<th>MW (kDa)</th>
<th>pi</th>
<th>p-value</th>
<th>Fold increase/Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>0008</td>
<td>Calmodulin (CaM)</td>
<td>P52204</td>
<td>42.95</td>
<td>5</td>
<td>51.87</td>
<td>16.8</td>
<td>4.22</td>
<td>0.042</td>
<td>0.418↓</td>
</tr>
<tr>
<td>1103</td>
<td>14-3-3 protein epsilon (14-3-3e)</td>
<td>P62259</td>
<td>51.76</td>
<td>11</td>
<td>81.91</td>
<td>29.2</td>
<td>4.74</td>
<td>0.057</td>
<td>0.480↓</td>
</tr>
<tr>
<td>1808</td>
<td>Neurofilament medium polypeptide (NFM)</td>
<td>P08553</td>
<td>19.22</td>
<td>15</td>
<td>116.58</td>
<td>95.9</td>
<td>4.77</td>
<td>0.048</td>
<td>0.275↓</td>
</tr>
<tr>
<td>4601</td>
<td>V-type proton ATPase catalytic subunit A (V-ATPaseA)</td>
<td>P50516</td>
<td>32.90</td>
<td>14</td>
<td>111.68</td>
<td>68.3</td>
<td>5.58</td>
<td>0.030</td>
<td>0.313↓</td>
</tr>
<tr>
<td>6305</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1 (MAPKK1, MEK1)</td>
<td>P31938</td>
<td>27.23</td>
<td>10</td>
<td>83.53</td>
<td>43.1</td>
<td>6.70</td>
<td>0.025</td>
<td>2.35↑</td>
</tr>
<tr>
<td>9201</td>
<td>Mitogen-activated protein kinase 1 (MAPK1)</td>
<td>P53085</td>
<td>16.20</td>
<td>6</td>
<td>35.50</td>
<td>41.2</td>
<td>6.98</td>
<td>0.037</td>
<td>3.39↑</td>
</tr>
</tbody>
</table>

4.4.2 Immunoprecipitation and Western blot validation experiments

To validate the changes in protein expression and phosphorylation as determined by the PDQuest analysis of the 2-D gels, immunochemistry and 1-D Western blotting analysis of PINK1(-/-) and WT samples was performed. Figures 4.3 A and B show the Western blot images from the immunoprecipitation experiments after probing with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies where the immunoprecipitated protein, MAPK1 and calmodulin, respectively, was used as the loading control. Figures 4.3 C & D display the corresponding histogram plot representations of the data.
**Figure 4.3 MAPK1 and CaM differential phosphorylation levels in brain of PINK1 KO mice.** Western blot validations and corresponding bar graph representations from immunochemistry experiments of (A & C) the significant increase in the phosphorylation of MAPK1 and (B & D) the significant decrease in the phosphorylation of Calmodulin (CaM) in the brains of PINK1 KO mice as compared to WT mice using the corresponding IP antibody as the loading control (n=5, p-value<0.05). Antibodies detected by chemiluminescence. Used by permission of John Wiley and Sons.
Figure 4.4 HSP70 and aldolase A expression in PINK1 KO brain. Western blot validations and corresponding bar graph representations of (A & C) the down-regulation of HSP70.2 and (B & D) the up-regulation of aldolase A in the brains of PINK1 KO mice as compared to WT mice using tubulin as a loading control (n=6, p-value<0.05). Antibodies detected by chemiluminescence. Used by permission of John Wiley and Sons.

The findings from these analyses confirmed a significant increase in the phosphorylation of MAPK1 (p=0.045) and a decrease in the phosphorylation of calmodulin (p=0.032) in the brain of PINK1 KO mice. Figures 4.4 A & B present
the Western blot images of \( PINK1^{(-/-)} \) and control samples after probing with antibodies for HSP70 protein 2 and aldolase A, respectively, where tubulin was used as the loading control. Figures 4.4 C & D are the corresponding bar graphs of the results. The results of the Western blot analyses confirmed a significant decrease in the expression of HSP70 protein 2 (\( p=0.047 \)) and a significant increase in the expression of aldolase A (\( p=0.049 \)) in the \( PINK1^{(-/-)} \) model. The \( p \)-values of most of these results are slightly higher than the values obtained from the 2-D proteomic studies. These differences may be attributed to the sensitivity of the Sypro Ruby staining of the 2-D images as compared to the range limitations of the chemiluminescent development of the 1-D Western blots.

4.5 Discussion

This current study focuses on protein expression and phosphorylation changes in the brains of 6 month-old \( PINK1^{(-/-)} \) mice compared to \( PINK1^{(+/+)} \) mice. Six month-old mice were selected based on the observation that at this age \( PINK1^{(-/-)} \) mice begin to exhibit a PD phenotype, including reduced dopamine levels, providing a model to study early changes occurring in brains with PINK1-related disease progression (Akundi, Huang et al. 2011). Proteomics studies revealed twenty-nine proteins with significantly altered expression or phosphorylation levels in the brains of PINK1 knockout mice (versus control mice). These proteins can be subdivided into the following categories that are discussed separately below: cellular signaling, energy metabolism, proteostasis
networks, oxidative stress, and neuronal plasticity, neurotransmission and structure.

### 4.5.1 Cellular signaling

Regulation of protein phosphorylation status by protein kinases and protein phosphatases is essential in the control of cellular signaling pathways. Ablation of the kinase PINK1 in mice revealed deregulated downstream phosphorylation events in the brain that may contribute to familial PINK1-related PD pathogenesis. Interestingly, lack of PINK1 is associated with increased phosphorylation of both MAP kinase kinase 1 (MEK1) and its downstream target MAPK1 that modulate vital functions including cell cycle, immunity, autophagy, apoptosis, and cell survival (Dzamko, Zhou et al. 2014). Interestingly, it has been reported that PINK1 deficiency resulted in increased p38 phosphorylation leading to dysfunction of astrocytes (Choi, Kim et al. 2013). In many neurodegenerative disorders, MAPKs display increased activity and can produce substantial physiological effects with only modest changes in their phosphorylation state (Zhu, Guo et al. 2003, Di Domenico, Sultana et al. 2011). Phosphorylated MAPKs have been shown to aggregate in the halo region of Lewy bodies and may have an early pathogenic role in PD (Zhu, Kulich et al. 2002).

Two additional ubiquitous signaling proteins were found to have decreased phosphorylation states in the brains of PINK1<sup>(−/−)</sup> mice: calmodulin (CaM) and 14-3-3 protein epsilon. CaM has been identified as a regulator of more than 100 proteins involved in numerous pathways regulating apoptosis,
neuronal plasticity, cytoskeletal organization, neurotransmitter release, cellular growth and proliferation and is a vital regulator of calcium homeostasis (DeLorenzo 1980, Benaim and Villalobo 2002, Xia and Storm 2005). 14-3-3 proteins are known to interact with more than 200 ligands including: kinases, phosphatases and transmembrane receptors and have been implicated in numerous neurological disorders (Fu, Subramanian et al. 2000, Foote and Zhou 2012). Aberrant phosphorylation of either of these identified proteins has the potential to cause a myriad of deleterious downstream effects.

Prohibitin (PHB), another ubiquitously expressed pleiotropic modulator of signaling pathways, was found to have significant down-regulated expression in the brains of PINK1 KO mice. One of PHB’s more interesting functions is as a chaperone in the assembly of the electron transport chain, and reduced production of ATP has been noted with the loss of PINK1 (Theiss and Sitaraman 2011). Additionally, PHB also has been reported to play a defensive role against oxidative stress, and decreased expression of PHB has been correlated to the aging process (Robinson, Joshi et al. 2011), both risk factors or mediators of PD. Further supporting the results of this study, PHB has been shown to be significantly decreased in brains of subjects with PD (Ferrer, Perez et al. 2007, Zhou and Qin 2013).

Decreases in expression were also found in acyl-protein thioesterase 1 (APT1) and peptidyl-prolyl cis-trans isomerase A (PPIaseA). APT1 catalyzes the removal of palmitate at CYS residues from the cytosolic surface of membrane
proteins (Dekker, Rocks et al. 2010). APT1 regulates protein-protein interactions, cell signaling, membrane localization, subcellular trafficking, vesicle transport and lysosomal degradation (Tian, McClafferty et al. 2012, Kong, Peng et al. 2013). PPIaseA, also known as cyclophilin A, is a multifunctional proteins that catalyzes the cis-trans isomerization at proline residues, playing a role in cellular signaling, inflammation response and protein trafficking (Lang, Schmid et al. 1987, Takahashi, Hayano et al. 1989).

4.5.2 Energy metabolism

A significant number of proteins identified as having altered expression are involved in energy metabolism pathways. We and other groups have shown that PINK1 deficiency impairs mitochondrial respiration, triggering metabolic adaptations including increased glycolysis (Diedrich, Kitada et al. 2011, Yao, Gandhi et al. 2012, Akundi, Zhi et al. 2013, Requejo-Aguilar, Lopez-Fabuel et al. 2014). The results of previous discoveries combined with the results of this current investigation provide a more detailed molecular mechanism for the increase of glycolysis noted in PINK1-related PD. Specifically, from this current study, we observed an up-regulation of several proteins related to glycolysis in PINK1-deficient mice. These include fructose-bisphosphate aldolase A (ALDOA), gamma-enolase (ENO2), D-3-phosphoglycerate dehydrogenase (3-PGDH), and transketolase (TK). Not only are ALDOA and ENO2 substrates directly involved in the glycolytic pathway, but ENO2 has been shown to be neuroprotective when up-regulated in microglial cells (Butterfield and Lange 2009, Hafner, Glavan et al. 2009).
Further, 3-PGDH is an oxidoreductase that catalyzes the transition of 3-phosphoglycerate to 3-phosphohydroxypyruvate, the first step in the serine biosynthesis pathway. L-serine is a crucial neurotrophic factor in the CNS as it is a precursor for nucleotides, neurotransmitters, sphingolipids, phosphatidylserine and L-cysteine (Ren, Qiang et al. 2013). Moreover, the increased levels of neuronal-specific ENO2 and 3-PGDH are likely a cellular stress response to prevent cell death in the absence of PINK1 (presence of mitochondrial dysfunction), consistent with the finding that blocking glycolysis in PINK1-deficient mouse embryonic fibroblasts led to rapid death of these cells (Akundi, Zhi et al. 2013). Additionally, TK is an enzyme that catalyzes two key reactions in the pentose phosphate pathway (PPP), resulting in the production of glyceraldehyde-3-phosphate and fructose-6-phosphate. Up-regulation of TK provides for more of these essential substrates to be fed into the glycolytic pathway. Moreover, the PPP provides NADPH, which is needed to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH). Loss of GSH is arguably the earliest neurochemical alteration in PD brain.

Dopaminergic (DA) neurons are reportedly more reliant upon mitochondrial energy metabolism than other types of neurons due, in part, to their reduced mitochondrial reserve (Kingsbury, Cooper et al. 2001, Van Laar and Berman 2013). In PD, depletion of ATP is major factor in the cascade leading to the neurodegeneration of these DA neurons (Mallajosyula, Chinta et al. 2009). Significant decreased levels of cytoplasmic malate dehydrogenase (MDHc), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were
observed in the brain homogenates of PINK1 KO mice. MDHc is a metabolic protein of the malate-aspartate shuttle that aids in the transfer of reducing equivalents of NADH into the mitochondria for consumption by complex I of the electron transport chain. A reduction of this key enzyme would result in impaired mitochondrial ATP synthesis. Moreover, reduced MDHc expression is a plausible contributor to the reduction of complex I activity that is reported in PD and PINK1 model organisms (Gautier, Kitada et al. 2008, Liu, Acin-Perez et al. 2011), as cytosolic NADH cannot cross the outer mitochondrial membrane without the enzymatic action of MDHc.

HPRT is another metabolic enzyme that is an essential player in the purine salvage pathway for the generation of purine nucleotides (Sculley, Dawson et al. 1992). Increasing nucleotide metabolism is essential for mitochondrial biogenesis fission events to maintain a pool of healthy mitochondria and has been shown to be neuroprotective in PINK1 models of PD (Tufi, Gandhi et al. 2014). Interestingly, HPRT deficiency is reported to dysregulate neurogenesis (Guibinga, Hsu et al. 2010), which in adults is responsible for generating new neurons for the olfactory bulb (Altman 1969) and the subgranular zone of the hippocampus (Eriksson, Perfilieva et al. 1998). Consistent with this observation, PINK1 deficient mice have anosmia (Glasl, Kloos et al. 2012). And, in PD, impaired olfaction occurs 2-7 years before onset of motor symptoms in over 75% of patients (Lang 2011). Furthermore, HPRT knockout mice were shown to have decreased levels of dopamine that correlated with age (Micheli, Camici et al. 2011). Thus, we suggest that decreased
expression of HPRT could quite possibly be a candidate as an early PD biomarker.

4.5.3 Proteostasis networks

Disruption of two intracellular degradation systems, the ubiquitin-proteasome system and autophagy-lysosome pathway, has been shown to play central roles in PD pathology (Lim 2007, Pan, Kondo et al. 2008). Dysregulation of these pathways causes accumulation and aggregation of abnormal proteins and damaged organelles, leading to cellular toxicity, dysfunction and neurodegeneration (Pan, Kondo et al. 2008). Without functional PINK1, mitophagy fails, resulting in accumulated dysfunctional mitochondria and elevated apoptotic rates (Lenzi, Marongiu et al. 2012). In this current study of PINK1 KO mouse brain, two subunits of V-type proton ATPase were found to be altered in either phosphorylation or expression: V-ATPaseA and V-ATPaseF. V-ATPase is a membrane transport protein whose function is to establish a proton gradient, creating an acidic environment in many intracellular compartments, including lysosomes (Beyenbach and Wieczorek 2006). Previously, we have shown that C. elegans expressing mutant α-synuclein and tau have altered V-ATPase and decreased autophagy (Di Domenico, Sultana et al. 2012). Further, when the function of V-ATPase is inhibited, autophagy may become dysfunctional because the acidic environment in lysosomes required for enzymatic hydrolysis cannot be generated (Pan, Kondo et al. 2008). Additionally, with diminished lysosomal function, more α-synuclein can accumulate and impair
cellular trafficking which may lead to neurodegeneration in PD (Dehay, Martinez-Vicente et al. 2013). The catalytic subunit A (V-ATPaseA) was found to have decreased phosphorylation. V-ATPaseA is known to be phosphorylated by protein kinase A at Ser-175; and, in liver cells, phosphorylation of subunit A leads to a decreased activity (Alzamora, Thali et al. 2010), possibly due to decreased binding affinity of ATP to V-ATPaseA. The F subunit of V-type proton ATPase (V-ATPaseF) was identified as having decreased expression, suggesting an overall decrease in vacuolar acidification and autophagy.

Additional proteins involved in the proteostasis network that were found to have significantly decreased expression were: proteasome subunit alpha type-2 (PSA2), heat shock-related 70 kDa protein 2 (HSP70.2), and voltage-dependent anion-selective channel protein 2 (VDAC2). PSA2 proteins compose the end rings of the 20S proteasome, an ATP dependent multi-protein assembly that is known to degrade oxidized proteins (Davies 2001). Hence, elevated oxidatively modified proteins in PD conceivably could be due in part to diminished function of the 20S proteasome. Moreover, since the 20S proteasome comprises the core of the 26S proteasome, the overall protein degradation abilities of ubiquitin-proteasome system could conceivably be diminished. Consistent with these results, decreased proteasomal function and expression has been noted in PD patients and PD animal models (Bukhatwa, Zeng et al. 2010, Ebrahimi-Fakhari, Wahlster et al. 2012, Martins-Branco, Esteves et al. 2012).
HSP70 is an ubiquitously expressed molecular chaperone that mediates the folding of newly translated proteins, stabilizes proteins against aggregation, aids in clathrin mediated endocytosis, exocytosis (including synaptic vesicles) and is an important mediator for relaying targeted proteins to the ubiquitin-proteasome system and autophagy-lysosomal pathways (Meimaridou, Gooljar et al. 2009, Alvarez-Erviti L and et al. 2010, Redeker, Pemberton et al. 2012). We have previously demonstrated that viral gene transfer of HSP70 protects against toxin-induced dopaminergic neuron loss in a sporadic model of PD in mice (Dong, Wolfer et al. 2005). In addition, HSP70 suppressed α-synuclein toxicity in a transgenic *Drosophila* model of familial PD (Auluck, Chan et al. 2002). Therefore, the finding of reduced HSP70 expression is intriguing and is consistent with the notion of an increased vulnerability of dopaminergic neurons to mitochondrial and proteotoxic stressors in the absence of PINK1.

VDAC2 is a mitochondrial membrane porin that opens at low or zero membrane potential, and allows diffusion of small hydrophilic molecules and ions and thus plays a role in mitochondrial metabolic processes (Blachly-Dyson and Forte 2001, Shoshan-Barmatz and Gincel 2003). VDACs are also involved in mitochondrial autophagy, possibly by recruiting Parkin to docking sites in defective mitochondrial membranes, tagging the organelles for degradation by lysosomes (Sun, Vashisht et al. 2012). A decrease in the expression of this protein in PD would inhibit the removal of malfunctioning mitochondria, contributing to an increase of cellular detritus and subsequent neuronal dysregulation.
4.5.4 Oxidative Stress

Increased levels of reactive oxygen species have been noted in PD and PD model organisms (Jenner 2003, Heeman, Van den Haute et al. 2011, Di Domenico, Sultana et al. 2012, Varcin, Bentea et al. 2012). Not only does aberrant mitochondrial function and dysfunction of proteostasis networks impact oxidative stress, but loss of neurotropic factors contributes as well. Consistent with this observation, two additional neuroprotective enzymes were found in the present study to have decreased expression in the brain of the PINK1 KO mouse: carbonyl reductase [NADPH] 3 (CBR3) and cytoplasmic isocitrate dehydrogenase [NADP+] (IDHc). CBR3 is an oxidoreductase that reduces oxidative stress by catalyzing the reduction of carbonyls to their corresponding alcohols (Miura, Nishinaka et al. 2008). This reduction of oxidative stress-mediated carbonyls leads to the creation of a less toxic species (Oppermann 2007). IDH also plays a defensive role combating oxidative damage as it contains a tagging sequence that can direct damaged proteins to peroxisomes for degradation (Xu, Zhao et al. 2004). Additionally, IDHc, utilizing NADP+ as a cofactor, generates NADPH, which noted above is an important cofactor for maintaining reduced glutathione, a key antioxidant. Supporting our data, knockdown studies of IDHc in PC12 cell lines demonstrated changes in cellular redox status, increased oxidative damage and apoptotic cell death (Yang and Park 2011). Further, cell lines that expressed lower levels of IDHc were shown to have increased lipid peroxidation, peroxide generation and oxidative damage to
DNA (Lee, Koh et al. 2002). Consequently, we opine that these proteins may contribute to the oxidative stress observed in PD brain.

4.5.5 Neuronal plasticity, trafficking and structure

Mitochondrial dysfunction is implicated in various neuronal degenerative diseases, leading to decreased neuroplasticity and neurite outgrowth (Cheng, Hou et al. 2010). Furthermore, PINK1 has previously been reported to be a possible contributor in the regulation of neurite outgrowth, and its deficiency causes dysregulation of this process (Samann, Hegermann et al. 2009). In the present study, we uncovered a significant decrease in two proteins involved in neurite outgrowth: elongation factor 2 (EF2) and dihydropyrimidase-related protein 2 (DRP2; CRMP2). EF2 mediates ribosomal translocation of peptidyl-tRNA from the A to the P site during protein translation, which has been shown to regulate neurite outgrowth in advancing growth cones (Nairn and Palfrey 1987, Iketani, Iizuka et al. 2013). When EF2 is down-regulated or inhibited by EF2 kinase, protein synthesis driving the growth cone is blocked; therefore, formation of new neuronal connections are inhibited (Sutton, Taylor et al. 2007). Furthermore, down-regulation of global protein synthesis has been linked to impaired chaperone and proteasome activity as cells lower protein synthesis when protein folding and/or degradation pathways are impaired to reduce the burden of aggregated and misfolded proteins (van Oosten-Hawle and Morimoto 2014), consistent with the observed decreases of HSP70 and proteasomal subunit expression in this study. Moreover, reducing protein synthesis has shown
to be beneficial in a *Drosophila* PINK1 model (Liu and Lu 2010). Thus, while reduced expression of EF2 may affect neurite formation it may also be an adaptive, overall neuroprotective response.

DRP2, also called CRMP2, is a signaling protein that interacts with binding partners and carries out multiple functions, some of which include: neurite outgrowth and retraction, growth cone guidance, kinesin-dependent axonal transport, neurotransmitter release, endocytosis, vesicular cycling, synaptic assembly, Ca$^{2+}$ homeostasis, organization of the dendritic field and neuronal differentiation (Hensley, Venkova et al. 2011, Khanna, Wilson et al. 2012, Tan, Ma et al. 2013). Even though DRP2 expression decreases with age, higher levels remain in areas of neurogenesis and neuroplasticity (Charrier, Reibel et al. 2003). Consequently, *PINK1*–/– mice deficient in CRMP2 would have severely diminished capacity in their brains to change and adapt.

In PD, multiple neuronal networks experience altered neurotransmission, a condition that is exacerbated by α-synuclein overexpression (Barone 2010, Nemani, Lu et al. 2010). In the *PINK1*–/– model in particular, there is a significant reduction in the neurotransmitter dopamine. Decreased expression of V-ATPase in the PINK1 KO mouse is a probable contributor in the breakdown in neurotransmission. Working in conjunction with the H+/neurotransmitter antiporter, V-ATPase assists in the concentration of neurotransmitters into synaptic vesicles (Beyenbach and Wieczorek 2006). Disruption of this process leads to decreased levels of neurotransmitters in the synaptic cleft. Further,
synaptic vesicle trafficking at the presynaptic cleft is maintained by a cycle of protein complex assembly and disassembly of the SNARE complexes; failure of this cycle can lead to loss of neuronal structure and function (Esposito, Ana Clara et al. 2012). A central protein involved in this process, vesicle-fusing ATPase, also known as N-ethylmaleimide-sensitive fusion protein (NSF), was found in the present study to be significantly decreased in the PINK1(−/−) brain. NSF is a membrane trafficking chaperone required for intracellular membrane fusion and vesicle-mediated transport (Whiteheart, Rossnagel et al. 1994, Bomberger, Parameswaran et al. 2005). Specifically for neurotransmission, NSF facilitates membrane fusion of SNARE complexes (in conjunction with SNAP) for synaptic exocytosis, subsequent disassembly of the complex and mobilization of the reserve neurotransmitter pool (Neuwald 1999, Lin and Scheller 2000, Whiteheart, Schraw et al. 2001), while also promoting re-sensitization of surface receptors on the plasma membrane (Bomberger, Parameswaran et al. 2005). Knockout of NSF leads to an accumulation of synaptic vesicles at the axon terminals and at the docking site (Whiteheart, Schraw et al. 2001). Consistent with our results and the above notion, PINK1 deficient Drosophila neurons show that rapid stimulation of synaptic vesicles is defective (Morais, Verstreken et al. 2009).

Loss of neuronal scaffolding is accompanied by decreased expression of structural proteins. In this study we found two structural proteins to be significantly decreased in the PINK1 KO brain: neurofilament light peptide (NFL) and actin-related protein 2/3 complex subunit 2 (ARPC2). NFL is a component in Lewy bodies and a major structural element in neurons, forming the backbone for
other neurofilaments (Fuchs and Cleveland 1998). In the substantia nigra of PD subjects, reduced levels ofNFL and NFL mRNA were found and correlated with the severity of the disease (Hill, Arai et al. 1993, Liu, Xie et al. 2011). ARPC2 is part of a structural protein complex that plays a role regulating the polymerization and branching of actin filament networks (Gournier, Goley et al. 2001, Spillane, Ketschek et al. 2012). Furthermore, in the current study, the phosphorylation state of neurofilament medium polypeptide (NFM) also was found to be significantly decreased. NFM is a structural protein that supports axonal caliber, and altered levels of NFM phosphorylation affect the function of larger neurofilaments. Additionally, phosphorylation of NFM produces inter-filament cross bridges that increase axonal structure; therefore, a decrease in NFM phosphorylation would be predicted to lead to a decrease in axonal caliber (Mukai, Toshimori et al. 1996). Thus, defects in neuronal structural proteins may contribute to the pathogenesis of recessive familial PD.

In conclusion, ablation of PINK1 in mice results in differential expression and altered phosphorylation states of multiple brain proteins with critical functions involved in early changes in cellular signaling pathways, energy metabolism, proteostasis networks, oxidative stress, neurotransmission and neuronal structural plasticity (Figure 4.5).
Figure 4.5 Summary of PINK1 KO proteomics and phosphoproteomics.

Schematic diagram summary of expression proteomics and phosphoproteomics profiles of the PINK1 KO mouse brain. We hypothesize that such changes contribute to the reputed and known changes in PD. Used by permission of John Wiley and Sons.

Our findings provide a starting point for future investigations of the impact that these differentially expressed and phosphorylated proteins and their regulated pathways may have on the pathogenesis of PD; particularly the role of potential
environmental triggers that interact with mutated PD susceptibility gene. In addition, further studies are required to determine if the proteins identified here may serve as therapeutic targets to interfere with the progression of PD or as part of a panel of potential biomarkers for PD.
QUANTITATIVE PHOSPHOPROTEOMIC ANALYSES OF THE INFERIOR PARIETAL LOBULE FROM THREE DIFFERENT PATHOLOGICAL STAGES OF ALZHEIMER DISEASE

5.1 Overview

Alzheimer disease (AD), the most common age-related neurodegenerative disorder, is clinically characterized by progressive neuronal loss resulting in loss of memory and dementia. AD is histopathologically characterized by the extensive distribution of senile plaques and neurofibrillary tangles, and synapse loss. Amnestic mild cognitive impairment (MCI) is generally accepted to be an early stage of AD. MCI subjects have pathology and symptoms that fall on the scale intermediately between ‘normal’ cognition with little or no pathology and AD. A rare number of individuals, who exhibit normal cognition on psychometric tests but whose brains show widespread postmortem AD pathology, are classified as ‘asymptomatic’ or ‘preclinical’ AD (PCAD). In this study, we evaluated changes in protein phosphorylation states in the inferior parietal lobule of AD, MCI, PCAD, and control brain using a 2-D PAGE proteomics approach in conjunction with Pro-Q Diamond phosphoprotein staining. Statistically significant changes in phosphorylation levels were found in 19 proteins involved in energy metabolism, neuronal plasticity, signal transduction, and oxidative stress response. Changes in the disease state phosphoproteome may provide insights into underlying mechanisms for the preservation of memory with expansive AD
pathology in PCAD and the progressive cognitive decline in MCI that escalates to the dementia and the characteristic pathology of AD brain.

5.2 Introduction

Alzheimer disease (AD) is neuropathologically characterized by the increasing accumulation of extracellular senile plaques (SPs), intracellular neurofibrillary tangles (NFTs), synapse loss, and increased neuronal cell death. SPs are insoluble fibrous material containing deposits of amyloid-β-peptide (Aβ) along with other proteins and dystrophic neurites. NFTs are formed following tau hyperphosphorylation, which leads to tau uncoupling from microtubules and aggregating with other proteins, thereby significantly hindering axonal transport. Distribution and quantity of SPs and NFTs and loss of synapses tend to correlate with disease severity (Thal, Holzer et al. 2000, Thal, Rub et al. 2002). Clinically, the pathology and underlying biochemical mechanisms bring about escalating memory loss and worsening cognitive function that culminates with severe dementia.

Mild cognitive impairment (MCI) is considered to be an intermediate disease state between persons with no cognitive impairment and individuals with AD (Petersen 2004). Clinically, MCI-diagnosed patients have non-age-related, failing cognition, yet do not exhibit signs of dementia, as primary day-to-day functional abilities are not overtly affected (Morris, Storandt et al. 2001). The amnestic MCI subtype, in particular, is considered to be a potential, preferential harbinger of AD (Portet, Ousset et al. 2006). Histopathologically, the MCI brain
tends to have intermediary quantities of NFTs, neuronal loss and atrophy than is associated with a control or AD brain (Gomez-Isla, Price et al. 1996, Kordower, Chu et al. 2001). Differences in SP deposition and density are generally intermediate to AD pathology, but this can widely vary (Morris, Storandt et al. 1996, Markesbery 2010, Villain, Chetelat et al. 2012). However, levels of Aβ oligomers, in particular Aβ(1-42), which is the most toxic Aβ (Butterfield and Kanski 2002, Klein, Stine et al. 2004), are reported to correlate more reliably to severity of cognitive impairment and loss of synaptic biomarkers (Pham, Crews et al. 2010). Diagnosis of disease stage must not only consider NFT and SP pathology and global cognition evaluations, but also biochemical changes which may relate to either cognitive decline or a compensatory disease response. Insights into underlying mechanisms of disease progression at earliest detectable stages may be critical in slowing disease progression.

While advanced SP distribution can be present at any stage of cognition, extensive distributions of NFTs are a rarity in people who are considered cognitively intact (Arriagada, Growdon et al. 1992, Erten-Lyons, Woltjer et al. 2009). These subjects, whose brains exhibit considerable SP and NFT pathology postmortem, yet were asymptomatic with no signs of failing cognition antemortem, are referred to as having preclinical AD (PCAD) (Abner, Kryscio et al. 2011). Interesting characteristics of PCAD brains include: larger brain volumes than in MCI or AD (Erten-Lyons, Woltjer et al. 2009), little or no neuronal loss (Price, Ko et al. 2001), increased neuronal hypertrophy (Iacono, O'Brien et al. 2008), overall increase in synaptic plasticity (O'Brien, Resnick et al. 2009),
and changes in proteins involved in Zn transport (Lyubartseva, Smith et al. 2010). Insights into neuroprotective mechanisms that prevent cognitive deficits despite heavy SP and NFT load may provide key insights into halting mental decline that leads to dementia.

Proposed contributing factors of AD include: oxidative stress, Aβ-induced toxicity, inflammation, and dysfunction of energy metabolism (Markesbery 1997, Butterfield and Kanski 2002, Hynd, Scott et al. 2004, Ho, Drego et al. 2005, Tuppo and Arias 2005). Accumulating evidence suggests that free radicals produced during oxidative stress play a key role in AD and other neurodegenerative diseases (Browne, Ferrante et al. 1999, Butterfield 2002, Zworschke, Mazurek et al. 2003, Lin and Beal 2006, Triplett, Zhang et al. 2015). Oxidative stress may lead to post translational modifications of proteins such as carbonylation, nitration and 4-hydroxyl-2-nonenal (HNE) adducts that change protein structure and can result in loss of function, protein unfolding and aggregation (Butterfield and Lauderback 2002, Butterfield, Reed et al. 2007). In AD, oxidative stress appears to be an early event as global levels of carbonyls, 3-nitrotyrosine and HNE are significantly increased in MCI brain; however, these global markers of oxidative stress in PCAD were generally similar to levels in control brains (Butterfield, Reed et al. 2006, Butterfield, Reed et al. 2007, Aluise, Robinson et al. 2010, Bradley, Markesbery et al. 2010, Aluise, Robinson et al. 2011).
Protein phosphorylation is a fundamental mechanism mediating diverse cellular functions that is strictly regulated by kinases and phosphatases; aberrant phosphorylation of neuronal proteins is a characteristic of several neurodegenerative diseases (Humbert and Saudou 2003, Hasegawa, Arai et al. 2008, Di Domenico, Sultana et al. 2011, Triplett, Zhang et al. 2015). Evaluating phosphorylation states in proteins in the three different pathological stages of AD may provide valuable insights into the biological pathways that contribute to or impede neurodegeneration in AD. In the current study, global protein phosphorylation levels in the inferior parietal lobule (IPL) were determined using 2-D PAGE in conjunction with a multiplexed proteomics technology (Steinberg, Agnew et al. 2003). By comparing the phosphoproteome of control, PCAD, MCI, and AD brain, 19 proteins were found with significant differential phosphorylation levels in the disease states. These proteins are involved in underlying biochemical processes that contribute to disease pathology and clinical presentation.

5.3 Materials and Methods

5.3.1 Materials

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. ReadyStrip IPG strips (pH 3-10), Criterion precast polyacrylamide gels (8-16% Tris/HCl or 4-12% Bis/Tris), TGS and MOPS electrophoresis running buffers, Precision Plus Protein All Blue Standards, mineral oil, Sypro Ruby protein stain, dithiothreitol (DTT),
iodoacetamide (IA), biolytes, urea, and nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA). Pro-Q Diamond phosphoprotein stain and anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine antibodies were obtained from Invitrogen (Grand Island, NY, USA). Re-Blot Plus Strong stripping solution and C\textsubscript{18} ZipTips were purchased from Millipore (Billerica, MA, USA). Amersham ECL IgG peroxidase-linked secondary antibodies, ECL Plus Western blotting reagents, and Protein A/G beads were purchased from GE Healthcare (Pittsburgh, PA, USA). Pierce BCA protein assay reagents were purchased from Thermo Scientific (Waltham, MA, USA). Modified trypsin digestion solution was obtained from Promega (Madison, WI, USA). Anti-CRMP2 and anti-SMP30 (senescence marker protein-30; regucalcin) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

### 5.3.2 PCAD, MCI, AD, and control subjects

Frozen IPL tissue specimens from well-characterized PCAD, MCI, AD, and respective age-matched control subjects (n=7 for each group) were obtained from the University of Kentucky Rapid Autopsy Program of the Alzheimer Disease Clinical Center, with an average post-mortem interval (PMI) of 2.62 h. All the subjects underwent annual physical and neurological exams. Control and PCAD subjects exhibited no history of dementia or other neurological disorders and had MMSE test scores in the normal range. Criterial categorizing the stage of disease progression has been previously described (Aluise, Robinson et al. 2011). Table 5.1 lists the relevant demographical data.
Table 5.1 Demographic data of PCAD, MCI, AD and control subjects. Raw and average data of subjects in this current dissertation study.

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<td>2.5</td>
<td>1183</td>
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5.3.3 Sample preparation

IPL sample homogenates were prepared using a Wheaton glass homogenizer (~40 passes) and diluted with ice-cold isolation buffer, pH 7.4 [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 0.2 µg/mL PMSF, 5
µg/mL aprotinin, 4 µg/mL pepstatin, 4 µg/mL leupeptin, and 10 µL/mL phosphatase inhibitor cocktail 2]. Protein concentrations of the homogenates were determined by the Pierce BCA method (Rockford, IL, USA) (Smith, Krohn et al. 1985).

5.3.4 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

2-D PAGE experiments were performed as described previously (Sultana, Boyd-Kimball et al. 2007). In short, 200 µg of each sample was suspended in rehydration buffer [8 M urea, 2 M thiourea, 50 mM DTT, 2.0% (w/v) CHAPS, 0.2% Biolytes, 0.01% bromophenol Blue] (200 µL total volume), applied to IPG strips and actively rehydrated for 18 h at 50 V and 20°C for 18 h. Next, proteins were isoelectricly focused at 20°C starting at 300 V for 2 h, 500 V for 2 h, 1000 V for 2 h, 8000 V for 8 h, and finishing at 8000 V for 10 h. IPG strips were immediately stored at -80 °C.

For the second dimension, IEF strips were thawed, equilibrated for 10 min in the dark with 4 mL of an equilibration buffer [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol] containing 0.5% DTT, and then re-equilibrated for 10 min in the dark with 4 mL of the same equilibration buffer containing 4.5% IA. Next, the strips were rinsed and placed into 11 cm Criterion precast polyacrylamide gels (8–16% Tris–HCl). Samples and Precision Plus Protein All Blue molecular standards were run at 200 V for 65 min in TGS running buffer.
5.3.5 Pro-Q Diamond and Sypro Ruby gel staining

Phosphorylated proteins were detected and normalized using Pro-Q Diamond and Sypro Ruby gel stains according to manufacturer's directions. In brief, gels were fixed in 50 mL of solution [10% (v/v) acetic acid, 50% (v/v) methanol] (30 min, two times) and washed with 100 µL of deionized (DI) water (30 min, three times). Next, gels were stained with 80 mL of Pro-Q Diamond for 90 min and then destained in 100 mL of solution [20% acetonitrile (ACN), 50 mM sodium acetate, pH 4] (30 min, four times). The gels were washed in 100 µL of DI water (30 min, three times) and scanned at 580 nm with ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA). Gels were then incubated in 50 mL of Sypro Ruby gel stain overnight (15 h). The Sypro Ruby stain was removed and the gels were rinsed in DI water. The gels were scanned at 450 nm with the ChemiDoc imager and then stored at 4 °C in DI water until protein spot extraction.

5.3.6 Image Analysis

2D-gel images of the stained gels were quantified using PDQuest 2-D analysis software (Bio-Rad), according to the manufacturer's instructions. Briefly, protein spots intensities were matched, measured, and normalized to the total spot density in the gel for the Pro-Q Diamond-stained images and then for the Sypro Ruby-stained images. Next, a high match analysis was conducted to match each Pro-Q Diamond stained sample to the corresponding Sypro-Ruby stained sample. The phosphoprotein spot densities were then normalized to the
Sypro Ruby spot densities so that a highly abundant but lightly phosphorylated protein could be differentiated from a lowly abundant but highly phosphorylated protein. Only protein spots identified in the PCAD, MCI and AD gels as having statistically significant, differential phosphorylation states from the control were considered for in-gel trypsin digestion and protein identification by MS/MS.

5.3.7 In-gel trypsin digestion/peptide extraction

Protein spots in PCAD, MCI, and AD gels identified as being statistically different from control group were excised, transferred to individual Eppendorf microcentrifuge tubes, and trypsin digested as described previously (Thongboonkerd, McLeish et al. 2002). Briefly, gels were incubated in 20 µL of 0.1 M ammonium bicarbonate (NH₄HCO₃) for 15 min. Next, 30 µL of ACN was added and incubated for another 15 min. After removal of the solvents, the gel plugs were dried under a flow hood and then incubated with 20 µL of 20 mM DTT in 0.1 M NH₄HCO₃ for 45 min at 56°C. The DTT/NH₄HCO₃ solution was removed and 20 µL of a 50 mM IA in 0.1 M NH₄HCO₃ solution was added for 15 min. The IA/NH₄HCO₃ solution was removed and replaced with 150 µL of a 50 mM NH₄HCO₃ solution for 15 min. Next, 200 µL of ACN was added for 15 min of incubation. The gel plugs were dried under a flow hood after removal of the solvents. The gel plugs were incubated with shaking at 37°C in 10 µL of modified trypsin solution in 50 mM of NH₄HCO₃ overnight (16 h). C₁₈ ZipTips were used to remove salts and contaminants from the tryptic peptide solutions. Tryptic peptide
solutions were reconstituted in 10 µL of 5% ACN/0.1% formic acid (FA) and stored at -80˚C until MS/MS analysis.

5.3.8 NanoLC-MS with Data Dependent Scan

A nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) platform using a data dependent scan mode was implemented in the analyses of the tryptic peptide solutions. An in-house packed capillary column (0.1 x 130 mm column packed with 3.6 µm, 200Å XB-C18) with a gradient using 0.1% FA and ACN/0.1% FA at 200 nL/min was used for separation. The orbitrap, operating at 30,000 resolution, was used to obtain MS spectra. For the MS/MS spectra of the six most intense parent ions, the orbitrap was operated at 7,500 resolution. To identify the protein in each peptide sample, the latest version of the Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4, Thermo Scientific) was used in the interrogation of data files. At least two high-confidence peptide matches were used for protein identification, and the false discovery rate was less than 1%. Proteins were reported as one protein group if they were matched with the same peptides. Initial verification of the MS/MS data was accomplished by the comparison of the expected molecular weight (MW) and isoelectric point (PI) of the identified protein to MW and PI of the extracted gel plug.

5.3.9 Immunoprecipitation and Western blotting validations

Individual brain homogenates (250 µg) were suspended in an IP buffer solution, pH 8 [8 M NaCl, 0.2 M KCl, 1.44 M Na₂HPO₄, 0.24 M KH₂PO₄, 0.05%
NP-40, aproprotin 5 µg/mL, leupeptin 4 µg/mL, pepstatin 4 µg/mL, and phosphatase inhibitor cocktail 10 µL/mL (500 µL total volume). Samples were shaken with Protein A/G agarose beads for 1.5 h at 4°C for preclearance of endogenous antibodies. Each sample was agitated overnight in IP buffer at 4°C with either anti-SMP30 or anti-CRMP2 antibodies (1:50 dilution). Protein A/G agarose beads were mixed with the sample for 1.5 h and washed with 500 µL of IP buffer (10 min, 5 times). The beads were suspended in 4X sample loading buffer [0.5 M Tris (pH 6.8), 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] (diluted to 1X with DI water). Samples were heated at 95°C for 5 min and cooled before loading into Criterion precast 18 well polyacrylamide gels (4-12% Bis-Tris). Using XT MOPS running buffer, samples and Precision All Blue MW marker were run at 80 V for 15 min and then at 120 V for 100 min. The in-gel proteins were transferred onto a nitrocellulose membrane with a Trans-Blot Turbo Blotting System (Bio-Rad) at 25 V for 30 min. After the transfer, membranes were blocked in solution (3% bovine serum albumin (BSA) in TBS-T [8 M NaCl, 2.4 M Tris, and 0.1% (v/v) Tween 20]) for 1.5 h. Anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies were added to the blocking solution (1:8000 dilution) and incubated for 2 h. Next, the blots were washed with TBS-T (5 min, three times) and incubated for 1 h with a peroxidase secondary antibody in TBS-T (1:3000). The membranes were washed in TBS-T (10 min, three times), developed with Clarity Western ECL substrate, scanned using Bio-Rad ChemiDoc XRS+ imaging system, and quantified using Image Lab software (Bio-Rad). The blots were then stripped with
Re-Blot Plus Strong solution, rinsed with TBS-T (5 min, three times each), probed with either anti-SMP30 or anti-CRMP2 antibodies (1:2000), developed and scanned.

5.3.10 Statistical analysis

All data are presented as mean±SEM. A one-way ANOVA statistical test with post hoc Tukey t-test was used in determining significance (p<0.05) in PDQuest analyses. A Student’s t-test was used in Western blot analysis. Protein spot fold-change values were calculated by dividing the average, normalized spot intensities of the PCAD, MCI, or AD group by the average, normalized spot intensities of the gels of the control group. Spot were extracted for MS/MS analysis if the fold change of the normalized spot density was 50% or more, whether elevated or decreased from controls. Proteins identified with the SEQUEST search algorithm were considered to be statistically significant if p<0.01.

5.4 Results

For the evaluation of phosphorylation levels of proteins in the IPL, the average, normalized protein spot densities from the PDQuest analysis of control, PCAD, amnestic MCI and AD samples were compared. Figure 5.1 shows representative Sypro Ruby-stained 2-D gels, while Figure 5.2 shows the same gels developed with Pro-Q Diamond phosphoprotein stain. Protein spots with statistically significant altered phosphorylation levels are labeled in the
appropriate gels. PDQuest analyses of all 2-D gels found 19 proteins whose phosphorylation levels differed in at least one of the comparisons.

Figure 5.1 Sypro Ruby-stained gels of control, PCAD, amnestic MCI and AD brain. Representative Sypro Ruby-stained 2-D gel images of isolated proteins from the IPL of (A) control, (B) PCAD, (C) amnestic MCI, and (D) AD subjects. Extracted protein spots of proteins whose phosphorylation levels were significantly altered (p<0.05) in each group are labeled in the images.
Figure 5.2 Pro-Q Diamond-stained gels of control, PCAD, amnestic MCI and AD brain. Representative Pro-Q Diamond-stained 2-D gel images of isolated proteins from the IPL of (A) control, (B) PCAD, (C), amnestic MCI, and (D) AD subjects. Proteins with significantly altered (p<0.05) phosphorylation levels in each group are labeled in the images.

Tables 5.2-5.4 summarize the PDQuest and MS/MS data and includes: the protein spot number as labeled by PDQuest, the SwissProt accession number, the percentage of the protein sequence identified by MS/MS analyses, the number peptide sequences identified, the confidence score of the protein, the expected molecular weight and predicted isoelectric point of the identified protein, and the p-values and fold-change levels of phosphorylation.
**Table 5.2** Significantly altered phosphoproteins of the IPL of PCAD and control subjects. PDQuest and MS/MS results of IPL proteins of control versus PCAD subjects with significantly altered phosphorylation states as compared to control subjects.

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<th>Coverage (%)</th>
<th>Number of Identified peptides</th>
<th>Score</th>
<th>MW (kDa)</th>
<th>p</th>
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**Table 5.3** Significantly altered phosphoproteins of the IPL of MCI and control subjects. PDQuest and MS/MS results of IPL proteins of control versus MCI subjects with significantly altered phosphorylation states as compared to control subjects.

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<th>Spot</th>
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Table 5.4 Significantly altered phosphoproteins of the IPL of AD and control subjects. PDQuest and MS/MS results of IPL proteins of control versus AD subjects with significantly altered phosphorylation states as compared to control subjects.

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Proteins were identified with two or more peptide sequences. To ensure correct identification of the proteins, the expected molecular weight and isoelectric point of the identified protein were visually compared to the spot of the extracted gel plug.

In comparing the IPL of PCAD and control brains (Table 5.2), 8 proteins found to have significantly altered phosphorylation levels: Heat shock 70kDa protein 12A (↑8.92-fold); isoform 2 of gelsolin (↓0.280-fold); regucalcin (↓0.0170-fold); L-lactate dehydrogenase b-chain (↑28.7-fold); septin 2 (↑2.89-fold); flavin reductase [NADPH] (↑8.86-fold); and isoform CNPI of 2’,3’-cyclic nucleotide-3’-phosphodiesterase (↓0.0083-fold). Analysis of the IPL of MCI and
control specimens (Table 5.3) revealed 5 proteins that had significantly different phosphorylation levels: citrate synthase (↑3.88-fold); transitional endoplasmic reticulum ATPase (↓0.0571-fold); isoform 2 of gelsolin (↓0.383-fold); regucalcin (↓0.0262-fold); EF-hand domain-containing protein D1 (↓0.0710-fold). The largest differences were observed in the comparison of the IPL of AD and control brains (Table 5.4), in which 14 proteins exhibited significant differential levels of phosphorylation. These 14 proteins were: voltage-dependent anion-selective channel protein 1 (↓0.149-fold); isoform 2 of voltage dependent anion-selective channel protein 2 (↓0.0829-fold); isoform 2 of gelsolin (↓0.179-fold); L-lactate dehydrogenase b-chain (↑34.8-fold); dihydropyrimidinase-related protein 2 [also called collapsing response mediator protein 2, CRMP-2] (↑3.51-fold); guanine nucleotide-binding protein G(o) subunit alpha (↑6.11-fold); EF-hand domain-containing protein D1 (↓0.0802-fold); transitional endoplasmic reticulum ATPase (↓0.258-fold); Cu/Zn superoxide dismutase (↓0.0097-fold); peroxiredoxin 1 (↓0.0489-fold); regucalcin (↓0.108-fold); stathmin (↓0.0454-fold); syntaxin-binding protein 1 (↓0.177-fold); and carbonyl reductase 1 (↓0.392-fold). Some of these proteins were found to have significantly different phosphorylation levels in multiple group comparisons and their relationships are illustrated in the Venn diagram of Figure 5.3.
Figure 5.3 Significant differentially phosphorylated proteins of the IPL in AD progression. Venn diagram of significant differentially phosphorylated IPL proteins illustrating the overlapping proteins involved in the progression of AD.

To validate MS/MS results, immunoprecipitation experiments were carried out. Figure 5.4 shows the images from the immunoprecipitation experiments after probing with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies in which the immunoprecipitated protein of each experiment, SMP30 or CRMP2, was used as the loading control.
**Figure 5.4 SMP30 and CRMP2 phosphorylation in IPL from control, PCAD, MCI and AD brain.** Western blots and corresponding bar graph representations from immunohistochemistry experiments evaluating changes in global phosphorylation levels of SMP30 and CRMP2 in the IPL of control, PCAD, amnestic MCI, and AD subjects (n=4 for each age group, *p<0.05). Immunoreactivity with specific antibodies was detected by chemiluminescence.

The findings from the SMP30 analysis confirmed a significant decrease in the global phosphorylation levels of SMP30 in the IPL in the following comparisons: PCAD versus control (p=0.0371), MCI versus control (p=0.0489), and AD versus control (p=0.00872). Analysis of the CRMP2 immunoprecipitation experiment verified the increased phosphorylation of CRMP2 in the AD brain.
versus control, \(p=0.00733\). Additionally, global levels of phosphorylated CRMP2 were also significantly increased in the IPL of MCI, \(p=0.0118\).

5.5 Discussion

Neuropathologically, AD progression is quantified by increasing SPs, NFTs, and synaptosomal loss. Clinically, the underlying biochemical mechanisms contributing to these pathologies result in progressive cognitive deficits, culminating in dementia. The PCAD brain is intriguing, as it possesses accumulation of SPs and NFTs, but does not have loss of synapses, cognition, or global changes in oxidative stress (Aluise, Robinson et al. 2011). In this current study, the phosphoproteome of the IPL of control, PCAD, MCI, and AD brains were compared to not only assess phosphorylation changes in brain proteins involved in disease progression from control to MCI and AD, but also to evaluate underlying biochemical pathways that may contribute to prevention of memory loss in the presence of abundant AD pathology. Significant changes in phosphorylation were found that may provide insights into clinical manifestations of underlying pathology. These insights are discussed in areas concerning: energy metabolism and mitochondrial dysfunction, oxidative stress and stress response, and neuronal plasticity, neurotransmission and cellular signaling.

Energy metabolism and mitochondrial dysfunction

Cognitive decline in AD is associated with cortical atrophy, reduced cerebral glucose metabolism and mitochondrial dysfunction and is reviewed in (Ferreira, Resende et al. 2010, Kapogiannis and Mattson 2011). The majority of
the energy generated by glucose oxidation is spent maintaining ion gradients, propagating action potentials, and the release and recycling of neurotransmitters (Attwell and Laughlin 2001). Therefore, regulating and maintaining high energy production is essential for neuronal survival.

Lactate dehydrogenase (LDH) is a metabolic enzyme that catalyzes the reversible production of lactate from pyruvate. Of the five different isoforms of LDH, LDHB activity preferentially promotes the conversion of lactate to pyruvate to provide a source of fuel (Newington, Harris et al. 2013). Lactate production has been shown to be increased in both sporadic and familial AD (Sims, Finegan et al. 1985, Hoyer, Oesterreich et al. 1988), especially in areas of AD pathology (Kapogiannis and Reiter). In the current study, phosphorylation levels LDHB were significantly increased in AD compared to control and MCI and also increased in PCAD as compared to control. Though the effect of phosphorylation of LDHB has yet to be determined, it is reasonable to speculate that phosphorylation may conceivably decrease LDHB activity, as increased lactate levels in AD brain have been confirmed by multiple groups (Sims, Finegan et al. 1985, Hoyer, Oesterreich et al. 1988). It is interesting that LDHB phosphorylation is significantly increased in the IPL of brains with extensive SP and NFT pathology, and it is reasonable to speculate that phosphorylation of LDHB could be a potential neuroprotective mechanism to induce the Warburg effect in an attempt to decrease mitochondrial throughput, thereby decreasing resultant ROS production and oxidative stress.
In the IPL of the MCI brain, on the other hand, citrate synthase (CS) had significantly increased levels of phosphorylation compared to control. Phosphorylation of CS has been reported to increase the enzymatic activity of this enzyme (Kojima and Numata 2002). Since CS is considered to be the TCA enzyme that drives the cycle (Wiegand and Remington 1986), the significant increase of phosphorylated CS may be an attempt to increase availability of ATP to meet cellular energy demands required to sustain functions in a deteriorating environment. Additionally, in AD brain, it has been previously shown that there is no change in the activity of CS (Canevari, Clark et al. 1999, Bubber, Haroutunian et al. 2005). Consistent with these results, the phosphorylation levels of CS in the AD brain in this current study were similar to control brain. Further, in contrast to the PCAD and AD brain, the MCI brain does not contain as many SP and as much NFT pathology. So perhaps, increasing energy production takes precedence over minimizing ETC-related oxidative stress in the MCI brain, though this is speculation at present.

Voltage dependent anion channels (VDACs) are the primary porins of the outer mitochondrial membrane that are involved the regulation of metabolic flux by modulating membrane permeability (Blachly-Dyson and Forte 2001, Shoshan-Barmatz and Gincel 2003). VDACs are key participants in numerous processes including: Ca$^{2+}$ signaling, synaptic plasticity, apoptosis, and mitophagy (Murgia, Giorgi et al. 2009, Sun, Vashisht et al. 2012). Therefore, dysregulation of VDACs may result in a considerable shift in cellular metabolism and homeostasis that promote neuronal dysregulation. Consistent with this notion, dysregulation of
VDACs has been reported in multiple neurodegenerative diseases, including AD (Ghosh, Pandey et al. 2007, Israelson, Arbel et al. 2010, Cuadrado-Tejedor, Vilarino et al. 2011, Triplet, Zhang et al. 2015). In the current study, phosphorylation levels of VDAC1 were decreased in PCAD and AD compared to control and isoform 2 of VDAC2 was decreased in AD brain compared to control. VDAC1, in particular, has been reported to be directly blocked by phosphorylated tau and Aβ (Manczak and Reddy 2012), impeding mitochondrial influx. The most abundant VDAC, VDAC1, has 11 known phosphorylation sites and VDAC2 has 4 known sites, but effects of phosphorylation at these sites remain largely undetermined for VDAC1 and completely unknown for VDAC2 (reviewed in (Kerner, Lee et al. 2012)). However, as both PCAD and AD brains contain high amounts of Aβ and phosphorylated tau, it is reasonable to speculate that global dephosphorylation of VDAC1 may serve to either enhance the interaction with Aβ and phosphorylated tau to inhibit influx through the pore to limit oxidative phosphorylation or to promote closure of the pore by phosphorylation itself to constrain ETC-related oxidative stress. It is hypothesized that the different isoforms of VDACs play distinct, although sometimes over-lapping roles (Messina, Reina et al. 2012). VDAC2 has been reported to complex with BAK, a pro-apoptotic protein, to inhibit the initiation of apoptosis (Cheng, Sheiko et al. 2003). And since phosphorylation of isoform 2 of VDAC2 was only significantly decreased in the IPL of AD brain, it is interesting to speculate that decreased phosphorylation of VDAC2 may diminish its affinity towards BAK, thereby promoting cell death.
Oxidative stress and stress response

Dysregulated energy metabolism and mitochondrial dysfunction can lead to increased levels of reactive oxygen species, in particular, superoxide radical. 

Cu/Zn superoxide dismutase (SOD1) catalyzes the conversion of superoxide to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \). Analysis of APP transgenic mice showed decreased activity of SOD1 which correlated to increased A\( \beta \) and contributed to increased oxidative damage (Schuessel, Schäfer et al. 2005). Similarly, SOD1 activity was reported to be significantly reduced in AD brain and is associated with increased oxidative stress (Marcus, Thomas et al. 1998). In the current study, phosphorylation levels of SOD1 in the IPL of AD brain were significantly decreased compared to control. Although little is known about how phosphorylation affects any one of the 11 known phosphorylation sites, considering the reported decrease in SOD1 activity in AD and the decrease in global SOD1 phosphorylation, it may be conceivable that decreased SOD1 phosphorylation promotes decreased activity of this important antioxidant, contributing to the increased oxidative stress reported in AD (Butterfield and Lauderback 2002).

Peroxiredoxin 1 (PRDX1) is another antioxidant enzyme that had decreased phosphorylation levels in the IPL of the AD brain compared to both control and PCAD brains. PRDX1 regulates the cellular redox state by reducing peroxides to water and the corresponding alcohol. By regulating H\( \text{H}_2\text{O}_2 \) levels, PRDX1 may also play a role in cellular signaling by regulating TNF\( \alpha \)-mediated inflammation (Kang, Chae et al. 1998) and modulating the peroxide-dependent
signaling of the ASK/p38 MAPK pathway (Jarvis, Hughes et al. 2012). Levels of PRDX1 are reportedly increased in AD and DS brain (Kim, Fountoulakis et al. 2001). Further, phosphorylation on Thr90 has been shown to decrease peroxidase activity by 80% and results in H$_2$O$_2$ accumulation (Chang, Jeong et al. 2002). Additionally, phosphorylation on Tyr-194 has also been shown to inactivate the protein (Woo, Yim et al. 2010). Therefore, the increased levels of PRDX1 as well as the decrease in phosphorylation levels likely increases protein activity as a compensatory response to the overwhelming levels of oxidative stress in AD brain.

Levels of protein carbonyls are the most widely-used indicator of oxidative stress as carbonyl levels are increased with age and age-related diseases (Aksenov, Aksenova et al. 2001, Dalle-Donne, Scaloni et al. 2005). Carbonyl reductase (CBR1) plays a protective role in an oxidative stress-induced environment by catalyzing the reduction of carbonyl-containing compounds as well as a variety of other substances, including HNE (Wermuth 1981, Forrest and Gonzalez 2000, Doorn, Maser et al. 2004). Interestingly, CBR1 has been shown to be oxidatively modified by HNE in the hippocampus of MCI brain (Reed, Perluigi et al. 2008). Additionally, CBR1 is biologically important because it can reduce the nitrosyl bond of S-nitrosothiols (GSNO) (Bateman, Rauh et al. 2008). GSNO plays an important function in NO signaling by acting as an intracellular NO reservoir. Therefore, CBR1 is a mechanism that terminates NO signaling that regulates many processes including: memory and learning, inflammatory response, neuronal plasticity, and apoptotic mechanisms, among
Pathways involving NO have been implicated in AD and (Togo, Katsuse et al. 2004). Expression and activity of CBR1 is reported to be increased in AD and DS, likely as a cellular stress response to increased carbonyls (Lemieux, Malfoy et al. 1993, Balcz, Kirchner et al. 2001). In the current study, phosphorylation levels of CBR1 were significantly decreased in AD as compared to both control and MCI brains. Implications of CBR1 phosphorylation are presently unknown; but, considering (i) PCAD brains exhibit SP and NFT pathology; (ii) phosphorylation levels were decreased in AD but not PCAD; and (iii) global measures of protein carbonyls have been shown to be similar in the IPL of control and PCAD brains (Aluise, Robinson et al. 2011), while carbonyls are increased in AD brain (Hensley, Hall et al. 1995, Aksenov, Aksenova et al. 2001), it is reasonable to speculate that decreased phosphorylation of CBR1 likely increases enzymatic activity as a stress response to carbonyl-related oxidative stress.

Interestingly, flavin reductase (FR), also known as biliverdin reductase B (BVR-B) (Shalloe, Elliott et al. 1996), was significantly increased in PCAD brain. BVR-B is a NADPH-dependent enzyme that catalyzes the reduction of flavins including the conversion of biliverdin to bilirubin, an antioxidant-scavenger of free radicals that promotes a reduced cellular state (Stocker, Yamamoto et al. 1987, Barone, Di Domenico et al. 2014). Moreover, both biliverdin and bilirubin have been reported to prevent oxidative stress and apoptosis (Jansen, Hortmann et al. 2010, Barone, Di Domenico et al. 2014). Further, BVR is a pleiotropic
(Ser/Thr and Tyr) kinase with roles in modulating phosphatidylinositol-3-kinase (PI3K) and MAPK signaling pathways which are involved in cell survival, synaptic plasticity and memory consolidation, among others (Maines 2007, Kapitulnik and Maines 2009). Phosphorylation is reportedly required for BVR activity (Salim, Brown-Kipphut et al. 2001). Therefore, the increase in phosphorylation levels of BVR-B in PCAD brain demonstrates a potential mechanism by which a brain, heavy with SP and NFT pathology, may conceivably still be able to maintain synaptic plasticity and normal cognitive function. In addition, another highly conserved BVR isoform, BVR-A, is reported to be extensively modified by oxidative stress, particularly by nitration, in AD brain (Barone, Di Domenico et al. 2011). Since phosphorylation and nitrosative modifications are in competition for the same Tyrosine hydroxyl groups on proteins, a highly oxidative environment such as exists in AD brain, could result in reduced activity of BVR, resulting in decreased synaptic plasticity and memory consolidation.

Protein aggregation can result in increased cellular oxidative stress leading to elevated expression of heat shock proteins and amplified intracellular Ca\(^{2+}\) levels that can lead to cell death (Stefani and Dobson 2003). Hsp70 chaperone proteins can respond to cellular stress by refolding misfolded proteins or leading them towards degradation, as well as obstructing apoptosis by blocking recruitment of procaspase-9 to the apoptosome (Beere, Wolf et al. 2000). In the current study, Hsp70 protein 12A (hsp70A12A) showed significantly increased phosphorylation in PCAD brain. The specific functionality of the hsp70A12A isoform within the Hsp70 family is currently unknown. However,
phosphorylation of Hsp70 by CK1, CK2 and GSK3β at the highly conserved C-terminal domain of Hsp70 reportedly inhibits binding to CHIP, a Ub-ligase that mediates protein degradation or aggregation, and promotes binding affinity to co-chaperone HOP, which promotes protein re-folding (Muller, Ruckova et al. 2013). Binding effects due to phosphorylation by other co-chaperones are currently being studied, but phosphorylation-dependent interactions with co-chaperones are critical regulators of Hsp70 activities. Therefore, as SPs and NFTs cause elevated Hsp70 production, the increased phosphorylation of the C-terminal domain likely promotes protein refolding to prevent further protein aggregation. Phosphorylation effects of other Hsp70 domains have yet to be elucidated; however, since PCAD brains contain high SP and NFT pathology and have no or low neuronal loss, global phosphorylation likely contributes to increased Hsp70 activity to promote proteostasis and cell survival.

The endoplasmic reticulum (ER) is sensitive to disturbances in cellular homeostasis and can either initiate signaling cascades or unfolded protein response to manage stress situations or induce apoptosis to save tissue from necrotic injury (Salminen, Kauppinen et al. 2009). In AD, ER stress can also lead to inflammation and AD pathology (Salminen, Kauppinen et al. 2009). The transitional endoplasmic reticulum ATPase (aka valosin-containing protein; VCP) is a pleiotropic protein involved in a wide array of cellular functions, including: ER-associated degradation (ERAD), ubiquitin-proteasome mediated proteolysis, autophagy, mediation of ER stress-induced apoptosis, vesicle transport and fusion, activation of transcription factors, and peroxisomal assembly (Dai, Chen
et al. 1998, Ogura and Wilkinson 2001, Wang, Song et al. 2004, Song, Wang et al. 2007). VCP has been connected to protein misfolding and aggregation-linked neurodegeneration (Weihl 2011). Previously, we showed VCP phosphorylation levels to be significantly decreased in the hippocampus in AD (Di Domenico, Sultana et al. 2011). In the current study, phosphorylation levels of VCP were significantly decreased in MCI brain as compared to control and PCAD brain and also decreased in AD brain relative to control. Phosphorylation of VCP has been reported to promote UPS-related degradation and prevent apoptosis (Yu, Yang et al. 2013). We opine that decreased VCP phosphorylation in MCI and AD IPL may be indicative of a mechanism to eradicate cells that are too damaged to rescue as a means to prevent necrosis. However, VCP has over 60 known phosphorylation sites and putative consequences of phosphorylation of various residues includes: ER assembly (Lavoie, Chevet et al. 2000), inhibition of ER-Golgi transport (Kano, Tanaka et al. 2004), degradation of misfolded glycoproteins (Li, Zhao et al. 2008), ERAD regulation (Klein, Barati et al. 2005), and regulation binding with some of over 40 known adaptor proteins (Kano, Kondo et al. 2005, Klein, Barati et al. 2005, Yeung, Kloppsteck et al. 2008, Madsen, Seeger et al. 2009), and others reviewed in (Ewens, Kloppsteck et al. 2010).

**Neuronal plasticity, neurotransmission and cellular signaling**

Mitochondria can be found concentrated at the synapse and in dendrites where they play key roles modulating Ca$^{2+}$ and providind ATP, thereby regulating
neuronal plasticity, cognition, and neuronal survival (Li, Okamoto et al. 2004). Calcium is controlled by a variety of cellular mechanisms including: G proteins and their receptors, Ca\(^{2+}\) binding proteins, transcription factors, organelle reservoirs, and other systems. In AD, the presence of Aβ peptides excitotoxicity and resultant dysregulated Ca\(^{2+}\) homeostasis can occur that can lead to impaired synaptic transmission and plasticity, cell death, and associated cognitive dysfunction (Mattson, Cheng et al. 1992).

Gelsolin (gsn) is a F-actin severing protein that remodels skeletal structure throughout the CNS, but is especially concentrated in growth cones where it is essential for Ca\(^{2+}\)-dependent actin motility (Tanaka, Kira et al. 1993, Sun, Yamamoto et al. 1999). Gsn is reportedly sequestered in an inactivated state by phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and activated by Ca\(^{2+}\) (Janmey, Iida et al. 1987). Gsn-PIP\(_2\) binding has been reported to enhance phosphorylation (De Corte, Gettemans et al. 1997); and this phosphorylation hinders association with actin filaments (De Corte, Demol et al. 1999). Further, gsn is cleaved by caspase-3 during apoptosis and the resultant carboxyl-terminal fragment is reportedly increased in AD brain, correlating with disease severity (Ji, Chauhan et al. 2009). Additionally, gsn has been shown to bind to Aβ 1-40 and Aβ 1-42, and this interaction is reported to be anti-amyloidogenic, sequestering Aβ thereby inhibiting Aβ fibrillization and Aβ-induced apoptosis and neurotoxicity (Ray, Chauhan et al. 2000, Qiao, Koya et al. 2005). In this study, phosphorylation levels of gsn were decreased in the IPL of all three stages of AD progression compared to control, suggesting decreased activity of the protein with
consequent decreased Aβ sequestering, promoting SP pathology, and, in a
neuroprotective process, by removal neurotoxic Aβ oligomers. Such a scenario
implies a cellular response to Aβ elevation. Furthermore, considering the
absence of apoptosis in PCAD, gsn dephosphorylation may also promote
neuronal hypertrophy to maintain actin structure in place.

Regucalcin (human senescence marker protein 30; SMP30; gluconolactonase) is a highly conserved, Ca\(^{2+}\) dependent protein that decreases
with age and plays a key role in Ca\(^{2+}\) homeostasis as an activator of Ca\(^{2+}\) membrane pumps (Fujita, Inoue et al. 1998). Since SMP30 is required for
synthesis of vitamin C, this protein also exhibits antioxidant properties (Kondo,
Inai et al. 2006). SMP30 deficiency has been reported to dysregulate Ca\(^{2+}\)
dependent kinases and phosphatases, RNA and DNA synthesis (Yamaguchi and
Sakurai 1991, Morooka and Yamaguchi 2002, Morooka and Yamaguchi 2002,
Yamaguchi, Morooka et al. 2002), increase oxidative stress (Son, Zou et al.
2006), decreased insulin secretion leading to diminished glucose tolerance
(Hasegawa, Yamasaki et al. 2010), NF-κB activation, and increased
inflammatory response (Jung, Lee et al. 2014). Further, SMP30 has been
suggested to be a potential biomarker for AD and PD (Yamaguchi 2014) as
levels were reported to be significantly decreased in animal models (Kim, Lee et
al. 2012). In the current study, SMP30 phosphorylation levels were significantly
decreased in the IPL of PCAD, MCI and AD, all compared to age-matched
control brains. Implications of SMP30 phosphorylation have yet to be elucidated,
though seven known phosphorylation sites have been identified. However,
considering the proposed functions of SMP30 and dysregulation of Ca\textsuperscript{2+} signaling in AD progression, it may be reasonable to speculate that dephosphorylation conceivably decreases SMP30 activity.

EF-hand domain-containing protein D1 (EFhd1; swiprosin 2) belong to the EF-hand superfamily of Ca\textsuperscript{2+} binding proteins that participate in a myriad of Ca\textsuperscript{2+} dependent processes. EFhd1 exhibits pro-survival activity in neurons and may act as a response to oxidative stress (Dutting, Brachs et al. 2011). Suppression of EFhd1 by siRNA has been reported to suppress neurite outgrowth and promote apoptosis (Tominaga, Kurihara et al. 2006). Based on these findings and the evidence that EFhd1 associates with the inner mitochondrial membrane, it is suggested that EFhd1 modulates mitochondrial functions (Tominaga, Kurihara et al. 2006). Measured phosphorylation levels of EFhd1 were significantly decreased in both MCI and AD brain as compared to control, suggesting a decrease in its activity. The literature concerning this protein is scarce and effect of phosphorylation is unknown, but considering what is known to date with the decrease in phosphorylation in both MCI and AD, it is rational to opine that dephosphorylation of EFhd1 decreases its activity.

Guanine nucleotide-binding protein G(o) subunit alpha (GNAO1), is a component of G protein transmembrane signaling and is involved in a multitude of cellular processes. Mutations in GNAO1 can result in epileptic encephalopathy, a severe neurological condition characterized by impaired behavior, progressive cognitive decline and cerebral atrophy (Nakamura, Kodera
et al. 2013). Further, it was believed that these mutations resulted in an impaired ability of the protein to localize to the plasma membrane (Nakamura, Kodera et al. 2013), suggesting that abnormal GNAO1 signaling can lead to cognitive decline. Earlier studies in G protein subunits showed that there was no difference in GNOA1 levels in AD brain as compared to control (O'Neill, Wiehager et al. 1994). Previously, work from our laboratory showed that phosphorylation levels of GNAO1 were significantly increased in the hippocampus of the AD brain (Di Domenico, Sultana et al. 2011). In this current study, GNAO1 phosphorylation was found to be significantly increased in the IPL of AD brains as compared to both control and PCAD brains, suggesting that GNAO1 phosphorylation may conceivably interfere with GTP binding, downstream coupling to beta and gamma G proteins or result in aberrant cellular localization. Further studies deciphering the particular residues phosphorylated, the kinases involved, and the impact on GNAO1 structure and function are needed to determine the role that phosphorylated GNAO1 may play in cognitive decline and dementia in the progression of AD.

Dihydropyrimidinase-related protein 2 (DRP2; collapsin response mediator protein 2, CRMP2) is a pleiotropic protein involved in: synaptic assembly, neurite outgrowth, guidance and collapse of the growth cone, axonal growth, neurotransmitter release, endocytosis, Ca\(^{2+}\) homeostasis, and organization of the dendritic field (Goshima, Nakamura et al. 1995, Byk, Dobransky et al. 1996, Uchida, Ohshima et al. 2005, Brittain, Piekarz et al. 2009, Hensley, Venkova et al. 2011, Ju, Li et al. 2013). CRMP2 is phosphorylated by a number of kinases
which result in breakdown of growth cones, neurite retraction, and hindrance of axonal expansion and is reviewed in (Khanna, Wilson et al. 2012). Interestingly, phosphorylated CRMP2 is a component found in NFTs (Uchida, Ohshima et al. 2005) and phosphorylation of CRMP2 has been suggested to be an early occurrence in AD progression based on studies of rodent models (Cole, Noble et al. 2007). In previous studies, we have shown that CRMP2 is oxidatively modified in AD (Castegna, Aksenov et al. 2002) and that phosphorylation levels of CRMP2 are increased in the hippocampus of AD brain (Di Domenico, Sultana et al. 2011). In this current study, the phosphorylation levels of CRMP2 were significantly increased in the IPL of AD brain as compared to control IPL. This result suggests that the decrease in synapses in AD may be directly related to the increased phosphorylation of CRMP2.

Septins are highly conserved cytoskeletal GTPases are involved in various cellular processes and signaling pathways, including: DNA response to cytoskeletal damage, protein scaffolding, membrane compartmentalization, cell division, vesicle trafficking, exocytosis and apoptosis (Barral, Mermall et al. 2000, Kremer, Adang et al. 2007, Spiliotis, Hunt et al. 2008, Hagiwara, Tanaka et al. 2011). It has also been suggested that 9 of the 14 known septins play a role in maturation of the dendritic field, as they may be involved in spine dynamics, synaptic transmission and connectivity (Tada, Simonetta et al. 2007, Xie, Vessey et al. 2007). Previous studies have shown that Septins contain multiple phosphorylation sights that are crucial in regulating functions of septin (Xue, Wang et al. 2000, She, Huang et al. 2004, Meseroll, Occhipinti et al. 2013).
Abnormal septin function has been noted in multiple neurological disorders such as AD, Down syndrome, Parkinson disease and Schizophrenia (Kinoshita, Kinoshita et al. 1998, Cheon, Fountoulakis et al. 2001, Barr, Young et al. 2004, Ihara, Yamasaki et al. 2007). Septin 2, in particular, is associated with synaptic vesicles and neurotransmitter release (Kinoshita, Kimura et al. 2004, Tokhtaeva, Capri et al. 2015). Further, it has been reported that septin 2 may regulate glutamate uptake by the excitatory amino acid transporter (EAAT1) in astrocytes, a mechanism that aids in the prevention of excitotoxicity and resultant neuronal death (Kinoshita, Kimura et al. 2004). In AD, septin 2 is associated with both NFTs and SPs (Kinoshita, Kinoshita et al. 1998, Pissuti Damalio, Garcia et al. 2012). Phosphorylation levels of septin 2 were significantly upregulated in PCAD as compared to all other groups, suggesting that increased phosphorylation of septin 2 may promote increased plasticity and neurotransmission. Further, maintaining normal synaptic activity, as in PCAD, has been suggested to protect the synapse against Aβ toxicity (Tampellini, Rahman et al. 2009).

Stathmin is an essential protein that regulates microtubule dynamics, playing key roles in neurite outgrowth, synaptic plasticity, learning, and memory by preventing microtubule assembly, promoting disassembly, and sequestering tubulin dimers (Belmont and Mitchison 1996, Jourdain, Curmi et al. 1997, Mori and Morii 2002, Grenningloh, Soehrman et al. 2004, Felkl and Leube 2008). Expression of stathmin is reportedly decreased in AD brain (Saetre, Jazin et al. 2011). Phosphorylation of stathmin decreases its binding affinity towards tubulin, thereby promoting microtubule assembly (Amayed, Pantaloni et al. 2002). We
found phosphorylation levels of stathmin significantly decreased in AD brain as compared to age-matched controls, demonstrating a mechanism for decreasing axon and synapse structure and decreasing LTP.

Syntaxin-binding protein 1 (stxbp1; Munc18) binds to syntaxin, thus controlling formation of the SNARE complex and neurotransmitter release (Misura, Scheller et al. 2000, Yang, Steegmaier et al. 2000). Additionally, stxbp1 has also been shown to regulate the filopodia of the growth cone to modulate presynaptic plasticity (Broeke, Roelandse et al. 2010). Protein kinase C phosphorylation on Ser-306 and Ser-313 has been reported to modulate neurotransmission by increasing rapid vesicle cycling and vesicle release (Barclay, Craig et al. 2003, Craig, Evans et al. 2003). In the current study, stxbp1 phosphorylation levels were significantly decreased in AD brain as compared to control, which correlates to decreased synaptic efficiency and implicates decreased stxbp1 phosphorylation in synapse loss in AD brain.

Protein levels of 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP) are mainly localized to oligodendrocytes and are considered to be a marker of myelination, as it is suggested that CNPI plays a role in maintain the myelin sheath (Thompson 1992, Lappe-Siefke, Goebbels et al. 2003). In AD, the activity of CNP is reportedly decreased in the hippocampus and putamen and increased in the parietal cortex, temporal cortex and parahippocampalis (Vlkolinsky, Cairns et al. 2001). In this study of the IPL, phosphorylation levels of CNP isoform CNPI were decreased in PCAD (with a near significant decrease in AD). Taken
together, this suggests that dephosphorylation of CNPI may conceivably increase the activity of the protein in an attempt to prevent synaptic and cognition loss.

In conclusion, this current study has identified significant changes in global phosphorylation levels of proteins in the IPL of PCAD, amnestic MCI and AD subjects, suggesting related alterations in protein activity to the underlying mechanisms that may contribute to aberrant changes in: \( \text{Ca}^{2+} \) signaling, energy metabolism, neuronal plasticity, signal transduction, and oxidative stress response. Further, we show how changes in phosphorylation may contribute to the progressive cognitive decline in MCI that escalates to the dementia of AD. Moreover, examination of the phosphoproteome of PCAD brain suggests potential mechanisms that may play key roles in preserving cognitive functions despite abundant AD pathology. The results are suggestive of changed activity of differentially phosphorylated proteins in all the states of AD. Further activity measurements are needed to confirm or refute these suggestions. Moreover, targeting the kinases and phosphatases involved in the regulation of these proteins may conceivably lead to promising treatment strategies to halt the cognitive decline in AD progression.
6.1.1 Overview

Naked mole-rats (NMRs) are exceptionally long-lived rodents that have sustained healthspans, exhibiting negligible senescence and a pronounced resistance to age-related disease. Uncovering insights underlying these extraordinary traits involved in successful aging may conceivably provide crucial clues to extend the human lifespan and healthspan. A prominent hallmark in age-related diseases, such as neurodegeneration and cancer, is the impairment and dysregulation of metabolic pathways. Using a 2-D PAGE proteomics approach, alterations in levels and phosphorylation of metabolic proteins in the brains of NMRs were evaluated in an age-dependent manner. We identified 14 proteins with altered levels and/or phosphorylation states that play key roles involved in metabolic functions. New insights into potential pathways involved in metabolic aspects of successful aging have emerged.

6.1.2 Introduction

Most aging research focuses on investigating a few well-characterized short-lived species including: *Caenorhabditis elegans*, *Drosophila melanogaster*, *Rattus norvegicus*, and *Mus musculus* (Finch and Austad 2001). While models with such short lifespans that provide the ability to perform experiments rapidly,
restraining biomedical research to very few models of aging or longevity may limit novel discoveries and scientific progression. Not surprisingly, there is a growing call for new models that may better mimic human aging and provide better translational predictability (Buffenstein, Nelson et al. 2014). Since humans are a long-lived species on the basis of their body size (Hulbert, Pamplona et al. 2007), it is hypothesized that species with naturally longer lives may yield better insights into diseases that afflict humans later in life than do short-lived models that commonly die before age-related diseases manifest. Such species likely also have evolved mechanisms which allow for extended healthy life spans, in contrast to those that do not, and as such provide more precise models for human aging (Lewis, Mele et al. 2012). Unlike most other rodents that live relatively short lives in absolute terms (as little as 2-3 months in the wild; 2-5 years in captivity), naked mole-rats (NMRs) live to exceedingly advanced ages (4-17 years in the wild and up to 30+ years in captivity), unrivaled by any other rodent species (Bobek 1969, David and Jarvis 1985, Buffenstein 2008, Edrey, Hanes et al. 2011). Perhaps even more extraordinary, NMRs exhibit a prolonged healthspan, seemingly resistant to age-related deterioration well into old age (Buffenstein 2008, Edrey, Hanes et al. 2011, Grimes, Reddy et al. 2014). Insights into mechanisms facilitating sustained physiology and metabolism in NMRs may have therapeutic implications for human aging and age-associated diseases.

NMRs are naturally found in the arid and semi-arid regions of the horn of Africa (Kenya, Ethiopia and Somalia). Here, they lead an exclusive subterranean existence, living in sealed burrows in large eusocial colonies of up to 300
individuals with a strict division of labor culminating in the presence of only one breeding female and 2-4 breeding males (Jarvis 1981). This extreme environment coupled with their eusocial life-style has led to the evolution of several adaptive traits including tolerance of variable oxygen and nutrient availability and concomitant metabolic effects (Buffenstein and Yahav 1991, Buffenstein and Yahav 1991, Larson and Park 2009).

NMRs present a number of unique phenotypes that may contribute to their unusually long lives. NMRs maintain a low basal metabolic rate throughout their lives (O'Connor, Lee et al. 2002). This is accompanied by low heart rates, decreased cardiac output, and low fasting blood glucose levels (Ables, Brown-Borg et al. 2014, Grimes, Reddy et al. 2014). Nevertheless, glucose tolerance tests reveal that a large dose of glucose will cause a prolonged blood glucose elevation, indicative of insulin insensitivity (Kramer and Buffenstein 2004). This insensitivity is characteristic of induced-diabetic animal models, yet healthy NMRs show no other signs of diabetic pathology (Dumm, Console et al. 1995, Tokuyama, Sturis et al. 1995, Kramer and Buffenstein 2004). Rather, animals appear to be extremely sensitive to insulin administration. Unlike mice treated in an identical manner, glucose levels in blood not only precipitously drop following treatment with human insulin but remain at these low levels for an extended period (Dumm, Console et al. 1995, Tokuyama, Sturis et al. 1995, Kramer and Buffenstein 2004). These findings suggest that in their normal daily lives they are extremely sensitive to insulin signaling. Novel and tightly regulated glucose management may play a critical role in lifespan extension as it has been shown
that genetic control of insulin signaling through the insulin/insulin-like growth factor signaling pathway may significantly expand the lifespan of an organism (Guarente and Kenyon 2000).

In this study, we explored not only the age-dependence of levels of brain proteins involved in energy metabolism, but also their global phosphorylation status as a means of gathering insights into the active proteome of NMRs during their life and development. The brain, in particular, is an essential organ on which to study, as cerebral insulin signaling is crucial in maintaining energy and glucose homeostasis, arguably key regulators of longevity and aging. Dysregulation of these pathways is associated with neurodegenerative diseases and metabolic syndromes leading to shortened lifespans and diminished quality of life (Butterfield, Di Domenico et al. 2014).

By evaluating the NMR proteome and phosphoproteome, the results obtained have yielded a number of insights on the salubrious aging process of NMR with regards to glucose utilization, proteostasis networks, signaling pathways, neuronal plasticity, and neuronal structure. A large number of brain proteins altered in aging have been identified, too many to be efficiently presented and discussed in a single manuscript. Accordingly, to facilitate the discussion of the implications of altered protein expression and phosphorylation and how they relate to the unusually long and healthy life spans of NMRs, we approached this effort by use of functional class of identified proteins. Here, we present the results related to metabolism.
6.1.3 Materials and Methods

6.1.3.1 Materials

The chemicals used in these studies were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Criterion precast polyacrylamide gels, ReadyStrip IPG strips (pH 3-10), TGS and MOPS electrophoresis running buffers, 0.2 nm nitrocellulose membrane, Precision Plus Protein All Blue Standards, Sypro Ruby Protein Stain, urea, biolytes, mineral oil, dithiothreitol, and iodoacetamide were obtained from Bio-Rad (Hercules, CA, USA). Modified trypsin solution was purchased from Promega (Madison, WI, USA). C18 ZipTips, and Re-Blot Plus Strong stripping solution were purchased from Millipore (Billerica, MA, USA). Pro-Q Diamond phosphoprotein stain, and anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine antibodies raised in rabbits were procured from Invitrogen (Grand Island, NY, USA). Amersham ECL rabbit IgG horseradish peroxidase-linked secondary antibody, ECL-Plus Western blotting detection reagents and protein A/G beads were obtained from GE Healthcare (Pittsburgh, PA, USA). Anti-malate dehydrogenase (cytosolic) and anti-lactate dehydrogenase antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-glucose-6-phosphate dehydrogenase antibody was obtained from Cell Signaling Technology (Danvers, MA, USA).
6.1.3.2 Animals

The brains from naked mole-rats used in this study, aged 2-24 years, were obtained from the colonies maintained by Dr. Rochelle Buffenstein at the University of Texas Health Science Center, San Antonio. These well-characterized colonies (Buffenstein 2005) were housed in fabricated burrow systems with climate conditions that mimicked their natural habitat (30°C and 30-50% relative humidity). Their diet consisted of fresh fruits and vegetables, provided *ad libitum*, and supplemented with a high protein and vitamin enriched feed (Pronutro, South Africa). NMRs were anesthetized with isofluorane and then euthanized via cardiac exsanguination. Brains were immediately harvested and flash frozen in liquid nitrogen. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, TX. The experimental groups of animals consisted of both male and female breeding and non-breeding adults. For the four age groups, n=5-9 individual brains.

6.1.3.3 Sample preparation

NMR brains were thawed and individual homogenates were prepared using a Wheaton glass homogenizer (~40 passes) with ice-cold isolation buffer [0.32M sucrose, 2mM EDTA, 2mM EGTA, 20mM HEPES, 0.2μg/mL PMSF, 4μg/mL leupeptin, 4 μg/mL pepstatin, 5 μg/mL aprotinin and 10 μg/mL phosphatase inhibitor cocktail 2]. Homogenates were vortexed on ice and sonicated for 10 s at 20% power with a Fisher 550 Sonic Dismembrator.
(Pittsburgh, PA, USA). Protein concentrations of the homogenates were determined by the Pierce BCA method (Smith, Krohn et al. 1985).

6.1.3.4 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Isoelectric focusing (IEF). 2-D PAGE experiments were conducted as described previously (Sultana, Boyd-Kimball et al. 2007). Briefly, 200 µg of each homogenate was shaken for 2 h at 22°C in 200 µL of rehydration buffer [8 M urea, 2.0% (w/v) CHAPS, 2 M thiourea, 50 mM DTT, 0.2% Biolytes, 0.01% Bromophenol Blue]. Samples were then sonicated for and dispensed into wells for application to 11 cm pH 3-10 ReadyStrip IPG strips for isoelectric separation of the proteins. Strips were actively rehydrated at 20 °C for 18 h at 50 V using a Bio-Rad Protean IEF Cell (Hercules, CA, USA). Isoelectrical focusing continued at a constant temperature of 20 °C beginning at 300 V for 2 h, 500 V for 2 h, 1000 V for 2 h, 8000 V for 8 h, and finishing at 8000 V for 10 h. IPG strips were then immediately stored at -80 °C.

SDS PAGE. IEF strips were thawed and then equilibrated for 10 min in the dark with 4 mL of equilibration buffer A [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 0.5% DTT]. Strips were then re-equilibrated for 10 min in the dark with 4 mL of equilibration buffer B [50 mM Tris–HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 30% v/v glycerol, 4.5% IA]. The IEF strips were rinsed in a 1X dilution of TGS running buffer and placed into Criterion precast polyacrylamide gels (8–16% Tris–HCl, linear gradient), 1 IPG + 1 Well Comb, 11cm. Precision Plus Protein All Blue molecular weight marker was added and
the wells were overlaid with agarose gel. The gels were run at a constant voltage of 200 V for approximately 65 min at 22°C in 1X Tris/Glycine/SDS running buffer using a Criterion Cell vertical electrophoresis buffer tank.

6.1.3.5 SYPRO Ruby and Pro-Q Diamond staining

Gel staining to detect proteins and phosphoproteins was carried out according to manufacturer's directions and as previously described (Triplett, Zhang et al. 2015). In brief, gels were fixed, stained with Pro-Q Diamond, scanned at 580 nm using a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA), stained with Sypro Ruby, and then scanned again at 450 nm. Gels were stored in deionized water at 4 °C until extraction of the protein spots.

6.1.3.6 Image Analysis

Expression Proteomics. Spot intensities from SYPRO Ruby-stained 2D-gel images of NMR samples were quantified by densitometry according to the total spot density using PDQuest 2-D Analysis Software (Bio-Rd, Hercules, CA, USA). Intensities of individual spots were normalized to the total density of the gel. Spot densities of the three older age groups (4-6 year-olds, 7-12 year-olds, and 15-24 year-olds) were independently compared to the spot densities from the earliest age group (2-3 year-olds) using a Student’s two-tailed t-test and a Mann-Whitney U statistical test, both at 95% confidence (i.e. p<0.05). Only spots with a significant difference in both tests were considered for in-gel trypsin digestion and subsequent identification.
**Phosphoproteomics.** Protein spots from Pro-Q Diamond-stained 2D-gel images of the NMR samples were quantified and matched together in the same manner as the SYPRO Ruby-stained gels. Next, the master gel from the Sypro Ruby matching and Pro-Q Diamond matching were used for a high match analysis. The PDQuest software provided numerical data based on the intensity of each protein spot. The phosphoprotein spot densities were then normalized to the Sypro Ruby spot densities and the resultant normalized phosphoprotein spot densities in the 3 older age groups were independently compared to the earliest age group using a Student’s two-tailed t-test and a Mann-Whitney U statistical test. Only spots that were significant (p<0.05) in both tests were considered for in-gel trypsin digestion and subsequent identification.

### 6.1.3.7 In-gel trypsin digestion/peptide extraction

Significant spots were excised from 2D-gels and transferred to individual Eppendorf microcentrifuge tubes for trypsin digestion as described previously (Thongboonkerd, McLeish et al. 2002). Briefly, gels were washed with 0.1 M ammonium bicarbonate (NH$_4$HCO$_3$) for 15 min, followed by incubation with acetonitrile (ACN) for 15 min. The solution was removed and gel plugs were allowed to dry under a flow hood. Next, the gel plugs were incubated in 20 mM DTT in 0.1 M NH$_4$HCO$_3$ for 45 min at 56°C. The DTT/NH$_4$HCO$_3$ solution was removed and 0.05 M IA in 0.1 M NH$_4$HCO$_3$ was added and allowed to incubate at 22°C for 15 min. The IA solution was removed and 150 µL of 0.05 M NH$_4$HCO$_3$ was added and incubated for 15 min at 22°C. Next, 200 µL of ACN was added
and allowed to incubate for 15 min at 22°C. The solution was then removed and
the gel plugs were allowed to dry under a flow hood. Then, 10 µL of modified
trypsin solution in 0.05 M NH₄HCO₃ was added and incubated with shaking
overnight at 37 °C. Salts and contaminants were removed from the tryptic
peptide solutions using C₁₈ ZipTips. Samples were stored at -80°C until MS/MS
analysis.

6.1.3.8 NanoLC-MS with Data Dependent Scan

Tryptic peptide solutions were reconstituted in 10 µL 5% ACN/0.1% formic
acid (FA). MS/MS analysis was performed by a nanoAcquity (Waters, Milford,
MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) system using a data
dependent scan mode. A capillary column packed in-house (0.1 x 130 mm
column packed with 3.6 µm, 200Å XB-C18). For separation, a gradient with 0.1%
FA and ACN/0.1% FA at 200 nL/min was used. The MS spectra were obtained
by the orbitrap at 30,000 resolution. The MS/MS spectra of the six most intense
ions in MS scan were acquired by the orbitrap at 7,500 resolution. Using the
Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4, Thermo
Scientific), data files of each sample were interrogated. For protein identification,
at least two high-confidence peptide matches were used (false discovery rate
<1%). Proteins that were matched with the same peptides were reported as one
protein group. Data from the MS/MS analysis was initially verified by comparing
the expected molecular weight and isoelectric point of the identified protein to
that of the extracted plug from the 2-D gel.
6.1.3.9 Immunoprecipitation and Western blotting validations

**Immunoprecipitation (IP).** Whole brain homogenates (250 µg) were suspended in 500 µL IP buffer [0.05% NP-40, leupeptin 4 µg/mL, pepstatin 4 µg/mL, aproprotin 5 µg/mL, phosphatase inhibitor cocktail 10 µg/mL] in a phosphate buffer solution, pH 8 [8 M NaCl, 0.2 M KCl, 1.44 M Na<sub>2</sub>HPO<sub>4</sub>, 0.24 M KH<sub>2</sub>PO<sub>4</sub>]. The samples were then precleared by incubation with Protein A/G - agarose beads for 1.5 h at 4°C and then incubated overnight with anti-malate dehydrogenase antibody (1:50 dilution) at 4°C. Samples were once again incubated with Protein A/G – agarose beads for 1.5 h at 4°C and then washed 5 times with IP buffer. The beads were preserved for a 1D-PAGE experiment.

**One-dimensional polyacrylamide gel electrophoresis (1D-PAGE).** Whole brain homogenized protein extracts (50 µg) or beads from immunoprecipitation experiments were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] diluted to 1X with distilled water. Samples were then heated at 95 °C for 5 min, cooled on ice, and loaded into a Criterion precast (4-12% Bis-Tris) polyacrylamide 18 well gel. Gels were placed in a Criterion Cell vertical electrophoresis buffer tank filled with XT MOPS running buffer and run at 80 V for 15 min and then at 120 V for approximately 100 min.

**1D-Western blotting.** 1D-gels were transferred to nitrocellulose membranes (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA, USA). After the transfer, membranes were blocked with 3% bovine
serum albumin (BSA) in Wash Blot [150 mM NaCl, 3 mM NaH$_2$PO$_4$, 17 mM NaHPO$_4$ and 0.04% (v/v) Tween 20] at 22˚C for 1.5 h. The membrane was incubated with a primary antibody (1:3000 dilution) in blocking solution with gentle rocking for 2 h at 22˚C. After washing the blots three times for 5 min each, the blots were incubated with a horseradish peroxidase (1:5000) secondary antibody in Wash Blot at 22˚C with gentle rocking. The membranes were washed three times in Wash Blot (10 min each) and developed chemiluminescently using Clarity Western ECL substrate. After developing in the dark for 5 min at 22˚C, membranes were scanned using Bio-Rad ChemiDoc XRS+ imaging system and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA). Blots were then stripped with Re-Blot Plus Strong solution and washed three times with Wash Blot (5 min each). The membranes were then blocked in 3% BSA for 1.5 h and incubated with an anti-tubulin antibody (1:5000) or with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies (1:6000) for 2 h. The membranes were washed, incubated with a secondary antibody, and developed as described above.

6.1.3.10 Statistical analysis

Initial statistical analyses of PDQuest data were performed conservatively by using both a two-tailed Student’s t-test and the Mann-Whitney U statistical test, comparing each age group independently to the earliest age group. A protein spot was considered statistically significant if p<0.05 for both tests. A one-way ANOVA with post hoc Bonferroni t-test was used in determining significance.
(p<0.05) between age groups for PDQuest data and Western blot analysis. Fold-change values of protein spots were calculated by dividing the average, normalized spot intensities of the gels of the older age group by the average, normalized spot intensities of the gels of the younger age group in the comparison. Spot extraction and MS/MS analysis was conducted only for spots with a 40% fold change or greater in normalized spot density. Identifications of proteins acquired with the SEQUEST search algorithm were considered to be statistically significant if p<0.01. All data are presented as mean±SEM.

6.1.4 Results

To evaluate age-related protein changes in the brain, the NMR were divided into four age groups: 2-3 year-olds (age group 1; young), 4-6 year-olds (age group 2; intermediate), 7-12 year-olds (age group 3; old) and 15-24 year-olds (age group 4; oldest). The average protein spot intensities from each age cohort were compared to determine significant differentially expressed and phosphorylated proteins.

Figure 6.1.1 shows representative examples obtained from SYPRO-Ruby-stained 2-D gel images of the isolated proteins from these four age groups with significant differentially expressed proteins labeled and Figure 6.1.2 shows characteristic 2-D gels of the isolated protein spots stained with Pro-Q Diamond showing significantly altered phosphorylation states identified in each age group. PDQuest and MS/MS analyses of all the significant proteins found 14 metabolic
proteins whose expression and/or phosphorylation states were significantly altered in NMR brains with age. These results are summarized in Table 6.1.1.

Figure 6.1.1 Sypro Ruby-stained 2-D gels of NMR metabolism-associated proteins with altered expression in brain with age. Representative Sypro Ruby-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-6 years (B), 7-12 years (C), and 15-24 years (D). Proteins whose expression was statistically significantly altered in the particular age group are labeled in the images (p<0.05).
Figure 6.1.2 Pro-Q Diamond-stained 2-D gels of NMR metabolism-associated proteins with altered expression in brain with age. Representative Pro-Q Diamond-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-6 years (B), 7-12 years (C), and 15-24 years (D). Proteins with statistically significantly altered phosphorylation states in the particular age group are labeled in the images (p<0.05).
Table 6.1.1 Metabolic proteins with significantly altered levels and/or phosphorylation states with age in the NMR brain. PDQuest and MS/MS results of NMR metabolic brain proteins with significant altered expression and/or phosphorylation states as a function of age.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein Identified/Location</th>
<th>Assignm ent</th>
<th>Coverage (%)</th>
<th>No of Peptides</th>
<th>MW (kDa)</th>
<th>pI</th>
<th>Age Groups Compared</th>
<th>p-Value Expression</th>
<th>Fold Change Expression</th>
<th>p-Value Phosphorylation</th>
<th>Fold Change Phosphorylation</th>
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<tr>
<td>3301</td>
<td>L-lactate dehydrogenase, brain</td>
<td>PD0128</td>
<td>26.15</td>
<td>8</td>
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<td>Glucose-6-P dehydrogenase</td>
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<td>6</td>
<td>12.50</td>
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<td>5311</td>
<td>Cytidine triphosphate kinase</td>
<td>K7HDBP</td>
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<td>4</td>
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<td>16.0</td>
<td>5.89</td>
<td>4 vs 3</td>
<td>0.0110</td>
<td>1.00</td>
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<tr>
<td>5563</td>
<td>Alpha enolase</td>
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<td>23.04</td>
<td>8</td>
<td>44.68</td>
<td>47.1</td>
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<td>4 vs 1</td>
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<td>0.158</td>
</tr>
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</table>

Additional information provided in the table includes: the PDQuest spot number, the SwissProt accession number, percentage of the protein sequence covered by matching peptides, the number of peptide sequences identified by the MS/MS analyses, the confidence score of the protein, the expected molecular weight and isoelectric point of the identified protein, the age groups compared with the corresponding p-values, and fold-change levels of expression and phosphorylation obtained from the PDQuest analyses. Two or more peptide sequences were used in the identification of all proteins; and the identified protein spots were visually compared against the expected molecular weights and isoelectric points to further ensure correct protein identification. The 14
metabolic proteins found to have differential expression and/or phosphorylation states in one or more age groups were: pyruvate kinase, pyruvate kinase PKM; glucose-6-phosphate 1 dehydrogenase; glucose-6-phosphate isomerase; malate dehydrogenase (both cytosolic and mitochondrial); L-lactate dehydrogenase b chain; cytosolic acyl CoA thioester hydrolase (2 different isoforms); fructose-bisphosphate aldolase; alpha enolase; cytosolic isocitrate dehydrogenase; aspartate aminotransferase; and ATP synthase subunit alpha (isoform 2).

To validate the identification of these proteins, immunoprecipitation and Western blot validation experiments were undertaken. Figure 6.1.3 shows the Western blot images using tubulin as a loading control verifying the age-related changes in expression of lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PD) with corresponding bar graph representations of the data. The results of the Western blot analyses of the expression of LDH confirmed a significant increase in the oldest age group as compared to the intermediate age group (p=0.0003) and to the old age group (p=0.0003). Additionally, the Western blot analysis also showed a statistically significant increase (p=0.024) in the oldest age group as compared to the youngest age group. Western blot analysis of G6PD confirmed a significant increase in the level of G6PD in the brains of the oldest age group as compared to the intermediate (p=0.0103) and old age group (p=0.014).
Figure 6.1.3 LDH and G-6-P dehydrogenase expression in brain of NMR with age. Western blot and corresponding bar graph representations from the validation experiments of the changes in the expression of lactate dehydrogenase (A & C) and glucose-6-phosphate dehydrogenase (B & D) in the brains of NMRs (n=4 for each age group, *p<0.05, ***p<0.001). Immunoreactivity with specific antibodies was detected by chemiluminescence.

Figure 6.1.4 shows the images from the immunoprecipitation experiments after probing with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies in which the immunoprecipitated protein, cytosolic malate dehydrogenase (MDH1), was used as the loading control.
Figure 6.1.4 Reduced MDH1 phosphorylation in NMR brain with age.

Western blot and corresponding bar graph representation from immunoochemistry experiments of the validation of the significant decrease in the phosphorylation of cytosolic malate dehydrogenase in the brains of NMRs (n=4 for each age group, *p<0.05). Immunoreactivity of anti-MDH1 antibody was detected by chemiluminescence.

The findings from this analysis confirmed a significant decrease in the phosphorylation of MDH1 in the NMR brain for the old age group (p=0.044) and in the oldest age group (p=0.03), both as compared to the youngest age group.

6.1.5 Discussion

Numerous alterations of metabolism are evident in a number of age-related diseases such as Alzheimer, Huntington and Parkinson diseases, cancer, atherosclerosis, and type 2 diabetes mellitus, to name but a few (Berg, Combs et
al. 2002, Kahn 2003, Hsu and Sabatini 2008, Cai, Cong et al. 2012, Butterfield, Di Domenico et al. 2014). Many of these metabolic diseases directly impact upon brain function for the brain is an organ with a high rate of metabolism. The brain consumes up to 20% of total glucose and 30% of inspired oxygen to maintain neuronal resting potentials, propagate action potentials, release neurotransmitters, and other energy-requiring processes (Madsen, Cruz et al. 1999). With age, energy metabolism in the brain undergoes a gradual decline and the efficacy of these essential brain functions correspondingly diminishes, thereby contributing to age related cognitive deficits (Petit-Taboué, Landeau et al. 1998, Poon, Shepherd et al. 2006, Navarro and Boveris 2007, Swerdlow 2007). Not only do changes in metabolism impact upon brain function and healthspan, but conversely, aging as well as many age-associated diseased states dramatically impact upon metabolism (Finkel, Serrano et al. 2007). For example, in cancer, activation of oncogenes and loss of tumor suppressor activity alters metabolism by inducing the Warburg effect, promoting glycolysis to support cancerous cell growth and survival (Dang 2012). Therefore, examining the age-related changes in metabolic proteins in the brains of the NMR and understanding the mechanisms that contribute to the resistance of senescence-related diseases in NMRs conceivably may provide crucial clues to metabolic mechanisms that promote successful aging and increased healthspan.

This current proteomics study revealed fourteen metabolic proteins with significantly altered expression and/or phosphorylation levels in the brains of NMRs as they aged. The NMRs were divided into four age cohorts: young,
The brain proteins identified suggest mechanisms by which the NMR brain responds to and adapts to the aging process and conceivably are clues to better understand how the NMR brain adapted to resist age-related degeneration. These 14 proteins that are significantly altered during aging are involved in glycolysis, the pentose-phosphate shunt, the malate-aspartate shuttle, the TCA cycle, and the electron transport chain.

Levels of pyruvate kinase (PK) were increased in the old NMR age group as compared to the youngest age group. PK is a key enzyme that generates ATP and modulates glycolytic activity. The expression of a specific isoform of PK, pyruvate kinase M (PKM), was lower in the intermediate age group relative to the youngest NMR group studied, and the phosphorylation state was decreased in both the intermediate and oldest age group in relation to the youngest. PKM is an essential glycolytic enzyme that exists as both a dimer and tetramer, and the ratio between these two forms determines whether glycolytic flux is directed towards catabolic or anabolic pathways (Iqbal, Gupta et al. 2014). Moreover, dephosphorylation of PKM has been shown to increase activity of PKM (Anastasiou, Yu et al. 2012). Increased PKM activity reportedly promotes anti-tumor activity by inhibiting tumor cell growth (Stetak, Veress et al. 2007, Anastasiou, Yu et al. 2012). Therefore, it is conceivable that the decreased phosphorylation state of PKM likely increases PKM activity in older NMRs and may contribute to their cancer resistance. In addition, PKM is a known co-activator of hypoxia-inducible factor 1α (HIF-1α), which modulates HIF-1α transcriptional activity, thereby regulating glucose transport and glycolytic
enzymes, among other pathways (Luo, Hu et al. 2011). Old NMRs have been previously shown to exhibit high levels of HIF-1α, compared to attenuated expression of HIF-1α in aged mice (unpublished data R.B.). Further, it is hypothesized that increased expression of HIF-1α is an adaptive response to modulate neurodegeneration by providing energy to sustain the cell through glycolysis in hypoxic conditions (Ogunshola and Antoniou 2009). Thus, the increased expression of PK in the old age cohort as well as the decreased phosphorylation of PKM in the oldest group, are mechanisms that conceivably contribute to sustaining the required energy demand during old age to maintain efficient cellular functioning.

Cytosolic acyl CoA thioester hydrolase, also known as brain acyl-CoA hydrolase (BACH), catalyzes the cleavage of acyl-CoA producing a fatty acid and coenzyme A (CoA-SH), thereby modulating many cellular processes. Activity of BACH is higher in the brain than in any other organ, and acyl-CoA, fatty acids and CoA-SH are involved in: cellular signaling, β-oxidation, inflammation, ion fluxes, protein and vesicle trafficking, allosteric regulation of enzymes, gene expression, lipid synthesis, and energy metabolism and regulation (Hunt and Alexson 2002, Yamada, Kuramochi et al. 2002, Yamada 2005). BACH’s role in regulating lipid metabolism may be a crucial factor affecting the aging rate by providing cell and membrane stability (Hulbert, Pamplona et al. 2007). The brain is rich in lipid content, and BACH is reportedly essential in the prevention of neurotoxicity by the dysregulation of neuronal fatty acid metabolism (Ellis, Wong et al. 2013). Additionally, BACH can scavenge free fatty acids in the cytosol by
linking CoA-SH to form fatty acyl-CoA. This fatty acyl-CoA can then be imported into the mitochondria, via the mitochondrial carnitine system, to participate in the TCA cycle (Kerner and Hoppel 2000). Analysis of BACH levels showed a significant increase from young to old NMR, which then significantly decreased in brains of the oldest age group. Expression of BACH isoform 2 was increased in the two oldest age groups relative to the youngest NMR, with a decrease in the phosphorylation state in the oldest age group. It is not yet clear what effect phosphorylation has on the activity of BACH; however, this is an important area to explore as BACH activity regulates the proper ratios of activated fatty acyl-CoA, free fatty acids and CoA-SH (Brocker, Carpenter et al. 2010). Such studies are underway in our laboratories.

Glucose-6-phosphate isomerase (GPI; a.k.a. neuroleukin; autocrine motility factor) is a pleiotropic protein performing different functions inside the cell and in the extracellular space. In the cytoplasm, GPI catalyzes the second step in glycolysis, converting glucose-6-phosphate to fructose-6-phosphate, regulates gp78-dependent mitochondrial fission and mitophagy (Fu, St-Pierre et al. 2013, Shankar, Kojic et al. 2013), and is reported to protect against ER stress and apoptosis by regulation of ER calcium release (Fu, Li et al. 2011). Outside the cell, GPI acts as a neurotropic factor for spinal and sensory neurons, contributing to maintenance of neuronal integrity (Haga, Niinaka et al. 2000). A decrease in phosphorylation of GPI has been reported to lead to an increase in activity (Yanagawa, Funasaka et al. 2005). Hence, the increased levels of GPI relative to the earliest age group and the decreased phosphorylation levels in the oldest
age cohort as compared to the intermediate, demonstrate a potential neuroprotective mechanism that may contribute to the NMR’s sustained lifespan and healthspan.

Two additional glycolytic enzymes were identified as being significantly altered in the NMR brain: fructose-bisphosphate aldolase (ALDO) and alpha enolase (ENO1). ALDO is a pleiotropic protein that not only catalyzes a key reaction in glycolysis but is also involved in: endocytosis, assembly and regulation of vacuolar H+-ATPase proton pump, mRNA stability, and assembly of the actin cytoskeleton (Kao, Noda et al. 1999, Lundmark and Carlsson 2004, Canete-Soler, Reddy et al. 2005, Lu, Ammar et al. 2007). Additionally, ALDO has been reported to act as a scaffolding protein, mediating the association of F-actin with GLUT4, an insulin-responsive transporter of glucose (Kao, Noda et al. 1999). Furthermore, the presence of ALDO’s substrates reportedly regulates glucose transport via GLUT4 by disrupting the aldolase-actin interaction (Kao, Noda et al. 1999). In this current study, the phosphorylation state of ALDO was decreased in the oldest age group as compared to the youngest. Considering the aforementioned roles of ALDO, it is tempting to speculate that an increase in ALDO activity via a decreased phosphorylation state may contribute to salubrious longevity in the NMR; yet, the functional effects of ALDO phosphorylation have yet to be elucidated. However, as ALDOC was previously reported to have an increase in phosphorylation of 4.1-fold in the hippocampus of Alzheimer disease (AD) brain (Di Domenico, Sultana et al. 2011) and NMR brains have very high levels of both beta amyloid (Edrey, Medina et al. 2013) and phosphorylated tau
(Orr, Salinas et al. 2014) (pathological hallmarks of AD), elevated ALDO phosphorylation in AD may be pathological and the decrease with age in this protein in NMR brains may reflect a potential mechanism used by NMRs in an attempt to better cope with these potentially toxic proteins.

ENO1 is primarily known in glycolysis and gluconeogenesis as the enzyme that catalyzes the conversion between 2-phosphoglycerate and phosphoenolpyruvate. However, enolase has been reported to have multiple regulatory functions, acting as: a neurotrophic factor, a hypoxic stress protein, a transcription factor in tumor formation and metastasis, a plasminogen binding protein in human diseases, an activator of plasminogen, and others (Butterfield and Lange 2009, Sedoris, Thomas et al. 2010, Yamada, Marutsuka et al. 2014). Previously, we have shown ENO1 to be oxidatively modified and differentially expressed in several age-related neurodegenerative disorders (Castegna, Aksenov et al. 2002, Perluigi, Fai Poon et al. 2005, Perluigi, Poon et al. 2005, Poon, Frasier et al. 2005). Further, ENO1 was shown to have an increased phosphorylation by 2.5-fold in the AD brain (Di Domenico, Sultana et al. 2011). Conversely, in the present study, the phosphorylation of ENO1 was shown to be decreased by 68% in the oldest NMR age group relative to the youngest age group. Therefore, we speculate that decreased phosphorylation of ENO1 may be a contributing factor to increased lifespan and healthspan in the NMR.

Lactate dehydrogenase (LDH) catalyzes the interconversion between pyruvate and lactate in an anaerobic environment. There are five different
isoforms of LDH, and LDHB activity preferentially favors the oxidation of lactate to pyruvate, generating energy (Newington, Harris et al. 2013). Moreover, levels of LDH are reportedly increased in senescent human fibroblasts (Zwerschke, Mazurek et al. 2003), and high amounts of lactate in brain, as a consequence of decreased LDHB, have been suggested to be an aging hallmark (Ross, Oberg et al. 2010). Therefore, given that NMRs live in a hypoxic environment in the wild, the increase in LDHB levels in the oldest NMR cohort as compared to both the intermediate and old cohorts conceivably may be a neuroprotective mechanism to increase energy output.

Malate dehydrogenase (MDH2) is a mitochondrial TCA enzyme that catalyzes the conversion of oxaloacetate to malate producing NADH. When compared to the youngest NMR, MDH2 levels were significantly increased in the oldest age group with a decrease in phosphorylation state. Phosphorylation of MDH has been reported to decrease its activity (Minard and McAlister-Henn 1994), suggesting that aged NMR brains may be effectively regulating the high levels of MDH2 through inhibitory phosphorylation and thereby maintain a physiologic level of activity. Malate dehydrogenase in the cytosol (MDH1) is a metabolic protein of the malate-aspartate shuttle that is necessary in the transference of reducing equivalents of NADH into the mitochondria for the use by complex I of the electron transport chain (ETC). MDH1 levels were greater in the old age group with a decrease in phosphorylation in the two oldest age groups, all as compared to the youngest cohort. An increased level of this key enzyme would result in increased mitochondrial ATP synthesis. Consequently,
the elevated levels of MDH1 and MDH2, along with their decreased phosphorylation states in brains from the oldest group of NMR are consistent with an increase in energy metabolism that may contribute to NMR health and longevity. Congruous with our results, MDH1 is reportedly decreased in aged human fibroblasts (Lee, Dho et al. 2012); and, in MDH1 knockdown cells, sirtuin 1 deacetylase, a regulator of cellular senescence, is decreased, suggesting MDH1 plays a role in the regulation of senescence (Lee, Dho et al. 2012) and could contribute to increased lifespan of these rodents.

Aspartate aminotransferase (AAT) is another enzyme involved in the malate-aspartate shuttle involved in the transfer of NADH reducing equivalents produced in glycolysis to the ETC in mitochondria (Panteghini 1990). AAT also plays a key role in amino acid metabolism by catalyzing an essential step in the biosynthesis of purines, pyrimidines, amino acids, and their derivatives (Wrenger, Muller et al. 2012), as well as the reversible transamination of α-ketoglutarate (α-KG) to glutamate. These steps are essential for the production of DNA and RNA, increased synthesis of ATP, and the formation of the major excitatory neurotransmitter, glutamate. The precise role of the altered phosphorylation state is unclear, but it was increased in the brain of the oldest NMR group relative to the youngest age group; and this increase may be consistent with the need for elevation of glutamate in extended aging and increased neuroplasticity (Segovia, Porras et al. 2001, Mattson 2008).
Isocitrate dehydrogenase [NADP+] cytoplasmic (IDH1) catalyzes the oxidative decarboxylation of isocitrate that produces α-KG. Further, this reaction regenerates NADPH, which contributes to cellular defense against reactive oxygen species (Bleeker, Atai et al. 2010). In the brain of NMR, the phosphorylation state of IDH1 was significantly decreased in the oldest age group, relative to the youngest age group, and a decreased phosphorylation state has been shown to increase the activity of the enzyme (LaPorte 1993). This is consistent with the notion that decreased phosphorylation of IDH1 contributes to the increased lifespan of NMRs. In contrast to the increased IDH1 activity in the senescent and disease-resistant NMR brain, the activities of IDHs are reported to be markedly decreased in aged cells (Samokhvalov, Ignatov et al. 2004), resulting in impaired insulin secretion (Reitman and Yan 2010).

Glucose-6-phosphate 1 dehydrogenase (G6PD) shunts glucose-6-phosphate from the glycolytic pathway to the pentose phosphate pathway, also producing NADPH as well as pentose phosphates for fatty acid and nucleic acid synthesis. G6PD levels were higher in the oldest age group as compared to both the intermediate and old age groups. Therefore, the upregulation of G6PD may promote a reduced cellular state to encourage a neuroprotective environment.

ATP synthase subunit alpha (ATP5A1) is among the catalytic subunits of complex V of the ETC. In the oldest NMR cohort, ATP5A1 (isoform 2) was found to have decreased inhibitory phosphorylation relative to both the young and intermediate age groups. Phosphorylation of ATP5A1 has been reported to
decrease activity as a consequence of decreased binding affinity to ATP (Alzamora, Thali et al. 2010). Therefore, our results are consistent with the notion that as NMRs age, ATP production is increased, perhaps as a compensatory mechanism.

Because this study involved both male and female and both breeding and non-breeding animals, a potential caveat for these results may be the contribution of breeding status, which could possibly introduce sample variation due to hormonal differences (Margulis, Saltzman et al. 1995). Future studies, with many more animals, will be necessary to estimate the contribution of breeding status to the results obtained. Further, it is tempting to speculate that the significant differences found between the youngest cohort and the other ages may represent an immature brain in its final steps of development. Studies dedicated to understanding and characterizing NMR brain development are needed to clarify this potential caveat common among this and many prior NMR studies.

In summary, this current study has identified significant alterations in protein levels and phosphorylation states of key metabolic proteins. The metabolic pathways in which these proteins are involved are illustrated in Figure 6.1.5.
Figure 6.1.5 Metabolism-associated pathways with differential protein levels and/or phosphorylation levels with age in NMR brain. Summary schematic diagram of expression proteomics and phosphoproteomics profiles of metabolic protein changes in the brain of the NMR as a function of age. Proteins with altered levels and/or phosphorylation states are highlighted in red.

Many of these proteins are pleiotropic, having many other interesting functions that may contribute to the exceptional longevity and disease resistance of the NMR. The results obtained using proteomics and phosphoproteomics have revealed important potential mechanisms for increased lifespan and increased healthspan of NMRs. Improved understanding of potential mechanisms that
facilitate an extended lifespan and an extended healthspan in the NMR conceivably may lead in humans to insights into the prevention of age-related diseases and may lead to therapies that aid in the delay of the onset and progression of the aging process.
CHAPTER 6.2

AGE-RELATED CHANGES IN THE PROTEOSTASIS NETWORK IN THE BRAIN OF THE NAKED MOLE-RAT: IMPLICATIONS PROMOTING HEALTHY LONGEVITY

6.2.1 Overview

The naked mole-rat (NMR) is a remarkably long-lived rodent that possesses several exceptional traits: marked cancer resistance, negligible senescence, prolonged genomic integrity, pronounced proteostasis, and sustained healthspans. The underlying molecular mechanisms that contribute to these extraordinary attributes are currently under investigation to gain insights that conceivably may promote and extended human lifespan and healthspan. The ubiquitin-proteasome and autophagy-lysosomal systems play a vital role in eliminating cellular detritus to maintain proteostasis and have been previously shown to be more robust in NMRs when compared to shorter-lived rodents. Using a 2-D PAGE proteomics approach, differential expression and phosphorylation levels of proteins involved in proteostasis networks were evaluated in the brains of NMRs in an age-dependent manner. We identified 9 proteins with significantly altered levels and/or phosphorylation states that have key roles involved in proteostasis networks. To further investigate the possible role that autophagy may play in maintaining cellular proteostasis, we examined aspects of the PI3K/Akt/mammalian target of rapamycin (mTOR) axis as well as levels of Beclin-1, LC3-I, and LC3-II in the brain of the NMR as a function of age.
Together, these results show that NMRs maintain high levels of autophagy throughout the majority of their lifespan.

6.2.2 Introduction

The bathyergid rodent, *Heterocephalus glaber*, more commonly known as the naked mole-rat (NMR) or sand puppy, is a strictly subterranean rodent indigenous to North East Africa. These rodents are arguably best known because of their unusually long, healthy life spans (15-30 years) when compared to that of the traditional rodent models such as mice and rats (1-3 years) (Buffenstein 2005, Buffenstein 2008, Edrey, Hanes et al. 2011, Ables, Brown-Borg et al. 2014). This prolonged healthspan has led to research involving many cellular systems thought to contribute to the aging process including: oxidative stress or damage of biomolecules such as proteins and nucleotides, mitochondrial dysfunction, and the autophagy/proteostasis network (Andziak, O'Connor et al. 2006, Lambert, Boysen et al. 2007, Lewis, Andziak et al. 2013, Edrey, Oddo et al. 2014, Zhao, Lin et al. 2014). The ability of the NMR to withstand the chronic insult of protein unfolding stressors has been attributed to the efficiency with which the rodent maintains the integrity of its proteome by way of a high functioning ubiquitin-proteasome system (UPS) and cellular autophagy (Perez, Buffenstein et al. 2009, Rodriguez, Wywial et al. 2011, Grimes, Chiao et al. 2012, Rodriguez, Edrey et al. 2012, Pride, Yu et al. 2015).

The UPS functions to maintain cellular proteostasis by degrading unwanted, misfolded or damaged proteins that could otherwise aggregate into potentially cytotoxic moieties, and UPS dysfunction has been implicated in
multiple neurodegenerative disorders (Leroy, Boyer et al. 1998, Fernandez-Funez, Nino-Rosales et al. 2000, Castegna, Thongboonkerd et al. 2004, Bennett, Shaler et al. 2007, Triplett, Zhang et al. 2015). The proteasome cleaves damaged proteins into smaller peptide fragments by the proteolytic center that contains trypsin-like (T-L), post-glutamyl peptide-hydrolyzing (PGPH), and chymotrypsin-like (ChT-L) specificities (Hanna and Finley 2007, Rodriguez, Osmulski et al. 2014). Liver and brain samples from NMRs when compared to mouse controls, have shown increased ChT-L and T-L activities, suggesting a more efficient UPS that may contribute to their inherent resistance to aging and age-related diseases (Rodriguez, Edrey et al. 2012, Edrey, Oddo et al. 2014). Moreover, human, mouse, and yeast proteasomes were demonstrated to have an increased activity when exposed to proteasome depleted cytosolic fractions containing a novel HSP-containing complex from NMR samples (Rodriguez, Osmulski et al. 2014).

The autophagy-lysosomal pathway (ALP) is an evolutionarily conserved catabolic process by which the cell removes and recycles complexes, protein aggregates and damaged organelles (Mizushima 2007). Often seen as a mechanism to address starvation to reduce energy output, autophagy provides the cell many important functions such as cellular differentiation, growth control, defense from xenobiotics, and general housekeeping and maintenance (Cecconi and Levine 2008). Thus, autophagy is generally thought of as a survival mechanism. ALP involves a number of proteins such as Beclin-1 and LC3 that are crucial in the initiation and recruitment of the autophagosome, which once
formed, engulfs the target prior to fusing with the lysosome for recycling (Klionsky 2005). Further, the PI3K/Akt/mTOR axis plays a central role in cellular proteostasis as mTOR activation inhibits autophagy, and mTOR is a direct target of the kinase Akt, which is regulated by PI3K. Dysregulation of these pathways has been linked to neurodegenerative diseases (Aguado, Sarkar et al. 2010, Glick, Barth et al. 2010, Martinez-Vicente, Talloczy et al. 2010, Winslow, Chen et al. 2010, Nixon and Yang 2011, Nixon 2013, Tramutola, Triplett et al. 2015).

A failing proteostasis network in the brain in particular increases vulnerability to dysfunctions in UPS and ALP due to the unique shape of neurons and their non-mitotic nature (Grimm, Hoehn et al. 2011). Further, these protein degradation mechanisms are essential in neuron function including neurotransmission and synaptic remodeling (Willeumier, Pulst et al. 2006, Yi and Ehlers 2007). Dysregulation of these proteostasis maintenance systems can lead to neurodegeneration, diminished quality of life and reduced lifespans (Tanaka and Matsuda 2014).

In the current age-related study of the NMR proteome and phosphoproteome, a large number of significantly altered brain proteins were identified, whose functions were related to metabolism, proteostasis networks, cellular signaling, structure, and neuronal plasticity. Too many proteins were identified to be efficiently discussed and expounded upon in a single manuscript; consequently, we facilitated discussion of the results by means of protein functionality. Here, we report on significant changes in proteins related to
proteostasis and autophagy systems in the NMR brain and their impact upon the unusually long and salubrious lifespan of the NMR.

6.2.3 Materials and Methods

6.2.3.1 Materials

Criterion precast polyacrylamide gels, TGS and MOPS electrophoresis running buffers, ReadyStrip IPG strips, mineral oil, Precision Plus Protein All Blue Standards, Sypro Ruby protein stain, biolytes, urea, dithiothreitol (DTT), iodoacetamide (IA), and nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA). Pro-Q Diamond phosphoprotein stain, and anti-phosphotyrosine, anti-phosphoserine, and anti-phosphothreonine antibodies were procured from Invitrogen (Grand Island, NY, USA). Protein A/G beads Amersham ECL IgG horseradish peroxidase-linked secondary antibodies, and ECL Plus Western blotting detection reagents were purchased from GE Healthcare (Pittsburgh, PA, USA). C\textsubscript{18} ZipTips and Re-Blot Plus Strong stripping solution were obtained from Millipore (Billerica, MA, USA). Modified trypsin solution was purchased from Promega (Madison, WI, USA). Pierce BCA protein assay reagents A and B were purchased from Thermo Scientific (Waltham, MA, USA). Anti-p-PI3K (Tyr\textsuperscript{508}) and anti-BAP1 (Ub carboxyl-terminal hydrolase) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Beclin-1, anti-p-mTOR (Ser\textsuperscript{2448}), anti-mTOR, anti-AKT, and anti-p-AKT (Ser\textsuperscript{473}) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-LC3-I and anti-LC3-II antibodies were purchased from Novus
(Littleton, CO, USA). Anti-VDAC2 antibody was purchased from Abcam (Cambridge, MA, USA). All other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.2.3.2 Animals

Brains from NMRs were obtained from well-characterized colonies (Buffenstein 2005) maintained by Dr. Rochelle Buffenstein at the University of Texas Health Science Center, San Antonio. The NMRs were housed in fabricated burrow systems under climate conditions that mimicked their natural habitat (30°C and 30-50% relative humidity). The NMRs were fed ad libitum a diet that consisted of fresh fruits and vegetables, which was supplemented with a high protein and vitamin enriched feed (Pronutro, South Africa). NMRs were anesthetized with isofluorane, euthanized by cardiac exsanguination and the brains were immediately harvested and flash frozen in liquid nitrogen. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, TX. Experimental animal groups consisted of 5-9 individual brains from both male and female individuals and of both subordinate and breeding status. NMRs were divided into four age groups for analysis: 2-3 year-olds (age group 1; young), 4-6 year-olds (age group 2; intermediate), 7-12 year-olds (age group 3; old) and 15-24 year-olds (age group 4; oldest).
6.2.3.3 Sample preparation

Naked mole-rat brain homogenates were prepared using a Wheaton glass homogenizer (~40 passes) and diluted with ice-cold isolation buffer [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 0.2 µg/mL PMSF, 5 µg/mL aprotinin, 4 µg/mL leupeptin, 4 µg/mL pepstatin, and 10 µg/mL phosphatase inhibitor cocktail 2]. Homogenate protein concentrations were ascertained by the Pierce BCA method (Rockford, IL, USA) (Smith, Krohn et al. 1985).

6.2.3.4 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Isoelectric focusing (IEF). 2-D PAGE experiments were performed as previously described (Sultana, Boyd-Kimball et al. 2007). In brief, 200 µg of each homogenate, suspended in 200 µL of rehydration buffer [8 M urea, 2 M thiourea, 50 mM DTT, 2.0% (w/v) CHAPS, 0.2% Biolytes, 0.01% bromophenol Blue], were sonicated and applied to 11 cm pH 3-10 ReadyStrip IPG strips. The strips were actively rehydrated and isoelectricly focused. At the end of the run, IPG strips were immediately stored at -80 °C.

SDS PAGE. IEF strips were thawed and equilibrated in DTT and IA-containing buffers. IEF strips were rinsed in TGS running buffer before placement into 11 cm Criterion precast linear gradient polyacrylamide gels (8–16% Tris–HCl). Precision Plus Protein All Blue molecular standards and samples were run at a constant voltage of 200 V for approximately 65 min at 22°C in Tris/Glycine/SDS running buffer.
6.2.3.5 Sypro Ruby and Pro-Q Diamond staining

After 2D-PAGE, gel staining was carried out according to manufacturer’s directions and as described previously (Di Domenico, Sultana et al. 2011). Briefly, gels were incubated in 50 mL of fixing solution [10% (v/v) acetic acid, 50% (v/v) methanol], washed in deionized (DI) water, and stained with 60 mL of Pro-Q Diamond for 90 min. Gels were then destained four times in 100 mL of solution [20% acetonitrile (ACN), 50 mM sodium acetate, pH 4] for 30 min each. The gels were washed three times in DI water (30 min each) and then scanned at 580 nm using a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA). Next, 50 mL of Sypro Ruby protein gel stain was added and allowed to incubate overnight (15 h). Gels were then rinsed in DI water, scanned at 450 nm with the ChemiDoc imager, and stored in DI water at 4 °C until protein spot extraction.

6.2.3.6 Image Analysis

Expression Proteomics. Spot intensities from SYPRO Ruby-stained 2D-gel images of NMR brain samples were quantified according to the total spot density using PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA, USA). Intensities of individual spots were normalized to the total gel densities. Normalized spot densities of the four age groups (2-3 year-olds, 4-6 year-olds, 7-12 year-olds, and 15-24 year-olds) were compared. Only spots with statistically significant differences (p<0.05) were considered for in-gel trypsin digestion and protein identification by MS/MS.
Phosphoproteomics. Protein spots from Pro-Q Diamond-stained 2D-gel images of the NMR brain samples were quantified and matched as described previously (Di Domenico, Sultana et al. 2011). Next, a high match analysis between the master gels from the Sypro Ruby matching and Pro-Q Diamond matching was conducted. The phosphoprotein spot densities were normalized to the Sypro Ruby spot densities and the resultant normalized spot densities in four age groups were compared and spots that were statistically significant (p<0.05) were considered for in-gel trypsin digestion and protein identification by MS/MS.

6.2.3.7 In-gel trypsin digestion/peptide extraction

Protein spots identifies as significantly altered from the earliest age group were excised from 2D-gels and transferred to individual Eppendorf microcentrifuge tubes for trypsin digestion as described previously (Thongboonkerd, McLeish et al. 2002). In brief, DTT and IA was used to break and cap disulfide bonds and the gel plug was incubated overnight at 37°C with shaking in modified trypsin solution. Salts and contaminants were removed from the tryptic peptide solutions using C_{18} ZipTips. Tryptic peptide solutions were reconstituted in 10 µL of a 5% ACN/0.1% formic acid (FA) solution and stored at -80°C until MS/MS analysis.

6.2.3.8 NanoLC-MS with Data Dependent Scan

Tryptic peptide solutions were analyzed by a nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) platform with a data dependent scan mode. An in-house packed capillary column (0.1 x 130 mm
column packed with 3.6 µm, 200Å XB-C18) was used for separation with a
gradient using 0.1% FA and ACN/0.1% FA at 200 nL/min. The spectra obtained
by MS were measured by the orbitrap at 30,000 resolution; and the MS/MS
spectra of the six most intense parent ions in the MS scan were acquired by the
orbitrap at 7,500 resolution. The latest version of the Swiss-Prot database by
SEQUEST (Proteome Discoverer v1.4, Thermo Scientific) was used to
interrogate the data files of each sample. At least two high-confidence peptide
matches were used for protein identification where the false discovery rate <1%.
Proteins that were matched with the same peptides were reported as one protein
group. Protein data reported from these analyses includes: the SwissProt
accession number, the percentage of the protein sequence identified by
matching peptides, the number of peptide sequences detected by the MS/MS
analysis, the confidence score of the protein, the expected molecular weight and
predicted isoelectric point (pI).

6.2.3.9 Immunoprecipitation and Western blotting

*Immunoprecipitation (IP)*. Individual NMR brain homogenates (250 µg)
were suspended in 500 µL of IP buffer [0.05% NP-40, aproprotin 5 µg/mL,
leupeptin 4 µg/mL, pepstatin 4 µg/mL, and phosphatase inhibitor cocktail 10
µg/mL] in a phosphate buffer solution, pH 8 [8 M NaCl, 0.2 M KCl, 1.44 M
Na₂HPO₄, and 0.24 M KH₂PO₄]. Samples were precleared by incubation with
Protein A/G agarose beads for 1.5 h at 4˚C. Next, each sample was incubated
overnight with anti-VDAC 2 antibody (1:50 dilution) at 4˚C. Samples were then
incubated with Protein A/G agarose beads for 1.5 h at 4°C and washed 5 times with IP buffer, preserving the beads for a 1D-PAGE experiment.

**One-dimensional polyacrylamide gel electrophoresis (1D-PAGE).** Sample homogenates (50 µg) or beads from VDAC2 immunoprecipitation experiment were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] (diluted to 1X with DI water) and then heated at 95°C for 5 min. Samples were cooled on ice and then loaded into Criterion precast 18 well polyacrylamide gels (4-12% Bis-Tris) or Criterion 12% TGX stain-free polyacrylamide 18 well gels. Using XT MOPS or TGS running buffer, gels were run at 80 V for 15 min and then at 120 V for approximately 100 min. Stain-free gels were scanned using a Bio-Rad ChemiDoc XRS+ imaging system to measure the total protein load before protein transfer to Western blots.

**1D-Western blotting.** In-gel proteins were transferred to a nitrocellulose membrane (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad, Hercules, CA, USA) at 25 V for 30 min. After the transfer, membranes were incubated in blocking solution (3% bovine serum albumin (BSA) in TBS-T [8 M NaCl, 2.4 M Tris, and 0.1% (v/v) Tween 20]) for 1.5 h. The membrane was then incubated with primary antibodies: VDAC2 and UCH (1:3000 dilution), tubulin (1:5000), and phosphoserine, phosphothreonine and phosphotyrosine antibodies (1:6000) and mTOR, p-mTOR (Ser2448), Akt, p-AKT (Ser473), p-PI3K (Tyr508) (1:1000 dilution), which were added to the blocking solution with gentle rocking.
for 2 h. The blots were washed three times with TBS-T (5 min each) and incubated with a horseradish peroxidase secondary antibody in TBS-T (1:5000) with gentle rocking. The membranes were then washed three more times in TBS-T (10 min each). Using Clarity Western ECL substrate, membranes were chemiluminescently developed in the dark for 5 min, scanned using the ChemiDoc XRS+ imaging system, and quantified using Image Lab software (Bio-Rad, Hercules, CA, USA). Blots were stripped up to two times with Re-Blot Plus Strong solution (15 min each) followed by three rinses with TBS-T (5 min each). Proteins were normalized either to total protein load, tubulin, or the protein itself in cases of phosphorylation measurements.

6.2.3.10 Statistical analysis

A conservative analysis was carried out on PDQuest data using both a two-tailed Student’s t-test and a Mann-Whitney U statistical test, independently comparing each age group to the youngest age cohort. Protein spots were considered statistically significant if p<0.05 in both tests. To further determine significant differences (p<0.05) between all of the various age groups, a one-way ANOVA with post hoc Bonferroni t-test was used. Protein spot fold-change values were calculated by dividing the average, normalized spot intensities of the gels of older age group by the average, normalized spot intensities of the gels of the younger age group in the comparison. For Western blot data, a one-way ANOVA with either a post hoc Bonferroni or Dunnett’s multiple comparisons test was used. All data are presented as mean±SEM. To ensure a rigorously
conservative approach, spots were extracted for MS/MS analysis only if the fold change was 40% or greater or smaller in normalized spot density. Identifications of proteins acquired with the SEQUEST search algorithm were considered to be statistically significant if p<0.01. At least two peptide sequences were used to identify each protein. To ensure correct identification of the proteins, a visual comparison was made between the expected molecular weight and isoelectric point of the identified protein to the spot of the extracted 2-D gel plug.

6.2.4 Results

6.2.4.1 Age-related changes in proteins of the proteostasis network

Figure 6.2.1 Proteins associated with the proteostasis network with altered expression with age in the NMR brain. Representative Sypro Ruby-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-
6 years (B), 7-12 years (C), and 15-24 years (D). Proteins whose expression and/or phosphorylation state was significantly altered (p<0.05) in the particular age group are labeled in the images.

Figure 6.2.2 Proteins associated with the proteostasis network with altered phosphorylation levels with age in the NMR brain. Representative Pro-Q Diamond-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-6 years (B), 7-12 years (C), and 15-24 years (D). Proteins with significantly altered phosphorylation levels (p<0.05) in the particular age group are labeled in the images.
Proteostasis network-related proteins with statistically significant changes with age in protein levels and phosphorylation states are labeled in the 2-D gel images of Figures 6.2.1 & 6.2.2, respectively. PDQuest analyses of all 2-D gels found 9 proteostasis network-related proteins with significant changes in the NMR brain as a function of age (Table 6.2.1).

Table 6.2.1 Proteins associated with the proteostasis network with significantly altered levels and/or phosphorylation states with age in the NMR brain. PDQuest and MS/MS results of NMR brain proteins involved in proteostasis networks with significant altered expression and/or phosphorylation states as a function of age.

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<th>Spot</th>
<th>Protein Identified</th>
<th>Accession #</th>
<th>Coverage (%)</th>
<th># of Peptides</th>
<th>Score</th>
<th>MW (kDa)</th>
<th>pl</th>
<th>Age Groups Compared</th>
<th>p-Value Expression</th>
<th>Fold Change Expression</th>
<th>p-Value Phosphorylation</th>
<th>Fold Change Phosphorylation</th>
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<td>VDAC1</td>
<td>P21769</td>
<td>36.04</td>
<td>8</td>
<td>39.96</td>
<td>50.8</td>
<td>8.54</td>
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<td>0.0122</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6303</td>
<td>VDAC2 (Isoform 2)</td>
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<td>30.26</td>
<td>30.4</td>
<td>7.20</td>
<td>1 v 2</td>
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<td>0.175</td>
<td>0.0004</td>
<td>0.165</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1 v 3</td>
<td>0.0004</td>
<td>0.152</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 v 4</td>
<td>0.0008</td>
<td>0.252</td>
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<tr>
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<td>Q9S277</td>
<td>12.01</td>
<td>3</td>
<td>13.86</td>
<td>30.6</td>
<td>8.66</td>
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<td>0.54</td>
<td>2</td>
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<td>26.5</td>
<td>7.20</td>
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<td>1 v 3</td>
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<tr>
<td>7004</td>
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<td>Q15813</td>
<td>15.10</td>
<td>2</td>
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<td>0.00417</td>
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</tr>
</tbody>
</table>

Many of these proteins are associated with the UPS. Significant elevation of heat shock protein (HSP) response with age was noted by the increased levels of HSP70 protein 4 (Figure 6.2.3A) and the decreased phosphorylation levels of HSP60 (Figure 6.2.3B).
Figure 6.2.3 HSP70, HSP60 and VDACs with age in the NMR brain. Trends in median protein levels and/or phosphorylation levels in NMR brain with age for (A) HSP70 protein 4, (B) HSP60, and (C) VDACs 1-3 (n=5-9 individual brains per age group comparison).

While the primary function of HSP is to maintain a protein’s native 3-D conformation, if a protein is terminally misfolded, some HSPs also function to chaperone the protein for UPS degradation.

The UPS, illustrated in Figure 6.2.4, depicts proteins that have significantly altered expression and/or phosphorylation levels with increasing age in the brain of the NMR. Two of these proteins are involved in protein ubiquitinylation: ub-like modifier-activating enzyme 1 (UBE1) and ub-conjugating enzyme E2 variant 2 (UBE2v2). Both exhibit altered phosphorylation levels in the older age cohorts. Further supporting the notion of increased UPS activity in the NMR with age is the increased expression of ub-carboxy-terminal hydrolase (UCH), the protein responsible for the removal of poly Ub chain, one Ub at a time from the C-terminal end, before entry into the proteasome.
Figure 6.2.4 Proteins associated with the Ubiquitin-Proteasome System with altered protein levels and/or phosphorylation levels in brain of NMR with age. Summary schematic diagram of expression proteomics and phosphoproteomics profiles of changes in proteins related to the ubiquitin-proteasomal system in the brain of the NMR as a function of age. Proteins with significantly altered protein and/or phosphorylation levels are indicated.

Interestingly, a component of the central proteasome itself, proteasome subunit beta type 1 (PSβ1), showed decreased phosphorylation levels in the intermediate and oldest age cohorts (Table 6.2.1).
Autophagy-related proteins with altered levels and/or phosphorylation states in one or more age cohorts includes all three isoforms of the voltage-dependent anion channel (VDAC): VDAC1, VDAC2, and VDAC3. VDACs are the major outer mitochondrial membrane porins and regulators of energy metabolism and mitophagy. Levels of both VDAC2 and VDAC3 were significantly increased in the old age group as compared to control (Table 6.2.1), while phosphorylation levels decreased with age for all VDACs (Figure 6.2.3C).

6.2.4.2 Immunoprecipitation and Western blotting validations

Immunoprecipitation and Western blot experiments were conducted on selected proteins to confirm MS/MS results. Western blot analysis of UCH (Figure 6.2.5A) confirmed a significant increase in the level of UCH in the brains of the oldest age group (p=0.049). Interestingly, the Western blot also showed a significant increase in UCH levels for the intermediate (p=0.011) and old age groups (p=0.013) when compared to the youngest age group.

The results of the Western blot analyses of the expression of VDAC2 (Figure 6.2.5B) verified a significant increase in the old age group (p=0.005), while also showing increased levels of VDAC2 in the oldest age group (p=0.025) both as compared to the youngest age group. Analyses of the immunoprecipitation of VDAC2 with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies confirmed a significant decrease in the phosphorylation of VDAC2 in the brain of the NMR for the intermediate age
group (p=0.04), the old age group (p=0.01), and in the oldest age group (p=0.048) as compared to the youngest age group (Figure 6.2.5C).

**Figure 6.2.5 UCH and VDAC2 in brain of NMR with age.** Western blot and corresponding bar graph representations from the validation experiments of the changes in the protein levels of (A) Ub carboxy-terminal hydrolase (UCH), (B) voltage-dependent anion channel 2 (VDAC2), and (C) the immunochemistry experiment validation of the significant decrease (*p<0.05) in the phosphorylation of VDAC2 in the brains of NMRs (n=4-6 for each age group). Immunoreactivity with specific antibodies was detected by chemiluminescence.
6.2.4.3 PI3K/Akt/mTOR axis in NMR brain with age

Figure 6.2.6 The PI3K/Akt/mTor axis and autophagy. Diagram summarizing the PI3K/Akt/mTOR axis signaling pathway leading to the nucleation and formation of the phagophore to initiate the autophagy-lysosomal pathway.

To further investigate the role that autophagy (illustrated in Figure 6.2.6) may play in the brain of the NMR with age, the PI3K/Akt/mTOR axis was examined. Western blots were probed for p-PI3K (Tyr508), Akt, p-Akt (Ser473), mTOR, and p-mTOR (Ser2448) (Figure 6.2.7). Analyses revealed that the p-PI3K protein level was found to be significantly increased from the early to intermediate age group (p=0.0001) and then significantly decreased from the intermediate to the old age group (p=0.0007). Additionally, there was a significant decrease from the intermediate to the oldest age group (p<0.0001).
Figure 6.2.7 PI3K/Akt/mTOR axis in NMR brain with age. Western blots and corresponding bar graph representations from the evaluation of the PI3K/Akt/mTOR axis in the brain of the NMR (n=5-8 for each age group, *p<0.05, **p<0.001, ***p<0.0001). Proteins were normalized to the total load of the gel. Immunoreactivity with specific antibodies was detected by chemiluminescence.

The p-Akt/Akt ratio was decreased from the intermediate age group to the old age group (p=0.031). Similar to the other proteins in the PI3K/Akt/mTOR axis, the p-Akt/Akt ratio increases from the youngest to the intermediate age group (data trended toward significance). There were no significant changes in the protein levels of mTOR; however the p-mTOR/mTOR ratio follows a
corresponding trend to that of p-PI3K, in which there is a significant increase from the early to intermediate age group (p=0.013) and a significant decrease from the intermediate age group to the old and oldest age groups (p=0.0009) and (p=0.0013), respectively.

**Figure 6.2.8 Beclin-1 and LC3 in NMR brain with age.** Western blots and corresponding bar graph representations of Beclin1 and the LC3-II/LC3-I ratio in the brain of the NMR (n=6-8 for each age group, ***p<0.0001). Proteins were normalized to the total load of the gel. Immunoreactivity with specific antibodies was detected by chemiluminescence.
We further analyzed the quantitative index of autophagy, the LC3-II/LC3-I ratio in all age groups. The LC3 ratio showed no significant changes with age (Figure 6.2.8). Additionally, we analyzed levels of the autophagy initiator protein, Beclin-1, which showed a significant decrease in the oldest age group (p<0.0001) (Figure 6.2.8).

6.2.5 Discussion

Not only are NMRs the longest-lived rodent, but they also maintain an extended healthspan. This extraordinary salubrious lifespan has been attributed to, in part, by mechanisms that contribute to maintaining proteostasis (Zhao, Lin et al. 2014). Processes that promote sustained cellular homeostasis, such as unfolded protein response and proteasome and autophagy pathways, remove damaged or unwanted proteins, macromolecules and organelles which can be cytotoxic and lead to neuronal death (Di Domenico, Head et al. 2014). Additionally, these proteostasis systems play a critical role in maintaining health by modulating protein levels in response to fluctuating physiological environments (Di Domenico, Head et al. 2014). Previous studies have shown that NMRs exhibit a more robust proteostasis as compared to shorter-lived rodents (Perez, Buffenstein et al. 2009, Rodriguez, Wywial et al. 2011, Grimes, Chiao et al. 2012, Rodriguez, Edrey et al. 2012, Zhao, Lin et al. 2014, Pride, Yu et al. 2015). In this current study, we evaluated age-related changes in the NMR proteome and phosphoproteome involved in proteostasis networks. These findings suggest that the NMR is able to maintain this health-sustaining, robust
proteostasis throughout the majority of their lifespan. Here, we discuss the implications of proteins with significant differential levels and phosphorylation states from the respective proteomics analyses (Table 6.2.1) as well as selected autophagy-related proteins evaluated via Western blot analyses.

Typically, susceptibility to age-related diseases correlates to a declining capacity to generate a stress response (Gutsmann-Conrad, Heydari et al. 1998, Njemini, Abeele et al. 2002). Consistent with this notion, in the brain of the NMR, levels of HSP70 (protein 4) were increased and phosphorylation of HSP60 decreased with age. HSP70 is a highly conserved pleiotropic protein that executes many cellular functions including: folding of newly synthesized proteins, prevention of protein aggregation, aiding in endocytosis, signal transduction, protein targeting, and relaying proteins to the ubiquitin-proteasome system and autophagy-lysosomal pathways (reviewed in (Meimaridou, Gooljar et al. 2009)). Moreover, HSP70 mediates proteasome assembly during stress (Grune, Catalgol et al. 2011), and together with its co-chaperone, HSP40, HSP70 is involved in mitigation of proteotoxic insults to the proteasome (Rodriguez, Osmulski et al. 2014). Further, overexpression of HSP70 has been shown to impede apoptotic mechanisms (Mayer and Bukau 2005, Arya, Mallik et al. 2007) and to curtail neurodegeneration and senescence (Bonini 2002, Klucken, Shin et al. 2004). Previously, HSP70 levels in NMRs have been shown to be higher in liver lysates compared to those in mice (Rodriguez, Osmulski et al. 2014), consistent with the idea that the increased levels of this important chaperone with age in the brain
observed in the current study may suggest a more robust and global proteostasis in these long-lived animals.

HSP60 primarily functions in the mitochondria to properly fold proteins. In addition, HSP60 has been reported to have anti-apoptotic properties as it can bind and inhibit pro-apoptotic proteins, Bax and Bak, to prevent neurodegeneration (Arya, Mallik et al. 2007, Chandra, Choy et al. 2007). Dephosphorylation of HSP60 has been reported to enhance chaperone functions (Khan, Wallin et al. 1998). Therefore, the data from our current study suggest that HSP60 may contribute to proper mitochondrial function in older NMR brains by preventing protein aggregation and by suppressing apoptotic mechanisms to impede neuronal loss.

Ubiquitinylation directs proteins to specific cellular targets, such as proteasomes or DNA, as well as regulating protein interactions and activity (David, Ziv et al. 2010). Protein ubiquitylation requires the activation and transfer of Ub to a protein in a three-step process. Two proteins involved in this ubiquitinylation process, UBE1 and UBE2v2, were found to have altered levels and/or phosphorylation states in the brains of NMR in different age groups. In the old age group, protein phosphorylation levels of UBE1 were found to be increased compared to the youngest age group. UBE1 not only catalyzes the first step in the Ub-proteasomal pathway, but it is also essential for the protein ubiquitinylation that modulates DNA double-strand break repair, suggesting an important role in policing genomic integrity and preventing disease pathogenesis.
Posttranslational modifications to UBE1 isoforms are still poorly understood (Schulman and Harper 2009). Known putative roles of UBE1 phosphorylation include targeting this protein to different subcellular locations and modulation of nucleotide excision repair (Stephen, Trausch-Azar et al. 1996, Nouspikel and Hanawalt 2006). However, with multiple UBE1 isoforms containing multiple phosphorylation sights in various domains of the protein, the implications of increased phosphorylation of UBE1 in this current study are as of yet, uncertain. However, as this critical enzyme is reported to be the apex of downstream signaling (Schulman and Harper 2009), we opine that UBE1 may be involved in maintaining a healthy genome and conceivably may be related to the dearth of cancer in this long-lived rodent.

Ubiquitinylation is a dynamic process and protein eventual protein destination depends on the lysine to which it is attached and whether it is monoubiquitinylated, homo-polyubiquitinylated, or hetero-polyubiquitinylated (Johnson, Ma et al. 1995, Shang, Deng et al. 2005). The UBE2v2 preferentially ubiquitinylates Lys63, which is reported to participate in chaperoning proteins for DNA repair, lysosomal degradation of epidermal growth factor receptors, and NF-κB activation by degradation of class I major histocompatibility complex molecules (Hofmann and Pickart 1999, Duncan, Piper et al. 2006, Huang, Kirkpatrick et al. 2006, Wu, Conze et al. 2006). Here, protein levels of UBE2v2 were increased in the intermediate age group, while phosphorylation levels were decreased in the intermediate and oldest age groups. The intricacies of UBE2 structure and function are complex; and as such, the implications of altered
phosphorylation states have yet to be elucidated, though we speculate that this protein may be involved in promoting the ability for NMR to maintain a healthy genome.

UCHs are a family of proteins responsible for the removal of the poly-Ub tags. Dysregulation of UCH may result in the accumulation of poly-Ub proteins; and this accumulation is reported in many chronic neurodegenerative diseases, as they are present in the senile plaques and neurofibrillary tangles in Alzheimer disease (AD) and in the Lewy bodies of Parkinson disease (Schwartz and Ciechanover 1999). Further, in AD, it is hypothesized that the UPS-mediated degradation of amyloid-beta (Aβ) is impaired, which leads to ubiquitinylated Aβ aggregating into neurotoxic plaques (Hong, Huang et al. 2014). Previously, we have shown that a particular UCH variant, UCHL1, is oxidized in AD brain, which could conceivably inhibit Aβ degradation (Castegna, Aksenov et al. 2002, Sultana, Boyd-Kimball et al. 2006). However, it has been reported that even though NMRs exhibit Aβ levels similar to that of 3xTg-AD mice, there is no accumulation of senile plaques in the NMR brain (Edrey, Medina et al. 2013, Edrey, Oddo et al. 2014). Moreover, in mice ubiquitinylated proteins accumulate with age; however, in NMRs levels of ubiquitinylated proteins of 2 year-old and 26 year-old rodents were similar (Perez, Buffenstein et al. 2009). Therefore, the increase of UCH levels in the brain of the oldest age group may function to maintain prolonged cellular proteostasis via availability to mediate disposal of increasing levels of neurotoxic proteins, such as Aβ.
When attached to the 26S proteasome and after the removal of Ub by UCH, the protein is degraded by threonine proteases in the core of the 26S proteasome (Voges, Zwickl et al. 1999). In this current study, PSB1, which is responsible for PGPH activity, was found to have decreased levels of phosphorylation in the intermediate and oldest age groups as compared to the youngest age group. Decreases in proteasomal function and/or expression have been reported in multiple neurodegenerative diseases including: AD, PD, Huntington disease, amyotrophic lateral sclerosis, and prion diseases (Keller, Hanni et al. 2000, Ciechanover and Brundin 2003, Bukhatwa, Zeng et al. 2010, Ebrahimi-Fakhari, Wahlster et al. 2012, Martins-Branco, Esteves et al. 2012). Moreover, PSB1, the variant identified in this current study, has been reported to promote anti-apoptotic activity of plasminogen activator inhibitor 2 (PAI2) (Fan, Zhang et al. 2004). While the consequences of the phosphorylation of this subunit are unclear, it has been shown that phosphorylation of β-subunits in the prokaryote *M. tuberculosis* can inhibit proteasomal assembly (Anandan, Han et al. 2014). While there are multiple β-subunits in eukaryotes, unlike prokaryotes that have only one type, the reduced phosphorylated states in the oldest NMR brains could suggest that there is an increased affinity towards proteome assembly and therefore, an increased degradation of unwanted or damaged proteins, clearing the cell of detritus to promote healthy cellular function. This observation would be consistent with the observed high levels of proteasome activity reported for brain lysates of the NMR (Edrey, Oddo et al. 2014).
Voltage dependent anion channels (VDACs) are outer mitochondrial membrane porins that are involved in mitochondrial metabolic processes by opening at low membrane potentials to regulate metabolic flux of small hydrophilic molecules and ions (Blachly-Dyson and Forte 2001, Shoshan-Barmatz and Gincel 2003). VDACs also participate in mitochondrial autophagy by recruiting Parkin to docking sites for the removal of defective mitochondria, targeting the organelle for degradation by lysosomes (Geisler, Holmstrom et al. 2010, Sheldon, Maldonado et al. 2011, Sun, Vashisht et al. 2012). Decreased levels of VDACs could lead to an increased presence of malfunctioning mitochondria, leading to increased protein oxidation and cellular detritus and ensuing neuronal dysregulation. However, in this study, the increased levels of VDACs suggest that the metabolic flux and the policing of mitochondrial function are improved in the aging brain of the NMR. VDACs are known to be phosphorylated by multiple kinases including: PKA, GSK3β, PKC, p38 MAP kinase, Nek1, and endostatin reduced hexokinase 2 (Chen, Gaczynska et al. 2010, Sheldon, Maldonado et al. 2011). Phosphorylation of VDAC1 by Nek1 has been reported to open the channel (Chen, Gaczynska et al. 2010). VDAC phosphorylation by GSK3β or PKA increases the interaction between VDAC and tubulin, blocking the channel (Sheldon, Maldonado et al. 2011). The consequences of the decreased phosphorylation levels of VDAC2 and VDAC3 in the aged NMR brain are unclear. Further investigations are needed to elucidate the implications of this reported global decrease in phosphorylation in brains of NMR rodents with age.
To further assess the role that autophagy may contribute to the sustained healthspan of the NMR by regulating cellular proteostasis, the PI3K/Akt/mTOR axis, Beclin-1 and LC3 were examined in the NMR brain as a function of age. Previous data suggested that the NMR, under basal conditions, maintains higher levels of autophagy, thereby removing potentially toxic proteins before they can negatively impact organ functionality (Rodriguez, Wywial et al. 2011) and that macroautophagy was shown to be substantially higher in NMRs than in shorter-lived mice (Rodriguez, Wywial et al. 2011, Pride, Yu et al. 2015). Further, when the autophagy markers LC3-I, LC3-II and Beclin-1 were measured in one-day-old NMRs and one-day-old mice, the NMRs were shown to have a higher LC3-II/LC3-I ratio, even though their Beclin-1 levels were lower, suggesting that NMRs have a significantly higher basal levels of autophagy than mice (Zhao, Lin et al. 2014). Although Beclin-1 plays a critical role in the regulation of autophagosome formation, it is also a shorter-lived protein involved in the formation of pre-autophagosomal structures. Consequently, it is generally accepted that the LC3-II/LC3-I levels usually correlate more reliably with the number of autophagosomes and can be used to monitor autophagosome formation (Klionsky, Abeliovich et al. 2008). Here, we measured the levels of Beclin-1 in the brain of the NMR as a function of age. Beclin-1 was significantly decreased in the oldest age group relative to the youngest age group. When the LC3-II/LC3-I ratio was measured, the levels of this quantitative index of autophagy did not significantly change, suggesting that NMRs do maintain a high level of autophagy throughout a vast majority of their lives.
The serine/threonine kinase, mTOR, is a major modulator of autophagy that receives inputs from many different signaling pathways (Cuyas, Corominas-Faja et al. 2014). One of the most important upstream positive regulators of mTOR is Akt. The hyperphosphorylation of Akt induces a complete inhibition of TSC2 and activation of mTOR through direct phosphorylation. In turn, the mechanism of activation of Akt is induced by another kinase, PI3K. All together, these proteins are recognized as the PI3K/Akt/mTOR axis, which plays a central role in controlling one of the processes of proteostasis, autophagy. Our results showed increased phosphorylation of Akt and PI3K (p85 subunit) at Ser$^{473}$ and Tyr$^{508}$, respectively, in the intermediate NMR age group. Surprisingly, the hyperphosphorylation of these two proteins do not appear to affect NMR aging negatively. Indeed, the increase of PI3K/Akt activity in intermediate-aged NMRs in this study could reflect the diversity of one of the several main downstream targets, mTOR. Consistent with this notion, our data showed a hyperphosphorylation of mTOR at Ser$^{2448}$ in the brain of the intermediate age group.

Taken together, these results demonstrate a plausible mechanism by which NMRs resist development of age-related diseases, even though they show high levels of oxidative damage to visceral tissues, even at young ages (Andziak and Buffenstein 2006, Andziak, O’Connor et al. 2006). Given that NMRs endogenously produce high levels of Aβ at a young age, yet live more than two decades with these high levels, it appears that this species is exceedingly tolerant of high Aβ levels and has evolved mechanisms to counter its
neurotoxicity (Edrey, Medina et al. 2013, Edrey, Oddo et al. 2014). One possible explanation could be the sustained activity of the autophagy pathway in the older age groups. Moreover, the slight elevation of mTOR activity in the intermediate age group could be a response to these increased levels of Aβ. In fact, a recent paper published from our lab, supporting earlier findings by LaFerla, Oddo and others, showed increased activity of the autophagy pathway in AD and down syndrome subjects, suggesting that Aβ could be one of the major causes causing hyperactivation of the pathway (Perluigi, Pupo et al. 2014).

Our results of this age-related study in the brain of the NMR expose significant alterations in protein levels and phosphorylation states of proteins involved in the functioning of the proteostasis network. Mechanisms that remove cellular detritus promote an efficient, functional environment. Additionally, by using long-lived species to identify specific proteins involved in these processes, targets for potential therapies are identified that may aid in the delay of the onset and progression of the aging process in humans.
CHAPTER 6.3


6.3.1 Overview

Aging is the greatest risk factor for developing neurodegenerative diseases, which are associated with diminished neurotransmission and neuronal brain structure and function. However, in the brain of the remarkable naked mole-rat (NMR), several salubrious traits have evolved to avert or delay age-related deterioration. Not only does the NMR hold the world record as the longest-lived rodent, but the NMR also possesses a extended healthspan, lasting 75-80% of its life span. Further, aged NMRs exhibit negligible senescence, pronounced genomic integrity, robust proteostasis, and a marked resistance to cancer. Using a proteomic approach, statistically significant changes with age in expression and phosphorylation levels of proteins associated with neurite outgrowth and neurotransmission were identified in the brain of the NMR. We hypothesize that such changes contribute to the extended lifespan and healthspan of the NMR.

6.3.2 Introduction

The naked mole-rat (NMR; sand puppy) is a subterranean rodent indigenous to the sub-Saharan region of North East Africa. Living in large, eusocial colonies in an underground and thermally stable ecological niche, NMRs have evolved several remarkable traits that have made this rodent an intriguing animal research model to investigate, some of which include: extremely long

Mitochondrial dysfunction and resultant decreased ATP production is implicated in various neuronal degenerative diseases and leads to decreased neuroplasticity and neurite outgrowth (Cheng, Hou et al. 2010). Moreover, in AD up to 50% of synapses are lost throughout the brain (Masliah, Terry et al. 1989). Uncovering proteins and related mechanism that prevent such catastrophic neuronal loss may conceivably identify potential therapeutic targets to halt or even ameliorate neurodegenerative damage.

Making new synaptic connections in the brain is dependent upon neurite outgrowth and neuronal pathfinding, processes in which the axon is extended to target cells by the growth cone. The growth cone is a highly motile, actin-based structure located at the tip of neuronal processes that contain lamellipodia and filopodia projections that respond to surrounding environmental cues to direct growth cone movement (Dent and Gertler 2003, Lowery and Van Vactor 2009).

Once the growth cone has finalized an axon terminal formation at the synapse, neurotransmitters can be repeatedly released, triggering enlargement
of the axonal spines. In order to maintain rapid neurotransmitter activity, efficient priming, releasing and recycling of synaptic vesicles is essential.

In this study, significant changes in the NMR brain proteome and phosphoproteome with age were identified. A large number of altered brain proteins were determined by proteomics and/or phosphoproteomics. To facilitate discussion of the various proteins that may be involved in promoting healthy longevity in the NMR, proteins were grouped according to their functionality. In this report, we report on significant changes with age in neuroplastic-related brain proteins and phosphoproteins in the NMR and their contribution to underlying mechanisms that may contribute to the unusually long and salubrious lifespan of this rodent.

6.3.3 Materials and Methods

6.3.3.1 Materials

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Criterion precast polyacrylamide gels, ReadyStrip IPG strips, TGS and MOPS electrophoresis running buffers, mineral oil, Precision Plus Protein All Blue Standards, Sypro Ruby protein stain, biolytes, urea, dithiothreitol (DTT), iodoacetamide (IA), and nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA). Pro-Q Diamond phosphoprotein gel stain, and anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies were purchased from Invitrogen (Grand Island, NY, USA). Protein A/G beads, Amersham ECL IgG horseradish peroxidase-
linked secondary antibodies, and ECL Plus Western blotting detection reagents were procured from GE Healthcare (Pittsburgh, PA, USA). C\textsubscript{18} ZipTips and Re-Blot Plus Strong stripping solution were obtained from Millipore (Billerica, MA, USA). Modified trypsin solution was purchased from Promega (Madison, WI, USA). Pierce BCA protein assay reagents A & B were purchased from Thermo Scientific (Waltham, MA, USA). Anti-septin7, anti-CRMP2 and anti-cofilin1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

6.3.3.2 Animals

Brains from NMRs, aged 2-24 years, were acquired from the well-characterized colonies (Buffenstein 2005) of Dr. Rochelle Buffenstein at the University of Texas Health Science Center, San Antonio. The fabricated burrow systems, which housed the NMRs, mimicked conditions of their natural habitat and were maintained at 30°C with 30-50% relative humidity. The NMR diet consisted of fresh fruits and vegetables (fed ad libitum), supplemented with a high protein and vitamin enriched feed (Pronutro, South Africa). NMRs of different ages were anesthetized with isofluorane and euthanized by cardiac exsanguination. Brains were immediately harvested and flash frozen in liquid nitrogen. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio, TX. Experimental animal groups consisted of 5-9 individual brains from both male and female individuals, of both subordinate and breeding status. NMRs were divided into the following four age groups for analysis: 2-3 year-olds
(age group 1; young), 4-6 year-olds (age group 2; intermediate), 7-12 year-olds (age group 3; old) and 15-24 year-olds (age group 4; oldest).

6.3.3.3 Sample preparation

NMR brains were homogenized using a Wheaton glass homogenizer (~40 passes) with ice-cold isolation buffer [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES, 0.2 µg/mL PMSF, 5 µg/mL aprotinin, 4 µg/mL leupeptin, 4 µg/mL pepstatin, and 10 µg/mL phosphatase inhibitor cocktail 2] and then sonicated for 10 s on ice. Protein concentrations of homogenates were determined by the Pierce BCA method (Rockford, IL, USA) (Smith, Krohn et al. 1985).

6.3.3.4 Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE)

Isoelectric focusing (IEF). 2-D PAGE experiments were performed as previously described (Sultana, Boyd-Kimball et al. 2007). Briefly, 200 µg of each sample was suspended in 200 µL of rehydration buffer [8 M urea, 2 M thiourea, 50 mM DTT, 2.0% (w/v) CHAPS, 0.2% Biolytes, 0.01% bromophenol Blue], applied to IPG strips (pH 3-10), actively rehydrated and isoelectrically focused. After completion of the run, IPG strips were immediately stored at -80 °C.

SDS PAGE. IPG strips were thawed and equilibrated in DTT and IAA-containing buffers. Strips were rinsed in TGS running buffer and placed into Criterion precast polyacrylamide gels (8–16% Tris–HCl). Precision Plus Protein
All Blue molecular standards and samples were run at a constant voltage of 200 V for approximately 65 min at 22°C in Tris/Glycine/SDS running buffer.

6.3.3.5 Sypro Ruby and Pro-Q Diamond staining

After completion of 2D-PAGE, gels were stained according to manufacturer's directions and as described previously (Di Domenico, Sultana et al. 2011). In brief, gels were fixed [10% (v/v) acetic acid, 50% (v/v) methanol] and stained with 60 mL of Pro-Q Diamond for exactly 90 min. Gels were incubated four times in destaining solution [20% acetonitrile (ACN), 50 mM sodium acetate, pH 4] (100 mL, 30 min each). The gels were scanned at 580 nm using ChemiDoc XRS+ imaging system (Bio-Rad). Next, gels were incubated overnight (15 h) in 50 mL of Sypro Ruby protein gel stain. Gels were imaged at 450 nm and stored in DI water in covered containers at 4 °C until protein spot extraction.

6.3.3.6 Image Analysis

Expression Proteomics. Spot intensities from SYPRO Ruby-stained gel images were quantified according to total spot density and normalized to total gel density using PDQuest analysis software (Bio-Rad). Normalized spot densities of the four age groups were compared and only spots with statistically significant differences in protein spot densities between age groups (p<0.05) were considered for in-gel trypsin digestion and protein identification by MS/MS.
Phosphoproteomics. Spot intensities from Pro-Q Diamond-stained gel images were quantified and matched as described previously (Di Domenico, Sultana et al. 2011). A high match analysis between the Sypro Ruby and Pro-Q Diamond-stained gels was conducted. Phosphoprotein spot densities were normalized to Sypro Ruby spot densities in order to differentiate between a lightly phosphorylated protein that is highly abundant and a protein of low abundance that is highly phosphorylated. The normalized spot densities were compared between the four age groups, and spots that were statistically significant (p<0.05) were considered for in-gel trypsin digestion and protein identification by MS/MS.

6.3.3.7 In-gel trypsin digestion/peptide extraction

Significantly differential protein spots were excised from 2D-gels and transferred to individual Eppendorf microcentrifuge tubes for trypsin digestion as previously described (Thongboonkerd, McLeish et al. 2002). In brief, DTT and IA were used to break and cap disulfide bonds and the excised gel plugs were incubated overnight (17 h) in modified trypsin solution with shaking at 37°C. Salts and contaminants were removed from the tryptic peptide solutions using C_{18} ZipTips. Tryptic peptide solutions were reconstituted in 10 µL of a 5% ACN/0.1% formic acid (FA) solution and stored at -80°C until MS/MS analysis.

6.3.3.8 NanoLC-MS with Data Dependent Scan

Reconstituted tryptic peptide solutions were analyzed by a nanoAcquity (Waters, Milford, MA)-LTQ Orbitrap XL (Thermo Scientific, San Jose, CA) platform using a data dependent scan mode and separated by a capillary column
(0.1 x 130 mm column packed in-house with 3.6 µm, 200Å XB-C18) with a
gradient using 0.1% FA and ACN/0.1% FA at 200 nL/min. Spectra obtained by
MS were measured by the orbitrap at 30,000 resolution; and the MS/MS spectra
of the six most intense parent ions were acquired by the orbitrap at 7,500
resolution. Swiss-Prot database by SEQUEST (Proteome Discoverer v1.4,
Thermo Scientific) was used to interrogate the data files for sample identification.
Proteins were identified by at least two high-confidence peptide-matched
sequences with a false discovery rate <1%. Proteins matched with the same
peptides were reported as one protein group. Tabular data reported from these
analyses includes: the SwissProt accession number, the percentage of the
protein sequence identified by matching peptides, the number of peptide
sequences sequenced in the MS/MS analysis, the confidence score of the protein,
the protein’s expected molecular weight (MW) and isoelectric point (pI).

6.3.3.9 Immunoprecipitation and Western blotting

*Immunoprecipitation (IP).* Brain homogenates (250 µg) were suspended
individually in 500 µL of IP buffer [0.05% NP-40, aproprotin 5 µg/mL, leupeptin 4
µg/mL, pepstatin 4 µg/mL, and phosphatase inhibitor cocktail 10 µg/mL] in a
phosphate buffer solution, pH 8 [8 M NaCl, 0.2 M KCl, 1.44 M Na₂HPO₄, and
0.24 M KH₂PO₄]. Samples were incubated with Protein A/G agarose beads in
500 mL of IP buffer for 1.5 h at 4˚C. Each sample was incubated overnight with
anti-cofilin1 antibody (1:50 dilution) at 4˚C. The next day, samples were
incubated with Protein A/G agarose beads for 1.5 h at 4˚C and washed with IP
buffer (500 mL, 5 times), preserving the protein-linked beads for a 1D-PAGE experiment.

One-dimensional polyacrylamide gel electrophoresis (1D-PAGE). Sample homogenates (50 µg) or beads from immunoprecipitation were suspended in 4X sample loading buffer [0.5 M Tris, pH 6.8, 40% glycerol, 8% SDS, 20% β-mercaptoethanol, 0.01% Bromophenol Blue] (diluted to 1X with DI water). Samples were heated at 95°C for 5 min, cooled on ice and loaded into Criterion precast 18 well polyacrylamide gels (4-12% Bis-Tris). Using MOPS running buffer, gels were run at 80 V for 15 min and then at 120 V for approximately 100 min.

1D-Western blotting. In-gel proteins were transferred to nitrocellulose membranes (0.2 nm) using a Trans-Blot Turbo Blotting System (Bio-Rad) at 25 V for 30 min. Membranes were blocked in solution (3% bovine serum albumin (BSA) in TBS-T [8 M NaCl, 2.4 M Tris, and 0.1% (v/v) Tween 20]) for 1.5 h. Membranes were then separately incubated with primary antibodies: CRMP2 and septin7 (1:3000 dilution), tubulin (1:5000), and phosphoserine, phosphothreonine and phosphotyrosine antibodies (1:6000) for 2 h. The blots were washed with TBS-T (3 times, 5 min each), incubated (1 h) with a horseradish peroxidase secondary antibody in TBS-T (1:5000), and washed again in TBS-T (3 times, 10 min each). Western blots were chemiluminescently developed (in dark, 5 min) with Clarity Western ECL substrate, scanned with the ChemiDoc and quantified.
using Image Lab software (Bio-Rad). Blots were stripped up to two times with Re-Blot Plus Strong solution (15 min each) for further probing.

6.3.3.10 Statistical analysis

An initial, conservative analysis was carried out on PDQuest data using both a two-tailed Student’s t-test and a Mann-Whitney U statistical test, independently comparing each age group to the youngest age group. Protein spots were considered significant if p<0.05 in both tests. Significant differences (p<0.05) between the age groups for PDQuest data were determined using one-way ANOVA with post hoc Bonferroni t-test. Fold-change values of proteins were calculated by dividing the average, normalized spot intensity of older age group by the average, normalized spot intensity of the younger age group in the comparison. For Western blot data, a one-way ANOVA with a post hoc Tukey multiple comparisons test was used. All data are presented as mean±SEM. Proteins identified by the SEQUEST search algorithm were considered statistically significant if p<0.01. At least two peptide sequences were used to identify each protein and a visual comparison was made between the expected MW and pI of the identified protein to the spot of the extracted 2-D gel plug.

6.3.4 Results

Age-related changes in neuroplasticity-related proteins

Neuroplasticity-related proteins with statistically significant alterations in protein and phosphorylation levels in the four cohorts are labeled Figures 6.3.1 & 6.3.2.
Figure 6.3.1 Proteins associated with neurite outgrowth and neurotransmission with altered expression and/or phosphorylation levels with age in the NMR brain. Representative Sypro Ruby-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-6 years (B), 7-12 years (C), and 15-24 years (D). Proteins whose expression and/or phosphorylation state was significantly altered (p<0.05) in the particular age group are labeled in the images.
Figure 6.3.2 Proteins associated with neurite outgrowth and neurotransmission with altered phosphorylation levels with age in the NMR brain. Representative Pro-Q Diamond-stained 2-D gel images of isolated proteins from the brains of NMRs, aged 2-3 years (A), 4-6 years (B), 7-12 years (C), and 15-24 years (D). Proteins with significantly altered phosphorylation levels (p<0.05) in the particular age group are labeled in the images.

PDQuest analyses of gels from all age groups identified 9 proteins related to neurite outgrowth and neurotransmission with significant changes in the NMR brain as a function of age (Table 6.3.1).
Table 6.3.1 Proteins associated with neurite outgrowth and neurotransmission with significantly altered levels and/or phosphorylation states with age in the NMR brain. PDQuest and MS/MS results of NMR brain proteins related to plasticity, structure and neurotransmission with significant altered expression and/or phosphorylation states as a function of age.

<table>
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<th>Spot</th>
<th>Protein Identified</th>
<th>Accession #</th>
<th>Coverage (%)</th>
<th># of Peptides</th>
<th>Score</th>
<th>MW (Da)</th>
<th>p-Value</th>
<th>Fold Change Expression</th>
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<th>Fold Change Phosphorylation</th>
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<tr>
<td>4710</td>
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These altered proteins were: cofilin-1; isoform 2 of dihydropyrimidinase-related protein 2, aka collapsin response mediator protein 2; destrin, aka actin depolymerizing factor; isoform 3 of spectrin alpha chain; septin-7; Syntaxin-binding protein 1; synapsin-2 isoform IIB; and both isoform 3 and 4 of dynamin1.

Immunoprecipitation and Western blotting validations

Immunoprecipitation and Western blot experiments were carried out on selected proteins to confirm MS/MS results and provide confidence of all proteomic or phosphoproteomic identifications. Western blot analysis of CRMP2 (Figure 6.3.3A) confirmed a significant increase in CRMP2 levels in the brain of
the oldest age group compared to the two younger age groups (p=0.008) and (p=0.011), respectively. The Western blot analyses of the levels of septin-7 (Figure 6.3.3B) verified a significant increase in the old age group (p=0.038) compared to the youngest, while also showing increased levels of septin-7 in the intermediate age group (p=0.034) also compared to the youngest age group. Analyses of the immunoprecipitation of cofilin-1 with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies confirmed a significant decrease in the phosphorylation of cofilin-1 in the brain of the NMR for the intermediate age group (p=0.039), the old age group (p=0.037), and in the oldest age group (p=0.037) all relative to the youngest age group (Figure 6.3.3C).

Figure 6.3.3 CRMP2, septin-7, and cofilin-1 in NMR brain with age.
Immunoprecipitation and Western blot analyses of (A) significant elevation of
CRMP2 protein levels in oldest age group (B) increased septin-7 protein levels in the middle age groups, and (C) levels of phosphorylated Ser/Tyr/Thr residues normalized to total cofilin-1 protein levels after immunoprecipitation showing significant decrease (*p<0.05) in the phosphorylation of cofilin-1 in the brains of NMRs (n=4-6 for each age group). Immunoreactivity with specific antibodies was detected by chemiluminescence.

6.3.5 Discussion

Evaluation of the proteins and phosphoproteins that significantly change with age in the brain of the NMR, which are related to mechanisms associated with neuroplasticity, fall into two different yet connected processes: neurite outgrowth and neurotransmission.

Neurite outgrowth

Rapid assembly and disassembly of the actin cytoskeleton at the leading edge of the cone is required for motility of the growth cone (Lin and Forscher 1995, Minamide, Striegl et al. 2000). Actin depolymerizing factor (ADF; destrin) and cofilin-1, members of the ADF/cofilin family, modulate actin dynamics in the growth cone by binding and depolimerizing F-actin at the negative end of the filaments (Lappalainen and Drubin 1997, Gungabissoon and Bamburg 2003), and ADF/cofilin regulate the rate at which these monomers separate from the actin filament (Maciver and Hussey 2002). Further, overexpression of cofilin has been reported to increase neurite outgrowth (Meberg and Bamburg 2000). In
fact, cofilin activity reportedly is essential for neurite extension by the growth cone (Endo, Ohashi et al. 2003). Interestingly, cofilins also are involved in cellular stress responses to heat shock and chemical stress, in which actin becomes saturated with cofilin inducing actin rod bundling (known as cofilin/actin rods), thereby halting actin polymerization and depolymerization (Munsie and Truant 2012). Upon dissipation of environmental stressors, the cofilin/actin rods dissipate. However, if the stressed environment persists, as in neurodegenerative diseases, the cofilin/actin rods block neuronal trafficking and lead to disruption of synapses and eventual apoptosis (Minamide, Striegl et al. 2000, Cichon, Sun et al. 2012). In the current study, NMRs exhibited increased ADF/cofilin-1 levels with significant elevation of ADF levels in the two oldest age cohorts compared to the youngest age group and increased cofilin-1 levels in the old age group. Additionally, ADF phosphorylation was decreased in the oldest age group, while cofilin-1 phosphorylation was decreased in all age groups as compared to the youngest. Phosphorylation at Ser-3 reportedly inactivates ADF/cofilin, while dephosphorylation activates actin depolymerization (Toshima, Toshima et al. 2001, Endo, Ohashi et al. 2003). Taken together, the increased levels and activity of ADF/cofilin suggest a possible mechanism by which the NMR brain maintains a high numbers of synapses, consistent with salubrious aging.

Dihydropyrimidinase-related protein 2 (DRP2; collapsin response mediator protein 2, CRMP2) is a pleiotropic protein that participates in a wide array of activity in and out of the growth cone, including: organization of the dendritic field,
guidance and collapse of the growth cone, neurite outgrowth, synaptic assembly, neurotransmitter release, endocytosis, and Ca\(^{2+}\) homeostasis (Goshima, Nakamura et al. 1995, Byk, Dobransky et al. 1996, Uchida, Ohshima et al. 2005, Brittain, Piekarz et al. 2009, Hensley, Venkova et al. 2011, Ju, Li et al. 2013). In previous studies, we have shown that CRMP2 is oxidatively modified in brain of subjects with Alzheimer disease (AD) (Castegna, Aksenov et al. 2002) and that phosphorylation levels of CRMP2 are increased in the hippocampus of AD brain (Di Domenico, Sultana et al. 2011). In this current study, levels of CRMP2 were significantly increased in the oldest NMRs compared to both the intermediate and old age groups. This increase in CRPM2 suggests that older NMR brains have increased neuronal plasticity and provides another mechanism by which the NMR is able to ward away cognitive decline with age.

Members of the septin family are highly conserved cytoskeletal GTPases involved in various cellular functions, including: dendritic field maturation, spine dynamics, synaptic transmission, vesicle trafficking, DNA response to cytoskeletal damage, protein scaffolding, membrane compartmentalization, cell division, and apoptosis (Barral, Mermall et al. 2000, Kremer, Adang et al. 2007, Tada, Simonetta et al. 2007, Xie, Vessey et al. 2007, Spiliotis, Hunt et al. 2008, Hagiwara, Tanaka et al. 2011). Septin7, in particular, is reportedly essential in the regulation of dendritic branching and spine morphology (Xie, Vessey et al. 2007). Additionally, septins can function cooperatively, forming hetero-filaments of 3 or more septins, the most notable of which being septin2/septin6/septin7 (Barral and Kinoshita 2008). Furthermore, septins have abnormal function in
several neurological disorders including AD and Parkinson disease (Kinoshita, Kinoshita et al. 1998, Cheon, Fountoulakis et al. 2001, Barr, Young et al. 2004, Ihara, Yamasaki et al. 2007). In this study of the NMR brain, expression of septin7 was significantly increased in the old age group with respect to the youngest age group, suggesting increased brain plasticity is a survival mechanism with aging of NMRs.

**Neurotransmission**

Rapid release and recycling of synaptic vesicles is one facet required for efficient neurotransmission. In the brain of the NMR, four proteins involved in this process were altered with age.

Syntaxin-binding protein 1 (stxbp1) apparently plays a role in both neurite extension and neurotransmission. Not only is stxbp1 reported to regulate the filopodia of the growth cone to modulate plasticity (Broeke, Roelandse et al. 2010), but stxbp1 also can bind to syntaxin and modulate the formation of the SNARE complex and subsequent neurotransmitter release (Misura, Scheller et al. 2000, Yang, Steegmaier et al. 2000). In the current study, the expression of stxbp1 was increased in the oldest age group compared to the youngest, and phosphorylation levels of stxbp1 were decreased in the two oldest groups relative to the youngest. Protein kinase C phosphorylation on Ser-306 and Ser-313 of stxbp1 reportedly modulates neurotransmission by increasing rapid vesicle cycling and vesicle release (Barclay, Craig et al. 2003, Craig, Evans et al. 2003). However, since PhosphoSite lists over 30 residues of stxbp1 that can be
phosphorylated, it is conceivable that the global phosphorylation events measured here may be responsible for modulation of other cellular activities, such as changing affinity to binding partners or cellular localization or other activities. Furthermore, based on the NMR’s long healthspan well into old age in addition to the implications of other proteins identified in this study, it may not be likely that the decreased phosphorylation of stxbp1 seen here would decrease synaptic activity. Further investigations into decreases of phosphorylation at particular phosphorylation sites are warranted.

Dynamin-1 (dnm1) is a brain-specific GTP-dependent motor protein that is abundant in the post-synaptic synapse (Raimondi, Ferguson et al. 2011). Interestingly, dnms are the only known molecular motor proteins to utilize a twisting motion, and dnm1 does so by pinching off synaptic vesicles from the plasma membrane during endocytosis (Soulet, Yarar et al. 2005, Roux, Uyhazi et al. 2006, Raimondi, Ferguson et al. 2011). Dnm1 phosphorylation plays a key role in regulating synaptic vesicle endocytosis (reviewed in (Smillie and Cousin 2005)). Dnm1 is activated by the dephosphorylation that occurs upon depolarization of the axon terminal, and then is deactivated by phosphorylation upon repolarization. We speculate that upregulation of dnm1 activity observed in the current study among the two oldest NMR age-groups compared to the youngest age group, may be the result of the brain’s defensive mechanism to ameliorate reduced neurotransmission by increasing the uptake of excitatory neurotransmitters into the post-synaptic synapse.
Spectrin is an α-β heterodimer that makes up to 2-3% of all proteins in the brain and is responsible for cross-linking actin filaments to form a resilient 3-D cellular matrix to increase stability of the cytoskeleton as well as to stabilize transmembrane proteins (Yan, Winograd et al. 1993, Zhang, Zhang et al. 2013). In addition to actin, spectrin also can cross-link membrane lipids and proteins (Baines 2009). Spectrin is found at greater concentrations at the presynaptic membrane and are thought play a role in synaptic transmission and organization, since spectrin mutations in *Drosophila* disrupted neurotransmission and resulted in aberrant synaptic protein localization (Featherstone, Davis et al. 2001). In the current study, expression of spectrin alpha chain, isoform 3 is increased in the brain of old-aged NMRs relative to the youngest, and in addition, the phosphorylation level of this protein is decreased in the brains of the two oldest NMR groups. Spectrin is known to be phosphorylated at numerous residues; however, the consequences of spectrin phosphorylation are not yet clear (Baines 2009). However, based upon the reputation of the NMR in terms of successful aging, we speculate decreased phosphorylation may contribute to the long healthspan and lifespan of the NMR.

In summary, the NMR, with its many unique traits associated with salubrious aging, is an exceptional model organism in the study of proteins to target for therapeutic interventions in the aging process. The findings of the current age-related study of brain from NMRs identifies changes in protein
expression and/or phosphorylation levels of key proteins involved in mechanisms that may be responsible for the increase in neuronal plasticity and the lack of senescence noted in the NMR.

**Figure 6.3.4 Proteins associated with neurite outgrowths that are increased in levels and/or activity in brain in older NMR age groups.** NMR brain proteins associated with neurite outgrowths that have significantly altered phosphorylation levels and/or protein levels with age.

These identified brain proteins are illustrated in figures 6.3.4 and 6.3.5. Further investigations into these proteomics-identified proteins may be warranted to
identify targets for potential therapies that conceivably may aid in the delay of aging onset and progression in humans.

Figure 6.3.5 Proteins associated with neurotransmission that are increased in levels and/or activity in brain in older NMR age groups. Proteins that play a role in neurotransmission that were significantly altered in phosphorylation and/or levels in the brain of the naked mole-rat with age.

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7.1 Conclusions

The studies in this dissertation examined the proteome and phosphoproteome in brain from the PINK1 KO model of familial PD, the IPL of PCAD, amnestic MCI and AD subjects, and changes with age in brain of the long-lived naked mole-rat. The majority of the significantly altered proteins identified in this dissertation work are involved in vital processes such as: energy production, disposal and recycling of cellular detritus, stress response, and neuroplasticity. Furthermore, the proteins identified with altered expression or phosphorylation in the neurodegenerative brain reflect increased oxidative stress, altered energy metabolism, diminished proteostasis networks, and decreases in neurotransmission and neurite outgrowth. Conversely, in the NMR brain, many alterations in protein levels and protein activities seemingly support underlying biochemical mechanisms that promote a healthy and efficient cellular state.

Depletion of ATP is a major factor in the cascade leading to apoptosis. In brain of AD subjects and PINK1 KO mice, declines in levels and/or activity of energy-related proteins associated with the mitochondria, such as MDH1, and VDACs were observed. Interestingly, VDACs were consistently identified as significantly altered in all of the studies put forth in this dissertation. VDACs are critical regulators not only of mitochondrial flux but of mitophagy as well.
Therefore dysregulation of VDACs may result in a considerable shift in metabolism and homeostasis that promotes a neurodegenerative environment.

Accumulation of cellular detritus, including the aggregation of proteins, is detrimental to proteostasis. In the IPL of AD brain and in brain of PINK1 KO mice, proteins involved in proteostasis networks are diminished in levels and/or activity, including heat shock proteins and proteasomal subunits to name a few. In contrast, in the brain of the senescent-resistant NMR, proteins associated with autophagy and proteasomal pathways are increased in expression and activity. In the NMR, these processes are thought to be a primary contributing factor in promoting a healthy lifespan.

Brain plasticity is critical to healthy brain functioning. In addition to PINK1’s role in policing mitochondrial integrity, it also contributes to neurite outgrowth. Consistent with this notion, the ablation of PINK1 leads to decreased levels of proteins associated with mechanisms of neuroplasticity, such as EF2 which mediates protein production in the growth cone, and CRMP2 which is a pleiotropic protein that plays roles in growth cone guidance and in neurotransmitter release and recycling, among other functions. Dysregulation of CRMP2 was also evident in the AD brain and associated with the decreased synapses present in AD brain, where in the NMR brain, increased CRMP2 activities were associated with negligible senescence.

Taken together, the work in this dissertation study has identified global mechanisms and specific key proteins involved not only in promoting
neurodegenerative environments, but also in encouraging sustained good health and a long lifespan. Further investigations into these key proteins may conceivably provide targets for potential global combatting of neurodegenerative diseases as well as providing insights into the prevention of age-related disease for an extended, healthy human lifespan.

7.2 Future Studies

Based on the results of the studies in this dissertation, future investigations may be warranted in the following areas:

1. For a great number of proteins identified with significantly altered phosphorylation levels, the implications of the global trends in phosphorylation were unclear. Further investigations are warranted to establish not only the identity of the exact amino acid residues with altered phosphorylation but also to determine how the phosphorylation or dephosphorylation of that residue impacts the function of the protein in order to determine if targeting upstream kinases and phosphatase could be a potential therapeutic strategy.

2. Mitochondrial dysfunction is present in numerous degenerative diseases, and as such, the PINK1 KO mouse model is a good general model organism to evaluate consequences of mitochondrial impairment. Therefore, it would be interesting to evaluate pathways associated with PINK1 in the NMR brain to examine if PINK1 is involved in promoting the healthy lifespan of the naked mole-rat.
3. Compare the changing levels of proteins and protein phosphorylation states that were seen in the brain of the NMR to the levels of proteins and protein phosphorylation in wild-type mice or in neurodegenerative animal models. Evaluating alterations in proteomes and phosphoproteomes with age within a species, such as rodents, may be key in discovering underlying mechanisms for the substantial difference in average lifespans within a species, such as between a mouse and a naked mole-rat.

4. NMRs reportedly have uncommonly high levels of oxidative stress, even at an early age compared to mice and these levels do not seem to be significantly altered over their lifespan. However it would be interesting to investigate whether individual proteins in the NMR brain have significant levels of oxidative modification.
## APPENDIX A

### DATA TO SUPPLEMENT TABLES AND FIGURES

Figure 4.2

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<th>p-NFM Spot Density</th>
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| WT average | 1.064 | 100 |
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| KO2        | 3130452 | 1717800 | 1.822 | 171.2 |
| KO3        | 3640788 | 2867175 | 1.270 | 119.3 |
| KO4        | 4689648 | 4029825 | 1.164 | 109.3 |
| KO5        | 3379536 | 2363025 | 1.430 | 134.4 |

| KO average | 1.383 | 130.0 |

| T-TEST | 0.0166 | 0.00605 | 0.0168 | 0.00909 | 0.0017 | 0.0091 |

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| WT average |             |             | 0.3622          | 100                                |
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| KO2        | 1603478      | 6557364     | 0.2445          | 67.50                              |
| KO3        | 1862208      | 6236028     | 0.2986          | 82.44                              |
| KO4        | 1189974      | 5058216     | 0.2353          | 64.94                              |
| KO5        | 674774       | 4547808     | 0.1484          | 40.96                              |

| KO average |             |             | 0.2583          | 71.31                              |

TTEST 0.036

Figure 4.4

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<td>CRMP2 Density</td>
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Unpaired t test

- **P value**
  - PCAD: 0.0371
  - MCI: 0.0489
  - AD: 0.0087

- **P value summary**
  - PCAD: *
  - MCI: *
  - AD: **

- **Significantly different? (P < 0.05)**
  - Yes
  - Yes
  - Yes

- **One- or two-tailed P value?**
  - Two-tailed
  - Two-tailed
  - Two-tailed

- **t, df**
  - PCAD: t=2.668 df=6
  - MCI: t=2.464 df=6
  - AD: t=3.824 df=6
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Unpaired t test

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P value summary

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### Figure 6.1.4

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<th>p-(S/T/Y)/MDH1</th>
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**2-3 average**

| 4-6               | 26105308          | 22387808     | 1.1661         |
| 4-6               | 20754607          | 20070612     | 1.0341         |
| 4-6               | 17815050          | 16167846     | 1.1019         |
| 4-6               | 21535826          | 18628516     | 1.1561         |

**4-6 average**

| 7-12              | 15132851          | 15639156     | 0.9676         |
| 7-12              | 19911379          | 20049768     | 0.9931         |
| 7-12              | 18341153          | 16362270     | 1.1209         |
| 7-12              | 20793016          | 17441028     | 1.1922         |

**7-12 average**

| 15-24             | 20203075          | 18940986     | 1.0666         |
| 15-24             | 23705433          | 18233750     | 1.3001         |
### Table: Bonferroni's multiple comparisons test

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<th>Age Group</th>
<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<tbody>
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<td>-0.03635 to 0.9457</td>
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<td>0.04211 to 1.024</td>
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<td>No</td>
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### Figure 6.2.3

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<th>Age Group (Years)</th>
<th>Normalized HSP70 Levels</th>
<th>Normalized HSP60 Phosphorylation</th>
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| 7-12 | 11.2367 | 12.98701 | 3.043172 |
|      | 28.618  | 17.546   | 29.3418   |
|      | 48.91094| 66.31009 | 58.77046  |
|      | 45.86103| 62.17523 | 55.10574  |
|      | 282.0846| 282.0846 | 23.08157  |
|      | 4.924471| 97.34139 | 60.84592  |
|      | 4.984894| 107.34139| 0.574018  |

| 15-24 | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |

| 15-24 | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |
|       | 1.3064 | 16.456 | 4.7044   |

209
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<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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Bonferroni's multiple comparisons test for HSP60

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<th>Significant?</th>
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<td>No</td>
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<td>&gt; 0.9999</td>
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<td>-73.87 to 75.72</td>
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<td>ns</td>
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### p-VDAC1

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<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
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<tbody>
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<td>-0.03835 to 0.3022</td>
<td>No</td>
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Bonferroni's multiple comparisons test of p-VDAC1

### p-VDAC2

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<th>Significant?</th>
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<th>Adjusted P Value</th>
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<td>0.2559</td>
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<td>2-3 years vs. 7-12 years</td>
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<td>2-3 years vs. 15-24 years</td>
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<td>0.08529 to 0.3791</td>
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<td>7-12 years vs. 15-24 years</td>
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Bonferroni's multiple comparisons test of p-VDAC2

### p-VDAC3

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<td>Tubulin Density</td>
<td>Normalized UCH / Tubulin</td>
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Figure 6.2.5
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### Dunnett's Multiple Comparisons Test

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<td>23857290</td>
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### Dunnett's multiple comparisons test

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<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<td>2-3 vs. 15-24</td>
<td>2-3 vs. 4-6</td>
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<td>2-3 vs. 4-6</td>
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<td>2-3 vs. 4-6</td>
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### Figure 6.2

**Normalized p-PI3K**

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<th>4-6 Years</th>
<th>7-12 Years</th>
<th>15-24 Years</th>
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<td>4-6 Years</td>
<td>7-12 Years</td>
<td>15-24 Years</td>
</tr>
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<td>648.</td>
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### Bonferroni's multiple comparisons test

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<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<td>2-3 vs. 15-24</td>
<td>2-3 vs. 4-6</td>
<td>2-3 vs. 7-12</td>
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<td>2-3 vs. 4-6</td>
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215
| 4-6 vs. 15-24 | 208.9 | 113.6 to 304.1 | Yes | **** | < 0.0001 |
| 7-12 vs. 15-24 | 44.21 | -46.01 to 134.4 | No | ns | > 0.9999 |

<table>
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<th>2-3 Years</th>
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<th>15-24 Years</th>
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<th>Significant?</th>
<th>Summary</th>
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<table>
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<td>4-6 vs. 15-24</td>
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Figure 6.2.8

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<tr>
<td>4-6 vs. 15-24</td>
<td>43.52</td>
<td>21.00 to 66.05</td>
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<td>7-12 vs. 15-24</td>
<td>27.24</td>
<td>4.714 to 49.76</td>
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</table>

2-3 Years | 4-6 Years | 7-12 Years | 15-24 Years
---|---|---|---
LC3-II/LC3-I | 87. | 80. | 105. | 82. |
| 94. | 76. | 104. | 83. |
| 102. | 106. | 84. | 80. |
| 95. | 85. | 103. | 79. |
| 108. | 95. | 84. | 100. |
| 113. | 111. | 103. | 90. |

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<th>Bonferroni's multiple comparisons test</th>
<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<tbody>
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<td>2-3 vs. 4-6</td>
<td>11.43</td>
<td>-7.067 to 29.93</td>
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<td>1.333</td>
<td>-16.31 to 18.97</td>
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<td>2-3 vs. 15-24</td>
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<td>-5.167 to 27.83</td>
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<tr>
<td>No</td>
<td>ns</td>
<td>Adjusted P Value</td>
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<td></td>
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<td>2-3 vs. 4-6</td>
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<td>2-3 vs. 7-12</td>
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<td>2-3 vs. 15-24</td>
<td>-6.724</td>
<td>-24.10 to 10.66</td>
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<td>4-6 vs. 7-12</td>
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<td>-18.43 to 16.33</td>
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<td>4-6 vs. 15-24</td>
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<td>-40.94 to -6.178</td>
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<td>7-12 vs. 15-24</td>
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<td>-39.89 to -5.126</td>
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<td>4-6</td>
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<td>7-12</td>
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<td>15-24</td>
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<tr>
<td>Treatment (between columns)</td>
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<td>Residual (within columns)</td>
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<td>Total</td>
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Tukey's multiple comparisons test

<table>
<thead>
<tr>
<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<tbody>
<tr>
<td>2-3 vs. 4-6</td>
<td>-52.34 to -3.647</td>
<td>Yes</td>
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<td>-63.22 to 48.06</td>
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<td>4-6 vs. 15-24</td>
<td>24.73 to 73.42</td>
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<td>25.36 to 74.05</td>
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Cofilin-1

<table>
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<th>Age Group</th>
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<th>7-12</th>
<th>15-24</th>
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ANOVA table

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<tr>
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<th>SS</th>
<th>DF</th>
<th>MS</th>
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Tukey's multiple comparisons test

<table>
<thead>
<tr>
<th>Mean Diff.</th>
<th>95% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<tbody>
<tr>
<td>2-3 vs. 4-6</td>
<td>31.94 to 62.42</td>
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<td>32.32 to 62.80</td>
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<td>0.3770 to 30.86</td>
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<td>0.3972 to 30.88</td>
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<td>-30.46 to 30.50</td>
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</table>
References


Grimes, K., Y. Chiao, M. L. Lindsey and R. Buffenstein (2012). "Cardiac function in an extraordinarily long-lived rodent, the naked mole-rat." Circulation 126.


Lemieux, N., B. Malfoy and G. L. Forrest (1993). "Human carbonyl reductase (CBR) localized to band 21q22.1 by high-resolution fluorescence in situ


Profile of Brain from PINK1 Knockout Mice: Insights into Mechanisms of Familial Parkinson Disease." J Neurochem.


Judy Carol Triplett was born in Frankfort, KY where she later attended Western Hills High School, graduating with honors. At the age of 18, Judy moved to Florida to pursue a career in the culinary arts, specializing in Caribbean cuisine. After working as a sous chef, restaurant manager and developing her own catering business, Judy enrolled at Saint Petersburg College in 2004 in Tarpon Springs, FL, earning her Associate of Arts degree in 2006. Subsequently, Judy enrolled at the University of South Florida in Tampa, FL where she earned her Bachelor of Science degree in Chemistry. In 2011, Judy moved back to her home state to pursue a doctoral degree in chemistry at the University of Kentucky (UK). Soon after enrollment, Judy joined a laboratory focused on studies in neurochemistry and neurodegenerative diseases under the aegis of Dr. D. Allan Butterfield.

Scientific Publications Stemming from this Dissertation Research


promoting healthspan involve neurite outgrowth and neurotransmission. *Manuscript submitted.*


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