Sex-Specific Behavioural Traits of the Sirtuin-3 Knockout Mouse Model

by

Elena Sidorova

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

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2019

Abstract

The NAD$^+$-dependent mitochondrial deacetylase sirtuin-3 (SIRT3) has been recognized to regulate a number of metabolic processes in peripheral tissues, however its role in the brain remains less well investigated. The results of this thesis show that sirtuin-3 displays a unique temporal and spatial pattern of mRNA and protein expression in the developing, adult, and aged brain, that encompasses both neurons and astrocytes. These data indicate sirtuin-3 is a potentially targetable product in the brain for pharmacological interventions. SIRT3 activity does influence brain function, as behavioural alterations were observed in both male and female Sirt3-KO mice. Interestingly, only female Sirt3-KO mice were found to display hyperlocomotor and exploratory behaviour in the open field, reduced anxiety-like behaviour in plus maze and light/dark paradigms, and impaired balance on the accelerated rotarod task. These observed phenotypes were also progressive across age. In addition, both male and female Sirt3-KO mice demonstrated hypersensitivity to low dose amphetamine treatment compared with wild-type animals, as increased locomotor responses and lowered anxiety responses were evident in the mutants but absent in wild-type mice. These behavioural alterations correlated with an upregulation of the mTORC1 signaling pathway in the cortex in the female Sirt3-KO mice, which could underlie the observed novelty-driven hyperactive phenotype. In summary, this thesis discloses an as-yet unrecognized role for sirtuin-3 in the regulation of behavioural activity, anxiety and motor functions that is sex-dependent and influenced by both age and environment.
Acknowledgments

First and foremost, I want to thank my supervisor Dr. James Eubanks for having me in your laboratory throughout both of my MSc and my PhD journeys. I can say with full confidence that I have learned so much from training in your lab. Thank you for all your expertise, guidance, and for being so understanding and patient when life got in the way. Thank you to my committee members: Dr. Les Buck, Dr. Zhengping Jia and Dr. Joanne Nash. I am very grateful for you taking the time to give me advice throughout my graduate studies. Dr. Joanne Nash, I also would like to extend my appreciation for being a supportive undergraduate Thesis supervisor, and for recommending me to Dr. Eubanks. A big gratitude to my parents, Larissa and Sergei for giving me such a beautiful life and for bringing me to Canada. I will never forget how hard you have worked for my brother and I to have a future in North America. To my grandfather Vasyl and my grandmother Ekaterina, thank you for raising me and instilling in me from a very young age that patience, hardwork and organization do pay off. My brother Sergei, sister-in-law Rosie, brother-in-law Demetri and parents-in-law Ted and Callie, thank you for your warm support and encouragement throughout my studies. I would like to say a big thank to my husband Peter for being such a big rock during my science journey, for enduring my all-over the place experiment schedule, my inadequacy with photoshop (for designing my diagrams much better than I could ever dream of) and for always being there whenever I needed you. To my daughter Kallie, thank you for all your warm smiles and hugs that melted away any stressful days in the lab. To the many faces who I have had the pleasure of knowing throughout my studies, it wouldn’t be the same without you all.
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Contributions

Elena Sidorova
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Rosa Summer
Compiled Table 1.4 for Chapter 1

Robert Wither
Contributed by assisting with running western blots for Chapter 3

Richard Logan
Contributed by assisting with subcutaneous amphetamine injections in mice for Chapter 6

Peter Darmos
Provided assistance with Figure compilation
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4E-BP1</td>
<td>factor-4E-binding protein</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>8-oxo-7,8-dihydroguanine</td>
</tr>
<tr>
<td>AceCS2</td>
<td>acetyl coenzyme A synthetase type 2</td>
</tr>
<tr>
<td>acetyl-CoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>AD</td>
<td>alzheimers disease</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPH</td>
<td>amphetamine</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>produce adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>BD</td>
<td>bipolar disease</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CGN</td>
<td>cerebellar granule neurons</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>COOH</td>
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<td>CoQ</td>
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<td>CoQH₂</td>
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<tr>
<td>CR</td>
<td>calorie restriction</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element-binding protein</td>
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Ct  critical threshold
CypD  cyclophilin D
cytc  cytochrome C
DA  dopamine
DMEM  dulbecco’s Modified Eagle Medium
DNA  deoxyribonucleic acid
DRP1  dynamin-related protein-dependent
DSM  diagnostic and Statistical Manual of Mental Disorders
E18  embryonic day 18
EDTA  ethylenediaminetetraacetic acid
EPM  elevated plus maze
ERK  extracellular signal–regulated kinases
ERRE  estrogen-related receptor (ERR)-binding element
ERRα  estrogen-related receptor α
ETC  electron transport chain
FAD  flavin adenine dinucleotide
FBS  fetal Bovine serum
FOXO  forkhead box O
$g$  relative centrifugal force
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GDH  glutamate dehydrogenase
GFAP  glial fibrillary acidic protein
Glu  glutamitergic
GR  glutathione reductase
GSH-Px  glutathione peroxidase
GSH-Px  glutathione peroxidase
GSK-3β  glycogen synthase kinase 3β
GSSG  oxidized glutathione
GTP  guanosine triphosphate
H⁺  proton
H₂O  water
H₂O₂  hydrogen peroxide
HAT  histone acetyltransferases
HBSS  hank’s Balanced Salt Solution
HD  huntington’s Disease
HDAC  histone deacetylases
HMGCS2  3-hydroxy-3-methylglutaryl-CoA synthase
HPRT1  hypoxanthine-guanine phosphoribosyltransferase
HRP  horseradish peroxidase
HS  horse serum
IDH2  isocitrate dehydrogenase
IMM  inner mitochondrial membrane
IMS  inter-membrane space
kDa  kilodalton
KO  knock out
LD  light/dark test
LCAD  long-chain acyl CoA dehydrogenase
LDH  lactate dehydrogenase
LRP130  leucine-rich protein 130
MAP2  microtubule-associated protein 2
MAPK  mitogen-activated protein kinase
MCAO  middle cerebral artery occlusion
MCT1  monocarboxylate transporters 1
MD  mitochondrial disease
<table>
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<td>MECP2</td>
<td>methyl CpG binding protein 2</td>
</tr>
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<td>mg</td>
<td>milligram</td>
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<td>MMP</td>
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<td>manganese superoxide dismutase</td>
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<td>mitochondrial processing peptidase</td>
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<td>mitochondrial transition pore</td>
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<td>MSOD₂</td>
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<td>O₂</td>
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<td>OAADPr</td>
<td>2'-O-acetyl-ADP-ribose</td>
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<tr>
<td>OF</td>
<td>open Field</td>
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<td>oxygen/glucose deprivation</td>
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<td>8-oxoguanine-DNA glycosylase 1</td>
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<td>OH−</td>
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<td>outer mitochondrial membrane</td>
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<td>optic atrophy 1</td>
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<td>polymerase Chain Reaction</td>
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<td>peroxisome proliferator-activated receptor gamma coactivator 1-α</td>
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<td>silent information regulator</td>
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<td>silent information regulator 2</td>
</tr>
</tbody>
</table>
SiRNA  small interfering RNA
SIRT  sirtuin
Sirt1  sirtuin-1 murine mRNA
SIRT1  sirtuin-1 murine protein
Sirt2  sirtuin-2 murine mRNA
SIRT2  sirtuin-2 murine protein
Sirt3  sirtuin-3 murine mRNA
SIRT3  sirtuin-3 human mRNA
Sirt3  sirtuin-3 murine protein
Sirt4  sirtuin-4 murine mRNA
SIRT4  sirtuin-4 murine protein
Sirt5  sirtuin-5 murine mRNA
SIRT5  sirtuin-5 murine Protein
Sirt6  sirtuin-6 murine mRNA
SIRT6  sirtuin-6 murine Protein
Sirt7  sirtuin-7 murine mRNA
SIRT7  sirtuin-7 murine Protein
SNP  single nucleotide polymorphism
SZ  schizophrenia
TCA  tricarboxylic acid cycle
TNF-α  tumor necrosis factor
TOM20  translocase of outer membrane 20
Tris  tris(hydroxymethyl)aminomethane
TRX  thioredoxins
Trx2  thioredoxin 2
TrxR  thioredoxin reductase
TSC  tuberous sclerosis
UCP  uncoupling protein
ULK1  UNC11like kinase 1
VLCAD  very long-chain acyl CoA dehydrogenase
WT  wild-type
$\Delta \Psi_m$  mitochondrial membrane potential
$\mu g$  microgram
$\mu L$  microliter
$\mu M$  micromole
“The history of science, like the history of all human ideas, is a history of irresponsible dreams, of obstinacy, and of error. But science is one of the very few human activities – perhaps the only one – in which errors are systematically criticized and fairly often, in time, corrected. This is why we can say that, in science, we often learn from our mistakes, and why we can speak clearly and sensibly about making progress there.”

—Sir Karl Popper
General introduction

1.1 History of silent information regulators

The complex functioning of an organism depends on the dynamic and cooperative activities of numerous cellular processes. Protein post-translational modifications (PTM) play a fundamental role in cell function through regulating their activity, localization and interactions with other substrates (Moore & Free, 1985; Freiman & Tjian, 2003; Stram & Payne, 2016). One of the most common PTMs is reversible lysine acetylation, which is the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the amino (NH$_2$-) group of a lysine residue. This mechanism was among the first covalent modification of histones to be described (Allfrey et al., 1964), however, lysine acetylation is also known to occur in many other non-histone protein targets (Freiman & Tjian, 2003). The alternation between the acetylated and deacetylated state of a protein can modify its activity level, protein–protein interactions, subcellular localization and stability. The dynamic balance of addition or removal of the acetyl group is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively.

Transcriptional silencing is associated with an increase of HDAC activity on histones, which results in a tightly wrapped deoxyribonucleic acid (DNA) around the histone cores. This compact structure is referred to as heterochromatin and because of its tightly condensed form it limits the accessibility and therefore binding of transcription factors to the DNA (Delcuve et al., 2009). On the other hand, an open and an active state is called euchromatin, which is associated with an increase in transcriptional activity. Therefore, the actions of HATs and HDACs play an imperative role in gene expression regulation. The targets of these enzymes also expand beyond histones and
involve other cellular factors that play a role in transcription, signalling, and metabolism (Freiman & Tjian, 2003; Lu et al., 2009). Advances in understanding the significance of reversible lysine acetylation have heightened the search for factors that could modulate this process. Mammalian HDACs are categorized into four classes (I, IIa, IIb, III and IV), which are further divided into two sub-types the “zinc-dependent” and the “nicotinamide-adenine-dinucleotide (NAD⁺)–dependent” deacetylases (Table 1.1), all of which differ in their structure, substrates, and subcellular localization (Seto & Yoshida, 2014). This thesis will focus specifically on class III NAD⁺-dependent HDACs.

A genetic screen in budding yeast (Saccharomyces cerevisiae) identified four genes that play a role in transcription silencing referred to as Silent Information Regulators (Sir1-4) (Ivy et al., 1986). DNA contains sequences that are associated with the silencing mechanism that recruits the SIR complex (Bose et al., 2004). The SIR complex binds to the histones, which results in a repressive heterochromatin structure and therefore hinders the binding of other proteins, such as transcription factors, which ultimately results in transcriptional silencing. Sir2 is the only catalytic member of the SIR complex, as it deacetylates histones through a NAD⁺-dependent mechanism (Imai et al., 2000). Specifically, Sir2 cleaves the glycosidic bond of the NAD⁺ molecule into nicotinamide (NAM) and 2’-O-acetyl-ADP-ribose (OAADPr) resulting in a deacetylation of a single lysine (Borra et al., 2004) (Figure 1.1). Sir2 deacetylates the NH₂-ermal tails of histones at the silent mating loci (Rine & Herskowitz, 1987) and telomeres (Gottschling et al., 1990; Xu et al., 2007), which therefore creates a binding site for the remaining SIR complex to attach to these structures (Hecht et al., 1995; Liou et al., 2005).
Table 1.1: Classification of Histone Deacetylases (HDAC)

<table>
<thead>
<tr>
<th>Class</th>
<th>Isoform</th>
<th>Cofactor</th>
<th>Cellular Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HDAC1</td>
<td>Zinc</td>
<td>Nucleus</td>
</tr>
<tr>
<td></td>
<td>HDAC2</td>
<td></td>
<td>Nucleus</td>
</tr>
<tr>
<td></td>
<td>HDAC3</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC3</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC8</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td>IIa</td>
<td>HDAC4</td>
<td>Zinc</td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC5</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC7</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC9</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td>IIb</td>
<td>HDAC6</td>
<td>Zinc</td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>HDAC10</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td>III</td>
<td>SIRT1</td>
<td>NAD⁺</td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>SIRT2</td>
<td></td>
<td>Nucleus, Cytoplasm</td>
</tr>
<tr>
<td></td>
<td>SIRT3</td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>SIRT4</td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>SIRT5</td>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td></td>
<td>SIRT6</td>
<td></td>
<td>Chromatin</td>
</tr>
<tr>
<td></td>
<td>SIRT7</td>
<td></td>
<td>Nucleolus</td>
</tr>
<tr>
<td>IV</td>
<td>HDAC11</td>
<td>Zinc</td>
<td>Nucleus</td>
</tr>
</tbody>
</table>
Sir2 homologues have now been identified in many organisms ranging from bacteria to humans (Brachmann et al., 1995). Converging studies show that SIR2 and its homologues may play a role in longevity (Imai et al., 2001; Dali-Youcef et al., 2007; Wierman & Smith, 2014). Gottlieb et al (1989) was the first report that suggested a role for SIR2 as a longevity factor (Gottlieb & Esposito, 1989). This study showed that SIR2 deacetylates and therefore suppresses ribosomal DNA (rDNA), which therefore inhibits rDNA homologous recombination in yeast. This inhibition results in attenuation of production of extrachromosomal rDNA circles – an accumulation of which cause accelerated senescence in ageing mother cells. Other reports have also supported the function of SIR2 and its homologues as longevity-promoting regulators of lifespan in Saccharomyces cerevisiae (Kaeberlein et al., 1999; Kim et al., 1999; Wood et al., 2004) C. elegans (Tissenbaum & Guarente, 2001) and Drosophila melanogaster (Wood et al., 2004). However, the role of SIR2 in promoting longevity has also been challenged (Burnett et al., 2011; Viswanathan & Guarente, 2011), and therefore more work remains to address whether sirtuins can directly regulate longevity. In summary, the SIR2 family regulates transcriptional silencing that feeds forward into diverse number of cellular processes.

1.2 Mammalian sirtuins

Seven descendants from the common ancestral yeast gene Sir2 have been identified and classified as sirtuins1-7 (Brachmann et al., 1995; Frye, 1999; Finkel et al., 2009;). Sirtuins are categorized by their highly conserved catalytic core and the NAD⁺ binding site of approximately 260 amino acids, but are distinct in their NH₂- and carboxy (-COOH) termini (Landry et al., 2000; Min et al., 2001; Marmorstein, 2004). Functionally, sirtuins are NAD⁺-dependent protein deacetylases (Imai & Guarente, 2010) (Figure 1.1), however they are now also known to possess other enzymatic functions (Laroche, & Renauld, 2001; Schwer et al., 2002; Liszt et al., 2005; Vakhrusheva et al.,
2008; Imai & Guarente, 2010; Du et al., 2011; Peng et al., 2011; Jiang et al., 2013; Bao et al., 2014; Mathias et al., 2014; Tan et al., 2014) (Table 1.2), however the physiological implications of all these processes are not fully understood. Sirtuins also differ in their subcellular localization and some are localized to more than one sub-cellular structure where they target a variety of substrates (Table 1.3).
Figure 1.1: Deacetylation. The acetyl-lysine residues of the target protein serve as substrates for sirtuin deacetylation. Sirtuins remove an acetyl group from the target molecule. This reaction consumes NAD+ by cleaving it into nicotinamide and ADP-ribose. The acetyl group is then added to the ADP-ribose, which then forms 2’-O-acetyl-ADP-ribose. The final products of the deacetylation reaction are: nicotinamide, 2’-O-acetyl-ADP-ribose, and a deacetylated substrate.
Table 1.2: Mechanisms of Action for Sirtuin Family Members

<table>
<thead>
<tr>
<th>Sirtuin</th>
<th>Catalytic Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>Deacetylase (Michishita et al., 2005),</td>
</tr>
<tr>
<td></td>
<td>Decrotonylase (Bao et al., 2014)</td>
</tr>
<tr>
<td>SIRT2</td>
<td>Deacetylase (North et al., 2003) (Perrod et al., 2001),</td>
</tr>
<tr>
<td></td>
<td>Decrotonylase (Bao et al., 2014)</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Deacetylase (Schwer et al., 2002),</td>
</tr>
<tr>
<td></td>
<td>Decrotonylase (Bao et al., 2014)</td>
</tr>
<tr>
<td>SIRT4</td>
<td>ADP-ribosyltransferase (Ahuja et al., 2007),</td>
</tr>
<tr>
<td></td>
<td>Lipoamidase (Mathias et al., 2014)</td>
</tr>
<tr>
<td>SIRT5</td>
<td>Deacetylase (Schlicker et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Demalonylase (Du et al., 2011) (Peng et al., 2011),</td>
</tr>
<tr>
<td></td>
<td>Desuccinylase (Du et al., 2011) (Peng et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Glutarylase (Tan et al., 2014)</td>
</tr>
<tr>
<td>SIRT6</td>
<td>ADP-ribosyltransferase (Liszt et al., 2005);</td>
</tr>
<tr>
<td></td>
<td>Deacetylase (Michishita et al., 2008),</td>
</tr>
<tr>
<td></td>
<td>Acylate (Jiang et al., 2013)</td>
</tr>
<tr>
<td>SIRT7</td>
<td>Deacetylase (Vakhrysheva et al., 2008)</td>
</tr>
</tbody>
</table>
Table 1.3: Sirtuin cellular targets

<table>
<thead>
<tr>
<th>Sirtuin</th>
<th>Localization</th>
<th>Selected Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT1</td>
<td>Nucleus, Cytoplasm</td>
<td>P53, PGC1-α, p300, FOXO, NF-kB</td>
<td>Bouras et al., 2005; Giannakou &amp; Partridge, 2004; Rodgers et al., 2005; Vaquero et al., 2004; Vaziri et al., 2001; Yeung et al., 2004</td>
</tr>
<tr>
<td>SIRT2</td>
<td>Cytoplasm, Nucleus</td>
<td>α-tubulin, H4, p53, FOXO</td>
<td>North, Marshall, Borra, Denu, &amp; Verdin, 2003; Peck et al., 2010; Vaquero et al., 2006; F. Wang, Nguyen, Qin, &amp; Tong, 2007</td>
</tr>
<tr>
<td>SIRT3</td>
<td>Mitochondria Nucleus</td>
<td>AccCS2, IDH2, MnSOD2, GDH, Complex I, PGC1α, ATPase</td>
<td>Ahn et al., 2008; Bao et al., 2014; Cimen et al., 2010; Schwer et al., 2002; T. Shi, Wang, Stieren, &amp; Tong, 2005; Someya et al., 2010; Tao et al., 2010; Y. T. Wu, Lee, Liao, &amp; Wei, 2013; W. Yu, Dittenhafer-Reed, &amp; Denu, 2012</td>
</tr>
<tr>
<td>SIRT4</td>
<td>Mitochondria</td>
<td>GDH, ANT, PDH</td>
<td>Haigis et al., 2006; Ho et al., 2013; Mathias et al., 2014</td>
</tr>
<tr>
<td>SIRT5</td>
<td>Mitochondria</td>
<td>CPSI, PK, IDH2, CytC</td>
<td>Nakagawa, Lomb, Haigis, &amp; Guarente, 2009; Rardin et al., 2013; Schlicker et al., 2008; Xiangyun et al., 2017; Zhou et al., 2016</td>
</tr>
<tr>
<td>SIRT6</td>
<td>Chromatin</td>
<td>PARP1, TNF-α, H3</td>
<td>H. Jiang et al., 2013; Z. Xu et al., 2015; B. Yang, Zwaans, Eckersdorff, &amp; Lombard, 2009</td>
</tr>
<tr>
<td>SIRT7</td>
<td>Nucleolus</td>
<td>p53, RNA polymerase I</td>
<td>Ford et al., 2006; Vakhrusheva et al., 2008</td>
</tr>
</tbody>
</table>
Like the ancestral SIR2, sirtuins SIRT1, SIRT6 and SIRT7 are primarily nuclear (Michishita et al., 2005), however SIRT3-5 are localized in mitochondria (Onyango et al., 2002; Ahuja et al., 2007; Nakamura et al., 2008) and SIRT2 is predominately cytoplasmic (Perrod et al., 2001; North et al., 2003). Given their dependence on intracellular NAD+ levels (Imai & Guarente, 2010), sirtuins are now recognized as key rheostats that govern the homeostatic balance of cellular environment, and play a role in a variety of biological processes including energy metabolism, cellular stress response, cytoprotection, apoptosis, metabolism and inflammation (Srivastava & Haigis, 2011; Houtkooper et al., 2012; Carafa et al., 2016; Mei et al., 2016; Vachharajani et al., 2016). The similarity of the sirtuin family to the longevity enhancing Sir2 gene has also spawned great interest in their potential role as mammalian longevity and neuroprotective factors (Outeiro et al., 2008; Guarente, 2011; Srivastava & Haigis, 2011).

1.2.1 Nuclear sirtuins

1.2.1.1 Sirtuin-1

SIRT1 is predominately a nuclear enzyme, however it can cross between cytosol and nucleoplasm under specific conditions (Michishita et al., 2005; Jin et al., 2007). SIRT1 enzymatic activity is predominately through deacetylation (Michishita et al., 2005), whereas one study also reported SIRT1 to have decrotonylase activity (Bao et al., 2014), which is the removal of a crotonyl from a protein, a modification that has been showed to regulate protein synthesis, folding and ubiquitin-dependent degradation (Sun et al., 2017). Sirtuin-1 is the most studied member of the mammalian sirtuins and holds the closest evolutionary homology to yeast SIR2 (Frye, 2000). Sirtuin-1 was first shown to promote chromatin silencing and transcriptional repression through histone deacetylation (Vaquero et al., 2004; T. Zhang & Kraus, 2010), however a large number of non-histone substrates for SIRT1 have now been identified (Elliott & Jirousek, 2008; Guarente, 2011; Haigis & Sinclair, 2010; Houtkooper et al., 2012). The regulation of these substrates feed forward
to a number of cellular processes such as stress resistance, mitochondrial biogenesis, apoptosis, insulin secretion, DNA repair and cell cycle regulation (Rahman & Islam, 2011). Studies in the brain showed that SIRT1 regulates neuronal differentiation (Hisahara et al., 2008; Prozorovski et al., 2008), axogenesis (Li et al., 2013), neurite outgrowth (Sugino et al., 2010) and cognitive functions (Michán et al., 2010), in addition to other roles in the central nervous system (CNS) reviewed in (Hisahara at al., 2005; Herskovits & Guarente, 2014; Ng at al., 2015).

1.2.1.2 Sirtuin-6

SIRT6 is enriched in heterochromatin, and is primarily characterized as a deacetylase (Michishita et al., 2008), however it was also shown to have very weak ADP-ribosylation (Liszt et al., 2005) and acylate activity (Jiang et al., 2013), which are the addition of an ADP-ribose and an acyl group to a protein, respectively. SIRT6 regulates DNA repair (Xu et al., 2015), plays both proinflammatory (Feng et al., 2018) and anti-inflammatory roles (Lappas, 2012), stress responses and genomic stability (Yang et al., 2009; Kaluski et al., 2017). Of all sirtuins, global sirtuin-6 deficiency in mice results in the most salient phenotype which include premature aging, DNA damage, and death within 4 weeks of birth (Mostoslavsky et al., 2006). Furthermore, a brain-specific sirtuin-6-knock out (KO) mouse model showed accelerated aging, accumulated DNA damage, as well as increased apoptosis (Kaluski et al., 2017). Taken together, these studies suggest that sirtuin-6 may play a role in the aging process. Furthermore, conflicting studies showed that over-expression of sirtuin-6 in the hippocampus impaired the formation of long-term contextual fear memory (Yin et al., 2016), as well as its over-expression decreased SH-SY5Y cell viability under hydrogen peroxide (H2O2)-induced oxidative stress (Shao et al., 2016). Therefore, more studies are required to assess the role of sirtuin-6 in the brain as it may be context-dependent.
1.2.1.3 **Sirtuin-7**

SIRT7 resides in the nucleolus, where it exhibits deacetylase activity (Vakhrusheva et al., 2008) and is the least characterized sirtuin. SIRT7 activates RNA polymerase I (Ford et al., 2006) and tumor suppressor protein 53 (p53) (Vakhrusheva et al., 2008), suggesting that it plays a role in DNA replication and apoptosis, respectively. Furthermore, immediately after a fear conditioning paradigm, *Sirt7*-KO mice displayed similar levels of freezing behaviour as control mice, however this stalling behaviour was significantly decreased within 24 hours in the mutant mice but not in the WT mice (Islam et al., 2018). Thus, these results suggest that sirtuin-7 may play a role in memory consolidation. Given that this was the only study that assessed sirtuin-7 role in the brain, further studies are necessary to understand the role of this enzyme in the CNS.

1.2.2 **Cytoplasmic sirtuin**

1.2.2.1 **Sirtuin-2**

SIRT2 mechanism of action is primarily through deacetylation (North et al., 2003), however decrotonylase activity has also been identified (Bao et al., 2014). SIRT2 resides primarily in the cytoplasm, where it was first shown to co-localize with microtubules and deacetylates α-tubulin (North et al., 2003), it also translocates to the nucleus during G2/Mitosis (Vaquero et al., 2006). These studies suggest that sirtuin-2 plays a role in cell motility and cell division. Other substrates of SIRT2 include transcription factors such as, forkhead box O3 (FOXO3) (Wang et al., 2007) and FOXO1 (Jing et al., 2007), because these factors regulate number of pathways such as cell cycle, apoptosis, and anti-oxidative defenses (Maiese, 2015), it is conceivable that SIRT2 may also be involved in such processes. In the brain, SIRT2 negatively regulates axonal and neurite outgrowth (Pandithage et al., 2008), enhances axonal degeneration in a Wallerian degeneration mouse model (Suzuki & Koike, 2007), and over-expression of sirtuin-2 results in cerebellar
granule cell death induced by low potassium treatment (Pfister et al., 2008). These data suggest that SIRT2 may be deleterious to neurons. However, a recent study showed that SIRT2 inhibition decreases cell proliferation and neuroblast differentiation in the mouse dentate gyrus (Jung et al., 2016). Taken together, these results suggest that SIRT2 role in the brain may be role and context specific.

1.2.3 Mitochondrial sirtuins

1.2.3.1 Sirtuin-3

SIRT3 is a nuclear encoded protein, however following translation it is targeted into the mitochondrial matrix (Onyango et al., 2002). In the matrix, SIRT3 NH2-terminus is cleaved by mitochondrial processing peptidase (MPP) and by mitochondrial intermediate peptidase (MIPEP) (Kobayashi et al., 2017), to generate an enzymatically active NAD⁺-dependent deacetylase (Schwer et al., 2002). Although SIRT3 was shown to be predominately a mitochondrial enzyme (Cooper & Spelbrink, 2008), contradicting reports have argued that SIRT3 may also reside and regulate substrates in the cytoplasm and the nucleus (Scher et al., 2007; Nakamura et al., 2008; Sundaresan et al., 2008; Iwahara et al., 2012). A number of studies have now identified several downstream factors that are regulated by SIRT3 and are involved in mitochondrial metabolism, ATP synthesis, and other biological processes essential for normal mitochondrial function (Guarente, 2011; Bause & Haigis, 2013; Salvatori et al., 2017). Although informative, most of our knowledge on sirtuin-3 stems from reports in non-neuronal cells, and the consequences of the interactions between SIRT3 and its targets in the CNS is just beginning to emerge. To date, studies have shown that elevating SIRT3 levels, or increasing its catalytic activity, has neuroprotective effects in a number of systems (Shulyakova et al., 2014; Zhang et al., 2016; Gleave et al., 2017; Qu et al., 2017), however, studies have also been emerging that show contrasting results; over-expression of SIRT3 in cerebellar granular neurons resulted in neuronal cell death (Pfister et al.,
and its ablation induces neuroprotection (Novgorodov et al., 2016; Verma et al., 2018). A detailed description of sirtuin-3 function will be discussed in later sections of this thesis.

1.2.3.2 Sirtuin-4

SIRT4 is also targeted to the mitochondrial matrix, where it is also proteolytically processed at the NH$_2$-terminus (Ahuja et al., 2007). SIRT4 primary function is ADP-ribosylation (Ahuja et al., 2007), however it can also act as a lipoamidase (Mathias et al., 2014), which is the removal of a lipoyl moiety from a protein. In the periphery, SIRT4 attenuates fatty acid metabolism in hepatocytes and myotubes, (Nasrin et al., 2010), downregulates insulin secretion in pancreatic β-cells (Haigis et al., 2006; Ahuja et al., 2007), represses tumour proliferation in lymphoma cell lines (Jeong et al., 2014) and protects cardiomyoblasts from apoptosis (Liu et al., 2013). Glutamate dehydrogenase (GDH) in pancreatic β-cells was the first target for SIRT4 to be identified (Haigis et al., 2006), GDH is a mitochondrial enzyme that converts glutamate to α-ketoglutarate and feeds the tricarboxylic acid (TCA) cycle to produce adenosine triphosphate (ATP) (Smith et al., 2017). Through ADP-ribosylation, SIRT4 inactivates GDH, which therefore results in a decrease of ATP levels. Furthermore, over-expression of sirtuin-4 in CTX8 cells, a radial glial cell line, drives cells away from astroglial phenotype (Komlos et al., 2013). These results suggest that SIRT4 may regulate the fate of brain development. In addition, Sirt4-KO hippocampal neurons were found to be more susceptible to excitotoxic kainic treatment (Shih et al., 2014). Shih et al. (2014) found that loss of SIRT4 results in attenuation of ATP, whereas the opposite was observed in peripheral tissues (Haigis et al., 2006), therefore further studies are required to understand the role of SIRT4 in the brain.
1.2.3.3 Sirtuin-5

SIRT5 is also translocated to the mitochondria (Michishita et al., 2005; Nakamura et al., 2008; Schlicker et al., 2008). Relative to SIRT1 and SIRT3, SIRT5 has weak deacetylase activity (Du et al., 2011), however strong desuccinylase (Du et al., 2011; Peng et al., 2011), demalonylase (Du et al., 2011; Peng et al., 2011), and deglutarylase (Tan et al., 2014) activities are observed. SIRT5 was first shown to deacetylate and activate carbamoyl phosphate synthase 1, an enzyme catalyzing the first step of the urea cycle for ammonia disposal (Nakagawa et al., 2009). A recent study showed that SIRT5 desuccinylates a large number of metabolic enzymes in liver mitochondria that are involved in the TCA cycle, and fatty acid metabolism (Rardin et al., 2013). These studies suggest that SIRT5 plays an important role in regulation of metabolic processes. Indeed, metabolic dysfunction is evident in Sirt5-KO mice, as they show dysregulated fatty acid metabolism in the liver (Rardin et al., 2013). In the brain, Sirt5-KO mice showed severe nigrostriatal dopaminergic degeneration following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment – a parkinsonian agent (Liu et al., 2015). The authors observed a larger decrease of manganese superoxide dismutase (MnSOD), a mitochondria-specific antioxidant enzyme, after MPTP induction. Collectively these results suggest that sirtuin-5 may play a metabolic and ROS regulatory role in the brain, however more studies are required to establish the role of SIRT5 in the CNS.

1.3 Mitochondrial sirtuins and the brain

Perturbations in mitochondrial homeostasis is considered to play a role in pathogenesis of a number of neurological and neuropsychiatric conditions (Martin, 2012; Morris & Berk, 2015), thus mitochondrial sirtuins are uniquely situated to regulate a number of molecular cascades in the CNS. Mitochondrial homeostasis is largely dependent on PTM (Stram & Payne, 2016), indeed one of the most common mitochondrial PTM is reversible lysine acetylation, and over 65% of the
mitochondrial factors are acetylated at nearly 2,200 sites (Hebert et al., 2013). Sirtuin-3 is the primary sirtuin deacetylase in mitochondria, as Sirt3-KO mice display globally elevated mitochondrial acetylation levels in liver, while Sirt4- or Sirt5-KO mice show no changes in mitochondrial proteome acetylation (Lombard et al., 2007). Moreover, a hyperacetylation of mitochondrial proteins was observed in myocardial tissue following infarction, and this change coincided with a downregulation of SIRT3 expression with both SIRT4 and SIRT5 expression levels being unaffected (Parodi-Rullan et al., 2012).

Furthermore, genomic association studies reported polymorphisms in the human SIRT3 gene, which may be linked to increased lifespan (Rose et al., 2003; Bellizzi et al., 2005). Hirschey et al (2011) also described a single nucleotide polymorphism in the human SIRT3 gene which encodes a point-mutation in the SIRT3 protein enzymatic activity that results in an age-related metabolic syndrome phenotype. Interestingly, the SIRT3 gene also resides on chromosome 11, a region that contains a number of longevity-associated genes such as Insulin-like Growth Factor 2, Proinsulin, and Tyrosine Hydroxylase (Rose et al., 2003). Because sirtuin-3 has been linked to longevity and it is the primary mitochondrial deacetylase, this spawned great interest in its potential role as a neuroprotective factor (Outeiro et al., 2008; Guarente, 2011; Srivastava & Haigis, 2011). It is now clear that sirtuin-3 plays a major role in regulating a host of mitochondrial molecular cascades involved in both normal and pathophysiological processes by regulating the acetylation state of the mitochondrial proteome (reviewed in (Outeiro et al., 2008; Giralt & Villarroya, 2012; Bause & Haigis, 2013; Lombard & Zwaans, 2014; McDonnell et al., 2015; Ansari et al., 2017). Given that sirtuin-3 is a regulator of mitochondrial homeostasis and that mitochondrial dysfunction underlies the pathogenesis of neurodegenerative and neuropsychiatric diseases, the idea that sirtuin-3 may be a bona-fide therapeutic target in neurodegenerative diseases arose. The rest of this thesis will focus on sirtuin-3 and its role in the brain.
1.4 Mitochondria

The mitochondrion is a cellular organelle thought to have evolved from a bacterial progenitor (Lang et al., 1999; Gray, 2012). Although there are competitive theories on the origins of mitochondria, the most widely accepted is the endosymbiotic theory (Margulis & Chapman, 1998), where the mitochondria are hypothesized to have evolved from free-living bacteria, that lived in symbiosis within a primitive eukaryotic host (Dover & Doolittle, 1980). This endosymbiotic theory is supported by the fact that mitochondria contain their own DNA and translational machinery, that is unique from that of the cytosol (Clayton, 1992). It is speculated that during the eukaryotic evolution, mitochondria evolved as organelles through an interplay between the deletion of the bacterial genome and transfer of nuclear DNA of the eukaryote to the forming mitochondria (Gray et al., 1999). Mitochondria take part in a variety of cellular functions which include, ATP production (Devin & Rigoulet, 2007), free radical formation and antioxidative defense system (Murphy, 2009; Kalogeris et al., 2014), metabolism (Vakifahmetoglu-Norberg & Norberg, 2017), apoptosis (Robertson, 2014; Vakifahmetoglu-Norberg et al., 2017), fatty acid oxidation (Bartlett & Eaton, 2004), calcium (Ca^{2+}) homeostasis (Vandecasteele et al., 2001) and cell signaling (Brookes et al., 2002; Finkel, 2011; Tait & Green, 2012).

1.4.1 Mitochondria structure

Mitochondria are double-membrane organelles consisting of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), which define two compartments, the inter-membrane (IMS) space and the matrix – both of which have distinct proteome environments (Sherratt, 1991; Kuhlbrandt, 2015). The OMM is a phospholipid bilayer that consists of protein structures called porins rendering the membrane permeable, where the IMM is largely impermeable and is folded into lamellae called cristae. The matrix is composed of a large number of factors such as: inorganic ions, organic molecules, ribosomes and mitochondrial
DNA (mtDNA). The mtDNA is a circular, double stranded DNA molecule of approximately 16.6 kB (kilo-base).

The mtDNA encodes a total of 37 genes that code for 2 rRNA, 22 transfer RNA and 13 polypeptides (Clayton, 1992; Taanman, 1999). Thus, more than 99% of mitochondrial proteins are encoded in the nuclear DNA (nDNA), which are then translated in the cytoplasm and further translocated to their respective mitochondrial compartment (Kang et al., 2017). The 13 polypeptides that are encoded by the mtDNA are designated as some of the components of the electron transport chain (ETC) (Clayton, 1992; Kuhlbrandt, 2015). The ETC in the mitochondrion consists of a series of protein complexes required for oxidative phosphorylation (OXPHOS), which combines electron transport with cell respiration to produce ATP. The components of the ETC consist of 4 membrane-bound complexes in the IMM (Complex I-IV). These transmembrane structures are connected by electron carriers in the IMM (ubiquionone coenzyme Q10, CoQ) and in the IMS (cytochrome c) (See Figure 1.2 and section 1.4.2 below for more detail). Complex I (NADH dehydrogenase, ubiquinone oxidoreductase) is a large multi-protein complex consisting of 45 sub-units, 7 of which are mtDNA-transcribed (Ryan & Hoogenraad, 2007). Complex II (succinate dehydrogenase) consists of 4 nDNA encoded protein subunits. Complex III (ubiquinol-cytochrome c oxidoreductase) consists of 11 subunits, cytochrome b is mtDNA-encoded and other 10 subunits are nDNA encoded (Ryan & Hoogenraad, 2007). Complex IV (cytochrome c oxidase) consists of 13 subunits, 3 are mtDNA-encoded and the remaining 10 are nDNA encoded. Complex V (ATP Synthase) consists of 16 subunits where 2 of them (ATP6 and ATP8) are mtDNA-encoded and the remaining are nDNA encoded (Ryan & Hoogenraad, 2007).
Figure 1.2: **Oxidative phosphorylation.** NADH₂ and FADH₂ are generated during glycolysis and the TCA cycle, transfer their electrons to Complex I and II, respectively. In the process, they are oxidized back to NAD⁺ and FAD. The electrons from NADH₂ and FADH₂ are passed down the ETC. The electrons are then transferred to and reduce Q, Complex III, cytochrome c, Complex IV and then to a terminal electron acceptor O₂ that results in the production of H₂O. The energy that the electrons release during the REDOX process is then used to pump H⁺ by complexes I, III, and IV across the membrane to the IMS. The H⁺ flow into the matrix through ATP synthase, which then catalyzes the addition of a P_i to ADP, producing ATP. ADP, adenosine diphosphate; ATP, adenosine triphosphate; H⁺, hydrogen; H₂O, water; P_i, phosphate; TCA, tricarboxylic acid cycle.
1.4.2 Mitochondrial ATP production

The brain has high-energy demand due to the need to maintain electrophysiological activity and cell signaling through sustaining transmembrane ion gradients, neurotransmitter cycling, and structural integrity (Kety 1957; Rolfe & Brown, 1997; Lenard & Berthoud, 2008; Herculano-Houzel, 2011; Mergenthaler et al., 2013; Bauernfeind et al., 2014). Therefore, processes regulating both ATP synthesis and ATP consumption in the brain play a fundamental role in physiological and pathophysiological conditions.

The mammalian brain relies on the bioavailability of glucose as its main source of energy, however fatty acids can also be utilized (Lenard & Berthoud, 2008; Freitas et al., 2017). Acetyl-CoA is the metabolic intermediate derived from glucose breakdown through glycolysis, and fatty acids through β-oxidation (L. Shi & Tu, 2015). Acetyl-CoA then enters the TCA cycle – a critical metabolic pathway that integrates carbohydrate and fatty acid metabolism. As acetyl-CoA is oxidized via TCA, the electrons removed the intermediate metabolic products are then used to reduce flavin adenine dinucleotide (FAD) and NAD⁺ to FADH₂ and NADH, respectively. NADH and FADH₂ pass their electrons through the ETC via a series of oxidation-reduction reactions (REDOX) (see Figure 1.2). In short, 2 electrons from either NADH or FADH₂ enter either Complex I or Complex II, respectively, which then reduce the electron carrier CoQ to ubiquinol (CoQH₂). The electrons are then transferred to and reduce Complex III, cytochrome c, Complex IV and then to a terminal electron acceptor, oxygen (O₂) that results in the production of water (H₂O). The energy that the electrons release during the REDOX process is then used to pump protons (H⁺) by complexes I, III, and IV across the membrane to the IMS. Because the concentration of H⁺ increases in the IMS, this then creates a pH gradient (ΔpHₘ, H⁺ concentration gradient) and mitochondrial membrane potential (ΔΨₘ, a charge or electrical gradient) (Kowaltowski et al., 2009) across the IMM. Thus, both the pHₘ gradient and ΔΨₘ, allow H⁺ to
flow back down their electrochemical gradient. The H\textsuperscript+ flow into the matrix through ATP synthase, which then catalyzes the phosphorylation of ADP to ATP (Leyva et al., 2003).

### 1.4.3 Mitochondria and ROS production

The complex pathogenesis of the neurodegenerative diseases remain largely unknown, however a plethora of studies now show that oxidative stress plays a critical role (Angelova & Abramov, 2018). Reactive oxygen species (ROS) are molecules derived from O\textsubscript{2} and contain one or more unpaired electrons, which can further generate other ROS species through enzyme- or metal-induced processes (Miller et al., 1990; Birben et al., 2012). Mitochondria are major source in ROS generation under physiological and pathophysiological conditions (Di Meo et al., 2016). The generation of mitochondrial ROS is a consequence of the REDOX reaction, as most steps involve single-electron reactions, further favouring the monovalent reduction of oxygen, which leads to an incomplete reduction of O\textsubscript{2}, which results in superoxide (O\textsubscript{2}\textsuperscript{−}) generation as a by-product (Andreyev et al., 2015). The main sites of premature electron “leak” have been suggested to occur at complexes I, II and III (Andreyev et al., 2015). The addition of one electron to O\textsubscript{2} results in a O\textsubscript{2}\textsuperscript{−}, which can be detoxified by the MnSOD to H\textsubscript{2}O\textsubscript{2} and is then converted to H\textsubscript{2}O by glutathione peroxidase (GSH-Px) (Morris et al., 2014). In the presence of reduced transition metals, H\textsubscript{2}O\textsubscript{2} can further be converted to hydroxyl radical (OH\textsuperscript{−}) (Miller et al., 1990; Birben et al., 2012; Andreyev et al., 2015). Other sources of mitochondrial ROS include the OMM (due to deamination of primary aromatic amines) (Reddy, 2006), elevated NADH/NAD ratio in the matrix may lead to H\textsubscript{2}O\textsubscript{2} increase from a α-ketoglutarate dehydrogenase (Tretter & Adam-Vizi, 2004) and xanthine oxidases (Rus et al., 2007; Vergeade et al., 2012).
1.4.4 Mitochondrial anti-oxidant defense

Mitochondria are equipped with an extensive anti-oxidant defense system that can combat the damaging effects of oxidants (Birben et al., 2012). These can be categorized into enzymatic and non-enzymatic systems. Enzymatic anti-oxidants include MnSOD, GSH-Px, catalase and thioredoxins (Trx). As described above, MnSOD is the mitochondrial matrix enzyme that reduces the harmful $\text{O}_2^-$ to $\text{H}_2\text{O}_2$, and $\text{H}_2\text{O}_2$ that is generated by MnSOD is further reduced to $\text{O}_2$ by GSH-Px (Morris et al., 2014), catalase, and the thioredoxin system (Pannala & Dash, 2015). GSH-Px uses GSH as an electron donor to neutralize mitochondrial $\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}$, $\text{O}_2$, and oxidized glutathione (GSSG). Glutathione reductase (GR) then uses nicotinamide adenine dinucleotide phosphate (NADPH), which is a by-product of the pentose phosphate pathway, to convert GSSG back to GSH (Flohe, 1988). Catalase also reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$, however although catalase is present in mitochondria (Salvi et al., 2007), its levels are low in the brain (Starkov et al., 2014). The thioredoxin system that is comprised of, mitochondrial thioredoxin 2 (Trx2), peroxiredoxin (Prx), and thioredoxin reductase (TrxR) also remove harmful $\text{H}_2\text{O}_2$ (Pannala & Dash, 2015). Specifically, Trx donates electrons to Prx to remove $\text{H}_2\text{O}_2$, and TrxR maintains the reduced Trx concentration with NADPH as the cofactor. In addition, non-enzymatic anti-oxidants are also found in mitochondria, which consist of: cytochrome c (Butler et al., 1975; Pasdois et al., 2011), coenzyme Q (DiNicolantonio et al., 2015), vitamin C (Wayner et al., 1986), vitamin E (Ham et al., 1995) and NADPH (Kirkman et al., 1999).

Another means by which mitochondria may control ROS levels is by lowering $\Delta \Psi_m$ (Sullivan et al., 2004). While higher potential may increase ATP production, it could potentially result in the genesis of unwanted ROS species. By inducing a small proton leak through the IMM, this process then converts mitochondrial ROS-producing sites to a more oxidized state thus lowering the probability of ROS production. Therefore, slower rate in OXPHOS would lead to a more
controlled and slower flow of the electrons through the ETC and potentially reduce the “leak”. This beneficial paradigm can partly be ascribed to the endogenous family of uncoupling proteins (UCP) (Andrews et al., 2005), that partially uncouple mitochondrial OXPHOS by allowing protons to leak back into the mitochondrial matrix, resulting in a decreased rate of ROS genesis without a significant effect on ATP production. Indeed, studies have demonstrated that increased expression and activity of UCPs are correlated with neuronal survival in neurodegenerative diseases such as stroke (Maragos & Korde, 2004; Mehta & Li, 2009).

1.5 Mitochondrial signaling pathways

It has become increasingly appreciated that mitochondria are involved in signaling pathways that extend beyond their localized metabolic functions. Accumulated studies have now demonstrated that mitochondria act as signaling organelles through both anterograde and retrograde signaling with the cytosol/nucleus. Interest in the mitochondrial role in cell signaling emerged from the discovery that mitochondria release cytochrome c from the IMM into the cytosol to initiate cell death cascade (Liu et al., 1996). Since then, numerous studies showed that mitochondria act as signalling organelles through release of ROS and other metabolites (Finkel, 2011).

1.5.1 Mitochondrial signaling through the ROS-axis

ROS are able to activate a number of molecular cascades that regulate a number cellular processes (Zhang et al., 2016). Specifically, ROS can reversibly oxidize protein cysteine thiols to sulfonic acid on some proteins to induce reversible, allosteric changes that would ultimately alter the function of that protein (Finkel, 2011). For example, one group of molecules that ROS target is the Ras superfamily (Sawyer et al., 2002; Kuster et al., 2005), which consists of guanosine triphosphate (GTP) binding proteins that are upstream of a number of signalling pathways that regulate apoptosis, morphology, cell cycle and growth. Deregulation of the Ras family signaling
is associated with a number of neurological, neuropsychological, cancer and cardiovascular diseases (Tidyman & Rauen, 2009; Stornetta & Zhu, 2011; Giancotti, 2014). ROS can also regulate Ca\textsuperscript{2+} signaling by oxidizing cysteine thiols of Ca\textsuperscript{2+} channels and or/pumps (Yan et al. 2006). Activation of these receptors results in an activation of a number of cellular events such as membrane excitability, neurotransmitter release, and gene expression (Clapham, 2007). Taken together, the role of ROS both as both toxic and signalling molecules adds to the complexity of mitochondrial function.

1.5.2 Metabolite signaling

Mitochondrial metabolites that are produced during energy generation can also act as cell-wide signaling molecules (Frezza, 2017). When there is a decrease in ATP production, as observed in many neurodegenerative-like related injuries, mitochondria can regulate signalling through upregulating AMP levels. An increase in AMP levels results in activation of the fuel sensor/regulator AMP-activated protein kinase (AMPK), which promotes ATP-producing and inhibits ATP-consuming pathways in various tissues (Herzig & Shaw, 2018; Mihaylova & Shaw et al., 2011). The most studied mechanism through which AMPK regulates cell growth is through inhibition of mechanistic target of rapamycin (mTOR) pathway (Xu et al., 2012). mTOR is a conserved serine/threonine kinase and is ubiquitously expressed in eukaryotic cell types (Brown et al., 1994; Sabatini et al., 1994; Sabatini et al., 1999) that exists in 2 distinct multi-protein complexes, mTOR-complex 1 and (mTORC1) mTOR complex 2 (mTORC2) (Laplante & Sabatini, 2012). This thesis will focus on mTORC1, as it is the principle regulator of cell growth and the functions of mTORC2 are less well researched than those of mTORC1 (Laplante & Sabatini, 2012). mTORC1 integrates extra- and intra-cellular signals from multiple pathways which include growth factors, nutrients, neurotransmitters and energy status to regulate cell growth, metabolism, and survival. Deregulation of mTORC1 signaling is associated with many
neurological and neuropsychiatric disorders (Costa-Mattioli & Monteggia, 2013). Under nutrient-rich conditions, such as elevated ATP levels, mTORC1 is activated and therefore drives cellular anabolic processes (Xu et al., 2012; Cargnello et al., 2015). mTORC1 can regulate protein synthesis through eukaryotic initiation factor-4E-binding protein (4E-BP1) and S6 kinase 1 (S6K1) pathways. In short, mTORC1 phosphorylates S6K1, that upregulates protein synthesis through various factors, including ribosomal S6 protein (Fonseca et al., 2014). Furthermore, by phosphorylating 4E-BP1, mTORC1 causes it to dissociate from eukaryotic initiation factor-4E (eIF-4E), which therefore promotes protein synthesis (Fonseca et al., 2014). In addition, active mTORC1 inhibits autophagy through phosphorylation of the downstream factor – UNC1like kinase 1 (ULK1), reviewed by (Jung et al., 2010). Whereas under nutrient-deficient conditions, such as low ATP production, mTORC1 is inhibited by AMPK which then results in decreased protein synthesis and increased autophagy (Xu et al., 2012; Saxton & Sabatini, 2017). Although it has been demonstrated that dysfunctional mitochondria result in a decrease in mTORC1 activity through the AMPK-axis (Herzig & Shaw, 2018), in some cases a mitochondria-mediated increase in oxidative stress may also underlie mTORC1 activation. As described above, ROS can activate Ras (Sawyer et al., 2002; Kuster et al., 2005), which is an upstream regulator of a number of signaling pathways involved in cell growth. Indeed, Ras is an upstream activator of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways, which regulate an array of downstream effector molecules, including mTORC1 (Mendoza et al., 2011). Activation of the Ras-MAPK-ERK pathway results in phosphorylation of the tuberous sclerosis complex 2 (TSC2) (Mendoza et al., 2011), which impairs its inhibitory activity on mTORC1. TSC2 is part of a complex that also consists of TSC1, which is required for the TSC complex stability and activity. Furthermore, although the precise mechanisms of activation are not elucidated, ROS can induce phosphorylation of Akt1 (Guha et al., 2010; Chetram et al., 2013), which is a serine/threonine kinase that plays a role in cell proliferation by
positively regulating mTORC1 signaling. Like the Ras/MAPK/ERK pathway, Akt1 can phosphorylate TSC2 and therefore also impair the inhibitory effect of the TSC complex on mTORC1 (Manning et al., 2002). Additionally, Akt1 phosphorylates the proline-rich Akt1 substrate of 40 kDa (PRAS40) resulting in mTORC1 activation (Sancak et al., 2007). PRAS40 functions as a negative regulator of mTORC1, however upon phosphorylation, PRAS40 dissociates from mTORC1 and the inhibitory constraint on mTORC1 activity is therefore lifted. Another negative regulator of mTORC1 is glycogen synthase kinase-3β (GSK-3β), a kinase that phosphorylates and activates TSC2. Indeed, Akt1 can also upregulate mTORC1 activity through phosphorylating GSK-3β, which inhibits the activity of GSK-3β and therefore lifts its inhibition on mTORC1. The simplified overview of the mTORC1 signaling network is presented in Figure 1.3.
Figure 1.3: Simplified diagram of the mTORC1 signaling pathway. Elevated ROS levels can activate mTORC1 signaling via Ras/MAPK/ERK and Ras/Akt1 pathways. mTORC1 activity is also tightly controlled by negative regulators including GSK-3β, AMPK and the TSC complex. Activated mTORC1 promotes protein synthesis through eIF-4E and S6K1 pathways. In addition, active mTORC1 inhibits autophagy through phosphorylation of the downstream factor – ULK1. AMPK, AMP-activated protein kinase; eIF-4E, eukaryotic initiation factor-4E; 4E-BP1, eukaryotic initiation factor-4E-binding protein; ERK, extracellular signal-regulated kinase; GSK-3β, Glycogen synthase kinase-3β; MAPK, mitogen-activated protein kinase; mTORC1, mechanistic target of rapamycin complex 1; S6, ribosomal S6 protein; S6K, S6 Kinase; TSC1/TSC2, tuberous sclerosis complex; ULK1, UNC1 like kinase 1.
1.6 Mitochondrial dysfunction

1.6.1 Mitochondrial dysfunction in neurodegenerative diseases

An imbalance between ROS levels and the biological ability to detoxify these species results in oxidative stress. While ROS are necessary for normal physiological cell processes such as signaling, gene expression, and neurotransmitter release (Finkel, 2011; Beckhauser et al., 2016), when in abundance they can cause cellular damage to protein, lipids, and DNA (Guo et al., 2013; Kim et al., 2015; Angelova & Abramov, 2018). The brain is particularly vulnerable to oxidative stress due to the high O₂ demand, the abundance of redox-active metals, high levels of polyunsaturated fatty acids and relatively low levels of antioxidants (Floyd & Carney, 1992; Hulbert et al., 2007; Chen, 2016). Toxicity of ROS contributes to mitochondrial dysfunction, which span from subtle changes in signaling pathways to apoptosis or necrosis (Kanduc et al., 2002; Valencia & Moran, 2004; Golstein & Kroemer, 2007; Nikoletopoulou et al, 2013).

Apoptosis is a programmed cell death that is initiated by a wide range of signalling molecules and/or stressors that are intrinsic or extrinsic in origin. These signals converge at the mitochondrial level to stimulate apoptosis. Dysregulation of mitochondrial ROS, Ca²⁺ and bioenergetics can trigger mitochondrial transition pore (mPTP) to open (Crompton, 1999). Mitochondrial permeabilization of the OMM leads to the release of proteins that result in cell death by caspase-dependent and independent pathways. In contrast to apoptosis, that is associated with a pre-programmed sequence of cellular events, necrosis is an unprogrammed or “accidental” cell death. Necrosis is a consequence of an acute interruption of cellular metabolism resulting in a rapid ATP depletion and ion deregulation leading to mitochondrial and cellular swelling and/or lysis (Golstein & Kroemer, 2007). Indeed, mitochondrial dysfunction is associated with both apoptotic and necrotic cell death, where evidence of both are observed in pathophysiology of
neurodegenerative and neuropsychiatric disorders (Kostrzewa, 2000; Broughton et al., 2009; Scaini et al., 2017).

### 1.6.2 Mitochondrial diseases and neurodegeneration

Mitochondrial diseases (MD) can manifest in both children or adults, with a prevalence of around 1:5,000 live births (Schaefer et al., 2008), and are associated with, but not limited to, neurological, neuromuscular, cardiovascular and metabolic disorders (Chaturvedi & Beal, 2013; Gorman et al., 2016). MD stem from mutations in mtDNA or nDNA that code for mitochondrial factors, these mutations collectively result in mitochondrial respiratory chain dysfunction which leads to impairment of energy production (Niyazov & Frye, 2016) and an increase in oxidative stress (Hayashi & Cortopassi, 2015). Because MD are caused by defects in numerous genes, these disorders are clinically diverse, and therefore pose a major challenge in their diagnosis as well as their treatment. MD have negative consequences in all tissues, however high energy demanding tissues such as the brain are the most affected (Chaturvedi & Beal, 2013). While there is considerable debate of whether MD is causal to, or consequential of, the progression of neurodegenerative diseases, there is general consensus that both mitochondrial and neurodegenerative diseases have similar pathological molecular cascades (Chaturvedi et al, 2013). Thus, improving mitochondrial homeostasis and its functional capacity could improve outcomes for many degenerative conditions.

### 1.7 Function of sirtuin-3

#### 1.7.1 SIRT3 is trafficked to mitochondria

Assessments of endogenous SIRT3 localization have demonstrated a mitochondrial residence for this enzyme in cells from different species (Schwer et al., 2002; Lombard et al., 2007; Scher et al., 2007). SIRT3 is expressed initially as a precursor protein of approximately 45 kilodaltons that
contains a mitochondrial localization sequence. (MLS) (P. Onyango et al., 2002). Upon entry into the mitochondrial matrix, this pro-form of SIRT3 is processed into a shorter form by mitochondrial matrix processing peptidase (MMP) (Onyango et al., 2002; Schwer et al., 2002). Recently, it was reported that this MMP-generated product is further cleaved by mitochondrial intermediate peptidase (MIPEP) to generate the mature enzymatically active 28 kilodalton form of SIRT3 present in the mitochondrial matrix (Kobayashi et al., 2017). However, while ubiquitously agreed that SIRT3 does reside within mitochondria, there remains debate regarding whether or not a population of some form of SIRT3 may also reside in nucleus or cytoplasm (Scher et al., 2007; Nakamura et al., 2008; Sundaresan et al., 2008). Indeed, nuclear localization of the endogenous SIRT3 precursor has been reported in U2OS and HeLa cells by immunocytochemical analysis (Scher et al., 2007; Iwahara et al., 2012) and both nuclear localization and nuclear deacetylase activity has been reported in HeLa cells and rat cardiomyocytes ectopically expressing a full-length human SIRT3 cDNA (Scher et al., 2007; Sundaresan et al., 2008). However, Cooper and Spelbrink (2008) failed to detect any nuclear SIRT3 using stringent subcellular fractionation techniques in HeLa, U2OS, or HEK-293 cells that ectopically expressed a full-length human SIRT3 cDNA. Onyango et al., (2002) reported no detectable nuclear signal in COS7 cells ectopically expressing a SIRT3-GFP reporter system (Onyango et al., 2002) and a lack of endogenous SIRT3 was observed in nuclear extracts isolated from rat skeletal muscle (Gurd et al., 2012). Thus, while it is clear that the processed form of catalytically active SIRT3 resides within the mitochondria where it targets a number of mitochondrial proteins (Bell & Guarente, 2011; Hirschey et al., 2011; Bause & Haigis, 2013; Ansari et al., 2017), it remains controversial whether additional subcellular expression patterns and non-mitochondrial protein targets exist for SIRT3 under normal conditions.
1.8 Sirtuin-3 and mitochondria

1.8.1 SIRT3 function in mitochondria

While there have been fewer studies of SIRT3 activity in neural cells or tissues, studies employing non-neuronal systems have uncovered a number of mitochondrial proteins targeted by SIRT3 that are also expressed in brain, and therefore it seems likely they will be targeted similarly by SIRT3 in nervous system settings. These include members of pathways involved in anti-oxidative defense systems (Jacobs et al., 2008; Tao et al., 2010; Bell et al., 2011; Chen et al., 2011; Ren et al., 2016), metabolism (Hallows, & Denu, 2006; Ahn et al., 2008; Schlicker et al., 2008; Salvatori et al., 2017), and mitochondrial biogenesis (Shi et al., 2005; Kong et al., 2010; Yang et al., 2010; Giralt et al., 2011; Dai et al., 2014; Zhao et al., 2016). Each of these pathways has direct relevance to brain physiology, and alterations in the function of each have been implicated as contributing factors in different neurodegenerative diseases or neurological disorders (Federico et al., 2012; Guo et al., 2013; Kim et al., 2015; Li et al., 2017). Visual overview of some of these pathways is outlined in Figure 1.4.

1.8.1.1 SIRT3 regulation of anti-oxidative defense systems

Of particular interest in neural cells, SIRT3 deacetylates and increases the catalytic activity of MnSOD (Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011), which as described above, is a primary mitochondrial enzyme that converts O$_2^-$ to H$_2$O$_2$, and the TCA cycle enzyme IDH2 (Schlicker et al., 2008; Someya et al., 2010; W. Yu et al., 2012), which converts NADP$^+$ to NADPH in mitochondria and helps maintain the anti-oxidant GSH in a reduced and active state. SIRT3 also physically interacts with the anxi-oxidant Trx-2 (Palacios et al., 2009). Indeed, converging studies show that cells and tissues deficient in sirtuin-3 show high indices of oxidative stress (reviewed in Bell et al., 2011; Bause and Haigis, 2013; Ansari et al., 2017). Interestingly
Parodi-Rullan et al., (2017) observed no difference in oxidative stress between WT and *Sirt3*-KO mouse hearts under basal conditions. Because there was an increase in SIRT4 expression in hearts of *Sirt3*-KO mice, these authors suggested that SIRT4 may serve as an independent anti-oxidative compensatory mechanism in these mice under normal conditions. However, following ischemia-reperfusion injury, *Sirt3*-deficient heart mitochondria showed elevations of ROS and oxidative damage relative to WT mitochondria, illustrating any potential compensatory sirtuin-4 activity has a ceiling relative to that of sirtuin-3. Future studies will be required to define the interplay between mitochondrial sirtuins and regulation of anti-oxidative systems in mitochondria under basal and various pathological conditions. Nevertheless, these results strongly suggest SIRT3 likely plays a key role in regulating ROS levels in neurons, where aerobic respiration is the primary mechanism for energy production.
Figure 1.4: SIRT3 influences oxidative phosphorylation and oxidative stress management. SIRT3 increases ETC efficiency by deacetylating and regulating the activities of mitochondrial complexes I, II, III, IV and V to increase ATP production. SIRT3 reduces mitochondrial oxidative stress directly by deacetylating IDH2 and MnSOD and also increasing GSH levels, and thereby enhancing ROS detoxification. SIRT3 can also negatively regulate ROS levels in some tissues indirectly by upregulating UCP expression, which lessens the driving force for mitochondrial ROS production by decreasing ΔΨm. Sirtuin-3 can also negatively regulate the opening of the mPTP by deacetylating CypD. SIRT3 also orchestrates mitochondria-nuclear cross-talk by deacetylating and activating mitochondrial FOXO3A, which then travels to the nucleus to activate the transcription of anti-oxidative genes, and by indirectly activating the transcription factor CREB, which activates genes whose products play roles in mitochondrial biogenesis. CREB: cAMP response element-binding protein, CypD: cyclophilin D, ETC: electric transport chain, FOXO3a: forkhead box O3, GSH: glutathione, IDH2: isocitrate dehydrogenase, MnSOD: manganese superoxide dismutase, mPTP: mitochondrial permeability transition pore, ROS: reactive oxygen species, UCP: uncoupling protein, ΔΨm: change in mitochondrial membrane potential.
SIRT3 over-expression lowers mitochondrial membrane potential (ΔΨm) and ROS levels in different non-neuronal cell types (Shi et al., 2005; Bell & Guarente, 2011; Y. Chen et al., 2011; Shulyakova et al., 2014; Liu et al., 2015; Pillai et al., 2016; Ren et al., 2016). While the exact mechanism of how SIRT3 decreases ΔΨm and ROS levels remain to be fully elucidated, a potential mechanism has been suggested for brown adipose tissue (Shi et al., 2005). In these cells, there is a SIRT3-dependent up-regulation of the transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) expression, a transcriptional co-activator that regulates the expression of numerous genes involved in mitochondrial function and detoxification pathways (Onyango et al., 2010). PGC1-α facilitates an increase in the expression levels of mitochondrial uncoupling protein 1 (UCP1), which in turn decreases ΔΨm by enhancing proton flux back across the inner mitochondrial membrane into the matrix (Klingenspor, 2003). The lower ΔΨm decreases the driving force for electron leak during oxidative phosphorylation, and thereby contributes to the decrease in ROS genesis (Sullivan et al., 2004). It is unlikely that this direct mechanism would apply to the brain, however, as UCP1 expression is low or undetectable in the CNS under normal conditions (Andrews et al., 2005) and therefore the function of SIRT3 in brain does not likely involve UCP1. It is worth noting though that other relatives of UCP1 are expressed in the brain (UCP2’, -4 and -5’), and their elevated expression and activity have also been correlated with neuroprotection (Andrews et al., 2005). It is therefore possible that these UCP family members could potentially substitute for UCP1 in neurons as targets for SIRT3. To date, though, this remains speculative, as direct SIRT3 regulation of any UCP family member in neurons remains undetermined.
1.8.1.3 **SIRT3 influences the expression of nuclear genes**

In addition to its direct activation of antioxidant target proteins by deacetylation, sirtuin-3 also indirectly regulates the expression of specific antioxidant enzymes. Jacobs et al., (2008) showed that the transcription factor FOXO3a is a substrate of SIRT3 in mitochondria, which upon deacetylation translocates into the nucleus to stimulate the transcription of specific antioxidative genes. As stated above, SIRT3 directly or indirectly also influences the expression of PGC-1α. Although the mechanism of SIRT3 regulation of PGC-1α is not fully understood, it has been speculated that SIRT3 indirectly increases the phosphorylation of the transcription factor, cAMP response element-binding protein (CREB) (Shi et al., 2005; Kong et al., 2010) which then facilitates PGC-1α gene expression. Thus, SIRT3 regulates mitochondrial proteins at both their expression and functional levels by indirectly regulating their gene-expression in the nucleus, and their catalytic/functional state directly in mitochondria through post-translational deacetylation. These results further illustrate how sirtuin-3 could be viewed as a key rheostat for maintaining mitochondrial homeostasis.

1.8.1.4 **SIRT3 influences mitochondrial permeability transition pore activation**

In pathological conditions and following different neural injuries such as stroke or trauma, the formation and opening of the mitochondrial permeability transition pore (mPTP) is a hallmark of end-stage mitochondrial dysfunction that allows for the release of pro-apoptotic factors from mitochondria that activate cell death pathways (Halestrap & Pasdois, 2009; Sims & Muyderman, 2010). Inhibiting mPTP opening has been proposed as a neuroprotective strategy for a number of neurodegenerative diseases (Rao et al., 2014), and one proposed mechanism for Sirtuin-3-mediated cytoprotection involves it inhibiting the opening of the mPTP (Hafner et al., 2010). In cardiac myocytes, Hafner et al (2010) showed that SIRT3 deacetylase activity inhibited the formation of the mPTP by decreasing the binding of cyclophilin D (CypD) to adenine nucleotide
translocase (ANT). The SIRT3-mediated regulation of CypD is evident in brain, as CypD hyperacetylation is observed in hippocampal tissues and cortical neurons isolated from Sirt3-KO mice (Cheng et al., 2016). These results suggest that SIRT3 activity in brain negatively influences mPTP opening by maintaining CypD in a deacetylated state. However, there may be context-specificity or limits to this action of SIRT3, as Novgorodov et al. (2016) found no difference in Ca²⁺-induced mPTP opening in mitochondria isolated from cerebral tissues that were harvested from ischemia-challenged Sirt3-KO and WT mice. However, the requisite role of acetyl-CypD on mPTP opening may also be context-dependent, as mPTP opening occurred without any CypD acetylation state changes in peroxide-treated H9c2 rat embryonic cardioblasts (Barreto-Torres et al., 2015), and Parodi-Rullan et al. (2017) found no difference in mPTP opening and cyclophilin D acetylation in hearts between WT and Sirt3-KO mice following ischemia-reperfusion injury. Further, CypD itself may influence mitochondrial acetylation levels, as mice lacking CypD show hyperacetylation of mitochondrial proteins without any change in SIRT3 expression or activity levels (Nguyen et al., 2013). These studies suggest that the cause-effect between CypD acetylation by SIRT3 and the opening of mPTP is complicated and may be context-dependent. Therefore, future studies are necessary to elucidate the role of SIRT3 on the opening of mPTP under normal physiological and different pathophysiological conditions.

1.8.1.5 SIRT3 influences mitochondrial biogenesis and mitochondrial dynamics

SIRT3 has also been implicated in mitochondrial biogenesis regulation (Shi et al., 2005; Kong et al., 2010; Giralt et al., 2011; Dai et al., 2014; Zhao et al., 2016), mitochondrial fusion/fission dynamics (Song et al., 2013; Samant et al., 2014; Morigi et al., 2015a), and related aspects of maintaining mitochondrial integrity. Mitochondrial biogenesis is a process through which new mitochondria are generated. This phenomenon is especially important for neural development and may also serve as a protective mechanism during neurodegenerative diseases (Onyango et al.,
Indeed, studies show that Sirtuin-3 expression is required for proper mitochondrial biogenesis in both mouse C2C12 myoblasts (Kong et al., 2010) and human oocytes (Zhao et al., 2016). Mitochondrial dynamics also play an important role in the brain, as mitochondria continuously undergo dynamic changes in their size, shape, number, and distribution to appropriately respond to fluctuating metabolic demands (Milone & Benarroch, 2012). Fission results in mitochondrial division, while fusion allows distinct mitochondria to exchange intramitochondrial metabolic products and mtDNA. This normal fission/fusion balance can be altered by various external factors, such as ROS elevation (Wu et al., 2011), reduced ATP levels (Mishra et al., 2016) and mitochondrial Ca\textsuperscript{2+} influx (Chen & Chan, 2009; Kaddour-Djebbar et al., 2010).

Samant et al. (2014) reported that SIRT3 preserves mitochondrial networking in cardiomyocytes by deacetylating and activating the GTPase activity of optic atrophy 1 (OPA1), which is an integral protein involved in promoting mitochondrial fusion. In addition, Morigi et al. (2015) reported that sirtuin-3 over-expression prevented the mitochondrial recruitment of the fission-promoting protein dynamin-related protein-dependent (DRP1) and also increased OPA1 expression in cultured human renal proximal tubular epithelial cells in response to fission-inducing cisplatin challenge. As mitochondrial fragmentation is evident in many neurodegenerative conditions such as Alzheimer’s disease (Wang et al., 2009), Parkinson’s disease (Van Laar & Berman, 2009), amyotrophic lateral sclerosis (Jiang et al., 2015), and at early stages following reversible ischemic stroke (Barsoum et al., 2006), it will be of considerable interest to determine whether sirtuin-3 can be harnessed to preserve mitochondrial integrity in these conditions and circumstances.

1.8.2 SIRT3 is a metabolic energy sensor

Accumulating evidence indicates SIRT3 enzymatic activity fluctuates in accordance with the metabolic state of the cell (Shi et al., 2005; Ferrer et al., 2009; Palacios et al., 2009; Hokari et
al., 2010; Li et al., 2011; Tauriainen et al., 2011; Dai et al., 2014; Brandauer et al., 2015; Cheng et al., 2016; Amigo et al., 2017). Because SIRT3 uses NAD$^+$ as a cofactor for deacetylation reactions, the ratio of NAD$^+$/NADH within the mitochondria can influence its overall deacetylase activity. NAD$^+$/NADH ratios can be dynamic within cells, particularly in metabolically demanding cells such as neurons (Cantó et al., 2015). As discussed in section 1.2, the deacetylation reaction catalyzed by SIRT3 results in the conversion of NAD$^+$ into nicotinamide and acetyl-ADP-ribose, high SIRT3 activity could deplete NAD$^+$ within mitochondrial microdomains, and thereby limit its own catalytic activity by removing its required co-factor. Moreover, nicotinamide itself is an endogenous inhibitor of the SIRT3 deacetylation reaction (Guan et al., 2014), which suggests the generation of nicotinamide by SIRT3 could feed-back to attenuate its own activity. Finally, it is also intriguing that NAD$^+$/NADH ratios tend to increase during metabolic states associated with CR and exercise (Hipkiss, 2008) – which are recognized for their neuroprotective and/or lifespan extension potential (Deslandes et al., 2013; Michan, 2014). In this regard, CR diets increase SIRT3 deacetylase activity in a number of tissues, which include brain (Someya et al., 2010; Amigo et al., 2017), liver (Someya et al., 2010) and inner ear (Someya et al., 2010) and many of the ascribed benefits of both CR and exercise are lost when SIRT3 activity is abrogated (Palacios et al., 2009; Someya et al., 2010; Cheng et al., 2016). These results therefore provide evidence for an intricate link between CR, exercise, increased NAD$^+$/NADH ratios, SIRT3 enzymatic activity, and cytoprotective effects associated with exercise and/or CR.

1.8.2.1  SIRT3 influences energy source utilization by mitochondria

SIRT3-mediated deacetylation and activation of several factors involved in mitochondrial energy production have been demonstrated. These include AceCS2, GDH, IDH2, and specific components of the ETC (Hallows et al., 2006; Schlicker et al., 2008; Someya et al., 2010; Yu et
AceCS2 catalyzes formation of acetyl-CoA from free acetate and CoA, which can then be used via the TCA cycle for ATP synthesis (Fujino et al., 2001). The function of AceCS2 is especially important during periods of diminished glucose availability, as in such times of need the liver can release significant amounts of stored acetate for use by extra-hepatic tissues that include the brain (Yamashita et al., 2001). SIRT3 activation of GDH enhances the production of TCA cycle intermediates α-ketoglutarate and NADH, which enhances the pool of electron donating substrates for oxidative phosphorylation (Schlicker et al., 2008). In this regard, SIRT3 has also been shown to deacetylate and regulate the activity of complex I in general (Kim et al., 2010), and specific mitochondrial respiratory chain complex components such as NDUFA9 of Complex I (Ahn et al., 2008); Succinate Dehydrogenase of complex II (Finley et al., 2011) and ATP synthase subunits α and β (Law et al., 2009; Wu et al., 2013; Rahman et al., 2014). In the absence of sirtuin-3, the activities of complex I, complex II, and complex V were each shown to be decreased relative to control (Ahn et al., 2008; Law et al., 2009; Finley et al., 2011; Rahman et al., 2014; Novgorodov et al., 2016), and the activity of complexes III and IV were also found to be decreased in liver extracts from Sirt3-KO mice maintained on a high fat diet (Kendrick et al., 2011). Consistently, decreased ATP levels were reported in several different organs of Sirt3-KO mice (Ahn et al., 2008). It should be noted, however, that these observations are not absolute, as Fernandez-Marcos et al. (2012) reported no changes in ATP levels in Sirt3-KO mouse muscle or liver, and Novgorodov et al. (2016) reported that complex I activity but not complex II or complex IV activity was reduced under basal conditions in Sirt3-KO mouse cerebral cortex. Interestingly, a decrease in basal mitochondrial oxygen consumption rate has been observed in primary cultured mouse dopaminergic neurons overexpressing sirtuin-3 (Gleave et al., 2017), suggesting that over-expression of this enzyme increases mitochondrial efficiency by stabilizing respiration at the electron transport level. While these results support SIRT3 as a factor regulating overall cellular energy production at different levels, additional studies are needed to better define
how changes in metabolic state affect the activity of SIRT3, and how SIRT3 influences mitochondrial respiratory activity in highly aerobic tissues such as brain.

1.8.2.2 SIRT3 influences the production of substrates

Brain metabolic demand is regulated by a complex interplay of behavioural state, substrate supply and availability, and hormonal signaling between periphery and tissues within the CNS (Lenard & Berthoud, 2008). Glucose is the primary substrate fueling brain metabolism, and tight regulation of its use is critical for normal brain physiology, structure integrity, and neuronal viability (Mergenthaler et al., 2013; Bauernfeind et al., 2014). However, when glucose availability becomes limited, the brain is able to utilize alternative sources from other sources in the body to support brain metabolism or ATP turnover (White & Venkatesh, 2011; Prins, 2012; Glenn et al., 2015; Proia et al., 2016). Glycogen reserves can be tapped to maintain blood-glucose levels acutely, and to meet the high-metabolic demands of the brain for a short period of time (Waitt et al., 2017). Once these glycogen reserves have been depleted, though, another alternative energy source must serve as substrates for brain metabolism. During such conditions, fatty acid reserves from adipose tissues can be catabolized, and used for fatty acid β-oxidation in the liver to generate acetyl-CoA, which is then converted by ketogenesis into ketone bodies (White & Venkatesh, 2011). These ketones are then carried to metabolically active tissues, such as the brain, where they are converted back to acetyl-CoA to serve as an energy source. Studies have suggested SIRT3 plays a vital role in all the major steps of this hepatic “glucose-sparing energy pathway”, as SIRT3 deacetylates and activates two key enzymes involved in fatty acid β-oxidation pathway initiation: long-chain acyl CoA dehydrogenase (LCAD) (Hirschey et al., 2010) and very long-chain acyl-CoA dehydrogenase (VLCAD) (Hallows et al., 2011; Y. Zhang et al., 2015). These interactions have been demonstrated in liver, but it is worth noting that both LCAD and VLCAD are also expressed in brain, and could therefore also be subject to SIRT3
regulation in the CNS. SIRT3 has also been implicated in activating L-3-hydroxyacyl-CoA dehydrogenase, short/branched-chain acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Hallows et al., 2011), which are each also involved in β-oxidation processes. Moreover, SIRT3 has also been shown to regulate the production of ketone bodies by targeting 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2), which is the limiting enzyme in the production of ketone bodies from acetyl-CoA and acetoacetyl-CoA (Shimazu et al., 2010). Intriguingly, SIRT3 not only promotes the synthesis of ketones, it also shifts the hepatic mitochondrial metabolism to meet the higher energy demand that is necessary for ketogenesis. This is accomplished by deacetylating and activating the mitochondrial transcription factor leucine-rich protein 130 (LRP130), who in turn stimulates the transcription of genes in the mitochondrial genome encoding factors required for oxidative phosphorylation cascades (Liu et al., 2011). Whether this occurs similarly in brain as demonstrated in liver remains to be determined.

In addition to ketone bodies, lactate is another alternative source of energy used by the brain under certain circumstances. Lactate can be transported from the periphery across the BBB by monocarboxylate transporters 1 (MCT1), and within the brain astrocytes can also serve a source of lactate for neurons (reviewed in Steinman et al., 2016). Neurons express MCT2, which transports lactate intracellularly where lactate dehydrogenase (LDH) converts it into pyruvate, which is then converted to acetyl-CoA by pyruvate dehydrogenase (PDH) (Pellerin et al., 2005). Although a direct link has not yet been established in neural tissues, SIRT3 can deacetylate both LDH (Y. Cui et al., 2015) and PDH in a number of cancer cells (293T, HCT116, HeLa, T47D, MMT, and MCF7) (Ozden et al., 2014) and mouse skeletal muscle (Jing et al., 2013). These data therefore provide another potential means through which SIRT3 could facilitate the use of alternative energy sources in the brain during times of need. The visual overview of how SIRT3 regulates energy source production and utilization is illustrated in Figure 1.5.
Figure 1.5: SIRT3 regulates energy source production and utilization. SIRT3 influences the use of alternative sources for energy production to meet high-metabolic demands of the brain. During low glucose availability, fatty acids from adipose tissue are catabolized in the liver via β-oxidation to generate acetyl-CoA, SIRT3 deacetylates and activates LCAD and VLCAD, which are two key enzymes involved in fatty acid β-oxidation. SIRT3 also deacetylates and activates HMGCS2, which is the limiting enzyme in the production of ketone bodies from acetyl-CoA. Ketone bodies travel to the brain, where they are converted back to acetyl-CoA for energy utilization. SIRT3 also deacetylates acetyl-CoA-synthetase 2, which can generate acetyl-CoA from free acetate. Lactate is another energy source derived from peripheral tissues and astrocytes. SIRT3 deacetylates and activates both LDH and PDH, which convert lactate to pyruvate and acetyl-CoA, respectively. HMGCS2: 3-hydroxy-3-methylglutaryl-CoA synthase, LCAD: long-chain acyl CoA dehydrogenase, LDH: lactate dehydrogenase, PDH: pyruvate dehydrogenase, VLCAD: very long-chain acyl-CoA dehydrogenase.
1.8.3 Sirtuin-3 expression in the brain

Analysis of mRNA and protein expression has revealed sirtuin-3 to be prevalent in tissues with high oxidative capacity such as brain, cardiac, hepatic, brown adipose tissue, and skeletal muscle, with lower levels found in tissues with lower-metabolic demand such as white adipose tissue, lungs, spleen, thymus, pancreas and small intestine (Shi et al., 2005; Lombard et al., 2007; Palacios et al., 2009; Braidy et al., 2015). Sirtuin-3 localization within the brain is not restricted to neurons, as clear sirtuin-3 expression has also been demonstrated in astrocytes and microglia (Kim et al., 2011; Weir et al., 2012; Dai et al., 2014; Cheng et al., 2016). Collectively, these studies do show that sirtuin-3 is expressed in the brain.

1.8.4 Role of sirtuin-3 in neurodegenerative diseases

Although sirtuin-3 has garnished considerable interest in neurodegenerative diseases, most of the currently available knowledge of sirtuin-3 stems from studies in non-neuronal cells, and the consequences of the interactions between SIRT3 and its targets in the CNS remain less well defined at present and its function in the brain is just at the beginning stages of exploration (Anamika et al., 2017). Below is the synopsis of current literature regarding the role that sirtuin-3 plays in neurodegenerative brain disorders, classified in a disease-specific manner (Table 1.3).

1.8.4.1 Sirtuin-3 in Alzheimer’s disease

Growing evidence has demonstrated mitochondrial dysfunction is a critical factor contributing to the initiation and progression of Alzheimer’s disease (AD) (Grimm et al., 2016), and recent data suggest sirtuin-3 may play a role in this process. Expression analysis has revealed changes in sirtuin-3 expression levels in post-mortem samples from AD patients and in experimental mouse models. For example, Weir et al., (2012) reported increased SIRT3 mRNA levels in human post-mortem midfrontal and temporal neocortical tissues from AD patients (Weir et al., 2012), while
in contrast, Yin et al., (2018) reported decreased sirtuin-3 mRNA and protein levels in human AD post-mortem cortical regions. The reason for the discrepancy in sirtuin-3 expression outcome is unclear, but the results do suggest an AD-related sirtuin-3 response does occur during the course of the disease.

In mouse models, sirtuin-3 expression responses have also been identified, but like in AD patients somewhat different outcomes have been observed in different models. Sirt3 mRNA levels were initially elevated in hippocampal tissues of early symptomatic 6 month-old AD PDAPP mice (which overexpress a human amyloid beta (Aβ) precursor protein (APP) carrying the V717F mutation), but this increase in sirtuin-3 expression returns to normal levels by 26 months of age (Weir et al., 2012). Both sirtuin-3 mRNA and protein levels were significantly diminished in APP/PS1 double transgenic mice at 12 months of age (Yang et al., 2015), and decreased SIRT3 expression was similarly found in PSEN1/APP/TAUP301L mice at 24-months of age (Han et al., 2014). The initial up-regulation of sirtuin-3 levels may represent an endogenous neuroprotective response early in disease pathogenesis that is then lost as the disease progresses. Furthermore, over-expression of SIRT3 prevented Aβ-42 induced tau accumulation in cultured cortical neurons derived from mice expressing a human tau transgene, while knockdown of SIRT3 in the same cultured neurons caused an elevation of tau accumulation (Yin et al., 2018). These results collectively suggest that SIRT3 activity may attenuate the over-production and accumulation of tau. While encouraging, further studies will be required to define the contribution and potential translational value of sirtuin-3 in AD pathogenesis.

1.8.4.2 Sirtuin-3 in Parkinson’s disease

Two agents widely used to induce experimental Parkinsonism disease (PD) in model systems are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone; both of which are believed
to cause PD-like degeneration through their induction of oxidative stress levels and impairment of mitochondrial function. In cultured SHSY-5Y cells, Cui et al. (2017) found that SIRT3 over-expression prevented the ATP loss induced by MPP+ (a metabolite of MPTP) treatment, and SIRT3 over-expression also diminished apoptosis, α-synuclein accumulation, and cell death following rotenone treatment of the same cells (Zhang et al., 2016). Consistently, SHSY-5Y cells lacking SIRT3 showed an increase in apoptosis, α-synuclein accumulation, and cell death following the same rotenone treatment (Zhang et al., 2016). In line with the in vitro studies, Sirt3-KO mice showed exacerbated degeneration of dopaminergic neurons compared to WT mice following MPTP treatment (L. Liu et al., 2015), and viral-mediated over-expression of SIRT3 protected dopaminergic neurons from α-synuclein-induced degeneration (Gleave et al., 2017). Shi et al. (2017) also observed an increase in oxidative stress and degeneration of SNc dopaminergic neurons from Sirt3-KO mice, which correlated with increased lysine-68 acetylation of MnSOD (MnSODK68). Importantly, SNc dopaminergic neuron degeneration was prevented by either reintroduction of functional SIRT3, or by introduction of a constitutively active deacetylation-mimetic form of MnSODK68 (Shi et al., 2017). Collectively, these studies suggest that the endogenous functions of SIRT3 may indeed play a protective role in PD pathogenesis, and suggest that sirtuin-3 may represent a therapeutic target for PD translational development, particularly since hyperacetylation of MnSODK68 has been observed in post-mortem midbrain tissues from PD patients (Shi et al., 2017).

1.8.4.3 Sirtuin-3 in amyotrophic lateral sclerosis

Although there have been few investigations of sirtuin-3 to date in amyotrophic lateral sclerosis (ALS) models, the available data suggest the actions of SIRT3 could influence ALS pathogenesis. As discussed above, (Song et al., 2013) showed the mitochondrial fragmentation and activation of apoptotic cascades in cultured motor neurons derived from SOD1G93A mutant mice could be
prevented by the ectopic over-expression of SIRT3. Similarly, Harlan et al (2016) found that SIRT3 over-expression in SOD1\textsuperscript{G93A} mutant mouse astrocytes rescued the degeneration of co-cultured SOD1\textsuperscript{G93A} mutant mouse motor neurons. Further studies are clearly required, but these results suggest that enhancing SIRT3 activity may provide a protective role in at least for some forms of ALS.

1.8.4.4 Sirtuin-3 in Huntington’s disease

Although the mechanism in neuronal degeneration is not fully understood in Huntington’s disease (HD), mitochondrial involvement has been speculated (Chen, 2011). To date, only one study has examined sirtuin-3 in the context of HD progression (Fu et al., 2012). Fu et al. (2012) showed that SIRT3 protein levels and deacetylase activity are diminished in a human striatal precursor cell line expressing mutant Htt, and the protective effects observed in these cells following treatment with the stilbenic compound \textit{ns}-(−)-\textit{ϵ}-Viniferin (viniferin) were lost when endogenous SIRT3 was knocked down. Although further studies are required, this initial study also suggest SIRT3 up-regulation or enhancement of enzymatic activity could slow the progression of HD.

1.8.4.5 Sirtuin-3 in stroke

To date, several studies have examined the role of sirtuin-3 in different \textit{in vitro} and \textit{in vivo} models of stroke. Kim et al. (2011) reported that the over-expression of sirtuin-3 in cortical neurons protected against NMDA-excitotoxicity; Dai et al. (2017) demonstrated lentiviral over-expression of SIRT3 protected cultured cortical neurons from an oxygen/glucose deprivation (OGD) challenge; Shulyakova et al. (2015) showed SIRT3 over-expression protected neuronally differentiated PC12 cells from OGD and from apoptotic degeneration induced by trophic withdrawal; Magnifico et al. (2013) found that transient over-expression of full-length mouse SIRT3 protected cultured mouse cerebellar granule neurons from axonal degeneration induced by
KCl depletion; and Yang et al. (2017) found that administration of the SIRT3 activator adjudin attenuated glial scar formation and improved functional recovery in mice following transient middle cerebral artery occlusion (MCAO), but that these effects were lost in Sirt3-KO mice. Collectively, these studies support the hypothesis that enhancing SIRT3 prevalence and/or activity can provide protection against a spectrum of insult-related challenges.

However, not all experimental data indicate that the over-expression or increased activation of SIRT3 is beneficial. In fact, quite contrasting outcomes have been observed in some experimental systems. For example, cerebellar granule neurons overexpressing SIRT3 displayed enhanced neuronal death in response to low potassium ion treatment (Pfister et al., 2008), and Sirt3-KO mice displayed smaller brain infarct volumes than wild type mice following reversible MCAO (Novgorodov et al., 2016), and Verma et al. (2018) also recently reported ischemia/reperfusion damage to be reduced in Sirt3-KO mice. In the Novgorodov et al. (2016) study, Sirt3 ablation was found to lessen the activation of ceramide synthases 1, 2 and 6 following reperfusion, which was hypothesized to help preserve the overall function of the challenged mitochondria by decreasing the generation and accumulation of toxic mitochondrial ceramides (reviewed in (Novgorodov & Gudz, 2011). The Verma et al. 2018 study employed a similar model, but speculated the observed protection may relate to a compensatory up-regulation of the SIRT1 in the brains of Sirt3-KO mice. These results do not necessarily contradict the anti-oxidant or anti-apoptotic capacity of SIRT3, as the severity or type of stroke challenge could differentially activate ROS-dependent or ceramide-dependent cell death cascades (Novgorodov & Gudz, 2011; Mencarelli & Martinez-Martinez, 2013), but they do highlight the possibility that SIRT3 may differentially regulate stroke-related outcomes in tissue- or context-dependent manners (Dittenhafer-Reed et al., 2015).
### Table 1.4: Sirtuin-3 and Neurodegenerative Diseases

<table>
<thead>
<tr>
<th>Disease Model</th>
<th>Experimental Model</th>
<th>Results</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Alzheimer’s Disease (AD)</td>
<td>Midfrontal and Temporal neocortical tissues of human AD patients AD PDAPP mice overexpressing human APP V717F mutation</td>
<td>Elevated \textit{SIRT3} mRNA levels in AD human tissues Elevated \textit{Sirt3} mRNA levels early stages (6 month-old mice) Wild-type levels of \textit{Sirt3} mRNA in late stages (26 month-old mice)</td>
<td>Weir et al., 2012</td>
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<td></td>
<td>APP/PS1 double transgenic mouse model</td>
<td>Decreased \textit{Sirt3} mRNA and SIRT3 protein levels (12 months)</td>
<td>Yang et al., 2015</td>
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<td></td>
<td>PSEN1/APP/TAUP301L mouse model</td>
<td>Decreased \textit{Sirt3} mRNA and SIRT3 protein levels (24 months)</td>
<td>Han et al., 2014</td>
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<td></td>
<td>Hamster ovary PS70 cells expressing mutant APP Treated with Honokiol (SIRT3 activator)</td>
<td>Attenuated ROS levels Increased mitochondrial membrane potential Elevated p-AMPK, p-CREB, PGC1α protein levels Decreased total intracellular amyloid beta (Aβ) protein levels</td>
<td>Ramesh et al., 2018</td>
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<td></td>
<td>Middle temporal gyrus, superior frontal gyrus, primary visual cortex and entorhinal cortex of human AD patients Primary cortical neurons from transgenic mice that carry human tau protein (hTau)</td>
<td>Decreased \textit{SIRT3} mRNA and SIRT3 protein levels in AD human tissues SIRT3 knockdown increased total tau expression in hTau mouse cortical neurons SIRT3 overexpression reduced total tau levels in in hTau mouse cortical neurons</td>
<td>Yin et al., 2018</td>
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<tr>
<td>Parkinson’s Disease (PD)</td>
<td>SHSY-5Y cells overexpressing hSIRT3 (MPP+ and rotenone models of PD)</td>
<td>Rescued ATP loss Diminished cell apoptosis, α-synuclein accumulation, and cell death</td>
<td>(X. X. Cui et al., 2017) Zhang et al., 2016</td>
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<tr>
<td></td>
<td>\textit{Sirt3}-KO mice (MPTP model of PD)</td>
<td>Exacerbated degeneration of dopaminergic neurons degeneration</td>
<td>Liu et al., 2015</td>
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<td></td>
<td>Viral-mediated hSIRT3 overexpression</td>
<td>Protective against α-Syn-induced dopaminergic neuron degeneration</td>
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<td></td>
<td>SNc dopaminergic neurons from \textit{Sirt3}-KO mice</td>
<td>SIRT3 overexpression prevented increase in oxidative stress, degeneration and lysine-68</td>
<td>Shi et al., 2017</td>
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<tr>
<td>Amyotrophic Lateral Sclerosis (ALS)</td>
<td>Cultured motor neurons derived from SOD1\textsuperscript{G93A} mutant mouse overexpressing hSIRT3</td>
<td>Prevents apoptotic cascades and mitochondrial fragmentation</td>
<td>(Song et al., 2013)</td>
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<td>SOD1\textsuperscript{G93A} mutant mouse astrocytes over-expressing mSIRT3 co-cultured with motor neurons</td>
<td>Rescued degeneration of SOD1\textsuperscript{G93A} mutant mouse motor neurons</td>
<td>Harlan et al., 2016</td>
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<td>Disease Model</td>
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<td>Huntington’s Disease (HD)</td>
<td>Human striatal precursor cell line expressing mutant Htt with SIRT3 knockdown</td>
<td>Loss of protective effects of viniferin in absence of SIRT3</td>
<td>(Fu et al., 2012)</td>
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<tr>
<td>Stroke</td>
<td>Mouse cortical neurons overexpressing mSIRT3</td>
<td>Protective against NMDA-induced excitotoxicity</td>
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<td>Rat cortical neurons overexpressing SIRT3</td>
<td>Protected from oxygen / glucose deprivation (OGD) challenge</td>
<td>Dai et al., 2017</td>
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<td></td>
<td>Neuronally differentiated PC12 cells over-expressing hSIRT3</td>
<td>Protected from oxygen / glucose deprivation (OGD) challenge</td>
<td>Shulyakova et al., 2015</td>
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<td></td>
<td>Cultured mouse cerebellar granule neurons transiently overexpressing mSIRT3</td>
<td>Protected from axonal degeneration induced by KCl depletion</td>
<td>Magnifico et al., 2013</td>
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<td>Transient MCAO in Sirt3-KO mice</td>
<td>SIRT3 activator adjudin attenuated glial scar formation and improved functional recovery in WT mice</td>
<td>Protective effects of adjudin lost in Sirt3-KO mice</td>
<td>Yang et al., 2017</td>
</tr>
<tr>
<td>Rat cerebellar granule neurons overexpressing SIRT3</td>
<td>Enhanced neuronal death in response to low potassium ion treatment</td>
<td></td>
<td>Pfister et al., 2008</td>
</tr>
<tr>
<td>Transient MCAO in Sirt3-KO mice</td>
<td>Smaller brain infarct volumes in Sirt3-KO mice</td>
<td>Decreased mitochondrial ceramides following stroke challenge</td>
<td>Novgorodov et al., 2016</td>
</tr>
<tr>
<td>Transient MCAO in Sirt3-KO mice</td>
<td>Neuroprotection 3 days post-ischemia/perfusion in Sirt3-KO mice</td>
<td></td>
<td>Verma et al., (2017)</td>
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1.9 **Rationale, hypothesis and specific aims of the thesis**

Mitochondrial diseases arise from acquired or inherited mutations in the mitochondrial or nuclear genomes which, in turn, cause impaired mitochondrial ATP production (Niyazov & Frye, 2016) and increased oxidative stress (Hayashi & Cortopassi, 2015). Although these conditions are highly heterogeneric, their clinical symptoms and molecular pathology frequently overlap (Gorman et al., 2016). However, the pathophysiology of genetic mutations and the consequential mitochondrial dysfunction in these conditions are still not fully understood, which greatly impedes the development of effective therapeutic treatments. Because neurons are susceptible to perturbations in energy supply, mitochondrial function has been suggested to play a pivotal role in the pathogenesis of a range of neurological and neuropsychiatric disorders and their associated behavioural disturbances (Johri & Beal, 2012; Filosa et al., 2014; Müller & Can, 2014; Valenti et al., 2014; Marin & Saneto, 2016). While there is considerable debate of whether mitochondrial dysfunction is causal to, or consequential of, the progression of these conditions, there is a general consensus that improving mitochondrial homeostasis and functional capacity could improve outcomes for many of these conditions.

SIRT3 is the predominant mitochondrial deacetylase, and SIRT3-mediated deacetylation provides one mechanism through which the activity of its target proteins are regulated. As SIRT3-targeted proteins are essential contributors to processes such as mtDNA repair (Cheng et al., 2013), calcium homeostasis, (Dai et al., 2014), anti-oxidative (Bell & Guarente, 2011; Bause & Haigis, 2013) and anti-apoptotic (Qiao et al., 2016) mechanisms, SIRT3 activity has been hypothesized to serve as a master regulator of mitochondrial homeostasis. Indeed, disturbances in these processes underlie both neurogenerative and neuropsychiatric conditions (Bossy-Wetzel et al., 2004; Martin, 2012; Berridge, 2014; Salim, 2014; Cha et al., 2015; G. H. Kim et al., 2015; Pei & Wallace, 2018;). As high correlation exists between neurodegenerative and neuropsychiatric
disorders through the mitochondrial-axis, (Lezi & Swerdlow, 2012; Pei & Wallace, 2018), it is conceivable that Sirtuin-3 may be one factor that regulates common molecular pathways involved in both types of conditions. Thus far, evidence derived from multiple studies have strongly suggested that Sirtuin-3 plays a role in different neurodevelopmental and neurodegenerative conditions (section 1.8.3), although, the link between this enzyme and the classical neuropsychiatric conditions, such as schizophrenia, major depressive disorder, and bipolar disorder has yet to be established.

Genetic mouse models for mitochondrial disease that can transcend a broad range of neurologic and neuropsychiatric symptoms are instrumental in understanding the complexity of such conditions. In order to elucidate the role of sirtuin-3 in the progression of these conditions, it is imperative to understand its role under basal conditions. To date, however, the majority of studies have focused on the role that sirtuin-3 function plays under pathological and various stress circumstances; its role under normal conditions – and particularly in energy-demanding tissues like the brain - has been relatively under-investigated. Therefore, the goal of my project is to expand on the current knowledge of the neurological role that SIRT3 plays in vivo under basal conditions.

In order to better understand the role that sirtuin-3 plays in normal physiology, knockout animal models were used to assess the function of this enzyme in vivo. My hypothesis is that the absence of sirtuin-3 in vivo will alter normal neuronal homeostasis by causing changes in molecular signaling cascades within the brain, and ultimately altering normal behavioural processes in Sirt3-KO mice. My hypothesis will be guided by the aims outlined below:
AIM 1: Despite recent data suggesting a key role for sirtuin-3 in the brain, only minimal information exists regarding its temporal and spatial prevalence patterns in the CNS (Shi et al., 2005; Lombard et al., 2007; Palacios et al., 2009). An understanding of its level and regional prevalence may aid in delineating how it influences brain function globally or in specific brain circuits. Because SIRT3 is a mitochondrial enzyme, and bioenergetic demands vary across brain areas (Magistretti & Allaman, 2015), I hypothesize that sirtuin-3 will display a unique spatial and temporal expression pattern in the CNS.

AIM 2: Initial behavioural characterization of Sirt3-KO mice failed to identify overt phenotypic alterations (Lombard et al., 2007; Liu et al., 2015). This seems surprising given that Sirt3-KO mice exhibit striking mitochondrial protein hyperacetylation in the brain and in the peripheral tissues (Lombard et al., 2007; Cheng et al., 2016). Furthermore, numerous tissues harvested from these animals show alterations in normal mitochondrial function (Ahn et al., 2008; Hafner et al., 2010; Kawamura et al., 2010; Bell & Guarente, 2011; Fernandez-Marcos et al., 2012; Bause & Haigis, 2013; Ansari et al., 2017), and recent work has shown that sirtuin-3 is highly prevalent in brain tissue (Shi et al., 2005; Lombard et al., 2007; Palacios et al., 2009). Therefore, it is perhaps surprising then that Sirt3-KO mice display only modest physiological or behavioural changes under baseline conditions (Lombard et al., 2007; Liu et al., 2015). Given these significant mitochondrial alterations, a more thorough behavioural assessment of Sirt3-KO mice to interrogate more specific types of behaviour than those evaluated in the initial studies would seem warranted. I hypothesize that sirtuin-3 deficiency will cause the development of behavioral impairments in mice that can be unveiled using a range of motor and cognitive tasks.

AIM 3: As described in section 1.5, mitochondria are involved in signaling pathways beyond their localized metabolic functions through release of ROS and other metabolites (Finkel, 2011).
Despite numerous studies illustrating that SIRT3 plays a key role in maintaining mitochondrial homeostasis, the consequences of how a sirtuin-3-deficiency in brain mitochondria can affect extra-mitochondrial signaling processes in the brain has not been assessed. Assessment of altered molecular mechanisms in Sirt3-KO mice will expand our knowledge on the role of sirtuin-3 in the cell as whole. I hypothesize that sirtuin-3 deficiency is sufficient to alter extra-mitochondrial biochemical signaling pathways in the brain.

Chapter 1 provides a general introduction. Chapter 2 states the materials and methods used in this thesis. Chapter 3 is dedicated to the assessment of sirtuin-3 expression in the rat brain relative to its sirtuin family members. Chapter 4 evaluates behavioural consequences arising in mice lacking Sirt3 at young and older stages of development. Chapter 5 investigates how energy-related signaling pathways are affected in the Sirt3-KO mouse brain to explore mechanisms for behavioural alterations observed in Chapter 4. Chapter 6 expands on the observations presented in Chapters 4 and 5 to assess whether Sirt3-KO mice display endophenotypes common to certain neuropsychiatric conditions. Chapter 7 includes a general discussion of the results and discusses implications and future considerations.
Materials and methods

2.1 Animal use

2.1.1 Statement of Ethics

Animal experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care, and protocols for animal use were reviewed and approved by the local University Health Network animal care committee before commencement of the study.

2.1.2 Animal models

For sirtuin expression study (see Chapter 3), male Wistar rats and timed pregnant female Wistar rats were purchased from Charles River Laboratories (Sherbrooke, Quebec). For expression assessments of sirtuins, Wistar rats were used as subjects. The consequences of sirtuin-3 deficiency on behaviour and molecular consequences were assessed using Sirt3-KO mice, which were originally developed by Dr. Schwer’s group (Lombard et al., 2007) and WT C57Bl/6 mice (purchased from Jackson Laboratories, Sirt3-KO mice, cat # 012755, WT mice, cat #000664). Sirt3-KO mice were originally on the 129S1/SvImJ background, which were backcrossed to the C57BL/6 background for 7 generations. Animals were housed at 5 mice per cage in a vivarium that was maintained at 22–23°C with a standard 12-hour light on/off cycle commencing at 6:00 with food and water provided ad libitum. All studies were conducted using young adult (3-8 months) and aged (18-24 months) Sirt3-KO and WT female and male littermates. For determination of body weight, animals were weighed on a laboratory scale.
2.1.3 Genotyping Sirt3-KO mouse model

Ear punches were collected and immersed in 75 µl of 25 mM Sodium Hydroxide / 0.2 mM Ethylenediaminetetraacetic acid (EDTA) solution and placed in Bio Rad. MJ mini Thermocycler at 98 °C for 30 minutes. After completion, 75 µl of 40 mM Tris(hydroxymethyl)aminomethane (Tris) Hydrochloric acid (HCL) (pH 5.5) was added to each sample to neutralize the solution (Truett et al., 2000). DNA samples were then stored in -20°C until further use. The collected DNA samples were used as template in Polymerase Chain Reaction (PCR) using a Bio Rad. MJ mini Thermocycler. Genomic DNA from WT mice was used as a positive control to detect the WT allele, and DNA from Sirt3-KO homozygous mouse was used to detect the absence of Sirt3. Amplification was carried out using the common primer 5’ -CTT CTG CGG CTC TAT ACA CAG -3’, the WT reverse primer 5’-TGC AAC AAG GCT TTA TCT TCC -3’, and Sirt3-KO reverse primer 5’- TAC TGA ATA TCA GTG GGA ACG-3’. The cycles are as follows: 35 cycles of denaturing at 94 °C for 30 seconds, annealing at 66 °C for 1 minute and extending at 72 °C for 1 minute. Following PCR, DNA loading dye samples (Thermo Fisher, Burlington, cat # R0611) was added to the and visualized on ethidium bromide-stained gel. A band of 562 base pairs (bp) reflects a WT mouse, one at 200 bp reflects a homozygous Sirt3-KO mouse and one and 562 bp and 200 bp reflect a heterozygous Sirt3-KO mouse.

2.1.4 Behavioural tests

All behavioural tests were conducted between 8:00 and 13:00 to minimize the effect of the circadian rhythms. The tests were performed in a sound attenuating room in the presence of 65 decibel ambient white noise to recapitulate the environment in the housing room. Prior to behavioural assessments, mice were allowed to habituate to the experimental room for 30 minutes. Between all trials, the behavioural equipment were cleaned with 70% alcohol and thoroughly aerated. All tests were performed and assessed by an experimenter blinded to the genotype.
2.1.4.1 Accelerating rotarod test

To test motor coordination and balance (Carter et al., 2001; Deacon, 2013), accelerating rotarod test was conducted on Panlab Rotarod (Harvard Apparatus) that accelerated linearly from 4 rotation per minute (rpm) to 35 rpm over a 5-minute period. Latency to fall was recorded in three daily trials. In total, mice were subjected to four consecutive days of testing. Same day trials were separated by at least 15 minutes to allow the animals to recover from physical fatigue. Rotarod apparatus was thoroughly cleaned with 70% alcohol between the trials.

2.1.4.2 Traversion test

Mice were subjected to the traversion test, which is directed to measure motor coordination (Carter et al., 2001; Luong et al., 2011; Deacon, 2013). Wooden dowels were fixed by a G-clamp and held above the floor by a wooden support column at each end. The columns are of different heights (60 cm start column, and 100 cm end column), creating a 45 degree incline. One flat wooden plank (2 cm wide) and one thinner wooden dowel (5 mm diameter) were used for training and experimental purposes, respectively. Both the plank and the dowel were 60 cm long. A mouse hut was placed on top of the end column as a motivational tactic to persuade the mice to traverse the dowel. Mice were trained to walk up the flat plank. Training consisted of two attempts to walk up the plank with no time limitations. On the test day, mice were initially assessed on the flat plank followed by assessments on the experimental dowel. The success of crossing the plank/dowel was recorded and marked as “complete” (up to a maximum of 1 minute). If mice did not reach the finish line within the allocated time, it was marked as incomplete. The beams were cleaned of mouse droppings and wiped with 70% ethanol before next mouse is placed on the apparatus.
2.1.4.3 Open field test

The open field (OF) assessment reflects the natural tendency of rodents to want to explore a novel environment while avoiding the brightly lit and open environment (Wilson et al., 1976; Choleris et al., 2001). It is a measure of motor activity as well as anxiety (Sestakova et al., 2013). Animals were evaluated in an OF arena using an automated activity monitoring system (Linton Instruments, UK) controlled by Amonlite activity monitor software. Briefly, this apparatus consists of a plexiglass box (20 x 30 cm²) which is surrounded by a frame (45 x 25 cm²). The apparatus contains 24 infrared beams that form a grid and detect motion. When an animal moves, it breaks a beam and a specific activity is recorded. Several behavioural parameters can be detected that depend on the location, level and speed of the beam breaks. For the behavioural assessments, mice were placed in a Plexiglas cage (20 cm x 30cm) and an automated movement detection system was used to record animal motor activity in 5-min increments over a 1 hour session. The following parameters were tested: total time spent active (total number of beam breaks), total rearing counts (total upper frame beam breaks), total center rearing counts (total number of beam breaks in the center field of the OF box), total distance travelled (distance covered by a mobile animal within a specified time), and total static counts (an animal is considered to be engaged in static activity when it is immobile however beams are still broken). Each mouse was examined in the OF only once to prevent acclimation to the apparatus. Plexiglass cages were thoroughly cleaned with 70% alcohol between the trials.

2.1.4.4 Light and dark placement preference test

Anxiety-like behavior was examined using light and dark (LD) preference test as described previously by (Bouwknecht & Paylor, 2002). Briefly, a mouse was placed into a plexiglass box consisting of a dark compartment (20 cm x 14 cm) and a light compartment (20 cm x 28 cm) connected through a single small opening (4 cm x 4 cm). The light compartment was illuminated
by a 60 Watt lamp. A mouse was initially placed in the light area of the apparatus facing the opening to the dark chamber. Mouse movements were video recorded for 5 minutes. The amount of time each mouse spent in the dark compartment, as well as the duration of head pokes out of the dark compartment was quantified. Risk assessment was calculated as the length of time a mouse spent head poking out of the dark compartment normalized to the total time spent in the dark compartment. Fewer risk assessments are indicative of anxiety-like behaviour. LD apparatus was thoroughly cleaned with 70% alcohol between the trials.

2.1.4.5 Elevated plus-maze test

Elevated plus maze (EPM) test was based on the procedures described by Pellow & File (1986), and it is a measure of anxiety (Lister, 1987; Sestakova et al., 2013). The apparatus is composed of two lit open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm × 19 cm). The arms radiated from a central platform (5 × 5 cm) and were raised at the height of 50 cm above the floor. A mouse was placed on the central platform, facing an open arm and was allowed to explore the arms for 5 minutes. Mouse movements were video recorded. When a mouse had its all four paws in one of the arms, it was assigned the corresponding compartment. The mouse was assigned a central platform location whenever its two paws were on it. The amount of time spent in the open area for the duration of the test was recorded. EPM apparatus was thoroughly cleaned with 70% alcohol between the trials.

2.1.4.6 Marble burying test

The marble burying test is a model of anxiety (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986). Clean mouse cages were used with approximate 5 cm deep bedding. 20 glass marbles (15mm diameter) were systematically placed at equal distances from each other. Test period consisted of a 1-hour exploration period. The number of uncovered marbles (>25% marble not covered by
bedding material) was quantified and averaged by two examiners who were blind to the mouse genotype.

### 2.1.4.7 Novel object recognition test

Mice were tested for the object recognition memory, based on the procedure described by (Vaucher et al., 2002). In short, each animal was first habituated to the testing environment, a clear plastic box (44 x 22 x 29 cm), for 15 minutes for seven days. On the test day, two objects that were similar in shape, color, and texture were placed at the two ends of the plastic box at an equal distance from the imaginary centerline. The duration spent exploring these objects was recorded, object exploration was considered to be any behaviour that involved touching the object with its forepaws and/or nose or sniffing at the object within a distance of 1.5 cm. Mice were allowed to explore the two objects for 10 minutes. Following a 5 min delay (short-term retention) mice were exposed to the original control object and a novel object for 5 minutes. After a 2 hour wait (long-term retention), mice were allowed to explore the original control object and a novel object for 5 min. Percentage of time spent with the novel object was calculated using the \([tN/(tF + tN)]*100\) formula, where \(tN\) = time spent exploring new object and \(tF\) = time spent exploring familiar object.

### 2.1.5 Telemetry

Experimental mice were implanted with wireless telemetry probes (TA11ETA-F10) purchased from Data Sciences International (DSI) to record core body temperature, overall activity and electroencephalography (EEG) waveforms. The surgical procedure was as previously described in (El-Hayek et al., 2011). In short, mice were anesthetized with 2% isoflurane and the wireless transmitter was implanted into their peritoneal cavity. The sensing and reference wires that were connected to the transmitter were subcutaneously routed rostrally toward the head. The sensing wire
was soldered to an intracranial EEG electrode that was positioned in the parietal cortex region (bregma -0.6 mm, lateral 1.5 mm, and depth 1.5 mm), with the reference wire placed at bregma -5 mm, lateral 1 mm, and depth 1.5 mm. To prevent potential infection, animals were treated with the antibiotic enrofloxacine (Baytril, Bayer HealthCare, Ontario, Canada), which was administered orally via drinking water at an estimated dose of 0.7 mg per day, 3 days before and 3 days after the surgery. To relieve post-surgery pain, animals received a subcutaneous injection of the analgesic metacam (Boehringer Ingelheim, Burlington, Ontario, Canada; 4mg/Kg). Following 1 month recovery post-surgery, telemetry recordings were performed on mice that were single-housed in their home cages for a continuous 24-hr period at 30-second intervals. EEG, gross motor activity and core body temperature were analyzed as previously described by (Wither et al., 2012).

2.1.6 Amphetamines

D-amphetamine sulfate (AMPH) (R&D Systems Europe, cat # 2813) was dissolved in saline (0.9% NaCl). AMPH was injected subcutaneously in a volume of 0.01 ml/g. Experimental animals received AMPH (1mg/kg) and control mice received saline vehicle injections. Mice were weighed, injected, and after 45 minute incubation placed in the OF apparatus for 1 hour session, followed by light/dark and plusmaze tests.

2.1.7 Rat and mouse model tissue collection

Animals were euthanized by decapitation while under isoflurane anesthesia, and specific brain and peripheral tissues were collected by dissection. For sirtuin expression assessments in rats, (see Chapter 3) (whole brain, cortex, hippocampus, cerebellum, brain stem, spinal cord, striatum, olfactory bulb, heart, liver, spleen, kidney, small intestine) at different ages (embryonic day 18 (E18), postnatal day (PN) 2, 7, 21 and 3, and 24 months) were collected. The sex of the subjects used for tissue collection was not specifically determined for E18, PN2, PN7 or PN21 stages,
while female rats were used for the 3 and 24 month tissue samples. For molecular assessments of Sirt3-KO mice (see Chapter 5), cortical tissues were collected in young male and female mice. All dissections were conducted on an ice-cold glass plate, and the isolated tissues snap frozen by submersion in liquid nitrogen. Frozen tissues were stored at −80°C until homogenization for RNA or protein examinations

2.2 Quantitative real time PCR

To quantify sirtuin mRNA expression levels for the sirtuin expression study (see Chapter 3), rats were sacrificed by decapitation, and specific brain regions isolated by dissection on an ice-cold glass plate. RNeasy Mini Kit (Qiagen, Valencia, CA, cat # 74104) was used to extract total RNA from the tissue as per manufacturer’s guidelines. Purified total RNA (1 μg) was then reverse transcribed into cDNA using the Superscript II First Strand Synthesis System (cat # 18064-014, Invitrogen). Pre-designed Taqman probe and primer mixtures (Applied Biosystems-Life Technologies, Burlington, Ontario) were used to assess mRNA expression: Sirt2 (cat # Rn01457502_m1), Sirt3 (cat # Rn01501412_m1), Sirt4 (cat # Rn01481485_m1), Sirt5 (cat # Rn01450559_m1), Sirt6 (cat # Rn01408249_m1), and Sirt7 (cat # Rn01471420_m1), using hypoxanthine-guanine phosphoribosyltransferase (Hprt1) (cat # Rn_01527831_g1) as a reference control. Because Taqman primers were not available for rat Sirt1, SYBR Green forward and reverse primers were designed that spanned an intron of the rat Sirt1 sequence (forward: 5’-cagcaacactcactgattgg-3’, reverse: 5’-tccctacggaagcagaaa-3’) and also for the reference Hprt1 (forward: 5’-gcagactttgctttgcttg-3’, reverse: 5’-cgagaggcttttcaccag-3’). We selected Hprt1 as the housekeeping gene for the mRNA quantification studies as it is expressed ubiquitously and stably in various tissues (Fischer, Skowron, & Berthold, 2005), and it has been used as an internal control for mRNA normalization in previous developmental studies (Franco-Montoya et al., 2011; Murta et al., 2013). All PCR samples were assayed in quadruplicate on a
384-well plate using Taqman master mix reagent kit (Applied Biosystems, cat # 4304437) or SYBR Green (Applied Biosystems, cat # 4368577) using 20 ng of cDNA generated from total isolated RNA as template in 10 µl reactions on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The thermal profile for the Taqman assays was as follows: one cycle of enzyme activation at 50°C for 2 min and template denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, primer annealing at 60°C for 1 min, and template extension at 72°C for 30 s. For SYBR Green assays, the thermal profile was: one cycle of enzyme activation at 50°C for 2 min followed by denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, primer annealing at 56 or 60°C for 1 min, and template extension at 72°C for 30 s. The amplification of all the primers was analyzed with using the Applied Biosystems Sequence Detection System software (SDS v. 2.2). For mRNA expression, critical threshold (Ct) cycle values were identified for each sirtuin being assayed relative the expression of the Hprt1 reference gene, and the results expressed as fold differences from Hprt1 using the $2^{-\Delta C_t}$ formula, where $\Delta C_t = C_t(Sirt) - C_t(Control)$. For mRNA expression comparisons between the astrocyte and neuronal enriched cultures, the expression of Hprt1 was used as a common reference. The Ct values for Hprt1 expression were equivalent between the two cultures (not shown).

2.3 Antibodies and western blotting

Quantification of protein concentration of the samples obtained was determined using the Bio-Rad protein assay (Biorad, Mississauga, Ontario, cat # 162-0115) and 30 µg of total protein samples were resolved using western blotting as outlined in Sidorova-Darmos (2014). The primary antibodies and dilutions used for the studies are outlined in Table 2.1. To allow normalization for minor variances in protein loading between sample lanes, the blots were re-probed for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Chemicon-Millipore cat # MAB374), Beta-actin (1:1000; Cell Signaling, cat # 51525), TOM20 (1:5000;
Santa Cruz, cat# FL-145) or stained with Coomassie blue (0.1% Coomassie Brilliant Blue R250, 10% acetic acid, Sigma-Aldrich). Coomassie staining was used to normalize peripheral tissue expression levels, as loading controls such as GAPDH have not proven reliable when comparing across different tissues and/or specific cell types from the same animal (Eaton et al., 2013). Horseradish peroxidase (HRP)-linked secondary antibodies used were: anti-rabbit raised in sheep (1:5000; GE Healthcare, cat # NA934V) or anti-mouse raised in donkey (1:5000; GE Healthcare, cat # NA931V). X-Ray Films were scanned, and the optical density of bands was quantified using Image J version 1.816 software (https://imagej.nih.gov/ij/), with film background subtracted to give the final densitometric values.
Table 2.1: List of primary antibodies

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<th>Antibody</th>
<th>Species</th>
<th>Dilution Used</th>
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</table>
2.4 Validation of sirtuin antibody specificity

The specificity of each individual sirtuin antibody was determined by assessing non-treated and siRNA-treated PC12 cells for the appropriate sirtuin. The specific siRNAs and concentrations used were: *Sirt1* (50 nM, Sigma-Aldrich, cat # SASI_Rn0200230695), *Sirt2* (13 nM, Ambion-Life Technologies, Burlington, Ontario, cat # S16750), *Sirt4* (13 nM, Ambion, cat # S156091), *Sirt5* (50 nM, Ambion, cat # S157785), *Sirt6* (50 nM, Ambion, cat # S152314), *Sirt7* (50–200 nM, Ambion, cat # S155507), and a scrambled siRNA control at matching concentrations (Ambion, cat # AM4611) was used as a negative control for each respective comparison. After 24 hour of transfection, whole cell lysates were collected and analyzed by western blotting. For confirmation for sirtuin-3 antibody specificity, cortical tissue from *Sirt3*-KO mice was used, as PC12 cells did not display readily discernible endogenous SIRT3 immunoreactivity under basal conditions (not shown). Cortical tissue was isolated and processed as described above.

2.5 Cell culture

2.5.1 Establishing neuron-enriched primary

Cortical tissue was isolated from fetuses obtained at embryonic day 18 (E18) from timed-pregnant Wistar rats (Charles River Laboratories). The head of each embryo was dissected away from the body and placed in 20 ml 1×Hank’s Balanced Salt Solution (HBSS) (Invitrogen-Life Technologies, Burlington, Ontario, cat # 14175103). Brains were removed and placed in a separate dish containing 20 ml supplemented HBSS. Cortices were dissected from whole brains using micro dissection forceps, and the collected tissue was incubated in 2 ml of 0.05% trypsin (Sigma–Aldrich, St. Louis, MO, cat # 59417C) at 37°C for 15 min. The tissue was then triturated by glass pipette 10–15 times to disperse the cells in seeding medium (Dulbecco’s Modified Eagle Medium (DMEM) /F-12) (Invitrogen, cat # 21041025) containing 10% horse serum (HS) and
then centrifuged for 5 min at 21,000 × g to pellet the cells. The cells were re-suspended in Neurobasal medium (Invitrogen, cat # 21103049) containing 2% B-27 supplement (Invitrogen, cat # 17504-044), 1% FBS, 0.5 mM L-glutamine (Invitrogen, cat # 25030-149) and cell counts done using Trypan Blue (Sigma–Aldrich, cat # T8154) as a viability indicator. The cells were then seeded in plating medium at a density of 30,000 cells/well in 96-well plates. After 24 hour of isolation, the plating media was exchanged with maintenance medium (Neurobasal medium containing 2% B-27 supplement, 0.5 mM L-Glutamine) containing 4 μM cytosine arabinoside (Sigma-Aldrich, cat # C6645) to inhibit proliferation of any residual non-neuronal cells. The primary cultures were fed with maintenance medium every 3–4 days, and the neurons were harvested for assay at 12–14 days after plating.

2.5.2 Preparation of astrocyte-enriched cultures

Astrocyte enriched cultures were generated from cortical tissue isolated from post-natal day 1-2 rat pups. In short, following decapitation, cortical tissue was isolated by dissection, placed in ice-cold HBSS, and the meninges removed by dissection. The tissue was then rinsed in HBSS, placed in a minimal amount of media, and chopped into 1 mm³ cubes by manual dissection. The brain cubes were then incubated with 0.05% Trypsin for 30 min at 37°C, and dissociated by triturating 12–15 times with polished Pasteur pipette. The cells were then collected by centrifugation at 21,000 × g for 5 min, re-suspended in Astrocyte Medium containing 1× N2 Supplement (Invitrogen, cat # 17502048), 2 mM Glutamax, Penicillin/Streptomycin, supplemented with 5 ng/ml EGF (Sigma-Aldrich, cat # SRP3052) as per manufacturer’s instruction. Trypan Blue was used to count viable cells and the cell suspension was then plated in at a density of 1,000,000 cells/well in 6-well plates. The cell suspension was then plated in Astrocyte Medium (1x N2 Supplement, 2 mM Glutamax, Penicillin/Streptomycin, supplemented with 5 ng/ml EGF). Cells were fed with this media
every other day until differentiation where cells were changed with differentiation media (Astrocyte Media plus 1 mM dibutyryl-AMP to induce astrocyte differentiation) (Sigma-Aldrich, D0627) twice weekly.

2.5.3 Establishing purity of neuronal and astrocyte cultures

To establish the purity in the neuronal (section 2.5.1) and astrocyte (section 2.5.2) cultures western blot was run on adult rat brain homogenates and in cultured rat neuronal and astrocytic whole cell lysates. The immunoreactivity for astrocyte, glial fibrillary acidic protein (GFAP), neuron-specific microtubule-associated protein 2 (MAP2) and oligodendocyte-specific (NG2) was assessed in these samples. Beta-actin immunoreactivity was used as a protein loading control.

2.5.4 PC12 culture

PC12 cells were grown in at 37°C in a humidified incubator under 5% CO₂ conditions. The culture medium was RPMI 1640 (Gibco-Life Technologies, Burlington, ON, cat # 11875-093), supplemented with 10% HS (Gibco, cat # 16050-122), 5% FBS (Gibco, cat # 16000-044), and 50 U/ml penicillin-streptomycin (Gibco, cat # 15240-062).

2.6 Mitochondrial purification

Mice were euthanized by decapitation while under isoflurane anesthesia. Cortical tissue was collected by dissection on an ice-cold plate. Mitochondrial purification was as previously described in Chinopoulos et al., (2011). In short, brain tissues were individually homogenized in MSEGTA Buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in water) that was supplemented with 0.2 mg/ml bovine serum albumin (BSA) and centrifuged at ~500 x g x 5 min. The supernatant was then centrifuged at 14,000 x g x 10 min. The resulting pellet was resuspended in 200 ul of 12% Percoll-MSEGTA solution (100% Percoll-
MSEGTA buffer: 225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in 100% Percoll), this suspension was then layered over 1ml of 24% Percoll-MSEGTA solution. The prepared density gradient was then centrifuged at 18,000 x g x 15 min. Following centrifugation, 700 µl was aspirated of the top portion of the sample and 1.2 ml of MSEGTA was added and mixed by inversion and centrifuged at 18,000 x g x 5 min. After centrifugation 1.5ml of supernatant was aspirated and 1.5ml of MSEGTA was added and mixed by inversion. The resulting sample was centrifuged at 14,000 x g x 5 min. The pellet was then lysed in lysis buffer consisting of 50 mM Tris—pH 8.0, 1% NP40, 150 mM NaCl, 1mM EDTA, 1mM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin, 2 mM Na3VO4 and supplemented with 1 tablet of protease inhibitor cocktail (Roche, Mississauga, Ontario, cat # 11836153001). The samples were then centrifuged at 21,000 × g for 10 min. The supernatant was then collected, and total protein concentration determined using the Bio-Rad protein assay (Biorad, Mississauga, Ontario, cat # 162-0115). The resulting protein samples were then stored at -80°C.

2.7 Statistical Analysis

All results are expressed as mean ± standard error of means (SEM) and p < 0.05 was set as the level of statistical significance. Statistical analysis was performed using student’s t-test, chi-square, and one-way or two-way ANOVA with a with a Bonferroni post-hoc test that was applied where there was a significant interaction to evaluate differences by genotype/Amphetamine treatment group. GraphPad Prism version 5.00 (GraphPad Software, San Diego CA) was used for all statistical assessments.
Differential expression of sirtuin family members in the developing, adult, and aged rat brain

3.1 Brief introduction and rationale

Although the brain is only 2% of total body mass, it utilizes approximately 20% of total body energy expenditure (Kety 1957; Rolfe & Brown, 1997; Herculano-Houzel, 2011). The high metabolic cost of this organ is largely due to the necessity of maintaining and reestablishing transmembrane ion gradients during and following an action potential, in addition to the “housekeeping” tasks, which include protein and lipid synthesis, structural integrity and mitochondrial proton leak (Kety 1957; Rolfe & Brown, 1997; Lenard & Berthoud, 2008; Herculano-Houzel, 2011; Mergenthaler et al., 2013; Bauernfeind et al., 2014; Engl & Attwell, 2015). Given that mitochondria play a pivotal role in ATP production, an understanding of the mitochondrial sirtuin-3 prevalence in this metabolically active tissue could aid in delineating its role in both normal physiological processes and also potentially in pathophysiological conditions. However, despite existing data illustrating the role of sirtuin-3 in mitochondrial homeostasis, relatively little information currently exists regarding its expression magnitude and spatial distribution in the brain. Furthermore, because sirtuin-3 is a mitochondrial enzyme, and bioenergetic demands vary across brain areas (Clay et al., 2011), I hypothesize that sirtuin-3 will display a unique spatial and temporal expression pattern in the CNS. Given that sirtuin-3 is one of the seven members of the sirtuin family, assessment of all the seven sirtuin members were also carried out. To begin addressing this issue, the endogenous mRNA and protein expression patterns of the seven sirtuin family members in the adult, developing and aged rat brain were examined, and compared their relative expression within the two predominant cell types of the brain, specifically neurons and astrocytes.
3.2 Results

3.2.1 Sirtuin antibody specifications

Assessing the specificity of sirtuin antibodies in rat whole cell PC12 lysates by using sirtuin-specific siRNAs (Figure 3.1). At the protein level, siRNA treatment identified: SIRT1 product of 110 kDa, SIRT2 protein product of 37 kDa, SIRT4 protein product with a mass of 30 kDa, SIRT5 product of 35 kDa and two specific SIRT6 protein products with masses of 36 kDa and 39 kDa. SIRT3 specific products were identified using cortical tissue derived from a Sirt3-KO mouse (Lombard et al., 2007), which identified one specific SIRT3 product of 28 kDa. No specific protein products for SIRT7 were identified.

3.2.2 Differential expression of sirtuins in adult rat whole brain

To compare sirtuin (Sirt1-Sirt7) expression in the whole brain of an adult rat, quantitative RT-PCR was used (Figure 3.2; Table 3.1) to measure sirtuin mRNA level relative to the ubiquitous housekeeping reference gene Hprt1 (Fischer et al., 2005) (Figure 3.3). Sirt1 was found to be one of the least expressed sirtuin family members in the brain. Sirt1 mRNA levels were significantly lower than Sirt2, Sirt3, and Sirt5 in the whole brain, and were present at similar levels to Sirt4, Sirt6, and Sirt7. Quantitative RT-PCR revealed that Sirt2 is the most abundantly expressed sirtuin in the rat brain at the mRNA level, where its expression was found to be more than 3-fold higher than the reference Hprt1 gene (Figure 3.3). Sirt3 was the third most expressed member of the sirtuin family in the whole brain. Sirt4 was found by quantitative RT-PCR to be the least abundantly expressed member of the sirtuin family in the adult rat brain. Quantitative RT-PCR revealed that Sirt5 mRNA is expressed at levels similar to Hprt1 in whole brain homogenates, making it the second most prevalently expressed member of the sirtuin family in the adult brain. Sirt6 mRNA was found at relatively low levels in the brain by quantitative RT-PCR, being
expressed at approximately 10% the level of the *Hprt1* reference gene. Quantitative RT-PCR revealed that *Sirt7* is also expressed at low levels in the adult brain relative to other sirtuin family members, being present at roughly 5% the levels of the *Hprt1* reference gene.
**Figure 3.1: Validation of sirtuin antibodies specificity using siRNA.** Representative Western blots showing the specificity of the immunoreactive products in rat whole cell PC12 lysates by using sirtuin- specific siRNA in (A) SIRT1 (B) SIRT2 (D) SIRT4 (E) SIRT5 (F) SIRT6 (G) SIRT7 and (C) in Sirt3-KO mice lacking SIRT3 expression. GAPDH immunoreactivity is shown as a protein loading control.
Figure 3.2: Sirtuins are expressed at different levels in the adult rat brain. Histogram showing Sirt1-7 mRNA expression levels in the whole adult rat brain as determined by qRT-PCR. The y-axis shows the relative sirtuin expression levels relative to the Hprt1 reference gene. Note the broken y-axis scale for Sirt2, Sirt3 and Sirt5. Data shown are based on the linear conversion of \( \Delta Ct \) values for each sample (\( n = 3 \), error bars denote SEM). Statistical comparisons between the expression levels of the different sirtuins in the adult brain are presented in Table 3.1.
Table 3.1: Comparison of sirtuin mRNA expression in 3 month-old rat whole brain homogenates

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.3: Representative Amplification Traces of Sirtuin Gene Expression Levels Relative to Hprt1. Representative Quantitative Real-Time PCR plots are shown for the mRNA expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 in the rat whole brain homogenates, the amplification profile of the reference transcript Hprt1 is also indicated.
3.2.3 Differential expression of sirtuins in adult rat brain regions

Within different adult rat brain regions, Sirt1 mRNA was found at similarly low levels, with only modest variability seen between different adult brain regions (Figure 3.4A; Table 3.2). The highest Sirt1 mRNA levels were in the cerebellum, while the lowest were in the spinal cord. SIRT1 protein prevalence paralleled its mRNA pattern, being highest in the cerebellum, intermediate in the cortex, hippocampus, striatum and the olfactory bulb, and lowest in the spinal cord (Figure 3.5A; Table 3.3). Within the different adult brain regions, Sirt2 mRNA was highest in the spinal cord, brain stem, and striatum, and at similar levels in the cortex, hippocampus, cerebellum and olfactory bulb (Figure 3.4B; Table 3.2). At the protein level, SIRT2 protein expression patterns were similar its mRNA patterns, being most pronounced in the spinal cord and brain stem, but still showing strong immunoreactive prevalence in the cortex, hippocampus, striatum and cerebellum (Figure 3.5B; Table 3.3). Sirt3 mRNA was expressed equivalently in each of the different adult brain regions examined except the cerebellum, where its expression was modestly lower (Figure 3.4C; Table 3.2). SIRT3 protein levels largely paralleled its mRNA expression patterns in the adult brain, being at similar levels within the cortex, hippocampus, striatum, spinal cord and brain stem, and at modestly lower levels in the cerebellum (Figure 3.5C; Table 3.3). Sirt4 mRNA expression levels ranged from 0.5 to 2% of the reference gene Hprt1 throughout the different regions of the brain, except for spinal cord, which had the highest level of Sirt4 expression at 5% of Hprt1 (Figure 3.4D; Table 3.2). SIRT4 protein expression was detected in each of the brain regions examined at relatively similar levels (Figure 3.5D; Table 3.3). Sirt5 mRNA was significantly higher in the brain stem, hippocampus, spinal cord, and olfactory bulb than in cortex and cerebellum (Figure 3.4E; Table 3.2). The adult mRNA expression profile was generally recapitulated at the protein level, although SIRT5 protein in the cerebellum was higher than in cortex and hippocampus (Figure 3.5E; Table 3.3). The highest mRNA expression for Sirt6 was observed in the olfactory bulb, and the lowest expression levels
were found in hippocampus and brain stem (Figure 3.4F; Table 3.2). SIRT6 protein expression for each of the isoforms was similar within the different adult brain regions examined (Figure 3.5F; Table 3.3). Within the different regions of the adult brain examined, no significant differences in Sirt7 mRNA expression was detected (Figure 3.4G; Table 3.2).
Figure 3.4: Sirtuins display differential gene expression levels within different regions of the adult rat brain. Histograms showing the mean and SEM of the mRNA expression levels for each individual sirtuin in the indicated brain regions as determined by qRT-PCR. The y-axis shows the mRNA expression levels for (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 relative to the Hprt1 reference gene in each of the denoted brain regions. Data shown are based on the linear conversion of ΔCt values for each sample (n = 3, error bars denote SEM). Statistical comparisons for the expression levels of each sirtuin in these adult brain regions are presented in Table 3.2.
Table 3.2: Relative mRNA expression pattern of specific sirtuin members in different regions of the adult rat brain

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.5: Sirtuin protein expression patterns are non-redundant in different regions of the adult rat brain. Representative Western blots showing the immunoreactive protein levels for (A) SIRT1 (B) SIRT2 (C) SIRT3 (D) SIRT4 (E) SIRT5 (F) SIRT6 in the different regions of the adult rat brain as denoted. The histograms in each panel show the densitometric mean and SEM for each specific sirtuin normalized to its corresponding GAPDH loading control (n=4 independent subjects). Statistical comparisons between sirtuin protein expression patterns in these adult brain regions are presented in Table 3.3.
Table 3.3: Relative protein expression pattern of specific sirtuin members in different regions of the adult rat brain

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<tr>
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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
3.2.4 Differential expression of sirtuins in adult rat peripheral regions

Relative to its expression in peripheral tissues, Sirt1 mRNA levels in the liver were similar to the whole brain; spleen and kidney expressed higher levels of Sirt1 mRNA than brain, while heart and small intestine expressed lower levels of Sirt1 than brain (Figure 3.6A; Table 3.4). This pattern was not recapitulated at the protein level, however, as SIRT1 protein expression was significantly higher in the brain than in any of these peripheral tissues (Figure 3.7A; Table 3.5). In adult peripheral tissues, Sirt2 mRNA and protein were expressed at varying levels relative to brain (Figures 3.6B, 3.7B; Table 3.4). SIRT2 protein prevalence in the heart, liver, and spleen were similar to its brain levels, while its protein prevalence in the small intestine and kidney were at lower levels than in the adult brain (Figure 3.7B; Table 3.5). The kidney displayed Sirt3 mRNA levels similar to those of whole brain, while each of the other tissues examined showed lower Sirt3 mRNA levels than brain (Figure 3.6C; Table 3.4). At the protein level, though, there was considerable variations in SIRT3 immunoreactivity in these tissues: SIRT3 protein prevalence in the heart was higher than in the brain, the spleen and small intestine had lower SIRT3 expression than in the brain, and the kidney and liver had similar SIRT3 levels as the brain (Figure 3.7C; Table 3.5). Sirt4 showed low level of expression in the peripheral tissues, where only the kidney expressed Sirt4 at higher levels than in the brain (Figure 3.6D; Table 3.4). At the protein level, SIRT4 prevalence in the heart, liver, and kidney were at levels similar to that of brain, while the spleen and small intestine displayed lower levels of SIRT4 protein than adult brain (Figure 3.7D; Table 3.5). In peripheral tissues, Sirt5 mRNA expression in the kidney was at levels similar to brain, while all of the other peripheral tissues examined expressed Sirt5 mRNA at levels lower than in the brain (Figure 3.6E; Table 3.4). The protein profile of SIRT5 in these peripheral tissues did not parallel its mRNA pattern, however, as SIRT5 protein was notably higher in the liver, spleen, and kidney than in whole brain, and present at lower or non-detectable levels in the small intestine and heart (Figure 3.7E; Table 3.5). Furthermore, low level of Sirt6 mRNA expression was observed in peripheral tissues, with only the
kidney displaying higher Sirt6 mRNA levels than brain, and the liver and heart showing lower expression than in the brain (Figure 3.6F; Table 3.4). In adult peripheral tissues, the expression levels of these two SIRT6 protein forms was variable, with the 36 kDa being expressed highest in the spleen, where its prevalence was greater than in brain, whereas the intestine expressed low or non-detectable levels of this 36 kDa form (Figure 3.7F; Table 3.5). In contrast, the 39 kDa form was expressed at levels similar to the brain, both in the heart and intestine, while liver displayed low to non-detectable levels of this 39 kDa SIRT6 form. Interestingly, and in contrast to the other sirtuin family members, Sirt7 mRNA expression was highest in the small intestine (Figure 3.6G; Table 3.4), where its expression levels were about 10-fold higher than its mRNA levels in the brain. Although lower than in the small intestine, both the spleen and kidney also showed significantly higher Sirt7 mRNA expression levels than in the brain. Summary of relative mRNA and protein expression patterns of specific sirtuin members in brain and peripheral tissues is presented in Table 3.6.
Figure 3.6: Sirtuins display differential gene expression patterns in adult peripheral tissues. Histograms showing the mean and SEM of the mRNA expression levels for each individual sirtuin in the indicated peripheral tissues and whole brain as determined by qRT-PCR. The y-axis of each histogram shows the relative mRNA expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 normalized to the Hprt1 reference gene. Data shown are based on the linear conversion of delta CT values for each sample (n=3 independent subjects done in quadruplicate). Statistical comparisons between the mRNA expression levels for each sirtuin in these tissues is presented in Table 3.4.
Table 3.4: Comparison of sirtuin mRNA expression in 3 month-old rat peripheral regions

<table>
<thead>
<tr>
<th></th>
<th>Sirt1</th>
<th>Sirt2</th>
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<td>Whole Brain vs. Liver</td>
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<td>Liver vs. Intestine</td>
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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.7: Sirtuin protein expression patterns in adult rat peripheral tissues relative to the brain. Representative Western blots showing the immunoreactive prevalence of (A) SIRT1 (B) SIRT2 (C) SIRT3 (D) SIRT4 (E) SIRT5 (F) SIRT6 in the indicated peripheral tissues of adult rats. The histogram in each panel shows the densitometric mean and SEM for the indicated sirtuin. For these calculations, Coomassie blue staining was used as a protein loading control due to variability in GAPDH expression within the peripheral tissues (n=3 independent subjects). The expression levels for each sirtuin in these peripheral tissues are presented relative to their expression level in brain. Statistical comparisons between sirtuin protein expression levels in these adult tissues are presented in Table 3.5.
### Table 3.5: Comparison of sirtuin protein expression in 3 month-old rat peripheral regions

<table>
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<tr>
<th></th>
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<th>SIRT6 36kDa</th>
<th>SIRT6 39kDa</th>
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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Table 3.6: Summary of relative mRNA and protein expression patterns of specific sirtuin members in brain and peripheral tissues

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<th>Sirt6</th>
<th>Sirt7</th>
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<td>+</td>
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<tr>
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<tr>
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<tr>
<td>Kidney</td>
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<tr>
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<th>SIRT6 (39 KDa)</th>
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<td>+</td>
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<tr>
<td>Kidney</td>
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<td>++++</td>
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<td>0</td>
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</table>

In the upper mRNA expression panel, “+” are assigned based on the specific range of ΔCt values for the Sirtuin expression levels, relative to the Hprt1 control gene, such as: ++++ = ΔCt >3; ++++ = ΔCt 1.5-3; +++ = ΔCt 0.5-1.5; ++ = ΔCt 0.05 – 0.5; and + = ΔCt < 0.05. In the lower protein expression panel, + are assigned based on their relative expression values for the sirtuin expression levels when compared to GAPDH control, such as ++++, very high; ++++, high; ++++, moderate; ++, low; and +, very low levels of expression. “0” was assigned to areas of no expression.
3.2.5 Differential development of sirtuins in rat brain regions

During brain development, Sir1 mRNA levels decreased progressively relative to the reference gene Hprt1 from the late embryonic stage in the cortex, hippocampus, and cerebellum (Figures 3.8A, 3.10A, 3.12A; Tables 3.7, 3.9, 3.11). At the protein level, this general pattern was recapitulated, as SIRT1 protein expression was significantly higher in the cortex and hippocampus at E18 and early postnatal stages compared to the adult and 24 month old stages (Figures 3.9A, 3.11A; Tables 3.8, 3.10). A slightly different pattern was observed in the cerebellum, as while SIRT1 protein levels were lower in the adult than in the perinatal stages, the decrease in its prevalence did not occur until after postnatal day 21 (Figure 3.13A; Table 3.12). In each brain region, SIRT1 protein prevalence at 3 and 24 months were similar, respectively, indicating the absence of an aging-associated decrease in SIRT1 protein prevalence. The developmental expression pattern of Sir2 mRNA relative to the Hprt1 reference gene in the cortex and hippocampus was highest at E18, and then remained fairly consistent across perinatal development and into adulthood (Figures 3.8B, 3.10B; Tables 3.7, 3.9). A spike of Sir2 mRNA was observed at PN21, but this increase was transient and not retained in the 3 month old cortex or hippocampus. The expression profile of Sir2 was somewhat different in the cerebellum, where Sir2 mRNA displayed a transient increase at PN7 and PN21 compared to E18 and PN2, and then decreased to consistent levels seen in both the adult and 24 month old tissues (Figure 3.12B; Table 3.11). Intriguingly, however, this mRNA profile for Sir2 was not recapitulated at the protein level, as SIRT2 immunoreactivity was minimal or at background levels in samples prior to the PN21 stage in the cortex, hippocampus, and in the cerebellum (Figures 3.9B, 3.11B. 3.13B; Tables 3.8, 3.10, 3.12). SIRT2 protein then rapidly appeared at PN21 in each of these brain regions, and remained at fairly consistent levels
until 24 months of age. The mRNA expression pattern of $Sirt3$ during development was relatively similar in the cortex, hippocampus, and cerebellum, with $Sirt3$ mRNA levels being fairly consistent from E18 until 24 months of age (Figures 3.8C, 3.10C, 3.12C; Tables 3.7, 3.9, 3.11). The pattern of SIRT3 protein expression was quite different, however, as a robust increase in SIRT3 protein prevalence was observed in the cortex and in the hippocampus between PN7 and PN21, and this level was maintained in the 3 and 24 month old samples (Figures 3.9C, 3.11C; Tables 3.8, 3.10). In the cerebellum, SIRT3 protein prevalence was similar between E18 to 3 months, but then increased significantly at 24 months of age (Figure 3.13C; Table 3.12). During development, $Sirt4$ mRNA levels in each of the brain regions examined were highest at the E18 stage, decreased significantly from this late embryonic level by PN2, and then remained at fairly consistent levels between PN7 and 24 months of age (Figures 3.8D, 3.10D, 3.12D; Tables 3.7, 3.9, 3.11). At the protein level, no significant differences in SIRT4 protein prevalence were noted in any of the brain regions examined throughout development, including the E18 time point where higher mRNA levels had been seen (Figures 3.9D, 3.11D, 3.13D; Tables 3.8, 3.10, 3.12). During development, $Sirt5$ mRNA levels decreased modestly in the cortex and in the hippocampus between E18 and PN7, and then remained at consistent levels until 24 months of age (Figures 3.8E, 3.10E; Tables 3.7, 3.9). In the developing cerebellum, $Sirt5$ mRNA levels decreased at 3 months of age, and this lower level remained in tissue from 24 month old rats (Figure 3.12E; Table 3.11). At the protein level, SIRT5 immunoreactivity did not significantly differ across development within either the cortex or hippocampus (Figures 3.9E, 3.11E; Tables 3.18, 3.10), although SIRT5 protein levels in the cerebellum were higher at 3 and 24 months when compared to levels seen at E18 or in the perinatal stages (Figure 3.13E; Table 3.12). In the developing brain, $Sirt6$ mRNA levels were highest in the cortex, hippocampus, and cerebellum at the E18 stage, and then decreased to levels at PN7 that were maintained at 24 months (Figures 3.8F, 3.10F, 3.12F; Tables 3.7, 3.9, 3.11). At the protein level,
the different forms of expressed SIRT6 protein displayed region-specific differences in expression. Throughout cortical and hippocampal development, the prevalence of the 36 kDa product decreased from E18 to PN21, and then remained at similar levels in these respective tissues at 3 and 24 months of age. This was not the case for the 39 kDa SIRT6 protein product, though, as its prevalence remained constant throughout cortical and hippocampal development (Figures 3.9F, 3.11F; Tables 3.8, 3.10). In the developing cerebellum, the 36 kDa SIRT6 product was found at similar levels throughout development, while the 39 kDa form increased in prevalence between E18 and PN7, and then remained at fairly consistent levels until 24 months of age (Figure 3.13F; Table 3.12). During cortical and hippocampal development, Sirt7 mRNA levels decreased between E18 and PN2, but then remained at similar levels throughout the remaining time points examined (Figures 3.8G, 3.10G; Tables 3.7, 3.9). In the cerebellum, Sirt7 mRNA levels remained at relatively similar levels throughout perinatal development, as well as in the adult and 24 month old brain (Figure 3.12G; Table 3.11). Summary of the relative developmental mRNA and protein expression patterns of sirtuin members in the cortex, hippocampus and in the cerebellum are outlined in Table 3.13.
Figure 3.8: Sirtuin mRNA expression patterns during rat cortical development. Histograms showing the mean and SEM of the mRNA expression levels for each individual sirtuin at the indicated stages of cortical development (E18 to 3 months) and in rats at 24 months of age. The y-axis of each histogram shows the relative expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 relative to the levels of the Hprt1 reference gene. Data shown are the linear conversion of ΔCt values for each sample relative to Hprt1 (n = 3 independent subjects done in quadruplicate). Statistical comparisons for the cortical expression levels of each sirtuin for the developmental times shown are presented in 3.7.
Table 3.7: Comparison of sirtuin mRNA expression during cortical development

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<tr>
<th></th>
<th>Sirt1</th>
<th>Sirt2</th>
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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.9: Sirtuin protein expression patterns during rat cortical development. Representative Western blots showing the prevalence of (A) SIRT1, (B) SIRT2, (C) SIRT3, (D) SIRT4, (E) SIRT5, and (F) SIRT6 in the rat cortex at the indicated stages of embryonic and postnatal development (n = 3 independent subjects). The corresponding GAPDH protein level for the same blot is shown, and serves as a protein loading control. The histograms in each panel show the densitometric mean and SEM for each specific sirtuin normalized to its corresponding GAPDH loading control. The level of expression for each Sirtuin in the 3-month old cortex was arbitrarily set to 100%, and different stages expressed as a percentage of this 3-month value. Statistical comparisons for the expression levels of each sirtuin for the developmental times shown are presented in Table 3.8.
### Table 3.8. Comparison of sirtuin protein expression during cortical development

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.10: Sirtuin mRNA expression patterns during rat hippocampal development. Histograms showing the mean and SEM of the mRNA expression levels for individual sirtuins at the indicated ages and stage of embryonic hippocampal development. The y-axis of each histogram shows the expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 relative to the levels of the Hprt1 reference gene. Data shown are based on the linear conversion of ΔCt values from qRT-PCR assays for each sample (n = 3 independent subjects done in quadruplicate). Statistical comparisons for the hippocampal expression levels of each sirtuin for the developmental times shown are presented in Table 3.9.
Table 3.9: Comparison of sirtuin mRNA expression during hippocampal development

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.11: Sirtuin protein expression patterns during rat hippocampal development. Representative Western blots showing (A) SIRT1 (B) SIRT2 (C) SIRT3 (D) SIRT4 (E) SIRT5 (F) SIRT6 protein levels in rat hippocampus at the indicated embryonic or postnatal stages (n = 3 independent subjects). The corresponding GAPDH protein level for the same blot is shown, and serves as a protein loading control. The histograms show the densitometric mean and SEM for each specific sirtuin normalized to its corresponding GAPDH loading control, with the 3-month value arbitrarily set as 100%. Statistical comparisons for the expression levels of each sirtuin for the developmental times shown are presented in Table 3.10.
### Table 3.10: Comparison of sirtuin protein expression during hippocampal development

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.12: Sirtuin mRNA expression patterns during rat cerebellum development. Histograms showing the mean and SEM of the mRNA expression levels for individual sirtuins in the cerebellum at the indicated ages and stage of embryonic development. The y-axis of each histogram shows the expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 relative to the levels of the Hprt1 reference gene. Data shown depict the linear conversion of ΔCt values from qRT-PCR assays for each sample (n = 3 independent subjects done in quadruplicate). Statistical comparisons for the cerebellar expression levels of each sirtuin for the developmental times shown are presented in Table 3.11.
### Table 3.11: Comparison of sirtuin mRNA expression during cerebellar development

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.13: Sirtuin protein expression patterns during rat cerebellum development. Representative Western blots showing (A) SIRT1 (B) SIRT2 (C) SIRT3 (D) SIRT4 (E) SIRT5 (F) SIRT6 protein levels in rat cerebellum at the indicated embryonic or postnatal stages (n = 3 independent subjects). The corresponding GAPDH protein level for the same blot is shown, and serves as a protein loading control. The histograms show the densitometric mean and SEM for each specific sirtuin normalized to its corresponding GAPDH control, with the 3-month value arbitrarily set as 100%. Statistical comparisons for the expression levels of each sirtuin for the developmental times shown are presented in Table 3.12.
Table 3.12: Comparison of sirtuin protein expression during cerebellar development

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Asterisks denote significance at *p < 0.05, **p < 0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Table 3.13: Summary of developmental mRNA and protein expression patterns of sirtuin members in cortex, hippocampus and cerebellum

In the upper mRNA expression panel, “+” are assigned based on the specific range of ΔCt values for the sirtuin expression levels, relative to the Hprt1 control gene, such as: +++++ = ΔCt >3; ++++ = ΔCt 1.5-3; +++ = ΔCt 0.5-1.5; ++ = ΔCt 0.05–0.5; and + = ΔCt < 0.05. In the lower protein expression panel, + are assigned based on their relative expression values for the sirtuin expression levels when compared to GAPDH control, such as +++++, very high; ++++, high; +++, moderate; ++, low; and +, very low levels of expression. “0” was assigned to areas of no expression.
3.2.6 Differential development of sirtuins in rat liver

Sir1 mRNA levels decreased progressively relative to GAPDH from the late embryonic stage in the liver (Figure 3.14A; Table 3.14). SIRT1 protein levels increased in the liver during perinatal development, and then remained at similar levels until the 24 month stage (Figure 3.15A; Table 3.15). During liver development, Sirt2 mRNA was expressed from PN2-24 months at similar levels, but was modestly higher at the E18 stage (Figure 3.14B; Table 3.14). SIRT2 protein in the developing liver largely paralleled this pattern, but unlike Sirt2 mRNA, SIRT2 protein displayed a significant increase in prevalence at the 3 month stage, before returning to levels similar to perinatal times at 24 months of age (Figure 3.15B; Table 3.15). Sirtuin-3 mRNA and protein expression in the liver across development displayed similarities to its brain expression pattern, as sirtuin-3 mRNA and protein levels increased dramatically between E18 to PN7, then decreased by PN21 to levels that remained similar at 3 and 24 months of age (Figures 3.14C, 3.15C; Tables 3.14, 3.15). Sirt4 mRNA levels decreased modestly between E18 to and PN2, and then remained fairly stable until 24 months of age (Figure 3.14D; Table 3.14). Similarly, SIRT4 protein levels did not significantly differ in liver from E18 to 24 months of age (Figure 3.15D; Table 3.15). During liver development, Sirt5 mRNA expression decreased between E18 and PN2, and then remained fairly consistent until 24 months of age (Figure 3.14E; Table 3.14). The pattern of SIRT5 protein differed from this mRNA profile in liver, however, as SIRT5 protein levels were found to increase progressively until PN21, and then remained at similar levels at 3 and 24 months of age (Figure 3.15E; Table 3.15). Sirt6 mRNA expression in the developing liver similarly decreased during early perinatal development from its late embryonic level and remained at similar levels until 24 months of age (Figure 3.14F; Table 3.14). The 36 kDa SIRT6 protein form was found at consistent levels throughout development, whereas the prevalence of the 39 kDa
product was at low or background levels for each of the developmental stages examined (Figure 3.15F; Table 3.15). Across liver development, *Sirt7* mRNA levels decreased modestly from E18 to PN21, and then remained at similar levels at 3 and 24 months (Figure 3.14G; Table 3.14).
Figure 3.14: Sirtuins gene expression patterns during liver development in rat. Histograms showing the mean and SEM of the mRNA expression levels for each individual sirtuin as determined by qRT-PCR at the indicated stages of liver development (E18 - 3 months) and in rats at 24 months of age. The y-axis of each histogram shows the relative expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 relative to the Hprt1 reference gene. Data shown are based on the linear conversion of ΔCt values for each sample (n=3 independent subjects done in quadruplicate). Statistical comparisons for the sirtuin expression levels for the developmental times shown are presented in Table 3.14.
Table 3.14: Comparison of sirtuin mRNA expression during liver development

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Asterisks denote significance at *p < 0.05, **p <0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
Figure 3.15: Sirtuins display differential protein expression patterns during liver development in rat. Representative Western blots showing the immunoreactive prevalence of sirtuins at the indicated stages of liver development (E18 - 3 months) and in rats at 24 months of age. The y-axis of each histogram shows the relative expression levels of (A) Sirt1 (B) Sirt2 (C) Sirt3 (D) Sirt4 (E) Sirt5 (F) Sirt6 (G) Sirt7 for the indicated stages of development. The histograms in each panel show the densitometric mean and SEM for each specific sirtuin normalized to its corresponding GAPDH loading control. (n=3 independent subjects). Statistical comparisons for the expression levels of each sirtuin are presented in Table 3.15.
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Asterisks denote significance at *p < 0.05, **p <0.01 and ***p < 0.001, One-way ANOVA, with Tukey post-hoc test (n=3). ns designates no statistical difference in expression.
3.2.7 Differential expression of sirtuins in adult brain cell sub-types

Analysis of sirtuin-1 expression between astrocytes and neurons revealed that while \textit{Sirt1} mRNA was detected in extracts from each of these respective cultures, its mRNA levels were higher in the astrocyte cultures than in the neuronal cultures (Figure 3.16A). Consistent with its low expression in the adult brain, \textit{Sirt1} mRNA was also relatively low compared to the other sirtuins except \textit{Sirt4} in each of these cultured cell types. At the protein level, though, SIRT1 prevalence differed somewhat from its mRNA profile in these cultures. Although SIRT1 protein was also detected in both the neuron and astrocyte-enriched cultures, its overall prevalence was higher in the neuronal cultures than in the astrocytes (Figure 3.16B). The analysis of sirtuin-2 expression in neuron and astrocyte cultures also yielded intriguing results. Consistent with its expression levels in whole brain, \textit{Sirt2} mRNA was the most prominently expressed sirtuin family member in each culture, although astrocyte cultures displayed more \textit{Sirt2} than neurons (Figure 3.16A). However, this mRNA pattern was not recapitulated at the protein level. In fact, while SIRT2 immunoreactivity was readily detected in extracts from the astrocyte-enriched cultures, its immunoreactivity in extracts from the neuronal cultures was at, or only slightly above, background levels (Figure 3.16C). Thus, despite having pronounced mRNA expression in neurons, SIRT2 protein was not readily detected in the same cultured neurons. Within the neuronal and astrocytes cultures, while \textit{Sirt3} mRNA was present in both cell types, Sirt3 mRNA levels were higher in the neuronal cultures than in the astrocyte cultures (Figure 3.16A). This pattern held at the protein level also, as SIRT3 protein was more prevalent in extracts from the neuronal cultures compared the astrocyte cultures (Figure 3.16D). Consistent with its low levels of expression levels in the brain and peripheral tissues, \textit{Sirt4} mRNA was also the least prevalently expressed sirtuins member in both the neuronal and astrocyte enriched cultures, and no significant difference in \textit{Sirt4} mRNA prevalence was observed between the neurons and astrocytes (Figure 3.16A). At the protein level, SIRT4 protein expression was also detected in each cell type,
although SIRT4 protein was higher in the neuronal cultures compared to astrocytes (Figure 3.16E). *Sirt5* mRNA was present at relatively high levels relative to other sirtuins in both the neuronal and astrocytic cultures, with its mRNA expression being moderately but significantly higher in neurons than in astrocytes (Figure 3.16A). At the protein level, SIRT5 prevalence was more skewed in its neuronal preference, being higher in the neuronal cultures than in the astrocyte cultures (Figure 3.16F). In the neuronal and astrocyte-enriched culture extracts, *Sirt6* mRNA was detected in both cell lineages, and at similar levels (Figure 3.16A). At the protein level, SIRT6 immunoreactivity was also found in both the neuronal and astrocyte-enriched cultures, although both the 36 kDa and the 39 kDa protein products displayed preferential expression in one cell type. The prevalence of the 36 kDa SIRT6 product was higher in the neuronal cultures than in the astrocytes (Figure 3.16G), while the prevalence of the 39 kDa SIRT6 protein form was higher in the astrocyte cultures compared to the neurons (Figure 3.16G). In the neuronal and astrocyte-enriched culture extracts, *Sirt7* mRNA was detected in both lineages, with its expression in astrocytes being higher than in neuronal cultures than in astrocytes (Figure 3.16A). Assessment of Purity of Neuronal and Astrocyte Cultures is shown in Figure 3.17, no astrocyte or neuronal contamination is visible in either of the cell lysates. Furthermore, no oligodendrocyte contamination is visible in neuronal cell lysates and minimal is observed in astrocyte lysate.
Figure 3.16: Sirtuin mRNA expression in mature rat astrocytes and neurons. (A) Histogram showing the mean and SEM of Sirt1-7 mRNA expression levels in primary cultures of astrocytes (gray bars) and neurons (black bars). The y-axis shows the relative mRNA expression levels relative to the endogenous housekeeping Hprt1 reference gene in each culture condition. Data shown depict the linear conversion of ΔCt values from qRT-PCR assays for each sample (n = 3 independent subjects done in quadruplicate). Asterisks denote significant differences in the relative expression of the specific sirtuin between astrocyte and neuronal cultures (p < 0.05, Student’s t-test). (B–G) Representative Western blots showing the relative immunoreactive levels of (B) SIRT1, (C) SIRT2 (D) SIRT3 (E) SIRT4 (F) SIRT5 (G) SIRT6 in cultured astrocytes and neurons. The histograms show the mean and SEM of the densitometric band intensity for each sirtuin normalized to its corresponding β-actin levels. Asterisks denote significant differences in expression levels for the indicated sirtuin between the neuronal and astrocyte cultures (n = 3, p < 0.05, student’s t-test).
**Figure 3.17:** Assessment of purity of neuronal and astrocyte cultures. Representative Western blot showing the immunoreactivity of astrocyte-specific (GFAP), neuron-specific (MAP2) and oligodendocyte-specific (NG2) markers in adult rat brain homogenate and in cultured rat neuronal and astrocytic whole cell lysates. Beta-actin immunoreactivity is shown as a protein loading control. No astrocyte or neuronal contamination is visible in either of the cell lysates. No oligodendrocyte contamination is visible in neuronal cell lysates and minimal is observed in astrocyte lysate.
3.3 Discussion

In this study, the expression patterns of seven sirtuin family members were assessed in specific regions of the developing, adult, and aged rat brain. Four principle findings emerge from this study. First, although each of the sirtuin family members is expressed within the adult and developing rat brain, there is considerable variability in the magnitude of mRNA expression between each of the individual sirtuins, with Sirt2 being the most prominently expressed and Sirt4 being the least expressed. Second, although there is some overlap between specific members, individual sirtuin family members display a unique pattern of temporal and spatial expression within the adult and developing brain. Third, specific members of the sirtuin family display preferential or even selective cell type expression patterns between neurons and astrocytes at the mRNA and protein levels. Fourth, there is notable discordance between the mRNA and protein expression patterns for sirtuin-2, indicating that its protein expression pattern is likely regulated by post-transcriptional mechanisms. Collectively, these results reveal a contrasting expression pattern for the seven sirtuin family members in the developing and adult brain, and suggest that metabolic processes and/or oxidative defense systems within different regions of the brain, and potentially within specific neural cell types, may be differentially regulated by specific combinations of sirtuins.

Although previous studies have indicated different sirtuins are expressed within the brain (Ford et al., 2006; Mostoslavsky et al., 2006; W. Li et al., 2007; Lombard et al., 2007; Ramadori et al., 2008; Nakagawa et al., 2009), my results indicate that there is considerable divergence amongst the family members in their spatial expression patterns and in the magnitude of their mRNA abundance within specific brain regions. Although none of the adult brain regions that were examined was devoid of expressing any sirtuin member, the three most prevalently expressed sirtuins within each brain region were Sirt2, Sirt3, and Sirt5. This general pattern was also recapitulated in the peripheral tissues we examined, although in some peripheral tissues Sirt7
mRNA was more prevalent that Sirt5 or Sirt3. In fact, Sirt7 mRNA levels were significantly higher in peripheral tissues than Sirt7 mRNA levels in any region of the adult brain we tested. This differential Sirt7 mRNA expression pattern may indicate a more prominent role for Sirt7 in tissues with high cell proliferation rates as suggested previously (Ford et al., 2006), as compared to its role within the largely post-mitotic brain.

### 3.3.1 Differential expression of nuclear sirtuins

During development, Sirt1, Sirt6, and Sirt7 mRNA levels were significantly higher in the late embryonic and early postnatal regions of the brain than in the corresponding adult brain regions. The relatively low mRNA levels observed for Sirt1, as well as for Sirt6 and Sirt7, in brain regions from 3 and 24 month old rats relative to their protein expression levels in the late embryonic and early postnatal times were somewhat surprising, as sirtuin-1 has been strongly implicated as a neuroprotective factor in models of Huntington’s disease (Jeong et al., 2011), multiple sclerosis (Khan et al., 2014), stroke (Yan et al., 2013) and Alzheimer’s disease (Qin et al., 2006). However, low basal expression levels could potentially be advantageous for these sirtuins in the brain, as sirtuin-1 levels have been shown to increase significantly in different cultured cell lines when they are exposed to conditions of energy/nutrient stress (Kanfi et al., 2008; Chen et al., 2011). Similarly, SIRT6 and SIRT7 have been implicated in the maintenance of DNA integrity, and in DNA repair following damage (Ford et al., 2006; Cardus et al., 2013), which is often a consequence of increased cellular stress. Therefore, the relatively lower levels of Sirt1, Sirt6, and Sirt7 present in the adult brain under normal conditions may allow for their pronounced induction under pathophysiological conditions to facilitate a beneficial compensatory response in times of need. It is worth noting that neither the mRNA levels or protein prevalence of either sirtuin-1 or sirtuin-6 in the 24 month old cortex or hippocampus differed from their respective levels at 3 months of age, suggesting that aging alone does not alter the expression of these sirtuins. While
this suggests the lack of an endogenous age-related compensatory response to aging, these results do confirm the expression of each sirtuin in the aged brain, which therefore validates their pharmacological targeting for age-related conditions. Indeed, brain-specific SIRT1 over-expressing transgenic mice show significant life span extensions (Satoh et al., 2013), suggesting enhancing SIRT1 activity pharmacologically in the brain may yield a similar effect. Likewise, the global over-expression of SIRT6 in transgenic mice similarly extended life span (Kanfi et al., 2012), suggesting SIRT6 may also be a rationale target for drug development. In this study, analysis of SIRT6 protein expression revealed two products at 36 kDa and 39 kDa, which comports with a recent study that also reported the presence of two distinct SIRT6 products (Lim et al., 2013). Interestingly, we show that these distinct SIRT6 forms displayed somewhat opposing expression patterns during brain development: while the 36 kDa product decreased during perinatal development, the 39 kDa product increased as the animal aged. While the reason for the divergent expression pattern remains unknown, these results indicate that an examination of the role played by each in temporal brain development and aging is warranted.

Analysis of the astrocytic and neuronal expression patterns of the predominantly nuclear residing sirtuin-1, sirtuin-6, and sirtuin-7, revealed that while each sirtuin is expressed in these two neural cell lineages, their relative expression levels are not equivalent between the two cell types. At the mRNA level, Sirt1, Sirt6, and Sirt7 displayed modestly (approximately 2-fold) higher expression in astrocytes than in neurons relative to the reference gene Hprt1. At the protein level, this pattern of more predominant astrocyte expression was not recapitulated by SIRT1, whose protein prevalence was found to be significantly higher in neurons than in astrocytes. This differential expression profile of sirtuin-1 mRNA and protein between cultured astrocytes and neurons suggests that post-transcriptional factors may play a role in establishing SIRT1 protein expression patterns in these specific cell types. Importantly, these results also indicate that glial cells should be considered as potential mediators of neuroprotective actions associated with SIRT1-activating
drugs (Khan et al., 2014). Somewhat surprisingly, SIRT6 protein expression was found to display isoform-specific preferences between neurons and astrocytes. This could indicate the two forms influence specific cell types in the brain in different or specific manners. My results indicate that the 36 kDa product is the form most predominately expressed in neurons, and is the only form reliably detected in the developing and adult liver. Taken together, my results confirm that both SIRT1 and SIRT6 proteins are expressed in varying levels in both neurons and astrocytes which therefore indicates drugs designed to enhance the catalytic activity of either SIRT1 or SIRT6 should elicit effects within both of these cell types—but potentially with differing cell-selective efficacies depending on the sirtuin, or the SIRT6 form, that is being pharmacologically targeted.

3.3.2  Differential expression of cytosolic sirtuin – Sirt2

In contrast to the relative low expression levels of the nuclear sirtuins, the mRNA expression levels of the cytosolic-nuclear Sirt2 were robust. High levels of Sirt2 mRNA were detected ubiquitously throughout the different regions of the adult and developing brain examined, and in each of the peripheral tissues investigated. These results are consistent with a recent study showing SIRT2 mRNA is expressed at high levels in the human cortex (Korner et al., 2013), and illustrate Sirt2 mRNA is expressed at higher levels than any of the other sirtuins in the adult or developing rat brain, peripheral tissues, and also in extracts from both the neuronal and astrocyte-enriched cultures, respectively. However, there is a pronounced disconnect between the high levels of Sirt2 mRNA expression in the embryonic and early postnatal brain, and the low to non-detectable amounts of SIRT2 protein detected by immunoblot in the same tissues. While isoforms of SIRT2 have been reported (Maxwell et al., 2011), the SIRT2 antibody we employed targets a carboxyl region of SIRT2 that is common to the different isoforms that have been characterized to date, suggesting that the differential expression we detect does not arise from the antibody we used missing an isoform-specific product lacking this epitope. This, together with the cell lineage
expression results discussed below, raises the possibility that post-transcriptional mechanisms may strongly influence the expression of SIRT2 protein in the developing brain and in specific cell contexts (Day & Tuite et al., 2008). When combined with quantitative PCR outcomes, these immunoblot results reveal an interesting phenomenon: although Sirt2 mRNA is clearly detected at high levels in both neuronal and astrocyte enriched cultures, SIRT2 immunoreactive protein is detected at robust levels only in the astrocyte-enriched culture extracts, with minimal or no protein detected in extracts from the neuronal cultures. While we did not examine oligodendrocytes specifically, our astrocyte enriched cultures displayed only minor immunoreactivity for the oligodendrocyte-specific marker Ng2 relative to whole brain samples, and Ng2 was not detected in the neuronal-enriched cultures (Figure 3.17). This argues against the SIRT2 protein we detect stemming from a contaminating oligodendrocytes presence in the enriched cultures. However, given that pronounced Sirt2 mRNA expression was detected in both the neuronal and astrocyte enriched cultures, the relative absence of SIRT2 protein in neurons suggests that SIRT2 protein expression must be negatively regulated in neurons by post-transcriptional mechanisms that either prevent protein from being generated from expressed transcripts or that rapidly destabilize and degrade SIRT2 protein in cultured neurons upon its synthesis.

3.3.3 Differential expression of mitochondrial sirtuins

The expression patterns of the mitochondrial sirtuins, sirtuin-3, sirtuin-4, and sirtuin-5 displayed a wide range of spatial and temporal divergence in the developing and adult brain, as well as considerable differences in magnitude of expression. Sirt5 and Sirt3 mRNA levels were found to be the second and third most abundant sirtuin transcripts in the developing and adult brain, in both the neuronal and astrocyte specific cultures, and in most of the peripheral tissues examined, respectively. The relatively higher expression levels observed for Sirt5 and Sirt3 mRNAs were not unexpected, as these sirtuins regulate mitochondrial homeostasis, metabolism and reactive
oxygen species genesis in multiple cell types (He et al., 2012; Webster et al., 2012). We found no evidence for significantly different expression levels of SIRT3, SIRT4, or SIRT5 protein in the brain tissues we assessed from rats at 24 months compared to 3 month of age, indicating an apparent lack of age-associated decrease in the brain expression of these mitochondrial sirtuins. This result contrasts with those of Zeng et al. (2014), who reported decreased SIRT3 protein levels in the aged rat auditory cortex, and (Di Loreto et al., 2014) who also reported a decrease in SIRT3 protein levels in the aged mouse cortex relative to young adult under conditions of acute food and water deprivation. The reason for these differing results is unclear, but could relate to cortical subfield specific changes in SIRT3, and/or the influence of different stress conditions presented to the subjects in the studies. Because mitochondrial dysfunction is heavily implicated in aging (Lee & Wei, 2012) and SIRT3 modulates mitochondrial metabolism and anti-oxidative defense systems (Kincaid & Bossy-Wetzel, 2013), the preserved prevalence of SIRT3, as well as SIRT4 and SIRT5, in the aged brain confirm that drugs selectively targeting any of these sirtuins would be expected to exert effects at later stages of aging. Further, as my results indicate each of these sirtuins are expressed in both neurons and astrocytes, the same drugs would likely affect both neural cell types. Interestingly, although SIRT3 protein has been reported to be expressed two predominant forms: a 45 kDa product corresponding in mass to its precursor form, and a 28 kDa product corresponding to its form following import into mitochondria after processing by mitochondrial processing peptidase (Onyango et al., 2002), we could only verify the 28 kDa product recognized with the antibody we employed as originating from SIRT3. It is not clear whether a 45 kDa SIRT3 product is also present in brain that is masked in the Sirt3-KO mouse brain by a cross-reactive co-migrating non-specific protein. As such, we only report the protein expression profile for the processed form of SIRT3, which could conceivably differ from the 45 kDa form that has been detected in the nucleus (Iwahara et al., 2012). Additional studies will be required to resolve this possibility. Finally, in contrast to the relatively high expression levels
observed for Sirt3 and Sirt5, Sirt4 was generally the least expressed member of the sirtuin family in all of the adult tissues and cell types examined. The notably higher level of Sirt4 mRNA expression in late embryonic stage cortex and hippocampus compared to perinatal or adult brain regions suggest Sirt4 may be more prominently expressed during embryogenesis, and that a closer examination of Sirt4 during embryonic brain development may be warranted.

### 3.3.4 Summary for the sirtuins

In summary, this study illustrates sirtuin family members display differential expression patterns within the developing and adult brain at both the mRNA and protein levels, with specific family members displaying preferential expression in either astrocytes or neurons. My results indicate that mRNA for each of the seven sirtuins is expressed in the brain throughout development. In all brain regions examined during development, Sirt2 was the most prevalently expressed sirtuin, and Sirt4 was the least expressed member of the family. No significant decreases in the expression of any sirtuin member were observed in any brain region between the 3 month old and 24 month old rats. However, the mRNA expression pattern for specific sirtuins did not always parallel its corresponding protein expression pattern. This was most evident for SIRT2, where protein expression was largely absent at early developmental stages despite the presence of pronounced mRNA expression. This differential expression was also noted in cell type specificity, where Sirt2 mRNA was detected in both astrocytes and neurons whereas SIRT2 protein was selectively observed in astrocytes. Sirtuin-5 also displayed similar differential cell type expression, as SIRT5 protein was preferentially observed in neurons despite its mRNA being present in both astrocytes and neurons. Collectively, these results add to the knowledge of the regulation of sirtuin expression within the brain at different developmental stages, and provide information on which specific sirtuins could potentially be targeted pharmacologically in the brain at times ranging from perinatal development to 24 months of age.
3.3.5 Summary for SIRT3

In summary, at the mRNA level, Sirt3 was the third most expressed sirtuin member in the whole brain. The expression level of this mitochondrial sirtuin is not surprising, given the high metabolic demands of brain tissue. Furthermore, SIRT3 was expressed at equivalent levels in the adult cortex, hippocampus, striatum, spinal cord and brain stem, and was expressed the lowest in the cerebellum. During development, Sirt3 mRNA expression levels were stable from E18 until 24 month of age in the cortex, hippocampus, and cerebellum. At the protein level, SIRT3 prevalence increased between PN7 and PN21 and then remained consistent into the 24-month age in the cortex and hippocampus. In the cerebellum, however, SIRT3 protein prevalence was similar between E18 to 3 months, but then increased significantly at 24 months of age. Furthermore, these results show that SIRT3 is expressed in both neurons and astrocytes, however its prevalence is higher in neurons at both the mRNA and protein levels.

In support of Aim 1 of this thesis, my results show that while sirtuin-3 is expressed throughout the brain, it displays unique spatial and temporal expression patterns between regions that were analyzed. This differential expression pattern suggests that the function of sirtuin-3 may be region- and age-specific. In addition, given that mitochondrial dysfunction is associated with aging (Lee & Wei, 2012) and sirtuin-3 prevalence is preserved in the aged brain, drugs that selectively target SIRT3 may exert their effects at later stages of aging. Taken together, this study is the first composite illustration of sirtuin-3 expression patterns in the CNS, and this knowledge provides an important foundation for future research to build upon with the goal of understanding the role that sirtuin-3 plays in the brain.
**Sirt3-KO mice display a sex-specific behavioural phenotype**

### 4.1 Brief introduction and rationale

Post-translation modifications (PTM) of mitochondrial proteins plays a crucial role in regulation of the bioenergetic function of mitochondria, with lysine acetylation representing one of the most prominent PTMs in this organelle. Up to 65% of mitochondrial proteome is acetylated at any given time (Hebert et al., 2013). As earlier discussed, SIRT3 plays the predominant role in regulating the acetylation state of the mitochondrial proteome (Lombard et al., 2007). As post-translational acetylation can influence the stability, enzymatic activity, and protein-protein interaction capacity of specific target proteins, alterations in SIRT3 levels would be expected to affect the functional activity of mitochondria. Depending on context, this could have a host of consequences at the cellular, systemic, and behavioural levels.

While the majority of knowledge relating to SIRT3 function and influence on mitochondrial function stems from *in vitro* investigations, studies using *Sirt3*-KO mice have begun to investigate how SIRT3 influences systemic physiology and behaviour. Somewhat surprisingly given the changes in mitochondrial proteome acetylation seen in peripheral (Lombard et al., 2007; Hirschey et al., 2011; Fernandez-Marcos et al., 2012) and central nervous tissues (Cheng et al., 2016) from *Sirt3*-KO mice, initial behavioural studies reported that *Sirt3*-KO mice display no overt behavioural differences compared to WT mice (Lombard 2007; Liu et al., 2015). It should be noted that these studies primarily examined male subject’s young adult stages of development, with sex-specific and/or progressive age-dependent impairments of behaviour remaining largely unexamined.
It is now appreciated that alterations in mitochondrial function impact behavioural performances (Streck et al., 2014), and that mitochondrial function is influenced by both age (Sun, 2016) and sex (Vijay et al., 2015). In Chapter 3, I found that SIRT3 protein expression increases significantly in rat forebrain regions between the perinatal and adult/aged stages of development, raising the possibility that behavioural impairments due to SIRT3 absence might not manifest until later stages of age. In this study we examine the potential for sex and age-dependent differences in behaviour in Sirt3-KO mice, whilst furthering our understanding of the role that sirtuin-3 plays in normal physiology in males and females.
4.2 Results

4.2.1 SIRT3 is differentially expressed in the cortex of male and female WT mice

Chapter 3 results show that sirtuin-3 is differentially expressed throughout development and aging, however potential sex differences in expression have yet to be investigated. I therefore assessed whether SIRT3 is expressed at different levels in male vs. female mice. Western blot analysis revealed sex differences in SIRT3 protein expression patterns in cortical extracts. In males, SIRT3 protein prevalence was found to be at similar levels between 3 and 8 months of age, but then decreased significantly between 8 and 24 months of age (Figure 4.1A). In the females however, SIRT3 protein prevalence decreased significantly between 3 and 8 months, and then remained at consistent levels at the 24 months of age (Figure 4.1B). In addition, SIRT3 protein expression levels were found to differ between male and female WT mice, where SIRT3 expression was higher in males than in the females, at both 3 months and 24 month-old stages (Figure 4.1C).

4.2.2 The magnitude of mitochondrial protein hyper-acetylation is greater in female Sirt3-KO mice compared to male Sirt3-KO mice

Given that SIRT3 prevalence levels differ between male and female WT mice, I next assessed whether the magnitude of acetylation levels in mitochondrial proteome of the Sirt3-KO mouse cortex is sex-dependent. Previous studies have demonstrated hyper-acetylation in the mitochondrial proteome of liver and muscle tissues in male Sirt3-KO mice relative to male WT mice (Lombard et al., 2007; Hirschey et al., 2011; Fernandez-Marcos et al., 2012), and in cortical and hippocampal brain regions of 6 month-old Sirt3-KO mice relative to WT mice (Cheng et al., 2016). Consistent with these observations, hyperacetylation was evident in cortical mitochondrial protein extracts isolated from both male and female Sirt3-KO mice when compared to their
respective male and female WT counterparts. Interestingly, the magnitude of increased acetylation was greater in female Sirt3-KO mice than in male Sirt3-KO mice (Figure 4.2).
Figure 4.1: Differential SIRT3 expression patterns in WT male and female mice. (A-B) Representative Western blots showing SIRT3 protein expression during mice cortical development in (A) male and (B) female mice at the indicated age stages. The 3-month value is arbitrarily set as 100% \((n = 4\) independent subjects). B-actin protein level for the same blot is shown, and serves as a protein loading control. (C) Representative Western blots of SIRT3 expression in the cortex between males and females at 3 months and 24 months of age \((n=4\) independent subjects). B-actin protein level for the same blot is shown, and serves as a protein loading control. Asterisks denote statistical significance \((p<0.05)\) between the indicated groups. One-way ANOVA with Bonferroni post-hoc was used for cortical development, student t-test was used for SIRT3 expression comparison between males and females.
Figure 4.2: Sex-dependent acetylation levels between WT and Sirt3-KO mice. Representative Western blots showing the immunoreactivity of anti-acetylated lysine antibody in the mitochondrial homogenates isolated from cortical tissues of male and female WT and Sirt3-KO mice (n=3 independent subjects). The corresponding mitochondrial TOM20 protein level for the same blot is shown, and serves as a protein loading control. The histograms show the densitometric mean and SEM for each specific animal homogenate expressed as a percent ratio over B-actin or TOM20. Asterisks denote statistical significance (p<0.05) between the indicated groups. Student t-test was used for acetyl-lysine immunoreactivity between WT and Sirt3-KO mice in male and in female mice.
4.2.3 General phenotypes are preserved in male and female \textit{Sirt3}-KO mice

Consistent with previous reports (Lombard et al 2007; Liu et al 2015), \textit{Sirt3}-KO male mice display no overt phenotypic differences when compared to their age matched control mice under normal housing conditions (Table 4.1). Similar to the male cohort, female \textit{Sirt3}-KO mice also show no overt phenotype under basal homecage environment. No visual signs of self-inflicted wounds, poor grooming, or cage aggressive behaviour were noted (not shown). Body weight comparisons at 3-8 and 18-24 months of age showed no differences between the sex and age matched \textit{Sirt3}-KO and WT mice, furthermore no epileptiform discharges, and no evidence was observed for sudden and unexpected death in either male of female \textit{Sirt3}-KO mice over the 24 months period of monitoring (Table 4.1). In addition, telemetry assessments showed no difference in overall general activity in the homecage environment either during the light or dark phases (Figure 4.3).
Table 4.1: General phenotypic assessments of *Sirt3*-KO and WT mice

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Sudden Deaths</th>
<th>Epileptiform Discharges</th>
<th>Average Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>young WT</td>
<td>0</td>
<td>0</td>
<td>28.5 ±0.64</td>
</tr>
<tr>
<td>young <em>Sirt3</em>-KO</td>
<td>0</td>
<td>0</td>
<td>30.0 ±0.70</td>
</tr>
<tr>
<td>aged WT</td>
<td>0</td>
<td>0</td>
<td>34.6 ±0.97</td>
</tr>
<tr>
<td>aged <em>Sirt3</em>-KO</td>
<td>0</td>
<td>0</td>
<td>37.4 ±1.29</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>young WT</td>
<td>0</td>
<td>0</td>
<td>23.6 ±1.30</td>
</tr>
<tr>
<td>young <em>Sirt3</em>-KO</td>
<td>0</td>
<td>0</td>
<td>23.8 ±0.64</td>
</tr>
<tr>
<td>aged WT</td>
<td>0</td>
<td>0</td>
<td>21.7 ±3.44</td>
</tr>
<tr>
<td>aged <em>Sirt3</em>-KO</td>
<td>0</td>
<td>0</td>
<td>25.3 ±2.30</td>
</tr>
</tbody>
</table>
Figure 4.3: *Sirt3*-KO mice display normal homecage activity. Histograms showing mean and SEM of the index of sum of activity in WT and *Sirt3*-KO mice in (A) male and (B) female mice for a full 24 hour telemetry assessment. No statistically significant differences were observed in any of the telemetry assessments, student t-test, male mice (WT n=3 young, n = 4 aged; *Sirt3*-KO n=4 young, n= 6 aged), female mice (WT n=8 young, n=5 aged; *Sirt3*-KO n=6 young, n=6 aged).
4.2.4 Sleep patterns are preserved in Sirt3-KO mice

Sirtuin-3 plays a role in sleep homeostasis, as sleep deprived Sirt3-KO mice show elevated indices of oxidative stress in the locus coeruleus – a region responsible for optimal alertness during wakefulness (Zhang et al., 2014). In addition, 2 month-old Sirt3-KO mice (sex not specified) showed an attenuation in EEG delta power during their recovery sleep (Zhang et al., 2014), which suggests that their sleep homeostasis is disturbed as these waves are associated with the deep sleep stage and are used as an index for the sleep state of animals (Franken et al., 1998). To expand on this study, I assessed whether normal sleep patterning is altered over the full 24-hour day in Sirt3-KO male and female mice. Both Sirt3-KO and WT mice show a strong inverse correlation between mobility and cortical delta power (Figure 4.4), which is consistent with the fact that delta power is a good predictor of sleep/immobility (Franken et al, 1998). These results suggest that under normal conditions, no overt differences are evident in general presentation or sleep/activity patterns in either male or female Sirt3-KO mice.
**Figure 4.4:** *Sirt3*-KO mice display normal inverse delta power and behavioural activity in homecage environment. (A-B) Scatter plots showing the Pearson’s product-moment correlation coefficients for delta power compared to activity in WT and *Sirt3*-KO for a full 24 hour telemetry assessment (A) male and (B) female mice. Each point represents the daily correlative strength for a single subject. The bar on the scatter plot indicates the mean for each set. Male mice (WT n=3 young, n = 4 aged; *Sirt3*-KO n=4 young, n=5 aged), female mice (WT n=7 young, n=4 aged; *Sirt3*-KO n=5 young, n=5 aged).
4.2.5  *Sirt3*-KO mice display normal body thermoregulation

Sirtuin-3 may play a role in thermoregulation, as a previous study showed that it regulates the expression of the uncoupling protein (UCP)-1 (Shi et al., 2005) - a factor responsible for cold tolerance. Given that internal body temperature fluctuates throughout the day, I assessed whether *Sirt3*-KO male (Figure 4.5) or female (Figure 4.6) mice would have irregular temperature regulation throughout the full 24-hour sleep-wake cycle. There was no significant difference between the average daily core body temperature (Figure 4.5A; Figure 4.6A), temperature range (Figure 4.5B; Figure 4.6B), temperature observed during periods of either activity (Figure 4.5C; Figure 4.6C) or inactivity (Figure 4.5D; Figure 4.6D) during the light or dark phases of the full 24-hour sleep-wake cycle between sex and age matched *Sirt3*-KO and WT mice.
Figure 4.5: *Sirt3*-KO male mice display normal temperature regulation. (A-D) Histograms showing mean and SEM telemetry assessments of different temperature parameters in young (grey bars) and aged (white bars) WT and *Sirt3*-KO male mice in their home-cage environment for the full 24 hour telemetry assessment, and for the light and dark phases of the day specifically. Temperature analysis includes: (A) average body temperature (B) core body temperature (C) average active body temperature (D) average inactive body temperature (WT n=3 young, n = 4 aged; *Sirt3*-KO n=4 young, n= 6 aged)
Figure 4.6: Sirt3-KO female mice display normal temperature regulation. (A-D) Histograms showing mean and SEM telemetry assessments of different temperature parameters in young (grey bars) and aged (white bars) WT and Sirt3-KO female mice in their home-cage environment for the full 24 hour telemetry assessment, and for the light and dark phases of the day specifically. Temperature analysis includes: (A) average body temperature (B) core body temperature (C) average active body temperature (D) average inactive body temperature (WT n=8 young, n=5 aged; Sirt3-KO n=6 young, n=6 aged).
4.2.6 Aged Sirt3-KO female mice display increased exploratory behaviour in a novel environment

Alterations in cortical mitochondrial proteome acetylation state could influence the efficiency of neural circuits and influence behavioural performance. I therefore compared the performance of Sirt3-KO mice relative to their age- and sex-matched WT controls in a battery of behavioural tests. To investigate whether locomotor responses in Sirt3-KO mice are affected by the stress of novelty, assessments were carried out in the open field (OF) arena. Consistent with a previous report (Kim et al., 2017), no significant differences in time spent active (Figure 4.7A), total distance travelled (Figure 4.7B), and cumulative static activity counts (Figure 4.7C) were observed for Sirt3-KO male mice at both 3-8 months and 18-24 months of age relative to their WT controls. Furthermore, although no significant differences were observed for any of the OF parameters that were assessed in 3-8 month old Sirt3-KO female mice, clear differences were evident in the 18-24 month-old female Sirt3-KO mouse cohort relative to the WT controls. Aged Sirt3-KO female mice displayed significant increases in total active time (Figure 4.7A), distance travelled (Figure 4.7B), and total activity counts (Figure 4.7C) relative to the 18-24 month old female WT mice.

4.2.7 Sirt3-KO female mice display poor balance on the rotarod

General balance was assessed using the accelerating rotarod test. In line with a previous study (Liu et al., 2015), no significant differences in latency to fall time were observed male Sirt3-KO mice when compared to their age-matched male controls (Figure 4.8A). Unlike Sirt3-KO male mice, young Sirt3-KO female mice displayed a shorter latency to fall when compared to their WT controls on the third and the fourth trial days (Figure 4.8A). Furthermore, aged Sirt3-KO female
mice revealed a larger deficit on rotarod performance, as the latency to fall for these mice was faster than for the age-matched female WT mice. Although Sirt3-KO female mice showed an improvement on rotarod performance during the trials within the same day, they however showed impaired performance between consecutive days.

4.2.8 Aged male and female Sirt3-KO mice showed impaired performance on the dowel traversal test

On the dowel traversal test, both male and female Sirt3-KO mice performed equivalently to WT controls at 3-8 months of age. Whereas, in the 18-24 month cohort, Sirt3-KO male and female mice displayed a higher failure rate than WT mice (Figure 4.8B).
Figure 4.7: *Sirt3*-KO female mice display altered locomotion in the open field. (A-C) Histograms showing the behavioural performances of young (grey bars) and aged (white bars) male and female mice in different open-field assessments during a 30 minute period. Parameters assessed were: (A) total activity, (B) distance travelled, and (C) total static counts. There was no statistical difference in performance between WT and *Sirt3*-KO male mice in either of the age cohorts (WT n=12 young, n=17 aged; *Sirt3*-KO n=12 young, n=20 aged). In female mice, there was no statistical difference in performance in the young cohort, however in the aged cohort *Sirt3*-KO mice had statistically higher activity, travelled a longer distance and had higher static counts relative to the WT mice (WT n=14, n=11 aged; *Sirt3*-KO n=12 young, n=20 aged). Asterisks denote (p<0.05) between WT and *Sirt3*-KO mice within the age groups. Student t-test was used for open field.
Figure 4.8:  *Sirt3*-KO female mice display altered motor performance. (A-B) Graphs showing the behavioural performances of young (grey bars) and aged (white bars) male and female mice in (A) The line graphs represent the performance of WT (solid line) and *Sirt3*-KO (dotted line) male and female mice on the rotarod test. Line graphs show the mean and SEM of latency for mice to fall from the accelerating rotarod. There was no difference between WT and *Sirt3*-KO male mice in the young or in the aged cohorts (WT n=16 young, n=19 aged; *Sirt3*-KO n=24 young, n=32 aged). Young female *Sirt3*-KO mice displayed a significantly shorter latency to fall on trial days 3 and 4, whereas aged *Sirt3*-KO female mice displayed a significantly shorter latency to fall on all trials (WT n=12 young, n=11 aged; *Sirt3*-KO n=21 young, n=22 aged). (B) Bar graphs represent the percentage of successful rod traversions for WT and *Sirt3*-KO male and female mice. Whereas there was no difference in performance on the rod traversions between young WT and *Sirt3*-KO sex-matched mice, aged male and female *Sirt3*-KO mice performed poorer than their WT counterparts. Males (WT n=13, n=17 aged; *Sirt3*-KO n=11 young, n=25 aged); females (WT n=11, n=11 aged; *Sirt3*-KO n=10 young, n=13 aged). Asterisks denote (p<0.05) between WT and *Sirt3*-KO mice within the age groups. Two-way ANOVA with Bonferroni post-hoc was used for the accelerating rotarod assessment. Chi-square analysis was used to compare the percentage of successful traversions on the rod.
Female *Sirt3*-KO mice display lowered anxiety-like behaviour

Recent research has demonstrated that mitochondrial function can contribute to anxiety behaviours (Streck et al., 2014; Hollis et al., 2015). Given that SIRT3 regulates mitochondrial function (Giralt & Villarroya, 2012; Ansari, 2017; Salvatori et al., 2017), I tested whether altered anxiety-like behaviour would be evident in *Sirt3*-KO mice. Performances were compared in four different tests: light/dark (LD) test, elevated plus maze (EPM), center rearing in the OF (Crawley, 1999), and marble burying paradigm (De Boer & Koolhaas, 2003). Consistent with the observations of (Kim et al., 2017), no differences in anxiety-like behaviour were seen in either young or aged male *Sirt3*-KO mouse cohorts relative to age-matched male WT mice (Figures 4.9A, 4.9B; 4.9C). In contrast, however, significant differences in anxiety-like behaviour were observed in both young and aged female *Sirt3*-KO mice in each of these tests. In the LD place preference test, *Sirt3*-KO female mice showed greater risk-assessment scores than their age-matched controls (Figure 4.9A). Likewise in the EPM assessment, *Sirt3*-KO female mice spent significantly more time in the open arms compared to their age-matched control mice (Figure 4.9B). Furthermore, in the center field rearing test, a significant increase in rearing was observed in the aged female *Sirt3*-KO mice relative to WT controls (Figure 4.9C). No difference in marble burying test was observed between *Sirt3*-KO and WT mice in either of the sex or age cohort groups (Figure 4.9D). Taken together, these data indicate that the deficiency in sirtuin-3 leads to decreased anxiety-like behavioural patterns in both young and aged female mice without significantly affecting this behaviour in male mice at either age.
Figure 4.9: *Sirt3*-KO female mice display decreased anxiety-like behaviour. (A-D) Anxiety measurements in WT and *Sirt3*-KO young (grey bars) and aged (white bars) male and female mice. (A) Histogram showing the mean and SEM of a LD preference test measuring the percentage of risk assessment behaviour (head poke) relative to time spent in the dark during a 5 minute period. No differences were observed between WT and *Sirt3*-KO male mice in the young or in the aged cohorts (WT n=11 young, n=16 aged; *Sirt3*-KO n=21 young, n=28 aged). Both young and aged *Sirt3*-KO female mice showed higher indices of risk assessment relative to WT (WT n=16 young, n=13 aged; *Sirt3*-KO n=14 young, n=21 aged). (B) Histograms showing the mean and SEM of the EPM test measuring the total amount of time mice spent in the open areas during a 5 minute test period. No differences were observed between WT and *Sirt3*-KO male mice in the young or in the aged cohorts (WT n=11 young, n=17 aged; *Sirt3*-KO n=18 young, n=31 aged). Both young and aged *Sirt3*-KO female mice spent longer in the open areas of EPM relative to WT mice (WT n=13 young, n=11 aged; *Sirt3*-KO n=9 young, n=22 aged). (C) Histograms showing the mean and SEM of total center rearing during a 30 minute open-field assessment. No differences were observed between WT and *Sirt3*-KO male mice in the young or in the aged cohorts (WT n=12 young, n=17 aged; *Sirt3*-KO n=12 young, n=20 aged). Aged *Sirt3*-KO female mice showed higher levels of center rearing relative to the WT mice (WT n=14 young, n=11 aged; *Sirt3*-KO n=12 young, n=20 aged). (D) Histograms showing the mean and SEM of the uncovered marbles during a 1 hour test. No differences were observed between WT and *Sirt3*-KO male (WT n=12 young, n=12 aged; *Sirt3*-KO n=13 young, n=7 aged) and female mice (WT n=12 young, n=9 aged; *Sirt3*-KO n=14 young, n=15 aged) in the young or in the aged cohorts. Asterisks denote statistical significance (p<0.05) between WT and *Sirt3*-KO mice within the age groups, student t-test.
4.2.10 \textit{Sirt3-KO} male and female mice exhibit normal object recognition memory

Impaired mitochondrial function has also been linked to impairments in memory (Picard \& McEwen, 2014). I therefore tested whether object recognition memory would be affected in male or female \textit{Sirt3-KO} mice using the novel object recognition task. Consistent with the results of Kim et al (2017), both young and aged male \textit{Sirt3-KO} mice spent similar amount of time exploring the novel object after a 5 minute delay and following a 2 hour delay (Figure 4.10A). In addition, female \textit{Sirt3-KO} mice also showed no difference in novel object discrimination index when compared to their age-matched female controls (Figure 4.10B). These results therefore indicate that sirtuin-3-deficiency does not interfere with the object recognition memory of either young or aged male or female mice.
Figure 4.10: *Sirt3*-KO mice display unaltered novel object recognition memory. Histograms showing the mean and SEM of the discrimination index (% of time), that (A) male and (B) female mice spent exploring the novel object after 5 minutes and 2 hour delay. No statistically significant differences were observed between WT and *Sirt3*-KO male or female mice in the young or in the aged cohorts, student t-test. Males 5 minutes (WT n=10 young, n=9 aged; *Sirt3*-KO n=19 young, n=17 aged), 2 hours (WT n=9 young, n=9 aged; *Sirt3*-KO n=16 young, n=16 aged); Females 5 minutes (WT n=11 young, n=10 aged; *Sirt3*-KO n=9 young, n=12 aged), 2 hours (WT n=11 young, n=10 aged; *Sirt3*-KO n=7 young, n=11 aged), student t-test.
4.3 Discussion

This study showed clear sex differences in sirtuin-3 expression levels as well as behavioural consequences of its deficiency. Five main findings emerge from this study. First, we show that under basal conditions, Sirt3-KO male and female mice do not differ from their WT counterparts in their weight, homecage activity, temperature regulation, and sleep patterns. This phenotype remained consistent in the aged animals as well. Second, when introduced to a novel environment, Sirt3-KO female mice displayed increased exploration and reduced anxiety-like behaviour. Third, sirtuin-3-deficiency results in impairment of motor coordination, however females were more severely affected. Fourth, sirtuin-3 deficiency leads to a more pronounced phenotype with advanced age. Fifth, SIRT3 expression in adult and aged WT mice is sex-specific.

4.3.1 Sirt3-KO mice show normal phenotype in their homecage environment

Converging studies have now convincingly demonstrated that mitochondria underlie the sexual dimorphism that is observed at the molecular, anatomical and behavioural levels (Demarest & McCarthy, 2015; Gaignard et al., 2017; Lejri et al., 2018). Based on that knowledge, I assessed whether sirtuin-3-deficiency would result in sex-specific behavioural phenotype in mice. In agreement with previous studies (Lombard et al, 2007; Liu et al., 2015), I found that under normal housing conditions, both young and aged Sirt3-KO male and female mice are healthy with no observed signs of abnormality, sudden deaths, and retain body weight similar to that of their WT counterparts. In addition, telemetry assessments of activity in their homecage showed no discernable difference between Sirt3-KO and WT male or female mice. These results concluded that in familiar environments Sirt3-KO and WT mice do not differ in their home cage activity and that this behaviour does not change as the animal ages.
Under basal conditions, I showed that Sirt3-KO mice did not differ from their WT counterparts in their temperature regulation over the 24-hour assessment window when compared to age and sex-controls. Interestingly, a previous study showed that following cold exposure (4°C for 6 hours), Sirt3-KO\textsuperscript{muscle} and Sirt3-KO\textsuperscript{liver} mice were indistinguishable from WT control mice in their body core temperature regulation (Fernandez-Marcos et al., 2012). The authors speculated that the thermoregulatory functions of brown fat tissue may be the reason for the preservation of temperature regulation in Sirt3-KO\textsuperscript{muscle} and Sirt3-KO\textsuperscript{liver} mice. Because my Sirt3-KO mice are whole-body knockouts, and therefore no SIRT3 tissue-specific compensation is possible, it would be interesting to assess temperature regulation in these mice following exposure to cold temperatures.

Recent studies have implicated that mitochondrial dysfunction can compromise normal sleep behaviour (Ramezani & Stacpoole, 2014). In line with this, Zhang et al (2014) demonstrated that sirtuin-3 deficiency results in sleep disruption, as the authors observed a reduction in EEG delta power waves in Sirt3-KO mice following forced extended wakefulness. This study suggests that sleep-associated dysfunction becomes apparent in Sirt3-KO mice following a stressor (Zhang et al., 2014). However, I showed that under basal conditions, normal patterns of cortical delta wave activity are comparable between Sirt3-KO and WT age and sex-matched mice over a 24-hour period. Therefore, the normal homeostatic balance of neural circuits involved in sleep of Sirt3-KO mice is preserved under basal conditions.

4.3.2 Sirtuin-3-deficiency results in impaired motor coordination in mice

Because mitochondrial dysfunction plays a role in motor impairments (de Haas et al., 2017) (Casadei et al., 2016), I assessed the motor performance of Sirt3-KO mice on rotarod and rod traversation tests. These behavioural paradigms evaluate motor coordination and also have been
demonstrated to be sensitive to brain dysfunction (Onyszchuk et al., 2007; Drucker-Colin & Garcia-Hernandez, 1991; Fleming et al., 2013). Motor learning capacity can also be assessed by the rotarod test. In short, as the speed of the rod rotation increases, it becomes more difficult for mice to retain their balance causing them to fall off from the rod. However, their performance would improve progressively with daily training, and mice learn to stay on the rod for a longer period of time. My results show that in concordance with a previous report, that there is no performance difference between Sirt3-KO and WT age-matched male mice on the rotarod test (Liu et al., 2015). Conversely, Sirt3-KO female mice display an impairment on the rotarod assessment, as the latency to fall was reduced in young Sirt3-KO female mice relative to the control mice, and this impairment was further exacerbated in aged Sirt3-KO female mice.

Furthermore, young male and female Sirt3-KO mice performed comparably to their WT counterparts in traversing the rod. On the other hand, both aged Sirt3-KO male and female mice showed poorer performance on this task when compared to their WT counterparts, which shows that sirtuin-3 deficiency leads to a more pronounced phenotype with advanced age. The impairments observed on the rotarod and traversion tests were not likely to be caused by muscle weakness, as Sirt3-KO mice did not show overt abnormalities in locomotion in their homecage and also performed similarly to WT mice on the traversion rod during the training period (there was no training period on the rotarod, to avoid premature motor learning).

Taken together, these results suggest that sirtuin-3-deficiency may play a role in specific brain regions involved in regulating motor-associated behaviours, such as: motor cortex, basal ganglia, and cerebellar circuits in a sex-dependent manner (Middleton & Strick, 2000). In addition, it appears that sirtuin-3-deficiency results in an age-progressive impairment of motor abilities,
which can be explained by the fact that mitochondrial dysfunction and oxidative stress can affect brain-regulated motor skills in an age-dependent manner (Forster et al., 1996).

4.3.3  _Sirt3-KO aged female mice show increased exploration in a new environment_

To test whether sirtuin-3 deficiency regulates locomotor activity in a novel environment, mice were subjected to the open field (OF) test. There were no pronounced differences between _Sirt3-KO_ and WT male or female mice in the young cohort on any of the assessed OF parameters. However, _Sirt3-KO_ aged female mice exhibited increased overall activity and travelled a larger distance when compared to the WT female mice. Similar to above, these results are also indicative of an age-progressing phenotype resulting from a sirtuin-3-deficiency. Such increases in exploration and locomotion indicate a mild-hyperactive phenotype that is driven by a novel environment, which are also generally interpreted as an attenuation of anxiety (Yen et al., 2013).

4.3.4  _Sirt3-KO female mice show reduced anxiety_

Recent research has demonstrated that mitochondrial function can regulate anxiety-associated behaviours (Streck et al., 2014; Hollis et al., 2015). Given that SIRT3 regulates mitochondrial homeostasis (Giralt & Villarroya, 2012), and the observation that _Sirt3-KO_ female mice display a mild hyperactive and exploratory phenotype in the OF, I tested whether altered anxiety-like behaviour would also be evident in _Sirt3-KO_ mice. Performances were compared in four different tests: elevated plus maze (EPM), light/dark (LD) place preference test, center rearing in the OF (reviewed in Crawley, 1999) and marble burying assessment. Consistent with the observations by Kim et al (2017), no differences in anxiety-like behaviour on any of the assessments were observed in _Sirt3-KO_ male mice relative to their age-matched WT controls. However, a different pattern emerged in the female cohort. In the EPM assessment, both young and aged female _Sirt3-KO_ female mice spent significantly more time in the open arms compared to their WT controls.
In the LD place preference test, both young and aged female Sirt3-KO mice showed greater risk-assessment scores than WT female mice. Furthermore, a significant increase in center rearing was observed in the aged Sirt3-KO female mice relative to their WT controls. However, no difference was observed in the number of buried marbles between Sirt3-KO and WT age and sex-matched mice. Interestingly, these results did not correlate with the results for the anxiety assessments stated above. These contrasting results can also be explained by Thomas et al. 2009, who demonstrated that the marble burying test is simply a reflection of repetitive digging behaviour and does not represent anxiety-related responses. One pitfall for the EPM and center rearing paradigms in the OF, is that the observed phenotype may primarily reflect an increase in locomotion (Section 4.3.3), which therefore may confound the interpretation for the anxiety assessments. However, the LD place preference test eliminates locomotion as the confounding variable, as the observed risk score is dependent on the duration of nose pokes relative to the time spent in the dark compartment.

Taken together, these data indicate that the deficiency in sirtuin-3 leads to decreased anxiety-like behavioural patterns in both young and aged female mice without significantly affecting this behaviour in males. These results suggest that sirtuin-3-deficiency may affect brain regions involved in anxiety regulation, such as: amygdala, hippocampus, and cortex in a sex-dependent manner (Steimer, 2002; Shin & Liberzon, 2010).

4.3.5 Sirtuin-3-deficiency has no effect on novel object recognition

Studies have shown a link between mitochondrial dysfunction and cognitive decline, such as memory (Picard & McEwen, 2014). To assess whether Sirt3-KO mice display cognitive deficits, such as object recognition memory, I assessed their performance in the novel object recognition task. No difference in the novel object recognition task was observed between Sirt3-KO and WT
mice in either of the sex or age cohorts. Interestingly, Kim et al (2017) found that Sirt3-KO male mice showed poor performance when compared to their WT counterparts using a Morris water maze test to assess spatial memory. It is therefore conceivable that sirtuin-3-deficiency affects only specific aspects of memory, such as remote spatial memory but has no effect on object-related memory. Interestingly, Broadbent et al (2004) demonstrated that while relatively mild hippocampal damage is sufficient to impair spatial memory, a much more extensive damage is required to impair recognition memory. Therefore, it is possible that the hippocampus in Sirt3-KO mice is mildly affected which results in spatial memory impairment (Kim et al., 2017), however it is not sufficiently damaged to affect object recognition memory.

4.3.6 **SIRT3 expression is sex-specific**

Males and females differ in their physiology, behaviour, brain structures and brain mitochondrial function – all of which become more apparent with age (Gaignard et al., 2017). Specifically, sex steroids influence numerous functions of mitochondria, such as: oxidative stress regulation, energy regulation, calcium homeostasis and apoptosis (Ueyoshi et al., 1991; Nilsen & Brinton, 2003; Irwin et al., 2008; Gaignard et al., 2017; Zárate et al., 2017). While the effects of testosterone and progesterone on mitochondrial function are less studied, the effect of estrogen on mitochondrial function has been gaining traction as estrogen receptors are found on brain mitochondria (Ueyoshi et al., 1991; Nilsen & Brinton, 2003; Irwin et al., 2008; Gaignard et al., 2017; Zárate et al., 2017). Indeed, studies have now demonstrated that co-regulation between mitochondria and estrogen is essential for maintaining proper physiology.

It is therefore conceivable that hormones may underlie the sex-specific SIRT3 expression levels in my study. To recap, I observed that temporal SIRT3 expression is sexually dimorphic. In detail, cortical SIRT3 protein expression levels in WT male mice were stable between 3 and 8 month-
old mice, but decreased in 24 month-old males. In females, SIRT3 protein levels dropped at an earlier stage – between 3 and 8-months and remained at stable levels in the aged female cortex. Interestingly, in both humans and rodents, aging is a salient period for hormone-related circuit reorganization (Sisk & Zehr, 2005; Wilson et al., 2011; Ladouceur et al., 2012). On average, female mice undergo reproductive senescence (the equivalent of pre-menopause) at around 10-15 months of age (Dutta & Sengupta, 2016). Consistently with this, I see a drop in SIRT3 protein prevalence in female mice at around the pre-menopause stage where the synthesis of estrogen begins to decline. It would be interesting to measure plasma estrogen levels during animal development as well as aging, to assess whether these levels correlate with SIRT3 expression.

Furthermore, in both young and aged cohorts, SIRT3 protein was more prevalent in the male than in the female mice. Interestingly, Kong et al (2010) found that the murine Sirt3 gene promoter region contains an estrogen receptor binding motif. However, the authors also found that the elevation or inhibition of estrogen alone had no effect on mouse Sirt3 promoter activity in absence of PGC-1α. The authors then concluded that SIRT3 mRNA expression is stimulated by PGC-1α, as this factor then co-operates with estrogen to induce transcription of SIRT3. Interestingly, Pgc-1α expression levels were found to be higher in male WT mice relative to female WT mice (Zawada et al., 2015). PGC-1α levels may therefore also dictate SIRT3 protein prevalence, and this would be consistent with my results that SIRT3 is expressed at a higher level in male WT mice relative to female WT mice under basal conditions. It would be therefore noteworthy to assess whether SIRT3 levels are attenuated in PGC-1α-KO mice.

In addition to the expression results, the findings from this Chapter demonstrate that sirtuin-3-deficiency also results in sex differences at the behavioural level. Interestingly, studies have demonstrated that estrogen has an influence on mood states, movement, and cognitive functions
(Gillies & McArthur, 2010). Effects of estrogen have also been linked to behaviours that involve a number of brain regions, such as: the cerebellum, amygdala, hippocampus, cerebral cortex and brainstem, as well as different neurotransmitter systems (Gillies & McArthur, 2010). Intriguingly, estrogen also plays a protective role in a number of neurodegenerative and neuropsychiatric conditions through the mitochondrial axis in a sex-specific manner (Gandy, 2003; Klinge, 2008). Therefore, it is possible that sirtuin-3-deficiency may results in the disruption of the crosstalk between mitochondrial function and estrogen action, which may alter neuronal homeostasis in these regions and therefore manifest in the observed phenotype in Sirt3-KO female mice. It is also important to note that since estrogens can be generated from periphery-derived androgens in the male brain (Miller & Auchus, 2011), male mice would not be as severely affected. These hypothetical speculations are intriguing and necessitate for further studies to investigate the role that sex-hormone fluctuations have on SIRT3 expression. However, it is important to keep in mind that differences between the sexes can also arise from diverse factors other than an estrogenic effect. For example, neuroendocrine responses within the hypothalamic-pituitary axis (Schroeder et al., 2018) and genetic and epigenetic influences (Ratnu et al., 2017) have also been shown to underlie emotion-driven behaviour.

### 4.3.7 Sirtuin-3-deficiency results in a phenotype that is age-progressive

This study showed that sirtuin-3-deficiency in mice resulted in progressive and age-related behavioural deficits. This is not surprising, as previous studies have demonstrated that Sirt3-KO mice are prone to age-related disorders and sirtuin-3 attenuation/ablation is associated with manifestation of several diseases of aging (McDonnell et al., 2015). Given that SIRT3 prevalence is preserved in the aged brain (section 3.2.5), it is presumable that SIRT3 is an important factor in regulation of mitochondrial homeostasis in the elderly. Indeed, converging data have implicated sirtuin-3 to regulate a number of biological functions that play an important role in longevity.
(Rose et al., 2003; Bellizzi et al., 2005; Hirschey et al., 2011; Kincaid & Bossy-Wetzel, 2013; Denu, 2017), cancer (Torrens-Mas et al., 2017; Li et al., 2018), and neurodegenerative diseases (Anamika et al., 2017). In addition, longevity-association studies reported polymorphisms in the human SIRT3 gene of the elderly, which suggest that this enzyme may be linked to increased lifespan (Rose et al., 2003; Bellizzi et al., 2005). Taken together these studies suggest that sirtuin-3 may be an indispensable factor that is required for normal aging, which is consistent with my results that aged Sirt3-deficient mice show a more pronounced phenotype when compared to the young Sirt3-KO cohort.

Taken together, and in support of the Aim 2, these results show that sirtuin-3 deficiency results in a development of sex-specific and age-related behavioural impairments. In summary, Sirt3-KO female mice, display increased hyperlocomotion in a novel environment, reduced anxiety-like behaviour, and impairment on motor learning and coordination, all of which are hallmarks of a mild hyperactive phenotype (Marazziti et al., 2002; Flaiser-Grinberg et al., 2010; Kaiser et al., 2015; Jul et al., 2015) and in this case driven by context-specific factors. The results from this Chapter therefore highlight the possibility that sirtuin-3 regulates molecular pathways which may play a role in a number of neurological and neuropsychiatric conditions that are demonstrated to have a sex-specific prevalence.
**Sirt3-KO female mice display mTORC1 pathway hyperactivation**

5.1 Brief introduction and rationale

Hyperactive behaviour, whether under basal conditions or influenced by a specific context, is a state of being abnormally active, and defined by: increased motor output, increased exploration, decreased attention to detail, involvement with high-risk activities and decreased anxiety. This behavioural phenotype is frequently prevalent in a number of neurological and neuropsychiatric disorders (Sakae et al., 2008; Miro et al., 2012; Yen et al., 2013; Jul et al., 2016). Although the molecular mechanisms causing a hyperactive phenotype are not yet fully elucidated, recent studies have begun to reveal the underpinnings of these processes which implicate hyperresponsive synaptic cascades (Du et al., 2004; Ramshaw et al., 2013). Several lines of evidence have now shown that some of these neuronal network alterations are due to the hyperactivation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway (Lasarge & Danzer, 2014; Lipton & Sahin, 2014). mTORC1, which is as described in section 1.5.2, is a conserved protein kinase that is ubiquitously expressed in eukaryotic cell types (Brown et al., 1994; Sabatini et al., 1994), which includes neurons (Sabatini et al., 1999). mTORC1 integrates signals from nutrients, energy status and growth factors to regulate many processes such as: synaptic organization, dendritic complexity, and overall cell proliferation (Costa-Mattioli & Monteggia, 2013; Lipton & Sahin, 2014; Perluigi et al., 2015; Ryskalin et al., 2018). Convergent studies show that mTORC1 activity is also associated with complex behaviours such as locomotor activity (Wang et al., 2012; Cornu et al., 2014) and anxiety (Saré et al., 2017). As described in section 1.5.2, mitochondria functions have either activating or inhibitory effects on mTORC1 which depend on the balance between ROS levels, nutrient supply, and the overall state of the cell (reviewed by (Heberle et al., 2015)).
In addition, mTORC1 function regulates mitochondrial processes through stimulating the protein synthesis of a number of nuclear-encoded mitochondrial factors (Groenewoud & Zwartkruis, 2013; Morita et al., 2015). Therefore, mTORC1 and mitochondria have a dynamic, bi-directional relationship, however the intermediate factors regulating this cross-talk are still being uncovered. Interestingly, Sundaresan et al (2009) demonstrated a link between sirtuin-3 and mTORC1, as Sirt3-KO cardiomyocytes showed an upregulation of the mTORC1 cascade. Unfortunately, the sex of the animals used in the Sundaresan (2009) study were not specified. However, these data suggest that, at least in the heart, sirtuin-deficiency results in an activation of the mTORC1 pathway. Given that Sirt3-KO female mice display increased novelty-driven hyperlocomotion, reduced anxiety-like behaviour, and impairment on motor learning and coordination, all of which are hallmarks of a mild hyperactive behavioural phenotype, I tested whether mTORC1 activity would be upregulated in a sex-dependent manner to reflect the observed behavioural phenotype.
5.2 Results

5.2.1 Glycogen synthase-3β and Akt1 display differential activation in male and female Sirt3-KO mouse cortex

Signaling components that are upstream and downstream of mTORC1 were assessed. While Akt1 and glycogen synthase kinase (GSK)-3β influence a host of intracellular signaling systems (Cohen & Frame, 2001; Manning & Toker, 2017), they are each upstream regulators of the mammalian target of rapamycin complex 1 (mTORC1) (Cohen & Frame et al., 2001; Hahn-Windgassen et al., 2010). In addition, (GSK)-3β and Akt1 signaling activities have been linked to behavioural hyperactivity and/or anxiety-like behavioural alterations (Yu et al., 2010; Mines & Jope, 2012; Crofton et al., 2017; Qiao et al., 2018). Given the divergent behavioural patterns seen in male and female Sirt3-KO mice, I tested whether the levels or activation states of cortical GSK-3β and Akt1 would differ in Sirt3-KO mice in a sex-specific manner. Cortex was assessed because it is recognized as a brain region that plays a role in anxiety (Steimer, 2002; Shin & Liberzon, 2010) and motor behaviours (Middleton & Strick, 2000), as this region receives and integrates convergent inputs from a number of limbic, sensory and motor structures. Consistent with the lack of behavioural changes seen in male Sirt3-KO mice, no changes in either the prevalence or phosphorylation state of GSK-3β Serine (Ser)-9 or Akt1 (Ser-473) were observed in the male Sirt3-KO mouse cortex compared to male WT cortex (Figures 5.1A; 5.1B). In contrast to the lack of change in phospho/total GSK-3β or Akt1 ratio in males, a robust increase in Ser-9 phosphorylation of GSK-3β, and an increase in Ser-473 phosphorylation of Akt1, was observed in Sirt3-KO female mice relative to WT female mice (Figures 5.1C; 5.1D). No changes in the total prevalence of either Akt1 or GSK-3β were observed in these tissues.
Figure 5.1: Sirtuin-3-deficiency in female mice increases the phosphorylation of upstream effector proteins of the mTORC1 pathway. Representative Western blots of phosphorylated Akt1 (Ser-473) and total Akt1 in (A) male and (C) female mice, and phosphorylated GSK-3β (Ser-9) and total GSK-3β in (B) male and (D) female mice. The histograms show the densitometric mean and SEM for each specific phosphorylated protein of interest expressed as a percent ratio its corresponding total protein. (A-B) No statistical difference between WT and Sirt3-KO mice in the ratio of phosphorylated Akt1 to total Akt1, and phosphorylated GSK-3β to total GSK-3β is observed in the male cohort. (C-D) The ratio between both phosphorylated Akt1 to total Akt1, and phosphorylated GSK-3β to total GSK-3β is significantly higher in Sirt3-KO female mice relative to WT controls. Asterisks denote statistical significance (p<0.05) between WT and Sirt3-KO mice, student t-test.
5.3 Female *Sirt3*-KO mice display hyperactivation of mTORC1 signaling pathway

mTORC1 signaling pathway was further assessed in cortical tissues of male and female *Sirt3*-KO mice by measuring the phosphorylation levels of its direct downstream target protein p70-S6 kinase (S6K) at the threonine (Thr)-389 site. The phosphorylation state of the ribosomal protein S6 at the Ser-240/244 site, a downstream target of S6K (Ruvinsky & Meyuhas, 2006), was also assessed. Western blot analysis revealed no significant changes in the total levels of either S6K or ribosomal protein S6 in cortical tissues from male or female *Sirt3*-KO mice relative to sex-matched WT mice (Figures 5.2A, 5.2B, 5.2C, 5.2D). However, the phosphorylation levels of S6K at Thr-389, and ribosomal protein S6 at Ser-240/244 in these same cortical samples were significantly elevated in *Sirt3*-KO female mice relative to female WT littermates (Figures 5.2B, 5.2D).
Figure 5.2: Sirtuin-3-deficiency in female mice increases the phosphorylation of upstream effector proteins of the mTORC1 pathway. Representative Western blots of phosphorylated S6K (Thr-389) and total S6K in (A) male and (C) female mice, and phosphorylated pS6 protein (Ser-240/244) and total S6 protein in (B) male and (D) female mice. The histograms show the densitometric mean and SEM for each specific phosphorylated protein of interest expressed as a percent ratio its corresponding total protein. (A-B) No statistical difference between WT and Sirt3-KO mice in the ratio of phosphorylated pS6K to total S6K, and phosphorylated pS6 protein to total S6 protein was seen in the male cohort. (C-D) The ratio between pS6K to total S6K, and phosphorylated pS6 protein to total S6 protein is significantly higher in Sirt3-KO relative to WT female mice. Asterisks denote statistical significance (p<0.05) between WT and Sirt3-KO mice, student t-test.
5.4 Discussion

Numerous lines of evidence implicate aberrant mTORC1 signaling to underlie complex behaviours such as changes in motor-associated activity (Wang et al., 2012; Cornu et al., 2014) and lowered anxiety (Saré et al., 2017). Indeed, the results of this study reveal that sirtuin-3 deficiency in the female but not in the male mice results in a hyperactive mTORC1 signaling pathway in the cortex. These molecular alterations are consistent with the behaviour observed in Sirt3-KO female mice (Chapter 4).

Elevated phosphorylation of Akt1 (Ser-473) was observed in the Sirt3-KO female mice. As discussed in section 1.5.2, Akt1 is a serine/threonine protein kinase and a key upstream modulator of mTORC1 activation. Akt1 can positively regulate mTORC1 through direct phosphorylation on Ser-2448, as well as through phosphorylation of a number of other regulatory factors upstream of mTORC1 (Hahn-Windgassen et al., 2005). Moreover, cortical tissues of Sirt3-KO female mice showed an increase in the phosphorylation of GSK-3β (Ser-9), which is indicative of its inhibition. GSK-3β a serine/threonine kinase that is an upstream inhibitor of mTORC1, and although many factors can phosphorylate GSK3-β at serine-9 and therefore inactivate it, one of them is in fact Akt1 (Cohen & Frame, 2001; Case et al., 2011). Interestingly, activation of the Akt1 (Ser-473)-GSK-3β (Ser-9) pathway has been observed in rodent models of hyperactivity (Mines & Jope, 2012; Yu et al., 2010; Crofton et al., 2017). Therefore, although mTORC1 has a complex regulatory network, the results in this Chapter suggest a model where in Sirt3-KO female mice, mTORC1 signaling pathway may be activated indirectly via phosphorylation of GSK3-β on Ser-9 by Akt1, and therefore relieving mTORC1 from inhibition by GSK3-β signaling.

One of the means by which Akt1 can be activated is via ROS (Chetram et al., 2013; Guha et al., 2010), which is in line with current literature, as sirtuin-3-deficiency has been demonstrated to
elevate oxidative stress (Sundaresan et al., 2009; Bell & Guarente, 2011; Pillai et al., 2016; Shi et al., 2017). Specifically, ROS can activate Ras (Sawyer et al., 2002; Kuster et al., 2005), a GTPase that is an upstream regulator of a vast number of signaling cascades associated with numerous cellular processes (Simanshu et al., 2017). Indeed, Ras is an upstream activator of MAPK/ERK and PI3K/Akt1 signaling pathways (Mendoza et al., 2011), both of which are involved in activation of mTORC1 (Mendoza et al., 2011; Lipton & Sahin, 2014).

Furthermore, hyperphosphorylation of the downstream mTORC1 effector proteins was also observed in the cortex of Sirt3-KO female but not male mice. Specifically, an increase in phosphorylation of S6K (Thr-389) and its downstream factor S6 ribosomal protein (Ser-240/244) was observed. These results are consistent with a recent publication that demonstrated an increase in phosphorylation in both S6K and S6 in Sirt3-KO MEFs relative to WT MEFS under basal conditions (Gonzalez Herrera et al., 2018). An increase in phosphorylation of both S6K and S6 is indicative of the overactivation of the protein translation pathway (Fonseca et al., 2014), and although an increase in protein translation is important in normal modification of neural circuits and behaviour, aberrant translation is associated with overactive signaling cascades at the synapses, increased neuronal proliferation, and overall imbalance in neuronal homeostasis (Santini & Klann, 2011; Topol et al., 2015; Jacquemont et al., 2018). Indeed, aberrant mTORC1 signaling disturbs the precise regulation of protein synthesis, which can result in pathological consequences for the nervous system development/function as well as predisposition to neurological and neuropsychiatric disorders (Loweth et al., 2010; Santini & Klann, 2011; Topol et al., 2015; Jacquemont et al., 2018). Thus, Sirt3-KO mice may have undergone excessive or abnormal neuronal activity during critical maturation periods, which may have influenced the wiring/network of neuronal circuits (Lipton & Sahin, 2014). Therefore, the resulting change in
neuronal topography and overactive signaling cascades may underlie the observed hyperactive behavioural phenotype.

### 5.4.1 Linking sirtuin-3-deficiency and mTORC1 activity

The mechanism of how sirtuin-3-deficiency in mitochondria leads to an upregulation in mTORC1 activity in our animal model remains to be determined. As explained in section 5.4.1, one possibility for the observed mTORC1 activation in Sirt3-KO mice may be through the ROS-axis. Sirtuin-3-deficiency results in an increase in oxidative stress (Bell & Guarente, 2011; Pillai et al., 2016; H. Shi et al., 2017; Sundaresan et al., 2009), which can activate a number of upstream pathways involved in mTORC1 activation (Sawyer et al., 2002; Kuster et al., 2005; Guha et al., 2010; Chetram et al., 2013; Zhao et al., 2017). Another way that SIRT3 can potentially regulate mTORC1 activity is through the AMP-activated protein kinase (AMPK) axis, which is a negative regulator of mTORC1 (Herzig & Shaw, 2018; Mihaylova & Shaw). More specifically, SIRT3 can deacetylate and activate liver kinase B1 (LKB1) (Pillai et al., 2010), a kinase that activates AMPK through direct phosphorylation (Woods et al., 2003; Zhang et al., 2013). Indeed, the absence of sirtuin-3 in murine cardiomyocytes showed an increase in LKB1 acetylation levels, which results in its de-activation, and subsequent decrease in AMPK phosphorylation and therefore a decrease in AMPK activity (Pillai et al., 2010). SIRT3 appears to also regulate AMPK at the level of expression (Huh et al., 2016), although the mechanism was not investigated, Huh et al. (2016) showed that Sirt3-KO osteoclasts had marked reduction of total protein and phosphorylation levels of AMPK. Thus, sirtuin-3-deficiency may result in a downregulation of AMPK pathway, which therefore can lead to an upregulation of the mTORC1 activity.

Another possibility is through the autophagy axis, as accumulating evidence show that dysfunctional autophagy is associated with both an overactive mTORC1 pathway (Jung et al.,
Autophagy is a process in which damaged organelles and other cellular constituents are sequestered in autophagosomes, which are then delivered to lysosomes for degradation, with the goal of restoring cellular homeostasis (Bento et al., 2016). Compromised autophagy can lead to the decrease of degradation of pre-synaptic vesicles, and as a consequence, this results in an overflow of neurotransmitters (NT) in the synaptic cleft (Hernandez et al., 2012). Interestingly, dysfunctional NT signalling has been demonstrated to underlie a hyperactive behavioural phenotype (Michael et al., 2003; Salahpour et al., 2008; Libert et al., 2011; Yamamoto et al., 2014; Ashok et al., 2017). Thus, ongoing research is trying to find ways to restore or increase autophagy in order to attenuate aberrant NT signaling (Rahman & Rhim, 2017). Although the role of sirtuin-3 in autophagy regulation has not been fully established, studies have now begun to uncover a relationship. It was recently demonstrated that SIRT3 positively regulates autophagy and therefore protects cortical neurons from an ischemia insult (Dai et al., 2017). The authors showed that an overexpression of sirtuin-3 in cortical neurons increased phosphorylation of AMPK, which therefore decreased mTORC1 signaling and consequentially upregulated autophagy. Furthermore, autophagy was impaired in SIRT3-KO human macrophages (Liu et al., 2018) and following SIRT3 knockdown in HT22 cells (Yan et al., 2018). Based on these studies, it is possible that elevated signaling of the mTORC1 pathway observed in Sirt3-KO female mice may result in a downregulated autophagy, which would result in an upregulation of NTs in synaptic cleft and consequently contribute to the hyperactive behaviour (Chapter 4). Future studies should investigate whether a relationship exists between SIRT3, mTORC1-autophagy axis, and pre-synaptic neurotransmission.

It remains unclear, however, whether the upregulation in the mTORC1 pathway is the primary or secondary effects of sirtuin-3-deficiency at this point. However, it is conceivable that sirtuin-3-
deficiency predisposes both sexes to mTORC1 activation, but sex-specific endogenous factors may act in synergy to potentiate mTORC1 activation in female mice (Baar et al., 2016). How sirtuin-3-deficiency preferentially upregulates the mTORC1 signaling cascades in the female sex is an important mechanistic question for future studies. In summary and in support of **Aim 3**, these results show that sirtuin-3 deficiency results in a sex-specific activation of extra-mitochondrial signaling pathways. This is the first study to show that sirtuin-3 deficiency in the brain results in alterations of mTORC1 pathway in the female and not in the male mice.
Sirt3-KO mice are sensitized to amphetamine treatment

6.1 Brief introduction and rationale

Sirtuin-3-deficiency in female mice appears to result in behavioural (Chapter 4) and molecular (Chapter 5) changes that reflect a mild-hyperactive behaviour. Specifically, Sirt3-KO female mice display a mild hyperactive phenotype in a novel environment, as evidenced by an increase in locomotor activity, exploration, and risk-taking behaviour. Furthermore, sirtuin-3-deficient female mice show an increase in the mTORC1 signaling pathway, an upregulation of which is also implicated to precipitate a hyperactive phenotype (Kim et al., 2013; Lipton & Sahin, 2014; Ryskalin et al., 2018). To further strengthen the correlation between sirtuin-3 and this phenotype, I assessed whether Sirt3-KO mice mimic behavioural symptoms of hyperactivity as a response to a psychostimulant. Amphetamine (AMPH)-induced hyperactivity in rodents is the most often used predictive pharmacological model of a hyperactive phenotype (Borison et al., 1978; Gould et al., 2001). AMPH is a psychostimulant that acts on different types of neurons and molecular targets. Although a variety of neurotransmitters (NT) are released following AMPH administration (Callaway et al., 1991; Rothman et al., 2001; Vieira-Brock et al., 2015; Underhill et al., 2014), catecholamine neurons are considered to be the main targets of AMPH, as dopamine (DA) and norepinephrine (NE) are the most affected NT systems (Wayment et al., 1998; Fleckenstein et al., 2007). Although the underlying mechanism of action for AMPH still remains uncertain (Sulzer et al., 2005), there is a consensus that AMPH competitively inhibits NTs reuptake by blocking their respective transporters, as well as by triggering the reverse transport of these NTs from the cell terminal to the synapse (Khoshbouei et al., 2004; Eiden & Weihe, 2011; G. M. Miller, 2011). In fact, overactivity of the NTs at the synapses has also been linked to a hyperactive phenotype (Kumakura et al., 2010), and implicated in a number of neuropsychiatric
and neurological disorders. I hypothesize that the sub-threshold dose of AMPH would aggravate hyperactive phenotype in Sirt3-KO mice, whereas it would have no effect on their WT-counterparts.
6.2 Results

6.2.1 Young Sirt3-KO mice treated with amphetamine showed hyperlocomotion in the open field

In Chapters 4 and 5, I uncovered that sirtuin-3 deficiency in female mice results in behavioural and molecular consequences that reflect a hyperactive phenotype. One way to strengthen the link between sirtuin-3-deficiency and this phenotype is to assess the response to AMPH treatment in Sirt3-KO mice and their WT counterparts. Because low dose of AMPH increases motor behaviours in rodents (Salahpour et al., 2008), I examined the activity of both WT and Sirt3-KO mice following AMPH and saline treatment in the open field (OF). Rodent behaviour, in the OF is often used as a tool to study the behavioural effects of AMPH and other stimulants (Schiorring, 1979; Stohr et al., 1998; Underhill et al., 2014). The 45-min interval between AMPH administration and OF exposure was chosen because it has proved to be the peak of activity in the OF following treatment of mice with 2mg/kg AMPH (Yates et al., 2007). Our results show that AMPH administration resulted in an increase of activity (Figures 6.1A, 6.2A), distance covered (Figure 6.1B, 6.2B), static counts (Figure 6.1C, 6.2C), but it had no effect on mobility rate (Figure 6.1D, 6.2D) in both AMPH-treated young male and female Sirt3-KO mice relative to their sex-matched Sirt3-KO group treated with saline. AMPH did not have an effect on WT mice in either female or male mice relative to their saline-treated WT controls. Furthermore. AMPH did not have an effect on activity (Figures 6.1A, 6.2A), distance covered (Figure 6.1B, 6.2B), or static counts (Figure 6.1C, 6.2C) in WT or Sirt3-KO male or female aged mice. In the aged cohort, Sirt3-KO male mice treated with AMPH showed an increase in mobility rate relative to the saline treated Sirt3-KO control group (Figure 6.1D).
Figure 6.1: Amphetamine treatment induced locomotor activity in Sirt3-KO male mice in the open field. (A-D) Histograms showing the behavioral performances of young and aged WT and Sirt3-KO male mice in different open-field assessments during a 30 minute period. Parameters assessed were: (A) total activity, (B) distance travelled, (C) total static counts, and (D) mobility rate. (A-D) There was no statistical difference in performance on any of the measured parameters between saline and AMPH treatment in the young cohort in the WT group. (D) AMPH treatment increased mobility rate of WT mice, relative to saline group in the aged cohort. Young Sirt3-KO mice treated with AMPH spent more time being (A) active, (B) covered greater distance, and (C) showed higher static counts than the Sirt3-KO group treated with saline. (D) There was no statistical difference between AMPH and saline treated Sirt3-KO mice on the rate of mobility in the young cohort. In the aged cohort, WT mice showed an increase in rate of mobility. No statistical difference in performance on any of the other measured parameters between saline and AMPH treatment in the aged cohort in Sirt3-KO group. Asterisks denote (p<0.05) between Saline and AMPH treated mice within Sirt3-KO or WT groups, student t-test, WT (young: saline n=8, AMPH n=7; aged: saline n=6, AMPH n=6), Sirt3-KO (young: saline n=6, AMPH n=7; aged: saline n=5, AMPH n=6).
Figure 6.2: Amphetamine treatment induced locomotor activity in Sirt3-KO female mice in the open field. (A-D) Histograms showing the behavioural performances of young and aged WT and Sirt3-KO female mice in different open-field assessments during a 30 minute period. Parameters assessed were: (A) total activity, (B) distance travelled, (C) total static counts, and (D) mobility rate. Young Sirt3-KO mice treated with AMPH spent more time being (A) active, (B) covered greater distance, and (C) showed higher static counts than the Sirt3-KO group treated with saline. There was no difference between AMPH and saline treated Sirt3-KO mice on the rate of mobility. (A-D) There was no statistical difference in performance on any of the measured parameters between saline and AMPH treatment in the young or aged cohort in the WT group. Asterisks denote (p<0.05) between Saline and AMPH treated mice within Sirt3-KO or WT groups, student t-test, WT (young: saline n=8, AMPH n=9; aged: saline n=6, AMPH n=7), Sirt3-KO (young: saline n=8, AMPH n=9; aged: saline n=6, AMPH n=8).
6.2.2 *Sirt3*-KO male and female mice display sensitized response to amphetamine treatment in the elevated plus maze paradigm

Changes in anxiety states is one of the frequent observed effects associated with psychostimulant administration (Pometlova et al., 2012; Vorspan et al., 2015). The elevated plus maze (EPM) has been used to assess effects of AMPH on rodent behaviour (Silva et al., 2002; Dawson et al., 2005). My results show that both young female and male APMH treated *Sirt3*-KO group spent more time in the open arms of the EPM, relative to the saline treated *Sirt3*-KO control group (Figure 6.3A; Figure 6.4A), which is suggestive of a lowered anxiety phenotype following AMPH administration. Whereas AMPH treatment did not have an effect in either the male or female WT group, relative to the saline treated group. Furthermore, AMPH did not have an effect in the either *Sirt3*-KO or WT aged group (Figure 6.3B; Figure 6.4B).
Figure 6.3: Amphetamine treatment reduced anxiety-like phenotype in Sirt3-KO male mice in the elevated plus maze paradigm. (A-B) Anxiety measurements in WT and Sirt3-KO young and aged male mice in the plus maze paradigm. Histograms show the mean and SEM of the plus maze test measuring the total amount of time mice spent in the open areas of the elevated plus maze during a 5 minute test period. AMPH treated (A) young male Sirt3-KO, but not WT, mice spent more time in the open arms than the saline treated group. (B) In the aged group, WT male mice treated with saline spent more time in the open arms. (B) In the aged group, there was no difference between AMPH and saline treatment in the aged Sirt3-KO groups. Asterisks denote (p<0.05) between saline and AMPH treated mice within Sirt3-KO or WT groups, student t-test, WT (young: saline n=8, AMPH n=9; aged: saline n=5, AMPH n=5), Sirt3-KO (young: saline n=6, AMPH n=7; aged: saline n=7, AMPH n=8).
Figure 6.4: Amphetamine treatment reduced anxiety-like phenotype in Sirt3-KO female mice in the elevated plus maze paradigm. (A-B) Anxiety measurements in WT and Sirt3-KO young and aged female mice in the plus maze paradigm. Histograms show the mean and SEM of the plus maze test measuring the total amount of time mice spent in the open areas of the elevated plus maze during a 5 minute test period. (A) AMPH treated young female Sirt3-KO, but not WT, mice spent more time in the open arms than the saline treated group. (B) In the aged group, there was no difference between AMPH and saline treatment in the aged Sirt3-KO or WT groups. Asterisks denote (p<0.05) between Saline and AMPH treated mice within Sirt3-KO or WT groups, student t-test, WT (young: saline n=10, AMPH n=13; aged: saline n=6, AMPH n=7), Sirt3-KO (young: saline n=7, AMPH n=9; aged: saline n=6, AMPH n=8).
6.2.3 Amphetamine treatment has no effect on Sirt3-KO mice in the light/dark paradigm

Another measurement of anxiety and risk-taking behaviour that has also been used with AMPH and other psychostimulants administration is the light/dark (LD) paradigm box (Hascoet & Bourin, 1998). AMPH treatment did not have an effect on the percentage of time spent in the dark compartment (Figures 6.5A, 6.6A) nor the percentage of risk-taking behaviour (Figures 6.5B, 6.6B) in young WT or Sirt3-KO male and female mice relative to their saline-treated control groups. In addition, AMPH did not have a significant effect on aged Sirt3-KO or WT male or female mice relative to their saline-treated counterparts (Figures 6.5A, 6.5B, 6.6A, 6.6B).
Figure 6.5: Amphetamine treatment had no effect on risk taking in Sirt3-KO male mice in the light/dark paradigm. (A-B) Anxiety measurements in WT and Sirt3-KO young and aged male mice in the Light/Dark paradigm. Histogram showing the mean and SEM of Light-Dark preference test measuring (A) time spent in the dark, and (B) the percentage of risk assessment behavior (head poke) relative to time spent in the dark during a 5 minute period. There was no statistical difference between AMPH and saline treatment in the young or aged WT or Sirt3-KO groups on the assessments, student t-test, WT (young: saline n=7, AMPH n=8; aged: saline n=5, AMPH n=5), Sirt3-KO (young: saline n=6, AMPH n=7; aged: saline n=7, AMPH n=8).
Figure 6.6: Amphetamine treatment had no effect on risk taking in Sirt3-KO female mice in the light/dark paradigm. (A-B) Anxiety measurements in WT and Sirt3-KO young and aged female mice in the Light/Dark paradigm. Histogram showing the mean and SEM of Light-Dark preference test measuring (A) time spent in the dark, and (B) the percentage of risk assessment behavior (head poke) relative to time spent in the dark during a 5 minute period. There was no statistical difference between AMPH and saline treatment in the young or aged WT or Sirt3-KO groups on the assessments, student t-test, WT (young: saline n=10, AMPH n=12; aged: saline n=6, AMPH n=7), Sirt3-KO (young: saline n=8, AMPH n=9; aged: saline n=6, AMPH n=7).
6.3 Discussion

In this study, I demonstrate that a single injection of AMPH (1mg/kg) is sufficient to induce an increase in exploration and a decrease in anxiety in both young male and female Sirt3-KO mice. Specifically, young Sirt3-KO mice treated with AMPH showed an increase in activity and distance coverage in the OF and spent more time in the open arms of the EPM. AMPH had no effect on mobility rates in the OF on either Sirt3-KO or WT cohorts, which is consistent with previous findings that speed is unaffected in rats following treatment with AMPH (Nickolson, 1981). The observed hypersensitivity to AMPH in Sirt3-KO mice strengthens the implication that the deficiency in sirtuin-3 may underlie a hyperactive behavioural phenotype.

Furthermore, AMPH treatment showed no difference on risk taking behaviour using the LD paradigm in young female and male Sirt3-KO and WT mice. Because AMPH increases risk-taking behaviour in humans (Zack & Poulos, 2004), the lack of an observed effect was surprising. However, there has been mixed findings in the literature, as some reports showed that AMPH-treated mice were indistinguishable from the saline-treated controls in the LD paradigm (Young & Johnson, 1991; Biala & Kruk, 2007), whereas other studies observed an effect (Hascoet & Bourin, 1998; Flaisher-Grinberg & Einat, 2010). This discrepancy among the different studies may be related to the dose used, route of AMPH administration, time between injection and testing, strain differences, and animal housing conditions.

The lack of an effect of AMPH in the aged group could be due to differences in AMPH sensitization in the aged brain. Specifically, the variable/non-consistent effects observed in the aged group may be eluted to alterations in NT systems (Morgan et al., 1987; Watanabe, 1987; Mattson et al., 2004; Kaiser et al., 2005; Zahr et al., 2008), as well as changes in permeability to AMPH (Sankar et al., 1983) in the aged brain.
Under basal conditions, I found that only female Sirt3-KO mice portray a hyperlocomotive and low anxiety phenotype in the novel environment (Chapter 4), however, treatment with AMPH unmasked a similar phenotype in male Sirt3-KO mice. These results suggest that the predisposition to the hyperactive behaviour in the novel environment is sex-dependent (Explained Section 4.3.6), whereas, following AMPH treatment, hyperactivity is sex-independent.

ROS and inflammatory responses have been linked to underlie dysregulated neurotransmission and elevated neuronal excitability, which may induce a hyperactive phenotype (Vezzani & Viviani, 2015; Lee et al., 2017). Indeed, a growing body of literature now shows that secondary to elevating NT release at the synapses, the neurochemical effects of AMPH can also upregulate both oxidative and pro-inflammatory factors, an elevation of which has been suggested to play a role in AMPH-induced hyperactivity (Kubaro et al., 2002; Brown & Yamamoto, 2003; Fukami et al., 2004; Yamamoto & Bankson, 2005). In line with this, mice deficient in sirtuin-3 display an increase in oxidative stress and inflammation in brain regions that play a role in regulating locomotor and anxiety behaviours (Middleton & Strick, 2000; Shin & Liberzon, 2009), such as cortex (Cheng et al., 2016; Shi et al., 2018; Tyagi et al., 2018), hippocampus (Cheng et al., 2016), striatum (Liu et al., 2015; Shi et al., 2017) and substantia nigra (Shi et al., 2017). Furthermore an increase in oxidative stress is also observed in dopaminergic neurons of Sirt3-KO mice (Liu et al., 2015; Shi et al., 2017), and viral-mediated over-expression of SIRT3 protected dopaminergic neurons from α-synuclein-induced degeneration (Gleave et al., 2017). Because the dopaminergic system is associated with regulating locomotor activity and anxiety processes (Kumakura et al., 2010; Zarrindast & Khakpai 2015), it is therefore conceivable that the absense of sirtuin-3 results in an impairment in dopaminergic neurons, which therefore manifests in the observed AMPH-induced hyperactivity. It is also important to note that the increase in oxidative burden and inflammation was also observed in male animals (Liu et al., 2015; Gleave et al., 2017; Tyagi
et al., 2018). Therefore, it is conceivable that AMPH-administration exacerbates ROS and inflammation in the brain regions and neuronal networks that underlie the observed hyperactive phenotype in both female and male Sirt3-KO mice.

However, given that Sirt3-KO female mice already display a phenotype without AMPH-administration (Chapter 4), one would expect that female Sirt3-KO mice would show more sensitization to the AMPH treatment, however both sexes demonstrated the same magnitude of hyperactivity following AMPH treatment. It is important to note that AMPH influence on behaviour is sex-dependent, as AMPH affects female Sprague-Dawley rats faster and to a greater degree than that of male rats (Milesi-Hallé et al., 2007). Therefore, one theory is that female Sirt3-KO mice may have shown sensitization to AMPH at an earlier time-point than that of male Sirt3-KO mice. Because my assessments were performed 45 minutes after AMPH administration, the full-time course of effects was not evaluated. It is possible that the critical period occurs outside of the observed window, where a phenotype in female Sirt3-KO mice manifested an earlier time-point than in male Sirt3-KO mice. Thus, assessment of the relationship between the timing onset of effects of AMPH treatment in Sirt3-KO mice and sex is warranted.

Taken together, these results show that Sirt3-KO male and female mice are sensitized to AMPH treatment. Given that AMPH majorly induces its effects through alteration of DA and NE systems, these data suggest that the loss of sirtuin-3 may induce imbalance in catecholaminergic systems and therefore precipitate the observed hyperactive behavioural phenotype. However further assessment of this hypothesis is warranted. Furthermore, it would be of interest to assess the response of Sirt3-KO mice following treatment with a mood stabilizer, such as lithium or valproate which are used to treat hyperactive episodes (Schloesser et al., 2012). Future studies
should examine whether treatment with a mood stabilizer would reverse AMPH-induced hyperactive phenotype in Sirt3-KO mice.
General Discussion and Conclusions

7.1 Summary of principal findings

The principal aim of this thesis was to investigate sirtuin-3 expression in the brain, and the consequences of sirtuin-3-deficiency in vivo at both behavioural and molecular levels. The major findings/discoveries reported in this thesis are:

Chapter 3: A detailed characterization of sirtuin-3 expression in the rat brain revealed that:

1. At the mRNA level, Sirt3 was the third most expressed sirtuin member in the rat brain
2. Sirtuin-3 mRNA and protein were present at equivalent levels in the adult cortex, hippocampus, striatum, spinal cord and brain stem, and was expressed the lowest in the cerebellum
3. Sirtuin-3 mRNA and protein levels in peripheral tissues were variable, however they were present at relatively similar levels in metabolic-demanding tissues such as the brain and heart
4. Sirt3 mRNA expression levels showed no significant changes between E18 until 24 months of age in the cortex, hippocampus, and cerebellum
5. SIRT3 protein prevalence increased between PN7 and PN21 and then remained consistent at the 24-months in the cortex and hippocampus. In the cerebellum, SIRT3 protein prevalence was similar between E18 to 3 months, but then increased significantly at 24 months
6. Sirtuin-3 mRNA and protein were expressed in both neurons and astrocytes, however its prevalence was higher in neurons at both the mRNA and protein levels
Chapter 4: Assessment of WT and Sirt3-KO male and female mice revealed that:

1. During adulthood, SIRT3 protein expression in the male brain was higher than in the female brain.

2. Under normal housing conditions, sex and age matched Sirt3-KO mice are phenotypically similar to WT mice, displaying no overt behavioural phenotype, sudden death or change in body weight compared to their respective WT counterparts.

3. Telemetry assessments in male and female Sirt3-KO mice showed no difference in gross activity, temperature regulation and delta wave/activity patterns under basal home-cage environment.

4. Overt phenotypes observed in Sirt3-KO female mice were:
   a. Poor motor learning on the rotarod and rod traversions assessments
   b. Exploratory behaviour in the open field test
   c. Attenuated anxiety in the light/dark place preference and elevated plus maze tests
   d. Age-progressive exacerbation of all above mentioned phenotypes

5. Overt phenotypes observed in Sirt3-KO male mice were:
   a. Poor motor learning on the rod traversions assessment

6. Sirt3-deficiency has no demonstrable effect on object recognition memory in male or female mice.

Chapter 5: Assessment of the mTORC1 pathway in male and female Sirt3-KO mice revealed that:

1. The mTORC1 upstream signaling factor Akt1 was hyper-phosphorylated on Serine-473 in Sirt3-KO female cortical lysates under basal conditions.

2. The mTORC1 negative regulator GSK-3β displayed hyper-phosphorylation on Serine-9 in Sirt3-KO female cortical lysates under basal conditions.

3. The mTORC1 downstream target protein p70-S6 kinase displayed hyper-phosphorylation on Threonine-389 in Sirt3-KO female cortical lysates under basal conditions.
4. The mTORC1 downstream target protein ribosomal protein S6 displayed hyper-phosphorylation on Serine-240/244 in Sirt3-KO female cortical lysates under basal conditions

5. No changes in either the prevalence or phosphorylation state of upstream factors: GSK-3β (Serine-9) and Akt1 (Ser-473), or downstream factors: p70-S6 (Threonine-389) and ribosomal protein S6 (Serine-240/244) in male Sirt3-KO mice under basal conditions

Chapter 6: Assessment of amphetamine administration in female and male Sirt3-KO mice revealed that:

1. Both male and female Sirt3-KO mice showed behavioural sensitization to amphetamine treatment in the open field and elevated plus maze tests, but not in light/dark place preference test

2. Amphetamine administration had no differential effects on behavior in aged Sirt3-KO or WT mice

7.2 General Discussion

Although neurodegenerative and neuropsychological diseases manifest with different clinical outcomes, their pathophysiology often converges at the level of mitochondrial dysfunction (Martin, 2012; Morris & Berk, 2015). Thus, understanding the common cellular mechanisms underlying the progression of neurological and neuropsychiatric conditions is critical in treatment development. The present study contributes to the growing body of evidence suggesting that deregulated mitochondrial activity plays an important role in neuronal functioning, as sirtuin-3-deficiency in vivo is sufficient to alter normal brain homeostasis observed at both behavioural and molecular levels.

In concordance with previous studies (Lombard et al., 2007; Liu et al., 2015), Sirt3-KO mice were indistinguishable from their WT counterparts in their familiar homecage environment. However,
when confronted with a mild stressor, in this case a novel environment, Sirt3-KO female mice showed decreased anxiety, increased risk-taking, and increased locomotive phenotypes – all of which indicate that Sirt3-KO female mice show a curious, exploratory and mild hyperactive phenotype that is driven by exposure to novel environments. A phenotype was also observed following energy-demanding tasks, as Sirt3-KO female mice showed impaired motor learning on the rotarod task, and both male and female Sirt3-KO mice performed poorly on the traversal task.

Chapter 3 results show that sirtuin-3 is expressed ubiquitously across multiple brain regions that are involved in regulating both emotional and motor-associated behaviours (Middleton & Strick, 2000; Steimer, 2002; Shin & Liberzon, 2010). Thus, sirtuin-3 deficiency may therefore alter the mitochondrial homeostasis in these regions resulting in the hyperactive behaviour that manifests in a context-specific manner (Chapter 4). Indeed, alterations in mitochondrial homeostasis can be associated with abnormal brain function, which can have an effect on stress, mood state, as well as motivation in novel and energy-demanding tasks (Marazziti et al., 2012; Allen et al., 2018; Picard & McEwen, 2018). Because sirtuin-3 deficiency results in an increase in oxidative stress and reduced systemic and cerebral anti-oxidant status (Sundaresan et al., 2009; Bell & Guarente, 2011; Pillai et al., 2016; Shi et al., 2017), this may create a permissive phenotype that manifests only in stressful, energy-demanding and novel environments (Nabeshima & Kim, 2013; Sharma, 2016; Howes et al., 2017). Therefore, placing an energetic burden on an already metabolically compromised system may explain the context-dependent association between sirtuin-3 deficiency and the appearance of the observed behavioural consequences.

Having demonstrated in Chapter 3 that sirtuin-3 is ubiquitously expressed throughout different brain regions, enriched in both neurons and astrocytes, and is expressed throughout maturation as
well as in the aged rat brain, it is conceivable that this enzyme plays a global role in healthy aging in mammals. Likewise, while attenuated anxiety was observed in both young and aged Sirt3-KO female mice, as shown by the light/dark and plus maze paradigms, enhanced locomotion in the open field did not become apparent until the animals were at least 18 months old. In addition, the impaired motor learning that was observed on the rotarod task, was further exacerbated in aged Sirt3-KO female mice. Therefore, sirtuin-3 deficiency may result in cumulative oxidative-damage and chronic mitochondrial dysfunction over time, which collectively may underlie the progressive behavioural phenotype observed in aged Sirt3-KO female mice. Thus, these data show that sirtuin-3-deficiency in mice results in progressive, age-related behavioral deficits, which implicates this enzyme as a mediator of brain function throughout aging. Indeed, my results are in concordance with other studies that suggest that sirtuin-3 plays a role in aging (Hirschey et al., 2011; Kincaid & Bossy-Wetzel, 2013), neurodegenerative diseases (Anamika et al., 2017; Outeiro et al., 2008; Salvatori et al., 2017), and is reported to affect human lifespan (Rose et al., 2003; Bellizzi et al., 2005; Hirschey et al., 2011; Denu, 2017).

As discussed in Chapter 6, sirtuin-3 deficiency may predispose both sexes to mTORC1 activation and subsequent hyperactive phenotype through elevation of oxidative stress and inflammation, however, females may be more susceptible due to an additive effect of hormones. Having observed an overt behavioural and molecular phenotype under basal conditions only in female Sirt3-KO mice, it is tempting to speculate that sex hormones may directly or indirectly influence this observed phenotype. As discussed in Section 4.3.7, estrogen plays a predominant role in the sex-specific neural circuit development, behaviour and sexual differentiation of mammalian brain (McEwen, 2012). Additionally, estrogen has been demonstrated to affect non-reproductive behaviours, such as anxiety and activity levels (Morgan & Pfaff, 2001; McEwen, 2012). Indeed, accumulating evidence has shown that estrogen can even protect the brain from a host of
neurodegenerative factors and that estrogen downregulation is observed in many neurodegenerative and neuropsychiatric conditions (Gandy, 2003; Klinge, 2008). Estrogen-mediated beneficial effects on mitochondrial function and general metabolism are now well recognized and given that mitochondria can also regulate estrogen biosynthesis (Velarde et al., 2014), it is possible that cross-talk between estrogen and mitochondria is important for maintaining cellular homeostasis. Intriguingly, the promoter region of the Sirt3 gene in mice houses an estrogen receptor binding motif (Kong et al., 2010), and estrogen-mediated effects on Sirt3 gene expression have been observed to indicate the motif is physiologically active. Therefore, the actions of estrogen on mitochondria are likely to involve at least some level of Sirt3 regulation. Given the wide spectrum of mitochondrial proteins governed normally by sirtuin-3 activity (Section 1.8.1), it is conceivable that sirtuin-3-deficiency in mice would therefore disrupt the co-regulation of mitochondria by both sirtuin-3 and estrogen, which may promote further mitochondrial dysfunction and oxidative stress. It is therefore would be of interest to assess whether Sirt3-KO female mice have higher indices of oxidative stress when compared to their Sirt3-KO male counterparts. If that proves to be the case, it would suggest that mitochondrial dysfunction in Sirt3-KO female mice would be exacerbated, which then would result in alteration in normal neuronal circuit activity and therefore manifest in the observed behavioural outcomes. In this context male mice would be less affected, as estrogens can be generated from androgens in the male brain, and thus the circuit homeostasis would not be as affected (Miller & Auchus, 2011). Furthermore, a decline in estrogen levels in the aged female brain could contribute to metabolic alterations and therefore explain why we see a progressive, age-dependent phenotype in Sirt3-KO female mice (Section 4.2). Such a model would comport with the lowered levels of estrogen that have been reported in many female patients displaying neuropsychiatric and neurodegenerative conditions (Kulkarni et al., 2008). Further studies will be required to test the merits of this hypothesis, however it is also important to keep in mind that there may be other
possibilities for the observed sex-specific phenotype in Sirt3-KO mice, as a simple PubMed search of “brain differences between males and females” generated ~57,000 publications. For example, neuroendocrine responses within the hypothalamic-pituitary axis (Schroeder et al., 2018) and genetic and epigenetic influences (Ratnu et al., 2017; Hamada & Matthews, 2018; Constantinof et al., 2019) can also play a role. Thus, it is clear that there are a plethora of theories and ongoing debates on various topics pertaining to sex-specific dimorphism, which should be carefully considered for future assessments in the Sirt3-KO model. Furthermore, the observed phenotype in Sirt3-KO female mice is interesting, as numerous studies have demonstrated that the presence of some neuropsychiatric (Seney & Sibille, 2014; Thibaut, 2016) and neurological (Attarian et al., 2015; Xing et al., 2015; Zagni et al., 2016) conditions is more prevalent in females than that of males. In summary, I have identified a quantifiable set of differences between males and females as a consequence of the disruption of a single autosomal gene. This illustrates that there are sex-differences in circumstances where sirtuin-3 expression would be impaired, which provides a potential mechanism that should be explored further in conditions where differences in males and females have been noted.

Furthermore, it is worth noting that novelty-driven hyperactive behaviour is highly prevalent in a number of conditions, as it is frequently associated with, but not limited to, bipolar disease (BD) (Hilty et al., 2006), schizophrenia (SZ) (Jones et al., 2011; Ramshaw et al., 2013), attention deficit hyperactivity disorder (ADHD) (Sharma & Couture, 2014), Alzheimer disease (AD) (Webster et al., 2014) and fragile X syndrome (Einfeld et al., 1991). To further strengthen the correlation between sirtuin-3 deficiency and novelty-driven hyperactive phenotype, I assessed whether Sirt3-KO mice will mimic behavioural symptoms of hyperactivity as a response to psychostimulants (Chapter 6). Following an amphetamine (AMPH) treatment, a predictive pharmacological model of hyperactive-like behaviour (Borison et al., 1978; Gould et al., 2001; Rothman et al., 2001),
unmasked a phenotype in both male and female Sirt3-KO mice. Specifically, both male and female Sirt3-KO mice displayed hyperlocomotion in the open field as well as attenuated anxiety in the plus maze paradigm, whereas WT mice of both sexes did now show sensitization to the AMPH treatment. As discussed in Section 6.3, AMPH may promote the observed behaviour in Sirt3-KO mice through the induction of catecholamine neurotransmitter (NT) overactivity at the synapses in both sexes. Indeed, enhanced catecholamine release has been demonstrated to underlie both hyperlocomotion in novel environments and lowered anxiety (Fowler et al., 2002), and deregulation in release in these NTs is associated with a number of neuropsychiatric and neurological disorders. Therefore, future assessments of the NT systems in Sirt3-KO mice are warranted.

While a number of studies have assessed the role of Sirtuin-3 in neurodegenerative conditions (Section 1.8.3; Table 2), to date, no studies exist, on the role of SIRT3 in the classical neuropsychiatric disorders. My results show that Sirt3-KO female mice recapitulate behavioural and molecular abnormalities which may be correlates of more than one neuropsychiatric condition. In order for Sirt3-KO mice to be considered a useful animal model for a specific condition, it must meet three validation criteria (Chadman et al., 2009): (1) face validity, it must display behavioural characteristics of a human disorder; (2) construct validity, it must recapitulate neuropathological hallmarks of the disorder; (3) predictive validity, the model must reliably respond to pharmacological treatments. Because assessments of Sirt3-KO mice are still in their infancy, the Sirt3-KO mouse model was not fitted to a specific disorder, rather, the results from this thesis were correlated to some of the key endophenotypes common to a number of conditions. The summary of sirtuin-3-consequences for face, construct and predictive validity in different neuropsychiatric conditions is briefly outlined in Table 7.1. As one can see from this table, many of the behavioural and molecular phenotypes observed in Sirt3-KO mice overlap with the
phenotypic spectra of more than one psychiatric condition. Indeed, although mental illnesses are distinctly outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM), a growing body of evidence shows that many of them share common underlying genetic, molecular and behavioural phenotypes with neurodegenerative conditions. Therefore, the Sirt3-KO mouse model may recapitulate some of the cardinal features of both neuropsychological and neurodegenerative disorders, which will contribute to a basic understanding of the pathophysiology of these conditions.

One common feature that is implicated as a point of convergence for many neurological and neuropsychological conditions is aberrant signaling of the mechanistic target of rapamycin complex 1 (mTORC1)(Hoeffer et al., 2010; Costa-Mattioli & Monteggia, 2013). As discussed in Chapter 5, several lines of evidence have now shown that an upregulation of mTORC1 results in overactive cellular networks, cellular protein overload, and depletion of energy stores, all of which have been observed in a number of neurological and neuropsychological conditions. Indeed, the importance of regulation of the mTORC1 pathway in such conditions is reflected in ongoing studies assessing mTORC1 inhibitors (Ryskalin et al., 2018). However, there are still considerable concern of the adverse consequences and unwanted side-effects of current available mTORC1 inhibitors (Pallet & Legendre, 2013). Thus, a search for a target that if activated or inhibited would attenuate mTORC1 pathway and at the same time incur minimal side effects is warranted. I believe that sirtuin-3 may be that target, as my and other studies have demonstrated that the absence of sirtuin-3 results in an overactive mTORC1 signaling cascades (Sundaresan et al., 2009; Gonzalez Herrera et al., 2018), whereas sirtuin-3 over-expression results in an attenuation of mTORC1 signaling (Dai et al., 2017; Zhao et al., 2018), but most importantly sirtuin-3 over-expression in vivo does not have negative complications (Ramachandran et al., 2017; Gleave et al., 2017). Thus, future studies should assess whether upregulating activity/levels
of sirtuin-3 would attenuate aberrant mTORC1 signalling and therefore improve neuronal and behavioural impairments associated with a number of neuropsychological and neurological conditions. In addition, because upregulation of the mTORC1 pathway and hyperactive phenotype was only observed in Sirt3-KO female mice, these results provides a rationale for future studies to assess whether polymorphisms in the sirtuin-3 gene contributes to susceptibility to neurological and neuropsychological conditions in women.
Table 7.1: Validity for Sirt3-KO Mouse as a Model for Psychiatric Disorders

<table>
<thead>
<tr>
<th>Validity</th>
<th>Phenotype</th>
<th>Examples of Psychiatric Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Face Validity</strong></td>
<td><em>Decreased anxiety</em></td>
<td>BD (manic phase), ADHD (Leo &amp; Gainetdinov, 2016), SZ (Jones et al., 2011)</td>
</tr>
<tr>
<td></td>
<td><em>Increased hyperactivity: increased exploration, increased locomotion</em></td>
<td>BD (manic phase), (Minassian et al., 2010), ADHD, (Leo &amp; Gainetdinov, 2013), SZ (Jaaro-Peled, 2009)</td>
</tr>
<tr>
<td></td>
<td><em>Impaired motor learning</em></td>
<td>ADHD, (Kaiser et al., 2015), BD (Lohr &amp; Caligiuri, 2006), SZ (Schwartz et al., 1996)</td>
</tr>
<tr>
<td></td>
<td><em>No change in circadian rhythm under normal conditions, disrupted circadian rhythm following forced wakefulness</em></td>
<td>ADHD, (Hvolby, 2015), BD (Gold &amp; Sylvia, 2016), SZ (Wulff et al., 2012)</td>
</tr>
<tr>
<td><strong>Constructive Validity</strong></td>
<td><em>Increased mTORC1 signaling</em> (Sundaresan et al., 2009)</td>
<td>ADHD (S. H. Kim et al., 2013), SZ (J. Y. Kim et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Increased ROS (Bell &amp; Guarente, 2011)</td>
<td>BD, (Yumru et al., 2009), ADHD, (Sezen et al., 2016), SZ (Kunz et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Inflammation (Zhang et al., 2018) (Kim et al., 2018)</td>
<td>BD, (Rosenblat &amp; McIntyre, 2017), ADHD, (Leffà et al., 2018), SZ (Müller et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Calcium dysregulation (Dai et al., 2014)</td>
<td>BD, ADHD, (D’Onofrio et al., 2017), SZ (Berridge, 2014)</td>
</tr>
<tr>
<td></td>
<td>ETC dysfunction (Ahn et al., 2008; Cheng et al., 2016)</td>
<td>BD, (Andreazza et al., 2010), ADHD (Verma et al., 2016), SZ (Ben-Shachar, 2017)</td>
</tr>
<tr>
<td></td>
<td>Decreased ATP levels (Ahn et al., 2008)</td>
<td>BD (Rosenblat &amp; McIntyre, 2017), ADHD (Kato, 2005), SZ (Deicken et al., 1995)</td>
</tr>
<tr>
<td><strong>Predictive Validity</strong></td>
<td><em>hypersensitivity to amphetamines</em></td>
<td>BD (manic phase) (Mamelak, 1978), ADHD (Majdak et al., 2016), SZ (Featherstone et al., 2007)</td>
</tr>
</tbody>
</table>

*Asterisks denote work done in this Thesis*
7.3 Molecular Working Model

By combining the results presented from this thesis with the evidence in current literature, I propose the following working model on the consequences of sirtuin-3 deficiency in vivo. (Figure 7.1). This model describes the effects of sirtuin-3 deficiency in the brain under normal conditions and following AMPH administration. My results show that under basal conditions, Sirt3-KO female mice show an increase in phosphorylation of GSK-3β (Serine-9) and Akt1 (Ser-473) factors, which regulate a number of cellular functions, including mTORC1 activity-level. Furthermore, downstream targets of mTORC1, p70-S6K (Threonine-389) and ribosomal protein S6 (Serine-240/244), which are factors involved in translation initiation (Fonseca et al., 2014), are activated. Protein synthesis activity is linked with overactive signaling cascades at the synapses, which has also been observed in context-dependent hyperactive behaviour (Topol et al., 2015; Jacquemont, 2018). Sirtuin-3 deficiency results in an increase in oxidative stress (Sundaresan et al., 2009; Bell & Guarente, 2011; Pillai et al., 2016; Shi et al., 2017), which may activate Ras and therefore activate mTORC1 through the Akt1/GSK-3β pathway (Sawyer et al., 2002; Kuster et al., 2005; Mendoza et al., 2011; Lipton & Sahin, 2014). In addition, ROS can also activate Akt1 signaling (Guha et al., 2010; Chetram et al., 2013). Autophagy, a process where old misfolded proteins are degraded, and is important for maintaining proper function of cells in all organs, including the brain (Bento et al., 2016). Inhibition of autophagy, which therefore impedes the clearance of misfolded, dysfunctional proteins, that have accumulated within the cell overtime, would result in altered cellular homeostasis. Compromised autophagy also results in a decrease of pre-synaptic vesicle degradation and, as a consequence leads to overflow of NTs resulting in overactive neuronal signaling at the synaptic cleft (Hernandez et al., 2012). Indeed, such deregulation at the synapses is observed in a number of neurodegenerative and neuropsychiatric diseases, and interestingly, a decrease in autophagy is also observed in models of increased locomotion, decreased anxiety and hyperactivity.
(Hernandez et al., 2012; Sarter et al., 2007). Thus, as discussed in Section 5.4.1, because overactive mTORC1 attenuates autophagy (Jung et al., 2010), it is conceivable that mTORC1 hyperactivity in Sirt3-KO female mice may result in an attenuation of autophagy. Thus, an increase in mTORC1 in Sirt3-KO female mice may inhibit autophagy, which would result in an increase in NT neurotransmission at the synapses and lead to the observed context-specific hyperactive phenotype. AMPH administration results in a hyperactive phenotype in both male and female Sirt3-KO mice but not WT mice, suggesting that the catecholamine system may be altered in Sirt3-KO mice (Kahlig et al., 2005). Because cross-talk between estrogen and mitochondria is important for maintaining cellular homeostasis, thus, it is possible that sirtuin-3-deficiency may result in the disruption of the crosstalk between mitochondrial function and estrogen action, which may alter neuronal homeostasis in these regions and therefore may explain why female Sirt3-KO mice display an overt phenotype.
**Figure 7.1: Proposed Working Model for how Sirtuin-3 Deficiency results in a Hyperactive Phenotype.** Sirtuin-3 deficiency in mitochondria results in an increase in systemic ROS levels and subsequent Ras activation. Ras then indirectly induces Akt1 phosphorylation through activating downstream effector proteins. ROS also directly activates Akt1. Activated Akt1 then phosphorylates and deactivates GSK-3β which then lifts off its inhibitory effect on mTORC1. Activated mTORC1 increases the phosphorylation of its downstream effector proteins S6K and S6, which then increase protein translation and ultimately inducing context-specific hyperactive phenotype. Increased mTORC1 signaling results in attenuated autophagy, therefore may cause synaptic overactivation promoting a hyperactive phenotype. AMPH also induces a hyperactive phenotype, possibly through the overactive catecholamine NT system. Sirtuin-3 deficiency in mitochondria may attenuate estrogen-mitochondria cross-talk, which then induces further mitochondrial dysfunction. AMPH, Amphetamine; GSK-3β, Glycogen synthase kinase-3β; NT, Neurotransmitters; ROS, Reactive oxygen species.
### 7.4 Future perspectives

The present study suggests that Sirt3-KO mice possess behavioural and molecular manifestations that share some commonalities with neuropsychiatric and neurodegenerative disorders; an interesting observation as these results open the possibility for further examination of sirtuin-3 signaling in these conditions. To further elucidate the usefulness of this model in neurological and neuropsychological disorders, there are various aspects at molecular and behavioural levels that need to be addressed.

I observed an increase in mTORC1 signaling pathway in the cortex (Section 5.2). Because activation of mTORC1 proceeds via various signalling pathways, further studies examining the mechanisms underlying the activation of mTORC1 pathway in Sirt3-KO mice are warranted. As an example, Sundaresan (2010) showed an activation of the mTORC1 pathway in cardiomyocytes of Sirt3-KO mice through activation of Ras. It would be interesting to assess whether or not Ras is hyperactivated in the brain in a sex-dependent manner in Sirt3-KO mice. Furthermore, although increased mTORC1 activation in the cortex has been linked to hyperactivity, anxiety, and motor-associated behaviour (Lipton & Sahin, 2014; Ryskalin et al., 2018) it would be useful to assess the mTORC1 signaling network in other relevant brain regions responsible for these behaviours. Specifically, in structures that are part of the limbic system (hippocampus, thalamus, hypothalamus, basal ganglia, cingulate cortex, and amygdala) which are responsible for anxiety and risk-taking behaviours, and in the striatum as it is responsible for voluntary motor control. Previous work has shown that mTORC1 is a critical factor of dendrite development (Di Polo, 2015), and that persistent hyperactivation of the mTORC1 signaling pathway results in an increased morphological complexity of neurons, defined by increased neuronal dendrite and soma size, as well as dendrite complexity (Kumar et al., 2005). In fact, mTORC1 hyperactivation contributes to aberrant circuit formation leading to neural disorganization which has been
implicated in both neurodegenerative and neuropsychiatric diseases (Costa-Mattioli & Monteggia, 2013). Because the mTORC1 signaling pathway is hyperactive in female Sirt3-KO mice (Chapter 5), future studies should assess whether these mice have increased neuronal complexity. Additionally, because Sirt3-KO female mice show a phenotype that is progressive, therefore age-related morphological changes should also be assessed. Given that only Sirt3-KO female mice show an upregulation of mTORC1 pathway, I hypothesize that they would display a more altered neuronal morphology than that of WT mice and Sirt3-KO male mice. Furthermore, because changes in connectivity or coherence between certain brain areas have also been heavily implicated in motor control and anxiety-related phenotypes (Fornito & Harrison, 2012; Iturria-Medina & Evans, 2015), functional neuroimaging and electroencephalography can be used to measure coherence across different brain region in Sirt3-KO mice. These assessments would give us the knowledge whether there are morphological and brain functional activity correlates to the observed behavioural and molecular phenotypes observed in Sirt3-KO female mice.

As discussed above, autophagy may be compromised in Sirt3-KO mice. Future investigations should assess whether autophagy-promoting proteins, such as ULK1, Atg13, and FIP200 (Jung et al., 2010; Bento et al., 2016) are inhibited under basal conditions in Sirt3-KO mice. Because upregulation of autophagy has been the focus of many therapeutic strategies for certain neurodegenerative diseases (Rahman & Rhim, 2017), upregulation of sirtuin-3 levels and activity may be one way to modulate autophagy in such conditions.

In addition, having observed behavioural and molecular consequences in female but not male Sirt3-KO mice, as discussed in Section 4.3.7, the possibility is raised that these phenotypes could stem from estrogen-related effects. Because it has been demonstrated that estrogen supplementation increases anxiety and decreases locomotion in mice (Morgan & Pfaff, 2001), a
study to assess whether estrogen supplementation would reverse the observed hyperlocomotive and lowered anxiety phenotypes in Sirt3-KO mice is warranted. These assessments are of particular importance, as they would demonstrate whether estrogen is involved in regulating behaviour through the sirtuin-3-axis.

Because hyperlocomotion under basal and AMPH-induced conditions is largely driven by limbic and striatal overactive catecholamine system, (Natarajan & Yamamoto, 2011), future studies should assess whether the catecholamine system is affected in Sirt3-KO mice. I hypothesize that pre-treatment with catecholamine antagonists by microinjecting them in limbic-associated and striatal regions prior to a single systemic injection of AMPH would attenuate the locomotion of Sirt3-KO mice to the levels of the saline-treated control group.

My results reveal that Sirt3-KO mice share common characteristics with a number of neuropsychological and neurological disorders. While of considerable interest mechanistically, it is also important to further scrutinize this model to assess whether it is better suited for a specific condition than others. For example, if Sirt3-KO mice also suffer from anhedonia, it may suggest that the Sirt3-KO model may be a better fit a BD model, or if there is a significant reduction in N-methyl-d-aspartate receptors, it may suggest that Sirt3-KO mice are more suited as a SZ model. Other factors such as timing of phenotype development should be considered, for instance, ADHD is a childhood onset disorder with symptoms of hyperactivity manifesting at an early age (Leo & Gainetdinov, 2013), whereas for Sirt3-KO female mice the hyperactive phenotype does not fully develop until the later stages of an animal’s life (Section 4.3.4). Therefore, the Sirt3-KO mouse may be a better suited model for conditions where the hyperactive phenotype develops at a later stage such as in SZ (Jones et al., 2011) and in AD (S. J. Webster, Bachstetter, Nelson, Schmitt, & Van Eldik, 2014). It is important to keep in mind that even if Sirt3-KO mice may not recapitulate
all the key endophenotypes of a particular neurological or neuropsychiatric condition, it can still be useful for understanding the biological basis of a number of diseases.

Finally, although the observed phenotype in Sirt3-KO mice is reminiscent of behaviour that is under the control of the central nervous system, it would be interesting to examine in greater detail the distinct and collective roles that different brain regions void of sirtuin-3 exert towards the observed behavioural alterations. Therefore, mouse models of sirtuin-3 deletions in different brain regions would help us better understand the role that it plays in regulating different circuitries of the brain.

7.5 Significance of the study

I report, for the first time, sex differences in sirtuin-3 expression and the consequences of its deficiency. Collectively these results reveal a sex-specific expression/function of sirtuin-3 and suggest that sirtuin-3 deficiency may differentially regulate molecular pathways in a sex-specific manner, which might contribute to the onset/outcome in a number of diseases that also show sex preference in relative rates. Furthermore, because overlap exists between neuropsychiatric and neurodegenerative diseases, it is possible that they may share a common trigger which results in compromised mitochondria and altered mTORC1 signaling through the sirtuin-3-axis. Thus, Sirt3-KO mice may be an animal model that can expand across different neuropsychiatric and neurodegenerative conditions, and therefore prove to be a valuable experimental tool to dissect overlapping pathophysiological mechanisms. This knowledge may simplify our understanding of the complex biology of mental and neurological illnesses. Furthermore, these results are interesting because, to date, no mitochondrial genetic model of novelty-driven hyperactivity is currently available. Taken together, although there is still much to be learned about the role of sirtuin-3 in the brain, my study shows that sirtuin-3 may represent a new therapeutic target in
neuropsychiatric and neurodegenerative conditions and further support a rationale for sex-specific diagnosis in mitochondrial disease.
References or Bibliography


Deslandes, A. (2013). The biological clock keeps ticking, but exercise may turn it back. *Arg Neuropsiquiatr, 71*(2), 113-118


Di Polo, A. (2015). Dendrite pathology and neurodegeneration: focus on mTOR. Neural Regen Res, 10(4), 559-561. doi:10.4103/1673-5374.155421


Fischer, M., Skowron, M., & Berthold, F. (2005). Reliable Transcript Quantification by Real-Time Reverse Transcriptase-Polymerase Chain Reaction in Primary Neuroblastoma Using Normalization to Averaged Expression Levels of the Control Genes HPRT1 and SDHA. *J Mol Diagn, 7*(1), 89-96


Kaddour-Djebbar, I., Choudhary, V., Brooks, C., Ghazaly, T., Lakshmikanthan, V., Dong, Z., & Kumar, M. V. (2010). Specific mitochondrial calcium overload induces mitochondrial fission in prostate cancer cells. *Int J Oncol, 36*(6), 1437-1444


Yu, W., Dittenhafer-Reed, K. E., & Denu, J. M. (2012). SIRT3 protein deacetylates isocitrate dehydrogenase 2 (IDH2) and regulates mitochondrial redox status. J Biol Chem, 287(17), 14078-14086. doi:10.1074/jbc.M111.355206


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Text in Chapter 3, Figures 3.1-3.17 & Tables 3.1-3.15


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