Oligodendrocytes of the adult hypothalamic median eminence

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DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared here and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other university or similar institution except as declared here and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other university or similar institution except as declared here and specified in the text. It does not exceed the prescribed word limit set by the Degree Committee for the Faculties of Clinical Medicine and Veterinary Medicine.

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Sara Kohnke

The median eminence (ME) of the hypothalamus is a dynamic structure able to rapidly respond to nutrient availability. Both immature oligodendrocyte precursor cells (OPCs) and mature myelinating oligodendrocytes (MOLs) are found in this region in adults. While proliferation of ME OPCs has been shown to be involved in bodyweight maintenance, not much is known about the functions of other subtypes of oligodendrocytes (OLs) in the region and how they might be involved in the ME response to food intake.

I first outline the use of single-cell RNA sequencing, single-molecule fluorescence in situ hybridization (FISH), and tissue clearing to characterize 3 subtypes of the OL lineage found in the ME: OPCs, MOLs, and an intermediate population designated ‘newly formed oligodendrocytes’ (NFOLs). I describe the molecular signatures of these cells and their unique organization within the ME.

I then detail the transcriptomic changes in these cells between the fasted and refeeding state: genes and pathways related to OL differentiation and myelination are upregulated with refeeding. These transcriptional changes translate to a rapid increase in OPC differentiation into NFOLs with refeeding, as shown by FISH, BrdU labelling, and OL-specific antibody labelling.

I explore the possible role of the mammalian target of rapamycin (mTOR) signalling pathway in translating increased energy availability to increased differentiation. I show mTOR signalling is transcriptionally regulated by refeeding, that mTOR is highly active specifically in OLs of the ME, and that certain nutritional stimuli can alter activation of the mTOR protein in OLs.

Finally, I discuss the development of mouse models to ultimately study the effects of OPC differentiation in the control of food intake and body weight. These tools will allow specific targeting of ME OLs.

These findings characterize a lesser-known population of OLs and provide evidence that these cells are nutritionally responsive.
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It has been an absolute pleasure to work with Clemence Blouet for the last 4 years. Not only has she provided regular support and pushed me to improve, she has also allowed me to be independent. Clemence is truly dedicated to and passionate about her work, and it has been exciting to share successes with her along the course of the PhD. I will be forever grateful for the opportunity to train and grow as a scientist in her lab.

I have also greatly enjoyed my time training in Giles Yeo’s lab. It was through working with him that I discovered my interest in coding and analyzing large datasets. I am orienting my career search around being able to use these skills.

It is without hyperbole that I say my PhD work would not have been nearly as successful had I not had the continual support of my husband, Simon Gfeller. He has helped improve every presentation I have given over the last 4 years, provided unending moral support, and championed the study of oligodendrocytes as well as if he were researching them himself!

The love and encouragement of my family and friends has also been influential in getting me to the end of this degree. They have supported me throughout the PhD, commiserating difficulties and celebrating every success.

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ABBREVIATIONS

3V – third ventricle
4V – fourth ventricle
AA – amino acid
AgRP – agouti-related peptide
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP – area postrema
APC – adenomatous polyposis coli
ARC/AN – arcuate nucleus of the hypothalamus
BBB – blood-brain barrier
Bcan – brevican
Bral1 – brain link protein 1
BrdU – 5-bromo-2’-deoxyuridine/bromodeoxyuridine
BSA – bovine serum albumin
C – Celsius
CART – cocaine- and amphetamine-regulated transcript
CC – corpus callosum
CCK – cholecystokinin
ChR2 – channelrhodopsin-2
CNTF – ciliary neurotrophic factor
Cnp – 2’,3’-cyclic-nucleotide 3’-phosphodiesterase
CNS – central nervous system
CR – calorie restriction
CRH – corticotrophin-releasing hormone
CSF – cerebrospinal fluid
CUBIC – Clear, Unobstructed Brain/Body Imaging Cocktails
CVO – circumventricular organ
CW – complex wheel
Abbreviations

DAB – 3,3’-diaminobenzidine
DAPI – 4’,6-diamidino-2-phenylindole
DEG – differentially expressed gene
DIO – diet-induced obesity
DREADD – designer receptor exclusively activated by designer drug
E – embryonic day
EAE – experimental autoimmune encephalomyelitis
EdU – 5-ethynyl-2’-deoxyuridine
FACS – fluorescence-activated cell sorting
FDR – false discovery rate
FFA – free fatty acid
FGF21 – fibroblast growth factor 21
FISH – fluorescence in situ RNA hybridization
fl – floxed
GABA – γ-aminobutyric acid
GFP – green fluorescent protein
GI – gastrointestinal
GLM – generalized linear model
GLP1 – glucagon-like peptide 1
GLUT1 – glutamate transporter 1
GPCR – G-protein-coupled receptor
HA – hyaluronan
HFD – high-fat diet
HIF – hypoxia-inducible factor
HPD – high-protein diet
HRP – horseradish peroxidase
icv – intracerebroventricular
IF – intermittent fasting
IGF1 – insulin-like growth factor 1
IHC – immunohistochemistry
ip – intraperitoneal
IPA – Ingenuity Pathway Analysis
ISH – in situ hybridization
Abbreviations

iv – intravenous
JAM2 – junction adhesion molecule 2
KO – knockout
lepR – leptin receptor(-expressing)
LFD – low-fat diet
LPD – low-protein diet
log₂FC – log₂-fold change
LV – lateral ventricle
MBP – myelin basic protein
MCT1/2 – monocarboxylate transporter 1/2
ME – median eminence of the hypothalamus
MOL – mature oligodendrocyte
MRI – magnetic resonance imaging
mSIN1 – mammalian stress-activated protein kinase interacting protein 1
mTOR – mammalian target of rapamycin
mTORC1 – mammalian target of rapamycin complex 1
mTORC2 – mammalian target of rapamycin complex 2
Myrf – myelin regulatory factor
Nav – sodium channels
NeuN – neuronal nuclei
NDS – normal donkey serum
NG2 – neural/glial antigen 2
NGS – normal goat serum
NFOL – newly formed oligodendrocyte
NMDAR – N-methyl-D-aspartate (glutamate) receptor
NPY – neuropeptide Y
NTS – nucleus of the solitary tract
OCT – optimal cutting temperature
OL – oligodendrocyte
OPC – oligodendrocyte precursor cell
PBS – phosphate buffered saline
PBST – PBS + Triton-X 100
Pcan – phosphacan
PDGFRα – platelet-derived growth factor α
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<tr>
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<th>Description</th>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
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<tr>
<td>pmTOR</td>
<td>phosphorylated mammalian target of rapamycin</td>
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<tr>
<td>PNN</td>
<td>perineuronal net</td>
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<td>POMC</td>
<td>pro-opiomelanocortin</td>
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<td>PRAS40</td>
<td>proline-rich Akt substrate of 40 kilodaltons</td>
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<td>protor</td>
<td>protein observed with rictor</td>
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<td>pS6</td>
<td>phosphorylated S6 protein</td>
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<td>pS6K1</td>
<td>phosphorylated S6 kinase 1</td>
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<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
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<td>ROI</td>
<td>region of interest</td>
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<td>RT</td>
<td>room temperature</td>
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<td>S6K1</td>
<td>S6 kinase 1</td>
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<td>scRNAseq</td>
<td>single-cell RNA sequencing</td>
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<td>SFO</td>
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<td>TGFβ</td>
<td>transforming growth factor β</td>
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<td>tuberoinfundibular dopamine</td>
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<td>Tnr</td>
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<td>TRH</td>
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<td>TSA</td>
<td>tyramide signal amplification</td>
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<td>TSO</td>
<td>template switching oligos</td>
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<td>tSNE</td>
<td>t-distributed stochastic neighbor embedding</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UMI</td>
<td>unique molecular identifier</td>
</tr>
<tr>
<td>V2</td>
<td>veriscan V2</td>
</tr>
<tr>
<td>VEGFA</td>
<td>vascular endothelial growth factor A</td>
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<td>VLMC</td>
<td>vascular and leptomeningeal cell</td>
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<td>VOLT</td>
<td>vascular organ of the lamina terminalis</td>
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<td>WFA</td>
<td>Wisteria floribunda agglutinin</td>
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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE HOMEOSTAT MODEL

In 1948, William Ross Ashby created a mechanical device that came to be known as a ‘homeostat.’ Made from spare military parts, the machine was capable of maintaining the position of vanes located on top of it. When the positions of the vanes were perturbed, the device adjusted other components within the system to return the vanes to their original position. The homeostat was one of the first machines able to regain a state of equilibrium after perturbation. In his book *Design for a brain*, Ashby describes the concept (which he called an ‘ultrastable system’) exemplified by the machine and how the model may also be applied to biological organisms (Figure 1.1). In the biological model, the organism is separate from the environment, but is able to interact directly with the environment (component R). When the environment changes, a sensor within the organism (D) detects whether the environmental change is outside of the normal range and causes changes in S, a set of parameters that alters how R interacts with the environment. The model also contains a mechanism by which the environment may interact directly with R, bypassing the loop. The components of this model are purely theoretical, but one could imagine how the biological process of maintaining energy homeostasis could fit within this framework.

Saving detailed background information for the introductions to chapters 3-6, here I will describe an example of neural control of energy homeostasis/food intake behavior in healthy animals using the homeostat model. I will then describe the study of different cell types involved in maintaining homeostasis, highlighting the lack of knowledge of the contribution of oligodendrocytes. Finally, I will state the overarching aim of the thesis.
Figure 1.1 The homeostat model
Adapted from Ashby, 1960. A conceptual model of a feedback loop between an organism and its environment to maintain homeostasis. When the environment changes, the organism senses the change and initiates an adaptive response to return to homeostasis. D = the component of the organism that senses changes in the environment, S = a set of parameters that affect R, R = the component of the organism that interacts directly with the environment.

1.2 MAPPING ENERGY HOMEOSTASIS MECHANISMS TO THE HOMEOSTAT MODEL

‘Energy homeostasis’ is the general term for the process that animals use to maintain bodyweight over time despite periods of dietary nutrient abundance or deficit (reviewed in Morton et al., 2014; Schwartz et al., 2000). When energy availability is altered, signals of this change reach sensor cells within the brain, which then alter signalling to centers of the brain responsible for behavior such as food seeking or food intake cessation. Here I will describe how specific biological mechanisms of energy homeostasis maintenance correspond to components of Ashby’s homeostat model (Figure 1.2a).

1 While it is true that energy expenditure is also a significant mechanism for maintaining energy balance, study has historically focused on neural control of food intake behavior as the main contributor to this homeostasis (Keesey and Powley, 2008). The role of food intake behavior in maintaining energy homeostasis will be the focus of this thesis as well.
1.2.1 Environment component

In an energy homeostasis system, Ashby’s environment component could represent an animal’s food availability. After periods of food deficit or abundance (perturbations to the environment), healthy animals are normally able to return their weight to baseline through changes in food intake. Historic studies showed that after force feeding of a high-calorie diet and significant weight gain, rats subsequently decreased their *ad libitum* food intake and eventually came to match the weights of control animals (Cohn and Joseph, 1962) (Figure 1.2b). Conversely, when rats were fasted and lost a significant amount of weight, animals increased food intake when it was available and returned to a baseline weight (Levitsky, 1970) (Figure 1.2c).

1.2.2 Energy availability signals

The signals entering the organism from the environment in Ashby’s model could equate to energy availability signals entering the brain of an animal. In healthy animals, signals of energy abundance or deficit enter the brain via various mechanisms. Signals of energy availability include, but are not limited to: 1) hormones that enter the circulation from adipose tissue and the pancreas (leptin and insulin, respectively), 2) stimulation of vagus and spinal nerves from gut-derived molecules (glucagon-like peptide 1 [GLP1] and cholecystokinin [CCK]) or stretch-receptor activation in the gastrointestinal (GI) tract, and 3) circulating nutrient substrates derived directly from food (such as glucose and free fatty acids [FFAs]). Ghrelin, produced by the stomach, is a signal of energy deficit (reviewed in Marx, 2003; Morton et al., 2014) (Figure 1.2d).

1.2.3 Component D (sensor)

Two well-studied populations of neurons in the brain can be considered akin to component D of the homeostat: the anorectic pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons and the orexigenic agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons. The cell bodies of these neurons are located in the arcuate nucleus of the hypothalamus (ARC). The adjacent median eminence of the hypothalamus (ME) acts as the gateway to the ARC. It lies outside of the blood-brain barrier (BBB) and thus is directly exposed to circulating signals of energy availability or energy deficit (Marx, 2003; Morton et al., 2014; Yin and Gore, 2010). It is thought that ARC neurons extend dendritic processes into the ME to sense these signals (Faouzi et al., 2007; Djogo et al., 2016) (Figure 1.2e).
Importantly, energy availability signals like leptin can activate POMC neurons while inhibiting AgRP neurons (reviewed in Morton et al., 2014).

1.2.4 Component S (parameters affecting R)
Component S could be represented by a well-known connection from the ARC to the brainstem. Both activation of POMC neurons and inhibition of AgRP neurons causes activation of melanocortin-4 receptor-expressing cells of the paraventricular nucleus of the hypothalamus (PVN) (reviewed in Schwartz et al., 2000). Neural connections between the PVN and the brainstem (specifically, the nucleus of the solitary tract, or NTS) have been shown to be important for food intake behavior: PVN oxytocin-releasing neurons that respond to leptin project directly to the NTS, and the anorexigenic effects of leptin require oxytocin signalling (Blevins et al., 2004) (Figure 1.2f).

1.2.5 Component R (environment interface)
The identity of the biological equivalent of component R in energy homeostasis maintenance remains a mystery. However, it has been postulated that the NTS/brainstem directly controls motor activity related to maintaining energy homeostasis (i.e. food seeking behavior or food intake cessation behavior) (Broberger, 2005) (Figure 1.2f). Linking well with the environment-component R interaction shown in Ashby’s model, energy availability signals such as CCK are able to directly affect food intake behavior via stimulation of vagal afferents to the brainstem, without transmission through the hypothalamus (Moran et al., 2001; reviewed in Schwartz, 2006) (Figure 1.2a, f).

From describing how well-known mechanisms of neural control of energy homeostasis/food intake behavior can fit to the homeostat model, it is clear that neurons have received the vast majority of study in this area. Studies conducted over the last decade, however, have made it clear that neurons are not the only cells involved in the neural control of food intake.

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2 The model in Figure 1.2a represents a very basic description of neural mechanisms of maintaining energy balance as a means of explaining the model framework. Other components of the system important for food intake behavior but outside the scope of the thesis (and thus excluded from the model) include sensory perception of food and reward-motivated feeding.
Figure 1.2 Mechanisms of energy homeostasis maintenance

(a) Mechanisms of neural control of food intake mapped onto the homeostat model (b) When allowed to eat ad libitum after force-feeding of a high-calorie diet, rats reduce food intake to lower their bodyweight. Adapted from Cohn and Joseph, 1962, reprinted with permission from YJB.M (c) Animals allowed to eat ad libitum after a 24- or 72-hour fast have a period of increased food intake before their bodyweight returns to the control level. Adapted from Levitsky, 1970, reprinted with permission from Elsevier (d) Energy signals that enter the brain include the hormones leptin and insulin, nutrients like glucose and free-fatty acids (FFAs), and activation of spinal and vagus nerves via stretch receptors or glucagon-like peptide 1 (GLP1) and/or cholecystokinin (CCK). Adapted from Morton et al., 2014, reprinted with permission from Springer Nature (e) Neurons (yellow) located in the arcuate nucleus of the hypothalamus (ARC) extend processes into the median eminence (ME), possibly to sense circulating factors. Adapted from Paxinos and Franklin, 2001, reprinted with permission from Elsevier.
1.3 CONTRIBUTION OF NON-NEURONAL HYPOTHALAMIC CELLS TO MAINTENANCE OF ENERGY HOMEOSTASIS

In the field studying neural control of food intake, glial cells are arguably underrated. While glia comprise around half of all cells in the brain (Herculano-Houzel and Lent, 2005), relatively few studies focus on the roles of glial cells in maintaining energy homeostasis (Figure 1.3). Tanycytes, microglia, astrocytes, and oligodendrocytes of the ME and ARC have all been found to play a role in energy homeostasis maintenance by regulating exposure of sensor cells to energy availability signals and by inhibiting/enhancing signalling downstream of sensor cells.

1.3.1 Tanycytes

Although the phenomenon is not specifically described in Ashby’s homeostat model, ability of energy availability signals to reach sensor cells has been shown to affect energy homeostasis maintenance. In the hypothalamus, tanycytes can regulate diffusion of peripheral/blood-borne signals into the ARC from the ME via vascular endothelial growth factor A (VEGFA)-mediated changes in ME capillary fenestration. In fasting, VEGFA secretion from tanycytes is increased which, in turn, increases capillary fenestration. This is thought to allow anorexigenic circulating hormone ghrelin to reach ARC neurons to initiate food-seeking behavior. When tanycytic VEGFA signalling is blocked via VEGF receptor inhibitor Axitinib, animals eat significantly less when refeeding after a fast (Langlet et al., 2013).

Another way tanycytes might affect the detection of energy availability signals is by acting as the conduit by which sensor cells receive the signals. One study has suggested that leptin is transported from the blood into ME tanycytes via the leptin receptor (lepR), which release the hormone into the cerebrospinal fluid (CSF) of the third ventricle (3V). From there, leptin is thought to diffuse into the ARC where it activates leptin-responsive neurons (Balland et al., 2014). The idea that leptin signalling requires transport to the CSF via tanycytes is controversial, and recent papers have challenged this idea: the Blackshaw group has showed...
that lepR is not highly-expressed in tanycytes, and complete ablation of ME tanycytes has no effect on the ability of leptin to activate ARC neurons (Yoo et al., 2019a and b).

### 1.3.2 Microglia
Microglia may affect energy homeostasis by changing the ability of sensor cells to sense energy availability signals (component D) and/or by altering their downstream signalling (component S). Two studies have shown that depleting resident microglia of the ARC/ME or preventing their expansion leads to reduced food intake and body weight gain on a high-calorie diet (André et al., 2017; Valdearcos et al., 2017). Conversely, activating microglia can cause increased food intake and bodyweight gain, even on a standard chow diet (Valdearcos et al., 2017). The exact mechanisms by which microglial activation (or lack thereof) causes changes in food intake are unknown; however, mice with increased microglial activation display decreased leptin sensitivity (Valdearcos et al., 2017). This indicates these cells play a role in the ability of ARC cells to sense energy availability signals, or else to signal detection of the signals to downstream cells.

### 1.3.3 Astrocytes
Astrocytes have been shown to affect the firing/signalling properties of neurons involved in energy homeostasis (component S), although their effect in controlling food intake is debated. Two studies have shown that when astrocytes were stimulated with a designer receptor exclusively activated by designer drug (DREADD, hM3D[Gq]) or channelrhodopsin-2 (ChR2), animals eat significantly less than control animals after fasting or when ghrelin (an orexigenic hormone) was administered (Sweeney et al., 2016; Yang et al., 2015). Yang et al. showed decreased activity in AgRP neurons when the astrocytes were stimulated, possibly mediating the effect on food intake behavior. However, another study showed that overall food intake and AgRP neuron activity are increased with hM3D activation in astrocytes (Chen et al., 2016). Clearly, the role of astrocytes in maintaining energy homeostasis still requires further study.

---

3 The effects of ‘stimulating’ astrocytes with DREADD or ChR2 activation are not fully characterized. DREADD activation via hM3D(Gq) induces calcium waves in astrocytes (Agulhon et al., 2013) while ChR2 activation can cause an extracellular accumulation of potassium and adenosine around the astrocyte, possibly affecting signalling of surrounding cells (Octeau et al., 2019; Sweeney et al., 2016).
1.3.4 Oligodendrocytes

Thus far, only a few studies have implicated oligodendrocyte lineage cells (OLs)\(^4\) in regulating food intake; OLs have been shown to regulate sensor cell detection of energy availability signals and to control downstream signalling from these cells. Ablation of proliferating oligodendrocyte precursor cells (OPCs) via 3V administration of mitotic blocker cytosine-β-D-arabinofuranoside (AraC) led to a significant increase in food intake and bodyweight gain. Importantly, ablation of OPCs in the ME, specifically, was responsible for bodyweight increase, as mice with ARC but not ME OPC ablation did not gain weight (Djogo et al., 2016).

Chang et al. (2012) also researched the role of OPCs in energy homeostasis by deleting neural/glial antigen 2 (NG2) proteoglycan, a process shown to reduce OPC proliferation (Kucharova and Stallcup, 2010). In this study, deletion of NG2 in Olig2-expressing cells (OLs) caused a significant decrease in bodyweight compared to wildtype controls. Interestingly, whole-body deletion of NG2 caused a significant increase in bodyweight in adulthood.

\(^4\) Throughout this thesis, ‘oligodendrocyte’ (OL) is used analogously to ‘oligodendrocyte lineage cell’ and refers to both pre- and postmitotic cells
Deletion of Gpr17, a G-protein-coupled receptor, in Olig1-expressing cells (OLs) also causes an overall reduction in food intake and bodyweight. Deletion of the receptor causes decreased \textit{Agrp} expression, increased \textit{Pomc} expression, and increased activation of POMC neurons, all of which may contribute to the food intake behavior (Ou et al., 2019).

Although they highlight the importance OLs play in maintaining energy homeostasis, these studies do not fully address the mechanisms that these cells might use to do so. For example, AraC administration via the 3V does not specifically affect OLs. Although OPCs make up a large majority of proliferating cells in the ME-ARC, AraC also ablates proliferating microglia in the region (Robins et al., 2013b). The study examining effects of NG2 deletion studies in-depth the mechanisms by which whole-body NG2 deletion causes body weight gain, but does not investigate the mechanisms by which OL-specific deletion causes reduced body weight. Finally, these studies do not investigate the differential roles of the various OL subtypes on the control of energy homeostasis maintenance.

1.4 Thesis Aim

The aim of this thesis is to explore the specific roles that OLs of the ME may play in maintaining energy homeostasis. To do this, I first characterize ME OL subtypes (chapter 3), detail the adaptive response of ME OLs to changes in energy availability (chapter 4), suggest mechanisms by which OLs may sense energy availability signals (chapter 5), and examine potential downstream effects of this signal detection (chapter 6). An overview of the topics, rationales, hypotheses, and results of these chapters is shown in Figure 1.4.
### Chapter 1 - General Introduction

#### Overview of the topics, rationales for study, hypotheses, and results of each chapter

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Topic</th>
<th>Rationale</th>
<th>Hypotheses</th>
<th>Results</th>
</tr>
</thead>
</table>
| Chapter 3 | What are OLs of the ME like? | • multiple subtypes of OLs exist in the CNS  
• 2 subtypes of OLs have been described in the ME | • More than 2 subtypes of OLs are present in ME  
• 3 subtypes of OLs are present in ME  
• MOLs/myelin are highly concentrated in the ME but absent in the ARC | • refeeding changes expression of genes related to OL differentiation  
• NFOL marker genes increase with refeeding  
• proliferation is unchanged with refeeding |
| Chapter 4 | How do ME OLs respond to a nutritional stimulus? | • ME cells respond to nutrient availability  
• OLs can respond to stimuli by increasing proliferation or differentiation | • OLS respond to refeeding after a fast with increased OPC proliferation or differentiation | • mTOR signalling in the hypothalamus is responsive to nutrient availability  
• mTOR is important for OPC differentiation and production of myelin  
• hormones and neurotransmitters can affect OPC differentiation |
| Chapter 5 | How do OPCs sense nutrient availability to increase differentiation? | • mTOR activity in OLs is regulated by dietary nutrient intake | • mTOR signalling in OLs is changed with refeeding and high-protein diet  
• it is unclear whether mTOR mediates OPC differentiation in fast-refeed  
• OPCs express plasma membrane proteins that may enable them to sense dietary manipulations | • increased OPC differentiation leads to an increase in myelination in the ME  
• Myrf KO will prevent OPC differentiation in the ME  
• injection next to the ME will allow targeting of the ME |
| Chapter 6 | What are the functional consequences of increased OPC differentiation? | • Myrf KO mice prevent OPC differentiation  
• brain injections enable site-specific Cre-recombination | • neither myelin thickness nor density changes within 2 h of refeeding  
• differentiation blockade leads to loss of postmitotic OLs in the ME  
• ME-ARC diffusion barrier is bidirectional |
CHAPTER 2

MATERIALS AND METHODS

2.1 MOUSE LINES

Male C57BL/6 mice obtained from Charles River at approximately 7-9 weeks of age were used for animal experiments conducted in-house, except where noted. Mice had free access to water and were maintained on a 12 h light/dark cycle. Except where specified, mice were given free access to normal chow (Safe Diets – Safe 105) and were group-housed (at least 2 animals per cage). Animals were handled (scruffed) regularly before experiments to reduce stress-related responses. Animals were treated in accordance with the UK Home Office (Scientific Procedures) Act (1986) with project approval from University of Cambridge.

2.1.1 Cnp-Cre mice

Cnp-Cre;Raptor\textsuperscript{floxed/floxed} (raptor knockout [KO]) and Cnp-Cre;Rictor\textsuperscript{floxed/floxed} (rictor KO) brain tissue (Lebrun-Julien et al., 2014) was provided by the Macklin lab at the University of Colorado Anschutz Medical Campus.

2.1.2 Aldh111-GFP mice

Aldh111-green fluorescent protein (GFP) mice (Gong et al., 2003) were initially provided by the Tchöp lab at the Helmholtz Diabetes Center & German Center for Diabetes Research and bred in-house. Genotypes were determined by PCR analysis of ear genomic DNA using primers to detect GFP: forward = 5’-TTCACCTTGATGCCGTCTTCT-3’ (forward) and 5’-GCCGCTACCCCGACCAC-3’ (reverse).
2.1.3 Myrf KO mice

Pdgfra-CreER\textsuperscript{T2};R26R-YFP;Myrf\textsuperscript{fl/fl} (Myrf KO) and Myrf\textsuperscript{+/-} littermate brain tissue (McKenzie et al., 2014) was provided by the Richardson lab at the University College London Wolfson Institute for Biomedical Research.

Contributions: Cnp-Cre mice were perfused by Hannah Hathaway (University of Colorado Anschutz Medical Campus). Aldh1l1-GFP mice were bred and genotyped by Debra Rimmington (University of Cambridge Wellcome Trust-MRC Institute of Metabolic Science). Takahiro Shimizu (University College London Wolfson Institute for Biomedical Research) bred, administered tamoxifen to, fasted and refed, and perfused the Myrf KO mice.

2.2 Dietary manipulations

2.2.1 Fast-refeed paradigm

Overnight fasting was performed as described previously by our lab (Burke et al., 2017). Before onset of the dark period, animals were moved to a new cage with enrichment (cardboard tube, wooden chew sticks and cotton) and a small amount of bedding from the old cage. Animals were fasted for approximately 16 h. Refed animals then received food pellets in the cage hopper and many pellets on the floor of the cage for 1 h before perfusion. For the single-cell RNA sequencing (scRNAseq) experiment, animals were exposed to fasting-refeeding one time before the final experiment (to remove novelty of the experience). For the bromodeoxyuridine (BrdU) labelling experiment only, animals began fasting at noon and refed animals were fed at 11 am the next day. For myelin studies, refed animals were fed for 2 h before sacrifice.

2.2.3 Low-fat diet/high-fat diet paradigm

Animals maintained on normal chow since weaning were changed to low-fat diet (LFD) or high-fat diet (HFD) from 4-5 weeks of age. The LFD diet consisted of 10% kcal fat (from soybean oil and lard), 70% kcal carbohydrate (from corn starch and sucrose), and 20% kcal protein (from casein and cysteine) (Research Diets - D12450H). The HFD diet consisted of 45% kcal fat (from soybean oil and lard), 35% kcal carbohydrate (from corn starch and sucrose – sucrose was matched to the LFD diet), and 20% kcal protein (from casein and cysteine).
(Research Diets - D12451). Animals were fed the new diets for 6 weeks before being subjected to the fast-refeed paradigm, then they were sacrificed.

2.2.4 Low-protein diet/high-protein diet paradigm
Animals were fed isocaloric low-protein diet (LPD) or high-protein diet (HPD) for 3-4 days (they had been maintained on normal chow diet since weaning). The LPD diet consisted of 20% kcal fat (from soybean oil), 73% kcal carbohydrate (from corn starch and sucrose), and 7% kcal protein (from casein) (Research Diets - D17030701). The HPD diet consisted of 20% kcal fat (from soybean oil), 35% kcal carbohydrate (from corn starch and sucrose), and 45% kcal protein (from casein) (Research Diets - D17030703). Animals were exposed to the new diet 3 days before completely switching to the new diet to avoid neophobia. Animals were sacrificed at the end of the 3-4 day diet exposure.

Contributions: Clemence Blouet assisted with perfusions of LPD/HPD-fed mice.

2.3 Substance administration

2.3.1 BrdU administration during fast-refeed
The dose and time course of BrdU administration for labelling proliferating cells in the brain has been described previously (Wojtowicz 2006). Animals were given intraperitoneal (ip) injections of BrdU (Sigma - B5002, 50 mg/kg in saline) 2 times during the afternoon of the initial day of the fast (after the fast had begun) and 2 times during the morning the second day of the fast before refeeding.

2.3.2 Rapamycin administration
Animals were fasted overnight. The following morning, animals were given an ip injection of rapamycin (BioVision - 1568, 10 mg/kg) in a vehicle of 5% ethanol, 5.2% Tween 80 (Sigma – P4780) and 5.2% polyethylene glycol 400 (AppliChem – A2203) in distilled water or vehicle alone as described previously (Lamming et al., 2012; Rensing et al., 2015). 30 minutes later, animals were refed for 1 h then perfused.

Contributions: Clemence Blouet assisted with perfusions of these mice.
2.3.3 Tamoxifen administration
For Cre induction in Myrf KO and littermate mice, tamoxifen was administered as previously described (McKenzie et al., 2014). Mice were given tamoxifen in corn oil (300 mg/kg) via oral gavage for 4 days then subjected to the fast-refeed paradigm 3 weeks later.

Contributions: Tamoxifen administration in Myrf mice was performed by Takahiro Shimizu.

2.3.4 ME injection pilot
Prior to surgery, all animals received a 5 mg/kg oral dose of analgesia (Meloxicam). Animals were anesthetized with 2% isofluorane and placed in a stereotaxic frame. Anesthesia was maintained at 2% isofluorane, 0.8 L/minute oxygen via a face mask affixed to the frame. After exposing the skull surface, a small burr hole was drilled at the following coordinates: anterior/posterior = -1.6 mm from Bregma, lateral = 2.4 mm from the midline. A steel guide cannula (PlasticsOne) was lowered into the brain to -4.4 mm from the surface at an angle of 20° from the midline. 100 nl of a Cre-independent tdTomato (red) labelled virus (AAV2-tdTomato, UNC Vector Core) was injected at a rate of 50 nl/minute via a stainless steel injector that extended 2 mm from the tip of the guide cannula. The injector and guide cannula remained in place in the brain for 5 minutes following the injection then were removed slowly. The scalp was then closed with resorbable surgical suture. After 2 weeks, animals were sacrificed.

2.4 SCRNASEQ
2.4.1 ME dissociation
Tissue dissociation for scRNAseq was performed as previously described by our lab (Lam, Kohnke et al., 2017). Ten P40-47 C57BL/6 mice were fasted overnight and half were refed for 1 h. Animals were sacrificed via cervical dislocation and brains were quickly extracted into a petri dish (CytoOne - CC7682-3340) filled with Neurobasal-A medium (ThermoFisher Scientific - 10888022) on ice. Under a dissection microscope, the ME was dissected from each brain by gently squeezing the hypothalamus with forceps and excising the ME with fine curved scissors (Fine Science Tools - No. 15010-11). Fast and refed animals were alternated in dissection. The MEs were placed in ice cold papain (Worthington Biochemicals - LK003160) in separate 1.5 ml Eppendorfs until all dissections were finished (total dissection time for all brains was one hour). The sections in papain were incubated at 37 °C (500 rpm) for 15-20
minutes, and the Eppendorfs were swirled every 5 minutes. The sections were aspirated into a pipette with as little papain as possible then placed in prewarmed DNase solution (Sigma-Aldrich - D4263) at 37 °C for 4 minutes. The tissue was tritured with a P1000 pipette tip ten times then placed back at 37 °C to settle. 200 ul supernatant was removed and placed in a fluorescence-activated cell sorting (FACS) collection tube on ice. The samples were tritured, settled, and supernatant removed once more. The remaining liquid was then tritured using a P200 tip and the rest of the sample/DNase solution placed in tubes on ice until Fluorescence-activated cell sorting (FACS).

2.4.2 FACS

FACS was used to remove debris from samples, to ensure single cells in suspension, to select for live cells, and to provide equal numbers of cells per condition. The cell suspensions from triturations were passed through a 40 µm cell strainer into fresh collection tubes. DraQ5 and DAPI were added to the samples in DNase solution to select for nuclei and exclude dead cells, respectively, then sorted with an Influx Cell Sorter (BD Biosciences) into 4 tubes containing 10 ul 0.4% bovine serum albumin (BSA) in Ca-/Mg-free PBS (two tubes for fasted and two tubes for refed samples). 3500 cells were sorted into each tube then kept on ice until sequencing.

Contributions: FACS was performed by Chiara Cossetti (Flow Cytometry Core of the Cambridge Institute of Medical Research), with input from Brian Lam (Wellcome Trust-MRC Institute of Metabolic Science).

2.4.3 Sequencing

The isolated cells from FACS were encapsulated in droplets with barcoded beads and enzymes to lyse cells (Figure 2.1a). For 10X sequencing, barcoded beads have an oligo d(T) tail to capture mRNA molecules from lysed cells. Each cell has its own barcode (10X Barcode) and each mRNA transcript will be linked to a unique molecular identifier (UMI) so that the number of transcripts per cell can be counted during sequencing (Figure 2.1b). cDNA libraries were made using a 10X Genomics Chromium instrument and 10X Single Cell 3’ V2 Reagent kit. Paired end sequencing was performed on an Illumina HiSeq 4000. The sequencing reads were mapped to the Genome Reference Consortium m38 (mm10) mouse reference genome and
counted using 10X Genomics Cell Ranger software version 2.0. The ME fast-refeed scRNAseq dataset is available on GEO (accession number GSE133890).

**Contributions:** Sequencing was performed by the Cancer Research UK Cambridge Research Genomics Core.
Chapter 2 – Materials and methods

Figure 2.1 scRNAseq
Images adapted from Salomon et al., 2019, reprinted with permission from the Royal Society of Chemistry. (a) For 10X sequencing, cells (from FACS) are combined with barcoded beads and lysis buffer, and encapsulated in oil. (b) Schematic of sequencing process: barcoded beads combine with mRNA from lysed cells, cDNA is synthesized, cDNA is amplified and fragmented, and sequencing libraries are created by attaching gene-specific Illumina adapters to the amplified cDNA. TSO = template switching oligos (for amplification)
2.5 BIOINFORMATICS

All code used for analysis of the scRNAseq dataset is found in the Appendix and in the open Github repository Kohnke-et-al-2019.

2.5.1 T-distributed stochastic neighbor embedding and clustering

R software (R Core Team) was used to analyse scRNAseq data. The R package ‘cellrangerRkit’ (supported by 10X Genomics) was used to perform t-distributed stochastic neighbor embedding (tSNE), a dimensionality reduction technique that allows mapping of all cells in 2 dimensions based on their transcriptomic profile. The package ‘NBClust’ (Charrad et al., 2014) was used to test the tSNE plot for the optimal number of clusters. Finally, cellrangerRkit was used to find the top defining genes per cluster.

2.5.2 Differential gene expression

The R package ‘edgeR’ (McCarthy et al., 2012; Robinson et al., 2010) was used to test for differentially expressed genes (DEGs) in particular clusters between fasted and re-fed conditions. This package fits gene expression data from one condition per cluster to a generalized linear model (GLM) then compares it to expression in the same cluster in the other condition. GLM fitting is beneficial for complex multifactor experiments. edgeR generated an output of log2-fold change (log2FC) in expression, p-values (adjusted for multiple testing), and false discovery rates (FDRs) for every gene of each cluster.

2.5.3 Pathway analysis

Ingenuity Pathway Analysis (IPA, Qiagen) was used for exploratory data analysis to identify pathways that are up- or downregulated in each cluster between experimental conditions. Log2FC values, p-values, and FDRs for DEGs found with edgeR were uploaded to the software.

2.6 MOUSE TISSUE PROCESSING

2.6.1 Perfusion fixation

Animals were anaesthetized with an ip injection of 50 µl pentobarbitol solution (Dolethal 200 mg/ml) then transcardially perfused as follows. For immunohistochemistry (IHC), tissue clearing, and RNAscope experiments, animals were perfused with 0.01 M phosphate buffered
saline (PBS) at room temperature (RT) until perfusate was completely clear (approximately 50 ml), then cold (4°C) 4% paraformaldehyde (PFA, Fisher Scientific – P/0840/53) solution in PBS (pH 7.4) until animals were completely rigid (approximately 50 ml). For experiments requiring resin embedding, animals were perfused with cold 4% glutaraldehyde (Generon – 23115.01), 0.008% CaCl₂ (Sigma – C5670) solution in PBS until animals were completely rigid (approximately 7 minutes).

Contributions: Per fusions with glutaraldehyde were performed by Chao Zhao (University of Cambridge Wellcome Trust-MRC Stem Cell Institute) with my assistance.

2.6.2 IHC on thin coronal sections

After perfusion, brains were postfixed in 4% PFA solution overnight then cryoprotected in 30% (weight/volume) sucrose solution in PBS for 24. Tissue was covered with optimal cutting temperature (OCT) media (CellPath – KMA-0100-00A) then sliced at 30 μm thickness using a Bright Series 8000 sledge microtome. For the present studies, tissue was always sliced in the coronal plane. For IHC studies, sections were taken from Bregma 0.62 to -7.76 mm (a region spanning the forebrain and hindbrain).

Antigen retrieval was used prior to antibody incubation. Sections were incubated in 10 mM sodium citrate (Fisher Scientific – S/3320/53) in distilled water at 80 °C for 20 minutes then washed 3 times in PBS. For stains including the BrdU antibody, sections were then incubated in 2 N hydrochloric acid (Sigma – 320331) in distilled water at 37 °C for 30 minutes. The acid was neutralized by washing sections in 0.1 M sodium tetraborate (Sigma – B9876) in distilled water with hydrochloric acid to adjust pH (final pH = 8.5) for 10 minutes, then sections were washed 3 times in PBS. For 3,3’-diaminobenzidine (DAB) labelling, sections were incubated in 0.5% hydrogen peroxide for 15 minutes at RT. For all experiments, sections were blocked in normal donkey serum (NDS, Vector Biolabs) or normal goat serum (NGS, Jackson ImmunoResearch – 017-000-121) in PBS plus 0.3% Triton X-100 (Sigma-Aldrich, 0.3% PBST) for 1 h prior to primary antibody incubation (antibodies were diluted in 0.3% PBST with or without block).

Sections were incubated in primary antibody solution (Table 2.1) for the appropriate time at 4 °C, then washed and incubated with appropriate secondary antibodies (Table 2.2) diluted at 1:500 in 0.3% PBST for 90 minutes at RT, followed by a final wash. Sections were mounted on slides (Clarity – C366) with mounting media containing 4’,6-diamidino-2-
phenylindole (DAPI, Life Technologies Corporation – P36971) and covered with thickness 1.0 coverslips (Marienfeld - 0101242). For DAB labelling, after biotinylated secondary antibody incubation, sections were incubated with avidin biotin complex (Vector Laboratories – PK-6100) for 1 h, washed, then incubated in DAB solution (Sigma-Aldrich – D4418) until cell bodies were visible. After washing and mounting on slides, DAB sections were dehydrated using the following ethanol gradient: 5 minutes 70% ethanol, 5 minutes 90%, 5 minutes 95%, 5 minutes 100%, 5 minutes 100%, 30 seconds xylene. DAB sections were covered with Vectashield mounting medium and coverslipped.

Conributions: Sophie Buller (Wellcome Trust-MRC Institute of Metabolic Science) performed IHC staining on LFD/HFD-fed tissues as part of her PhD rotation project training
# Materials and methods

## Table 2.1 Primary antibodies used in IHC labelling

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Serum concentration</th>
<th>Manufacturer and code</th>
<th>Antibody dilution</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC (clone CC1)</td>
<td>mouse</td>
<td>3%</td>
<td>Millipore - OP80</td>
<td>1:200 in block</td>
<td>24 h</td>
</tr>
<tr>
<td>BrdU</td>
<td>rat</td>
<td>3%</td>
<td>Biorad - OBT-0030</td>
<td>1:200 no block</td>
<td>24 h</td>
</tr>
<tr>
<td>GFP</td>
<td>chicken</td>
<td>5%</td>
<td>Abcam - ab13970</td>
<td>1:500 no block</td>
<td>24 h</td>
</tr>
<tr>
<td>MBP</td>
<td>rat</td>
<td>5%</td>
<td>Abcam - ab7349</td>
<td>1:1000 no block</td>
<td>24 h</td>
</tr>
<tr>
<td>NEUN (clone A60)</td>
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<td>5%</td>
<td>Millipore - MAB377</td>
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<td>24 h</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>rabbit</td>
<td>10%</td>
<td>Cell Signaling Technology - 3164</td>
<td>1:500 in block</td>
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<tr>
<td>pmTOR (Ser 2448, clone 49F9)</td>
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<td>1%</td>
<td>Cell Signaling Technology - 2976</td>
<td>1:65 no block</td>
<td>48 h</td>
</tr>
<tr>
<td>pmTOR (59,Ser 2448)</td>
<td>mouse</td>
<td>5%</td>
<td>Santa Cruz – sc-293133</td>
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<td>48 h</td>
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<tr>
<td>RFP</td>
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<td>24 h</td>
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<tr>
<td>SOX10 (to human)</td>
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<td>R&amp;D Systems - AF2864</td>
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<tr>
<td>Vimentin</td>
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<td>5%</td>
<td>ab5733 Millipore</td>
<td>1:1000 no block</td>
<td>24 h</td>
</tr>
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</table>

Abbreviations used: APC = adenomatous polyposis coli, GFP = green fluorescent protein, MBP = myelin basic protein, NEUN = neuronal nuclei, PDGFRα = platelet-derived growth factor receptor α, pmTOR = phosphorylated mammalian target of rapamycin, RFP = red fluorescent protein, SOX10 = SRY-Box 10.

## Table 2.2 Secondary antibodies used in IHC labelling

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
<td>biotinylated goat anti-rabbit</td>
<td>Thermofisher Scientific – 65-6140</td>
</tr>
<tr>
<td>donkey anti-chicken Alexa Fluor® 488-conjugate</td>
<td>Jackson ImmunoResearch - 703-545-155</td>
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<td>Thermofisher Scientific - A21206</td>
</tr>
<tr>
<td>donkey anti-rat Alexa Fluor® 488-conjugate</td>
<td>Thermofisher Scientific - A21208</td>
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2.6.3 Multiplex fluorescence in situ hybridization – mouse tissue

After perfusion with PFA and extraction of the brain from the skull, brains were postfixed in 4% PFA solution overnight then cryoprotected in 30% sucrose solution in PBS until the brain sank to the bottom of the container. Tissue was covered with OCT media then sliced at 16 μm thickness using a Leica CM1950 cryostat directly onto Superfrost Plus slides (ThermoScientific – J1800AMNZ) in an RNase free environment. Slides were then stored at -80 °C. For the present studies, tissue was always sliced in the coronal plane. Sections were taken from Bregma -1.58 to -2.30 mm (a region that captures the entire rostrocaudal length of the ME).

Multiplex fluorescence in situ hybridization (FISH) was performed as previously described (Bayraktar et al., 2018; Wang et al., 2012) (Figure 2.2). After manual epitope retrieval and dehydration, sections on slides were processed for FISH using the RNAScope LS Multiplex Assay (Advanced Cell Diagnostics) then IHC on a Bond RX robotic stainer (Leica). ‘Z-probes’ to *Pdgfra* (1:1), *Bmp4* (1:50), *Tcf7l2* (1:50) and *Plp1* (1:400) were used to detect mRNA transcripts in the tissue.

Samples were first permeabilised with heat in Bond Epitope Retrieval solution 2 (pH 9.0, Leica - AR9640) at 95 °C for 2 minutes then incubated in protease reagent (Advanced Cell Diagnostics) at 42 °C for 10 minutes. To inactivate endogenous peroxidases and the protease reagent, samples were incubated in hydrogen peroxide for 10 minutes.

Samples were then incubated in z-probe mixtures for 2 hours at 42 °C and each slide was then washed 3 times. DNA amplification trees were built through incubations in AMP1 (preamplifier), AMP2 (background reducer), then AMP3 (amplifier) reagents (Leica) for 15-30 minutes each at 42 °C. Between incubations, LS Rinse buffer (Leica) was used to wash the slides. After, samples were incubated in channel-specific horseradish peroxidase (HRP) reagents for 15 minutes at 42 °C, tyramide signal amplification (TSA) biotin or TSA fluorophores for 30 minutes and HRP blocking reagent for 15 minutes at 42 °C. The following TSA labels were used to visualize z-probes: Atto 425-streptavidin (Sigma - 40709, 1:200), Opal 520 (1:500), Opal 570 (1:500), and Opal 650 (1:2500) fluorophores (Perkin Elmer).

Directly following the FISH assay, tissue was incubated with anti-SOX10 antibody in blocking solution for 1 h (Abcam - AF2864, 1:100). To develop the antibody signal, samples were incubated in donkey anti-goat HRP (Thermo fisher Scientific - A15999, 1:200) for 1 h, TSA biotin (Perkin Elmer - NEL700A001KT, 1:200) for 10 minutes, and streptavidin-conjugated Alexa 700-streptavidin (Sigma - S21383, 1:200) for 30 minutes.
Contributions: tissue was cryosectioned onto slides by Sophie Buller. FISH was performed by Staffan Holmqvist (Wellcome Trust-MRC Stem Cell Institute).

Figure 2.2 RNAScope single-molecule FISH
Image from Wang et al., 2012 (published under a Creative Commons Attribution-NonCommercial-No Derivatives License). Schematic showing how RNAScope z-probes enable multiplex single-molecule labelling amplification. HRP = horseradish peroxidase

2.6.4 Clearing of thick coronal sections
After perfusion with PFA and extraction of the brain from the skull, brains were postfixied in 4% PFA solution overnight. Whole brains were washed in PBS 2 times for 2 h. Brains were trimmed in the coronal plane using a Leica VT1000s vibrotome until approximately Bregma -1.58 mm. Four to six 50 µm sections were then sliced from each brain and placed in a 24-well plate (Costar) in PBS. Tissue clearing was performed using the Clear, Unobstructed Brain/Body Imaging Cocktails (CUBIC) method as published (Susaki et al., 2014, 2015), with minor modifications (Figure 2.3).


2.6.4.1 Preparing reagents
CUBIC1 solution - 25% weight/volume urea (VWR Chemicals – 443874G), 28.8% distilled water, 31.2% Quadrol (Aldrich - 122262, diluted to 80% in distilled water), and 15% Triton X-100 (Fisher Bioreagents – BP151) - was made as follows: urea, water, and Quadrol were mixed on a hot plate at 150 °C for 15 minutes then allowed to cool to RT. Triton-X 100 was then added to the solution and mixed without heating. CUBIC2 solution is comprised of 25% (weight/volume) urea, 50% saccharose (VWR Chemicals – 443815S), 15% distilled water, and 10% triethanolamine (Sigma – 90279). Urea, saccharose, and water were mixed on a hot plate at 150 °C for 30 minutes then allowed to cool to RT. Triethanolamine was then added to the solution and mixed without heating.

2.6.4.2 Tissue clearing – CUBIC1
PBS in the well plate was replaced with a mixture of 1:1 CUBIC1 solution and distilled water plus Hoechst stain (Life Technologies – H3570, always used at 1:2000) (approximately 1 ml per well). The seam of the well plate was sealed with Parafilm and the well plate was placed in a shaking waterbath at 37 °C for 3 h. The CUBIC1/water solution was then discarded and replaced with 100% CUBIC1 solution plus Hoechst and kept at 37 °C overnight. The following day, the samples were washed in PBS three times for 1 h on a shaker (the samples, made transparent by the CUBIC1 solution, became opaque again at this point). The samples were then placed in 30% sucrose in PBS until the sections sank to the bottom of the wells. Finally, the sections were immersed in OCT and frozen at -80 °C at least overnight.

2.6.4.3 IHC labelling of cleared tissue
The samples were thawed and washed in PBS three times for 1 h on a shaker. The samples were placed in a new 24-well plate and covered with an anti-MBP antibody (Abcam - ab7349, 1:1000) diluted in PBS plus 2% Triton X-100 (2% PBST) and 10% NDS for 48 h at 4 °C on a shaker. Samples were then washed in 0.3% PBST at RT three times for 1 h. The samples were then placed in donkey anti-rat Alexa Fluor® 488-conjugate (Thermofisher Scientific - A21208, 1:500) diluted in 2% PBST and 10% NDS for 48 h at 4 °C on a shaker. Samples were then washed in 0.3% PBST at RT three times for 1 h.
2.6.4.4 Matching refractive index – CUBIC2

IHC-labelled samples were immersed in a mixture of 1:1 CUBIC2 solution and PBS without Hoechst. The well plate was sealed with Parafilm then placed in a shaking waterbath at 37 °C for 3 h. The CUBIC2/water solution was then discarded and replaced with 100% CUBIC2 solution without Hoechst and kept at 37 °C at least overnight, but maximum 72 h. The day before imaging, samples were placed in a 1:1 mixture of mineral oil (Sigma - M8410) and silicone oil (Sigma - 175633).

![Diagram of tissue clearing process](image)

**Figure 2.3 Tissue clearing with the CUBIC method**

(a) Schematic of tissue clearing process. After PFA fixation to cross-link (red lines) proteins (red) in a tissue sample, CUBIC reagent 1 is used to remove lipid membranes (blue). Fluorescent antibodies (green) are thus able to deeply penetrate tissue to label proteins. Then, refractive index matching with CUBIC reagent 2 increases transparency of the tissue for large-volume imaging (b, c) Representative results of whole brain clearing (b) and thick coronal section (c) clearing

2.6.5 Resin embedding for myelin studies

After perfusion with glutaraldehyde and extraction of the brain from the skull, brains were postfixed in 4% glutaraldehyde for 24 h then moved to PBS. 1 mm-thick sections containing the ME were sliced by hand from the brains and were stained with 2% osmium tetroxide (Oxkem) for 24 h at 4°C. Sections were washed with water three times then dehydrated by placing in an ethanol gradient as follows, on a rotator: 50% 2 times for 15 minutes, 70% 2 times for 15 minutes, 90% 2 times for 15 minutes, 95% 2 times for 15 minutes, 100% 3 times
for 10 minutes. Sections were then placed in propylene oxide (Agar Scientific) for 20 minutes. Sections were incubated in a mixture of 1:1 propylene oxide and resin (TAAB) for 6 h on a rotator, then in 100% resin for 24 h. Sections were mounted in resin in plastic molds (TAAB) and incubated at 60 °C for 24 h.

2.6.6 Toluidine blue labelling of resin embedded tissue for light microscopy
Resin blocks were trimmed with a microtome (Leica RM-2065) to expose the tissue, then 0.75 µm-thick sections were placed on a water droplet on a slide. Slides were heated on a hotplate to evaporate the water, then toluidine blue (0.5%, Merck) was applied to the sections for 30 seconds before washing off with distilled water.

Contributions: Chao Zhao assisted with resin embedding and toluidine blue labelling.

2.6.7 Post staining of resin embedded tissue for transmission electron microscopy
Resin embedded tissues were trimmed around the ME and semi thick slices were cut to create sections that only contained the ME. Then, 70 nm ultrathin sections were sliced on an ultramicrotome (Reichert-Jung - Ultra-cut 701701 Ultra Microtome) with a diamond knife (Diatome - Ultra 45). Sections were placed on mesh copper grids (size 300) and were post stained with aqueous uranyl acetate for 6 minutes then lead citrate for 2 minutes.

Contributions: post staining of resin embedded tissue was performed by Danae Nuzzaci (Wellcome Trust-MRC Institute of Metabolic Science).

2.7 HUMAN TISSUE
Human hypothalamic tissues used for this study were from donors to the Cambridge Brain Bank. Donors gave informed written consent for the use of brain tissue for research, and tissues obtained were used in accordance with the Research Ethics Committee Approval number 10/H0308/56. Samples were from an 83-year-old with no neuropathology. 59 h after death, hypothalamic samples were collected and stored in 10% neutral buffered formalin at RT for 24 h, transferred to 70% ethanol, and processed into paraffin. 6 µm sections were cut and
mounted onto Superfrost Plus slides (Thermo-Fisher Scientific) in an RNase free environment, and then dried overnight at 37 °C.

2.7.1 Multiplex FISH – human tissue

Using a Bond RX robotic stainer (Leica), slides were deparaffinized, rehydrated, treated with Epitope Retrieval solution 2 88 °C for 15 minutes, and with ACD Enzyme from the Multiplex Reagent kit at 40 °C for 10 minutes. Z-probes (PDGFRA 1:50, ACD - 604488-C4; BCAS1, 1:50, ACD - 525788-C3; PLP1, 1:1, ACD – 499278) were used to detect mRNA transcripts in the tissue.

Probe hybridisation and signal amplification was performed according to manufacturer’s instructions (Figure 2.2). The following TSA labels were used to visualize z-probes: TSA plus-Cy5 (1:750 to detect PLP1, Akoya Biosciences - NEL745001KT), TSA plus-Fluorescein (1:300 to detect BCAS1, Akoya Biosciences - NEL741001KT) and Opal 620 (1:300 to detect PDGFRA, Akoya Biosciences - FP1495001KT). After completion of the FISH assay, slides were removed from the Bond RX and mounted using Prolong Diamond (ThermoFisher - P36965).

Contribution: paraffin-embedded tissue was sectioned by James Warner (Histopathology Core of the Wellcome Trust-MRC Institute of Metabolic Science). FISH was performed by Julia Jones (Histopathology Core of the Cancer Research UK – Cambridge Institute).

2.8 Imaging

2.8.1 Confocal microscopy

Thin immunolabelled mouse sections (40x oil objective) and human tissue labelled with FISH (10x dry and 20x oil objectives) were imaged using a Leica SP8 confocal microscope. For mouse tissue, sections were imaged at multiple points in the z plane (z-stacks) at intervals of 3.3 µm to collect signal from the entire depth of the tissue for the region of interest (ROI). ROIs for mouse tissue included the vascular organ of the lamina terminalis, subfornical organ, subgranular zone, subventricular zone, ME, ARC, corpus callosum (CC), and area postrema. For human tissue, sections were imaged at multiple points in the z plane (z-stacks) at intervals of 5 µm. ROIs for human tissue were the ME and ARC at 20x and the full coronal hypothalamic section at 10x. Gain and laser power settings remained the same between experimental and control conditions within each experiment.
2.8.2 High-content confocal microscopy

Mouse tissue labelled with FISH was imaged using a spinning disk Operetta CLS (Perkin Elmer). Sections were imaged in confocal mode using a sCMOS camera and a 40x automated-water dispensing objective. Sections were imaged with z-stacks at intervals of 1 µm. ROIs included the ME and CC. Gain and laser power settings remained the same between experimental and control conditions within each experiment.

2.8.3 Light microscopy

Toluidine blue-labelled tissue was imaged using a Nikon Eclipse E600 light microscope with 40x and 100x (dry) objectives. The ROI was the ME.

2.8.4 Spinning disk confocal microscopy

Thick cleared mouse tissue was imaged using an Andor Dragonfly spinning disk confocal with a 20x objective. Sections were placed in a glass-bottom dish (MatTek – P-35G-0-14-C) with a small amount of 1:1 oil mixture to coat the interface of the glass and tissue. Sections were imaged with z-stacks at the software-recommended interval. The ROI was the ME and ARC – the large field of view of this microscope allowed both structures to be imaged at once without tiling.

Contributions: high-content confocal microscopy of mouse FISH sections was performed by Katherine Ridley (Wellcome Trust-MRC Stem Cell Institute)

2.9 IMAGE ANALYSIS

For histological data, the person assessing the images was blinded to experimental condition and image order for quantification was randomized.

2.9.1 IHC, human FISH, toluidine blue labelling

For immunolabelled and toluidine blue-labelled mouse sections and FISH labelled human sections, Fiji software was used to analyse colocalization, distribution, and counts/density of markers (Schindelin et al., 2012). For images in z-stacks, individual images were first projected into a single image (a ‘Z-Project’ at maximum intensity) so all cells could be counted at once, and to eliminate double-counting. Areas of ROIs were measured by setting the image scale
according to scale bars imprinted on images during acquisition, then tracing the ROI with the freehand tool and measuring. Borders of the ME, ARC, and other ROIs were determined using the Paxinos and Franklin Mouse Brain Atlas (2001). The Fiji manual cell counter was used to count marker-positive cells or axons.

*Contributions:* Sophie Buller counted labelled cells in tissues of LFD/HFD-fed mice. Sophie Buller or Clemence Blouet also quantified cell labelling in other tissues to confirm my results.

### 2.9.2 Cleared tissue visualization

Videos of thick cleared tissue were made using Imaris software (Oxford Instruments).

### 2.9.3 Myelin thickness

To assess differences in g-ratio between fasted and refed conditions, at least 100 distinct transverse axons were measured per animal. Using Fiji, the cross-sectional area of axons was measured by tracing the outside of the axon with the freehand tool. Similarly, the outside of the myelin sheath was traced to determine the area of the myelin+axon. Diameters of the axon and the myelin+axon were back-calculated from the areas, assuming the cross sections were perfect circles. The g-ratio was then calculated by dividing the axon diameter by the myelin+axon diameter.

*Contributions:* Danae Nuzzaci also measured g-ratios in this tissue to confirm results.

### 2.9.4 Automated spot counting for mouse FISH

Harmony software (Perkin Elmer) was used to automatically quantify number of labelled RNA molecules (spots) per cell, intensities of spots, and area of spots.

*Contributions:* Clemence Blouet and Greg Strachan optimized the automated cell count script and collected the data.
2.10 STATISTICAL ANALYSIS AND DATA VISUALIZATION

2.10.1 scRNAseq

All code used for analysing data and creating figures related to scRNAseq is found in the Appendix and in the open Github repository Kohnke-et-al-2019. Ranking of cluster-defining genes and statistical significance of DEGs was determined by the cellrangerRkit (10X) and edgeR packages in R (McCarthy et al., 2012; Robinson et al., 2010). A gene was considered ‘differentially expressed’ if it had a $p$ value of less than 0.05 and an FDR of less than 0.25, as previously described (Campbell et al., 2017). Statistical significance of pathways changed and upstream regulators of DEGs between fasting and refeeding was determined using IPA (Qiagen). Figures relating to scRNAseq data were created using the cellrangerRkit, ggplot2, tidyr, and GOplo packages in R (Wencke et al., 2015; Wickham, 2014, 2016).

2.10.2 Histology experiments

R and GraphPad Prism 8 (GraphPad Software) were used to perform statistical analysis on all other experiments. For experiments with two conditions, data was assessed for normality using a Shapiro-Wilk test. Normal samples were assessed for equality of variance with Bartlett’s test, and non-normal samples were assessed for equality of variance with Levene’s test. Significant differences between experimental conditions were determined with a paired t-test, Student’s t-test, Welch’s t-test, Wilcoxon signed rank test, or Mann-Whitney U test. For experiments with two factors, two-way ANOVA and Sidak’s multiple comparisons test were used to determine significant differences. Difference between linear regression lines was calculated with ANCOVA. Figures relating to histological data were created using GraphPad Prism 8. In all graphs, error bars indicate mean ± SEM. These experiments were the initial studies in our lab in the field of dietary nutrient intake and OLs. As such, meaningful effect sizes and standard deviations were unknown in order to conduct a priori power analyses to determine sample size. Samples sizes were chosen based on our lab’s previous work in detecting neural effects of different diets. Effect sizes and standard deviations from these studies will enable a priori sample size calculations for future experiments.
CHAPTER 3

OLIGODENDROCYTE SUBTYPES OF THE MEDIAN EMINENCE

3.1 INTRODUCTION

The median eminence (ME) has garnered a lot of interest from those studying neural control of food intake. This is largely due to its function as gatekeeper to the arcuate nucleus of the hypothalamus (ARC), where neurons responsible for integrating signals of nutrient availability are located. Understanding the organization of cell types located in the ME is important for inferring functions of the cells. In this introduction, I will describe this organization, including the placement of two subtypes of OLs that have been described previously. Then, I will review previous studies that use scRNAseq to characterize transcriptomes of individual hypothalamic cells, including OLs. Finally, as the presence of multiple OL subtypes in the adult brain suggests functions other than facilitating axonal conduction, I will describe a few of these putative roles.

3.1.1 Background

3.1.1.1 Unique cellular architecture of the ME

The ME is a circumventricular organ (CVO) that serves as a bidirectional gateway between the hypothalamus and the periphery. The fenestrated capillaries lining the base of the ME allow all cells within the structure to have direct access to circulating hormones and nutrients. Likewise, factors such as hormones that are released or secreted in the ME are able to directly enter the blood (Yin and Gore, 2010).
Derived from the embryonic ventral forebrain, the ME forms the anterior portion of the infundibulum, the structure comprising the ME, pituitary stalk, and the posterior lobe of the pituitary. The infundibulum forms at embryonic day 9.5 (E9.5) in mice, and is situated directly above Rathke’s pouch, which will later give rise to the anterior pituitary. The infundibulum is the source of several ME cell types (Rizzoti and Lovell-Badge, 2017).

Early electron microscopy studies have allowed detection of four distinct zones within the adult murine ME: the third ventricular zone (I), the myelinated axon zone (II), the neural profile zone (III), and the capillary zone (IV) (Yin and Gore, 2010) (Figure 3.1a). Layer I consists of tanyocytes and ependymal cells lining the interface between the third ventricle (3V) and the rest of the ME. Layer II contains myelinated vasopressin and oxytocin neurons. Layer III is comprised of unmyelinated processes of releasing hormone neurons that terminate in layer IV – the zone containing fenestrated capillaries. There are very few neuronal cell bodies in the ME, but they are mostly located in layer I.

Layer I tanyocytes are thought to be descendants of embryonic radial glia: in the perinatal period, most radial glia will become astrocytes in the region, but some will differentiate into tanyocytes. Tanyocytes retain many features of radial glia, such as a cell body that contacts the CSF and extends into the parenchyma, and expression of markers GFAP, vimentin, and nestin (reviewed in Rodríguez et al., 2005). In the adult mouse brain, layer I tanyocytes extend their processes through the other layers and contact fenestrated capillaries in layer IV with their endfeet. Through their endfeet, tanyocytes may act as surveyors of blood content: they are known to be sensitive to conditions of low glucose (Frayling et al., 2011). Tanyocyte endfeet also surround terminals of some of the releasing hormone neuronal processes and can regulate release of hormones such as gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH) into the capillaries (Lazcano et al., 2015; Parkash et al., 2015). Intriguingly, several studies have shown that adult genesis of hormone-responsive neurons can occur in the adult murine hypothalamus and that tanyocytes may be the source of these cells (Haan et al., 2013; Lee et al., 2012a). However, one study has also identified ME/ARC oligodendrocyte precursor cells (OPCs) as potential neuronal progenitors (Robins et al., 2013a).

As mentioned, layer II consists of myelinated magnocellular vasopressin and oxytocin neurons. The cell bodies of these neurons lie in the paraventricular and suprachiasmatic nuclei, and some of their axons pass through the ME on their way to terminate in the posterior pituitary gland (Low, 2016) (Figure 3.1a-b).
The cellular processes residing in layer III that terminate in the ME are those of releasing hormone and inhibiting hormone neurons whose cell bodies lie in several hypothalamic nuclei (Kandel et al., 2013). Corticotrophin-releasing hormone (CRH), growth hormone-releasing hormone, dopamine, TRH, and GnRH all release hormones/neurotransmitters into the portal blood. From there, the hormones reach the anterior pituitary to control secretion of hormones such as adrenocorticotropic hormone (to increase cortisol production from the adrenal gland), growth hormone (to stimulate growth and regeneration), prolactin (to stimulate milk production), thyroid-stimulating hormone (to control production of thyroid hormones), and follicle-stimulating and luteinizing hormones (to regulate reproductive function in males and females) (Gross and Weindl, 1987; Marques et al., 2000; Mullur et al., 2014; Sonntag et al., 1985; Voogt et al., 2001).

Releasing hormone neurons, especially GnRH neurons, possess unique properties. Studies of GnRH terminals in the ME revealed that the processes can function as both axons and dendrites, earning them the moniker ‘dendrons’ (Herde et al., 2013). Unmyelinated processes of leptin receptor-expressing (lepR) neurons are also present in layer III (Djogo et al., 2016). Interestingly, leptin signalling is required for proper regulation of pituitary GnRH receptor expression. A lack of leptin signalling can significantly impair fertility in female mice (Odle et al., 2017). OPCs are another cell type located in layer III (although they can be found in other layers as well). Here they make contact with and provide trophic support to neuronal processes (Djogo et al., 2016).

The fenestrated capillaries of layer IV are unique in that they are part of the brain’s only portal system (a system of capillaries that drain blood into other capillaries via veins rather than draining directly to the heart). Because of this, the ME can transport factors such as hormones in high concentrations to the anterior pituitary gland, and vice versa, as blood does not have to enter the heart first to circulate to the new location (Ciofi et al., 2009; Yin and Gore, 2010). As will be described in detail in chapter 4, capillary fenestration in adults is plastic and can be responsive to states of nutrient deficiency or abundance.
Figure 3.1 Layered structure of the ME

(a) Schematic of a coronal section containing the ME and the cell types present there. Cells largely segregate into 4 layers (b) Midline sagittal section of the mouse brain (adapted from Paxinos and Franklin, 2001, reprinted with permission from Elsevier) – vasopressin and oxytocin neurons pass through the ME on their way to the posterior pituitary gland. The vertical line indicates the location of the coronal section in (a).
3.1.1.2 scRNAsequencing to study hypothalamic cells

Several previous studies have used scRNAseq to provide transcriptomic characterization of hypothalamic cells (Campbell et al., 2017; Chen et al., 2017; Lam, Kohnke et al., 2017; Marques et al., 2016). scRNAseq is a method of next-generation RNA sequencing that allows analysis of transcriptomes of individual cells. As the total concentration of mRNA in one cell is too small for sequencing, the mRNA is first reverse-transcribed to complementary DNA then amplified using polymerase chain reaction (Tang et al., 2009). Single-cell tagged reverse transcription — the method of scRNAseq we employed in this study — allows for single cDNA strands with UMIs to be transcribed from each mRNA molecule; thus, the total amount of mRNA within a cell can be easily estimated (Islam et al., 2011, 2014). Although not a perfect proxy, studies have found that (depending on the organism) anywhere from 36 to 76% of a protein’s concentration variability can be correlated to mRNA concentration (Maier et al., 2009; de Sousa Abreu et al., 2009; Vogel and Marcotte, 2012). scRNAseq data can, therefore, be a useful tool for directing future studies to examine proteomic differences between cells and can provide insight into differing functions of cells within the ‘same’ population.

Within the last 5 years, scRNAseq has been used extensively to study gene expression in individual OLs of mice and humans (Campbell et al., 2017; Chen et al., 2017; Darmanis et al., 2015; Habib et al., 2016, 2017; Hochgerner et al., 2017; Jäkel et al., 2019; La Manno et al., 2016; Lake et al., 2018; Marques et al., 2016; Patel et al., 2014; Tasic et al., 2016; Zeisel et al., 2015). The studies have shown that OLs are heterogeneous, with one study showing the presence of 5 main subtypes of OLs throughout the central nervous system (CNS) (Marques et al., 2016). These subtypes represent different stages of maturity of OLs rather than different lineages of the cells: the authors of the study were unable to find region-specific subpopulations of OPCs, and clusters representing more mature OLs were the progeny of these OPCs.

Definition of subtypes by gene expression is achieved by testing for enrichment of genes within a particular cluster. The top DEGs can be thought of as defining genes. In accordance with pre-RNAseq literature, the most developmentally immature OL lineage cells in the scRNAseq studies are termed oligodendrocyte precursor cells (OPCs) and are demarcated from other clusters by their expression of Pdgfra and/or Cspg4 (Table 3.1). OPCs (sometimes referred to as ‘NG2-glia’) are multipotent proliferative cells. While most OPCs commit to the OL lineage, one study has shown that some OPCs become neurons in the hypothalamus (around 4-8% of NG2 glia/OPCs express neuronal markers within 60 days in the adult murine hypothalamus) (Robins et al., 2013a). Marques et al. showed that OPCs also express markers Fabp7 and Tmem100, markers of radial glia/astrocytes. This reflects their
origins and ability to differentiate into astrocytes, especially in injury (reviewed in Dimou and Götz, 2014).

Identification and nomenclature for other OL subtypes is not as clear as it is for OPCs. The most developmentally mature OLs have been referred to as myelin-forming OLs, myelinating OLs, mid-late stage OLs, or mature OLs (MOLs) in previous scRNAseq studies (Table 3.1). Marques et al. (2016) described possible functional heterogeneity in this population, as some MOLs are enriched in genes related to lipid synthesis and myelination, while others are enriched in genes for synapse formation.

Lying developmentally between OPCs and myelin-producing OLs is a population of intermediate-stage OLs. Depending on their gene expression, these have been referred to as differentiation-committed OPCs, newly formed OLs (NFOLs), or early stage OLs (Table 3.1). While gene and protein expression can distinguish between OPCs and postmitotic OLs fairly clearly, it can be difficult to determine the difference between NFOLs and MOLs. For example, NFOLs can produce the myelin-related gene Mbp but may not have begun producing myelin proteins (Xiao et al., 2016). RNA sequencing has been useful for discovering new markers specific to NFOLs, including Enpp6, and creation of antibodies to NFOL-specific proteins is improving (Fard et al., 2017; Marques et al., 2016; Zhang et al., 2014). The presence of immature, intermediate, and mature OLs in the adult brain could indicate that OPCs continuously produce OLs throughout adulthood or that OLs have functions other than simply insulating axons.
Table 3.1 Comparison of OL subtype terms from scRNAseq studies containing hypothalamic OLs

<table>
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<tr>
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<td>Klk6, Pmp22</td>
<td>late stage OLC</td>
<td>Gm21984, Mag</td>
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Subtype terms from different studies and gene expression that defines the subtype. OL/OLC= oligodendrocyte, OPC = oligodendrocyte precursor cell, POPC = proliferating OPC, COP = differentiation-committed OPC, NFOL/NFO = newly formed OL, MFOL = myelin forming OL, MO/MOL = myelinating/mature OL

3.1.1.3 Non-conduction/non-myelination roles of OLs

In addition to their canonical function of myelinating to facilitate axonal conduction, other roles of OLs have been described. MOLs are known to provide trophic support to the axons they ensheath. Monocarboxylate transporters located in the axonal membrane and myelin sheath allow transport of lactate from OLs to the axon for ATP production (Lee et al., 2012b). Increased glucose import into OLs (and presumed increased glycolysis, producing lactate) can be stimulated by activation of NMDA receptors on OLs, thought to be caused by glutamate release along an active axon (Saab et al., 2016). Substrate transport to axons through OLs may be important in regions of the brain where highly myelinated axons have limited access to extracellular milieu (Saab and Nave, 2017) (Figure 3.2a).

Non-myelinating OLs are also able to communicate with neurons. Prior to (or in absence of) formation of myelin, factor(s) secreted by OLs can cluster sodium channels at regularly spaced intervals along an axon in vitro (Freeman et al., 2015; Kaplan et al., 1997, 2001). Evenly spaced clusters of sodium channels can increase conduction velocity along an axon, even in the absence of myelin (Freeman et al., 2015). While the identity of the clustering factor is unknown, one study has reported the factor is not glial cell-derived neurotrophic factor, brain-derived neurotrophic factor, ciliary neurotrophic factor (CNTF), leukemia...
inhibitory factor, nerve growth factor, adhesion molecule Nfasc186, or electrical activity from neurons (Freeman et al., 2015) (Figure 3.2b).

In certain parts of the brain, OPCs are integral to maintaining the BBB. OPCs make contact with pericytes that surround capillaries and secreted factors from OPCs can increase pericyte viability in culture (Maki et al., 2015). OPCs may also regulate tight junctions in endothelial cells via TGF-β signalling, as OPC-specific deletion of TGF-β1 causes loss of BBB integrity (Seo et al., 2014) (Figure 3.2c).

Trophic support of neuronal processes by OLs has also been described in the ME. OPCs in the ME contact processes of neurons expressing the leptin receptor (lepR). When OPCs are ablated through blocking proliferation via AraC or X-irradiation of the ME, lepR processes degenerate (Figure 3.2d).

Interestingly, the exact function of myelin in this region is also unknown. Its presence in the ME is presumably to facilitate conduction of magnocellular neuron signals to the posterior pituitary gland, but effects of myelin disruption in this region (without affecting non-myelinating OLs) have never been studied.
Chapter 3 – Oligodendrocyte subtypes of the median eminence

Figure 3.2 Alternative roles of OLs
(a) In response to glutamate released from active axons, OLs can increase glucose import and glycolysis to provide lactate to myelinated axons. (b) Prior to (or in absence of) myelination, non-myelinating OLs can secrete factors to induce sodium channel clustering along axons. (c) Unknown secreted factors from OPCs can increase pericyte viability and TGFβ1 from OPCs is necessary for integrity of endothelial cell tight junctions. (d) OPCs provide trophic support to leptin receptor-expressing neurons in the ME. OPC ablation causes degeneration of unmyelinated neuronal processes. GLUT1 = glutamate transporter 1, lepR = leptin receptor-expressing, MCT1/2 = monocarboxylate transporter 1/2, NaV = sodium channels, NMDAR = NMDA (glutamate) receptor. Images were inspired by Djogo et al., 2016; Freeman et al., 2016; O’Brown et al., 2018; Saab and Nave, 2017.
3.1.2 **Hypothesis**

The knowledge that multiple OL subtypes exist throughout the brains of mice and humans introduces the possibility that multiple subtypes also exist in the ME. For this section of work, I hypothesized that more than the two previously described subtypes of OLs (myelinating OLs in layer II and OPCs in layer III) exist in the ME of mice and humans.

3.1.3 **Chapter 3 experimental objectives and design**

1. I used scRNAseq to obtain a high-resolution transcriptomic characterization of cell types in the adult murine ME. Samples were from fasted and refed mice. Transcriptomic changes between fasting and refeeding are covered in detail in chapter 4.

2. FISH was used to map the distribution of OL subtype markers in the ME and ARC. Samples were from fasted and refed mice. Fasted and refed samples were pooled to compare densities between the ME and ARC in this chapter, but differences between fasting and refeeding are discussed in chapter 4.

3. To confirm the location of postmitotic OLs in the ME, I studied antibody labelling of myelin and a postmitotic OL marker. I used both thin and cleared thick coronal sections from adult *ad libitum* fed mice to study distribution of those markers.

4. I again used FISH to map distribution of human-specific OL subtype markers in the human ME and ARC.
3.2 RESULTS

3.2.1 Quality control checks for samples in scRNAseq experiment

To study the transcriptomic profiles of cell types present in the ME, I used scRNAseq. I excised the MEs from 10 mice (n=5 fasted, n=5 fasted then refed for 1 h) then passed individual cells through FACS to select for: 1) individual cells, not doublets (small pulse width, Figure 3.3a); 2) nuclei-containing particles, not debris (membrane permeable DRAQ5⁺, Figure 3.3b); and 3) live cells, not dying cells (membrane impermeable DAPI⁻, Figure 3.3c). Of 14,000 cells captured via FACS, 5,982 cells were sequenced using the 10X platform Single Cell 3’ v2. These cells had UMI counts (molecular tags corresponding to unique mRNA transcripts) greater than 2,000 (Figure 3.3d-e). Neuroanatomical specificity of dissections was later confirmed by measuring expression of genes specific to the ME (as determined with the Allen ISH differential gene search compared with the ARC). The scRNAseq dataset contains high expression of ME-specific genes (Figure 3.3f). Lack of contamination from the adjacent ARC was confirmed by the absence of expression of genes specific to the ARC (as determined with the Allen ISH differential gene search compared with the ME) (Figure 3.3g).
Figure 3.3 Quality control checks for scRNAseq samples

(a-c) Dissociated cells from ME samples were sorted with FACS to select for: single cells (a, particles with small pulse width), nuclei-containing cell particles (b, particles containing membrane permeable DNA dye DRAQ5), and exclusion of DAPI (c).

(f-g) Gene expression analysis showing ME versus ARC-specific genes (Gpc3, Igfbp4, Mgat5) and ARC versus ME-specific genes (Epha8, Gira1, Egr4).
3.2.2 Eight cell types are present in the murine ME

After the high RNA-count cells were sequenced, I used the cellrangerRkit package in R to map the cells on a t-distributed stochastic neighbor embedding (tSNE) plot which groups cells with similar gene expression. I used the NBClust package in R (Charrad et al., 2014) to perform silhouette analysis on the tSNE plot to determine that the plot optimally segregates into 9 clusters (Figure 3.4a). K-means clustering produced the cluster assignments shown in Figure 3.4b. I then used cellrangerRkit to find the genes that distinguish each cluster from one another (Table 3.2). Based on cell-type markers described in previous scRNAseq studies of the hypothalamus (Campbell et al., 2017; Chen et al., 2017), I was able to define each cluster as follows: 1 = tanyocyte ($Rax^+$), 2 = astrocyte ($Agt^+$), 3 = oligodendrocyte (type 1, $Ermn^+$), 4 = microglial cell ($C1qc^+$), 5 = neuron ($Snhg11^+$), 6 = oligodendrocyte (type 2, $Cd9^+$), 7 = vascular and leptomeningeal cell (VLMC, $Dcn^+$), 8 = ependymocyte ($Elof1^+$), 9 = endothelial cell ($Itm2a^+$) (Figure 3.4b-c). Plotting the top 5 genes from each cluster against expression levels in all cells shows that gene expression is distinct in each of the 9 clusters (Figure 3.4d). Besides OLs, other cell types did not separate into distinct clusters on the tSNE plot. OL type 1 and type 2 separate into 3 distinct groups on the tSNE plot. The middle group expresses genes found in OPCs ($Cspg4$), the top group expresses genes found in COPs/NFOLs ($Bmp4$), and the bottom group expresses genes associated with MOLs ($Mal$) (Table 3.1, Figure 3.4e-f).
Figure 3.4 Eight cell types of the ME
(a) Optimal cluster analysis using the silhouette method (b) tSNE plot of scRNAseq data. Numbers next to cluster IDs indicate the number of cells in the cluster. VLMC = vascular and leptomeningeal cell (c) Violin plot showing UMI count distribution of one defining gene per cluster (variable scales per gene) (d) Heatmap of log2 UMI counts (mRNA transcripts) per cell for the top 5 defining genes per cluster (e) Expression of OPC marker Cspg4 in the ME tSNE plot (f) Expression of NFOL marker Bmp4 in the ME tSNE plot (g) Expression of MOL marker Mal in the ME tSNE plot. Red = high expression, grey = low expression
### Table 3.2 Top defining genes per cluster of ME cell types

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OL = oligodendrocyte, VLMC = vascular and leptomeningeal cell
3.2.3 Three subtypes of OL lineage cells are present in the ME

In order to better define the OL clusters in relation to one another (versus compared to the entire dataset) the 1,107 cells present in cluster 3 (oligodendrocyte 1) and cluster 6 (oligodendrocyte 2) were pooled and re-analyzed. I used silhouette analysis to find that 3 clusters best represent the entire population of OLs in the ME (Figure 3.5a). K-means clustering grouped cells based on their position in the tSNE plot as shown in Figure 3.5b. Again, cellrangerRkit was used to find the defining genes per cluster (Table 3.3, Figure 3.5c). Cells in OL cluster 2 were oligodendrocyte precursor cells (OPCs, express Pdgfra and Cspg4). Cells in cluster 3 – newly formed oligodendrocytes (NFOLs) – expressed genes associated with a transient population of OLs committed to OL lineage that are not yet fully myelinating: Bmp4, Fyn, and Tcf7l2. Cells in cluster 1 were mature oligodendrocytes (MOLs) as they expressed Opalin, Mal, and Klk6 (Table 3.1, Figure 3.5c,e). Plotting the top 10 genes from each cluster against expression levels in all OLs shows that gene expression is distinct in each of the 3 clusters (Figure 3.5d). Expression of genes specific to different OL subtypes indicate that the position of OLs in the tSNE plot is determined by the cell’s developmental ‘age,’ which follows a curve in the plot (Figure 3.5e).
Figure 3.5 Three subtypes of OLs in the ME
(a) Optimal cluster analysis using the silhouette method (b) tSNE plot of scRNAseq data. Numbers next to cluster IDs indicate the number of cells in the cluster.
Figure 3.5 Three subtypes of OLs in the ME, continued
(c) Violin plot showing UMI count distribution of five defining genes per cluster (variable scales per gene) (d) Heatmap of log₂UMI counts (mRNA transcripts) per cell for the top 10 defining genes per cluster (e) tSNE plots of current dataset showing expression of defining OL markers used in other studies. Red = high expression, grey = low expression

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3.2.4 OL subtype distribution in the mouse and human ME

3.2.4.1 Greater density of cells expressing NFOL and MOL genes in the mouse ME

Single-molecule FISH was used to map expression of OL subtype markers in the murine ME and ARC. A probe to \textit{Pdgfra} was used to identify OPCs, \textit{Bmp4} and/or \textit{Tcf7l2} labelled NFOLs, and \textit{Plp1} labelled MOLs. Antibody labelling of SOX10 (a pan-OL marker) was used to confirm that gene labelling was present in OLs. OPCs, identified by coexpression of \textit{Pdgfra} and SOX10, are evenly distributed throughout the ME and ARC, with similar density of cells in both structures (1.23e-04 cells/µm² in the ME versus 1.38e-04 cells/µm² in the ARC, \( p = 0.52 \), paired t-test) (Figure 3.6a, b). SOX10\(^+\) cells labelled with probes to \textit{Bmp4} and/or \textit{Tcf7l2} had a much higher density in the ME than in the ARC (6.54e-05 cells/µm² in the ME versus 2.18e-05 in the ARC, \( p = 0.020 \), Wilcoxon signed rank test) (Figure 3.6a, b). \textit{Plp1}\(^+/\)SOX10\(^+\) cells are concentrated in the upper third of the ME and are significantly more dense in the ME than the ARC (1.54e-04 cells/µm² in the ME versus 6.08e-05 cells/µm² in the ARC, \( p = 0.012 \), Wilcoxon signed rank test) (Figure 3.6a, b).

![Figure 3.6 OL subtype gene expression in the mouse hypothalamus](image)

(a) FISH plus IHC for detection of OL subtype marker gene expression in mouse brain: red = SOX10 (IHC, pan-OL marker), green = \textit{Pdgfra} (OPC marker), grey = \textit{Bmp4} (NFOL marker), blue = \textit{Tcf7l2} (NFOL marker), purple = \textit{Plp1} (MOL marker). Scale bar = 100 µm (b) Measurement of densities of OPCs (\textit{Pdgfra}\(^+/\)SOX10\(^+\)), NFOLs (\textit{Bmp4}\(^+\) and/or \textit{Tcf7l2}\(^+\)/SOX10\(^+\)), and MOLs (\textit{Plp1}\(^+/\)SOX10\(^+\)) in the mouse ME and ARC. \(*\) indicates \( p < 0.05 \), error bars depict mean ± SEM, \( p \) values determined by paired t-test or Wilcoxon signed rank test, ME \( n = 9 \) animals, ARC \( n = 9 \) animals.
3.2.4.1 Proteins of postmitotic OLs have unique distribution in the mouse hypothalamus

The discrete location of MOLs is supported by antibody labelling of myelin basic protein (MBP), a marker of myelin: it also is located in the upper portion of the ME and extends laterally under the base of the ARC (Figure 3.7a, Video 3.1). Antibody labelling with postmitotic OL marker APC (clone CC1) also labels OLs in the upper portion of the ME (Figure 3.7b, Video 3.2 – tanycyte marker vimentin is also present here). Throughout the rest of the brain, APC labelling is found in the subfornical organ and area postrema but not the vascular organ of the lamina terminalis or pituitary gland (Figure 3.6c), indicating that not all CVOs contain postmitotic OLs. Plp1, MBP, and APC labelling all indicate that the ARC is largely devoid of MOLs and myelin (Figure 3.6a-b, Figure 3.7a-b).

Figure 3.7 OL protein expression in the mouse hypothalamus

(a) MBP protein (myelin marker, green) in thin coronal sections (left, scale bar = 100 µm) and in thick cleared ME tissue (right, screenshots from Video 3.1). (b) APC protein (postmitotic OL marker, red) in thin coronal sections (left, scale bar = 100 µm) and APC and vimentin (tanycyte marker, green) in thick cleared ME tissue (right, screenshots from Video 3.2) (c) Schematics of coronal sections containing CVOs (adapted from Paxinos and Franklin, 2001, reprinted with permission from Elsevier). APC labelling (green) in thin coronal sections of circumventricular organs. 4V = fourth ventricle, AP = area postrema, LV = lateral ventricle, SFO = subfornical organ, VOLT = vascular organ of the lamina terminalis. Scale bars = 100 µm
3.2.4.2 Greater density of cells expressing NFOL and MOL genes in the human ME

FISH was also used to compare densities of young (PDGFRA$^+$), intermediate (BCASI$^+$), and mature OLs (PLP1$^+$) in the ME and ARC of human tissue (Figure 3.8a-c) (Fard et al., 2017; Jäkel et al., 2019; Wilson et al., 2006). In the sample, PDGFRA$^+$ cells were similar in density in the ME and ARC (2.16e-05 cells/µm$^2$ in the ME, 1.31e-05 in the ARC). However, as in mice, the densities of presumed NFOLs (BCASI$^+$) and MOLs (PLP1$^+$) were much higher in the ME than in the ARC (2.76e-05 and 6.38e-05 cells/µm$^2$ in the ME versus 7.88e-06 and 1.45e-05 cells/µm$^2$ in the ARC, respectively) (Figure 3.6c).

![Figure 3.8 OL subtype gene expression in the human hypothalamus](image)

(a) DAPI stain in human hypothalamus. OT = optic tract, scale bar = 3.75 mm. (b) Probes to PDGFRA, BCASI, and PLPI label putative OPCs, NFOLs, and MOLs of the human hypothalamus, respectively. Arrows indicate probe labelling (c) Measurement of densities of OPCs (PDGFRA$^+$), NFOLs (BCASI$^+$), and MOLs (PLP1$^+$) in the human ME and ARC. (n = 1 human)
3.3 DISCUSSION

In summary, these studies provided evidence that three subtypes of OLs exist in the mouse ME. Multiple types of OLs may also exist in the human ME. Additionally, results revealed specific spatial organization of OLs in the ME. In this discussion, I will explore my analysis of the scRNAseq data for defining subtypes of OLs, recommend next steps for study of OLs in the human ME, and suggest the possible significance of MOL/myelin distribution in the ME/ARC.

3.3.1 Number of OL clusters

When Marques et al. (2016) examined clusters of OLs, they found five main OL subtypes in their dataset. There are a few possible reasons why my dataset clustered best into three versus more subtypes. These include differences in sample size/location of collected tissue and clustering method used. Contrary to my dataset, the Marques et al. dataset examined OLs from throughout the CNS. Due to the regional diversity in the samples, it is possible the greater number of OL subtypes in that study reflects the variety of OLs throughout the brain. As well, the overall larger OL population in that dataset (5,072 OLs versus 1,107 in my dataset) might have provided larger samples of smaller subdivisions and brought out their differences more clearly. For example, my NFOL population contained 132 cells but has similar gene expression to the committed OPC (COP) and NFOL population described by Marques (Table 3.1). Additionally, there may be two groups of MOLs in my dataset (those that express Man1a versus those that express Pmp22 and Klk6, Figure 3.5e). These two groups could correspond to Marques’ ‘myelin forming OL’ (MFOL) and ‘mature OL’ (MOL) populations, respectively (Table 3.1). More cells in my sample or a different clustering method might have distinguished the two groups better.

The methods used for clustering scRNAseq data are highly variable in the literature (reviewed in van Bruggen et al., 2017). While I used K-means clustering (a centroid clustering approach), Marques et al. used BackSPIN (a biclustering approach), and Campbell et al. used DBSCAN (a density-based clustering method). Other clustering methods that have been used for scRNAseq data include graph based and hierarchical clustering (van Bruggen et al., 2017). Advantages of K-means clustering are the relative ease of implementation and shorter time required to perform the algorithm. However, determination of cluster number is not unsupervised via this method like it is in other methods (Seif, 2019) (I have tried to mitigate this by performing secondary analysis to find the ideal number of K-means clusters).
DBSCAN is able to calculate the ideal number of clusters through use of the algorithm, but it is not as able as other methods to handle clusters of different densities (Seif, 2019). Biclustering methods have been recommended for scRNAseq experiments with relatively small cell numbers and large read ‘depth,’ or number of reads per cell (Menon, 2017). Ultimately, there is no one best way to cluster this type of data and several methods can be tested. Often the researcher’s eye will be a good tell of whether a clustering method fits his or her data well (scikit-learn, 2019; Seif, 2019). For this thesis, I have focused on studying the three clusters of OLs in my dataset, but our lab plans to study potential subclustering of other cell types from this ME dataset in the future.

### 3.3.2 Human ME NFOLs

This work is the first to my knowledge that examines OLs of the human ME. Obtaining sections that span the midline is difficult due to normal processing of donations to brain banks (normally half the brain is fixed and half the brain is frozen). We have a pathologist in our lab who is able to section brains so that the ME remains intact.

As I found through trial and error, antibodies and probes that work for mice often do not work for humans. Compounded with the relative lack of antibodies and probes to NFOLs, studying OL subtypes in human tissue in situ can be difficult. BCAS1 protein has only recently been shown to distinguish NFOLs from NG2+ OPCs and TPPP/p25+ MOLs in human tissue. In the adult human brain, BCAS1 expression remains relatively stable in frontal cortex grey matter throughout life. Additionally, a small proportion of BCAS1+ cells have a myelinating morphology even into old age (Fard et al., 2017). While probes to BCAS1 do label cells well in the ME, double labelling with a marker of OL lineage, such as SOX10 will be necessary to confirm that the gene is expressed solely in OLs.

scRNAseq of human tissue is difficult due to the rapid degradation of tissue during the postmortem interval before collection. However, new technology is advancing the ability to perform high-quality single-cell sequencing by analyzing mRNA of nuclei instead of whole cells (Del-Aguila et al., 2019). A recent study using single-nucleus sequencing of human OLs found several new gene markers defining the intermediate OL population, including APOE and CD74 (Jäkel et al., 2019). To discover appropriate OL markers in our ME-containing tissue, we are currently collaborating with a research group in Cambridge who are optimizing single-nuclei sequencing in the human hypothalamus.
3.3.3 Unique MOL/myelin distribution in the ME and ARC may indicate a non-insulating function of OLs

3.3.3.1 Lack of myelin in the ARC

One of the more interesting findings was the absence of MOLs and myelin in the ARC. By definition this would indicate the greyest of grey matter – a structure so full of neuronal cell bodies that there are no neuronal processes to myelinate. This region is indeed densely packed with neurons: tuberoinfundibular dopamine (TIDA) neurons (control secretion of prolactin), kisspeptin neurons (induce pulsatile release of GnRH), growth hormone releasing neurons (control release of growth hormone), AgRP/NPY neurons (stimulate feeding behavior), POMC/CART neurons, (inhibit feeding behavior), and possibly a few others all have their cell bodies located in the ARC (Dungan et al., 2006; Lyons et al., 2012; Morton et al., 2014; Rodier et al., 1990). Video 3.3 and Figure 3.9 are images from the cleared tissue of NPY-GFP mice, and they show the high density of just NPY-expressing cells in the ARC.

![NPY-GFP](image1)

3.3.3.2 Myelin/OLs are located at the ME-ARC diffusion barrier

Another remarkable finding was the localization of MOLs and myelin at the border between the ME and the ARC. Figures 3.10a-b show labelling of Mbp, a myelin gene, from the Allen ISH Atlas. Compared with the rest of the tissue section, expression of Mbp in the ME is strikingly high. When compared with images how far blood-borne factors are able to enter the hypothalamus (measured via intravenous [iv] injection of Evans blue dye), myelin gene

![Figure 3.9 Neurons are extremely dense in the ARC](image2)
expression seems to demarcate the blood-permeable area of the hypothalamus from the non-permeable area (Mullier et al., 2010) (Figure 3.10b-c).

Some have postulated that tanycytes, whose processes lie between the ME and ARC, adhere to one another to form bundles and prevent blood-borne factors from reaching the ARC and cerebrospinal fluid (Rodriguez et al., 2010) (Figure 3.10d). However, a very recent study has shown that ablation of tanycytes in the ME does not lead to an increase in diffusion of blood-borne factors into the ARC (Yoo et al., 2019b) (Figure 3.10e). This suggests that another cell type is responsible for maintaining the ME-ARC diffusion barrier.

Figure 3.10 ME-ARC diffusion barrier
(a) Section from the Allen ISH Atlas (1.94 mm posterior to Bregma) showing expression of myelin gene Mbp. Black square denotes inset (b). Reprinted from the Allen Institute (a free open resource). (c) Image from Mullier et al., 2010 showing how far blood-borne Evans blue dye (white) is normally able to enter the hypothalamus through the ME, reprinted with permission from John Wiley and Sons (d) Image from Rodriguez et al., 2010 showing their hypothesis that tanycytes act as a barrier preventing diffusion of blood-borne factors into the ARC, reprinted with permission from Elsevier (e) Image from Yoo et al., 2019b showing that tanycyte ablation has no effect on the integrity of the ME-ARC diffusion border, reprinted with permission from the Blackshaw group.
3.3.3.3 NFOLs/MOLs express genes to putative diffusion barrier proteins

Recently, a group has described the presence of perineuronal nets (PNNs) in the border between the ME and the ARC in mice and humans. *Wisteria floribunda* agglutinin (WFA) labels PNN-component chondroitin sulfate chains (Mirzadeh et al., 2019) (Figure 3.11a). PNNs are matrices of extracellular proteins mainly known to surround neuronal cell bodies (Celio 1994). While the exact role of PNNs remains unclear, they may buffer ions or molecules that reach neurons they surround or act to maintain high concentrations of molecules such as growth factors around a neuron (rather than the factors diffusing into the extracellular space) (Brückner et al., 1993; Celio and Blumcke, 1994). In addition to surrounding neuronal cell bodies, PNN-like structures have been found to enmesh nodes of Ranvier, where they may act to pool cations around the unmyelinated axonal segment (Bekku et al., 2009) (Figure 3.11b). As the ME-ARC barrier is dense with myelinated axons, the PNN structures could be regulating diffusion of ions or molecules or protecting exposed axonal segments from potentially cytotoxic circulating factors.

It is currently unclear whether PNNs are responsible for limiting diffusion of blood-borne factors from the ME to the ARC, but it is interesting that the group of cells in my dataset expressing NFOL markers also express the gene to PNN component tenascin-R (*Tnr*) more than any other ME cell type (Figure 3.11c). In chapter 1 I mentioned that tanycyte-mediated changes in capillary fenestration lead to changes in food intake behavior via regulating access of energy availability signals into the ARC. This data shows that OLs may also be involved in this mechanism of energy homeostasis maintenance.
Figure 3.11 PNNs are located at the ME-ARC diffusion border
(a) Image from Mirzadeh et al., 2019 showing WFA-labelled component of PNNs near the ME-ARC border, reprinted with permission from Springer Nature (b) Image from Bekku et al., 2009 showing placement of PNN proteins surrounding a node of Ranvier. Bcan = brevican, Bral1 = brain link protein 1, HA = hyaluronan, Pcan = phosphacan, V2 = veriscan V2, reprinted with permission from John Wiley and Sons (c) Expression of PNN component gene Tnr in the ME scRNAseq dataset. Red = high expression, grey = low expression
CHAPTER 4

OLIGODENDROCYTES OF THE MEDIAN EMINENCE ARE REGULATED BY NUTRITIONAL STIMULI

4.1 INTRODUCTION

To design experiments described in this chapter, I have had to draw upon knowledge from two fields of research: studies of hypothalamic responses to dietary manipulations, and studies of OL adaptive plasticity. In this introduction, I will describe how cell types of the ME respond to nutrient availability, and also how OLs (throughout the brain) respond to stimuli such as learning. Finally, I will bring the fields together with an exploration of how dietary manipulations have been used to stimulate OL adaptive plasticity.

4.1.1 Background

4.1.1.1 Dynamic nutritional regulation of the hypothalamus/ME by dietary manipulations

As described in chapter 1, healthy animals can respond to alterations in nutrient availability with appropriate changes in food intake behavior (Keesey and Powley, 2008). Acute manipulations such as overnight or 24 h fasting then refeeding or more chronic manipulations like high-fat diet (HFD) feeding or long-term calorie restriction [CR]) are examples of environmental changes that alter nutrient availability. As shown in the energy homeostasis homeostat model, however, ‘responses’ to environmental changes do not only refer to the final

1 ‘Nutrient availability’ is equivalent to ‘energy availability signals’ discussed in chapter 1, except here I refer to changes in these signals caused by dietary manipulations.
outcome of behavioral change. Entry of nutrient availability signals into the brain, detection of signals, and changes in cellular activity are also mechanisms of energy homeostasis maintenance.

Fasting-refeeding

Experiments in this chapter use the fast-refeed paradigm to study how ME OLs respond to nutrient availability in a short time frame. Previous studies have examined responses of other ME cell types to this paradigm. I mentioned in chapter 1 that inhibition of tanycytic VEGFA signalling caused a significant decrease in food intake when animals were refed after fasting (Langlet et al., 2013). In those studies, fasting also caused a change in how far blood-borne particles were able to enter the hypothalamus. Normally, peripheral (iv) injection of Evans blue dye only causes labelling in CVOs, including the ME (Figure 3.11c). After a 24 h fast, however, the dye can reach past the ME into the adjacent ARC (Figure 4.1a). The authors suggest that this could allow greater access of energy deficit signal ghrelin into the ARC, where its detection could lead to pathways initiating food seeking behavior (Langlet et al., 2013).

In addition to being used to characterize cell populations, scRNAseq has been used to study cells’ detection of and response to nutrient availability. Chen et al. (2017) compared RNA expression in hypothalamic neurons of ad libitum fed mice with expression in 24 h fasted mice and found heterogeneous responses in these cells. The group even discovered a new type of neuron responsive to nutrient deprivation (mammillary body neurons). This group (and others before them) attributed the detailed resolution of scRNAseq to allow them to detect specific transcriptomic changes in subpopulations of neurons.

Responses of unmyelinated releasing hormone neurons to fasting has also been described. In fasted rats, tanycytes of the ME increase secretion of pyroglutamyl peptidase II, an enzyme that degrades TRH. Fasting also causes decreased pro-TRH expression in the cell bodies of putative TRH neurons, suggesting TRH release into the capillaries of the ME decreases in a state of low nutrient availability (Lazcano et al., 2015; Sánchez et al., 2009) (Figure 4.1a).

High-fat diet/calorie restriction

In contrast to the acute fast-refeed paradigm, more chronic feeding manipulations like HFD feeding or chronic CR have been used to study how the brain adapts to altered feeding behavior over a longer period of time. HFD feeding can increase inflammatory markers and reactive gliosis in the ME/ARC within 1-3 days in mice and rats (these effects preceded weight gain),
and gliosis is present in the hypothalamus of obese animals and humans (Souza et al., 2019; Thaler et al., 2012) (Figure 4.1b). Inflammation is thought to be a response to neuronal injury induced from the HFD. Indeed, HFD can cause apoptosis of neurons of the hypothalamus, disrupting detection of anorexigenic hormones and contributing to weight gain (Moraes et al., 2009). Feeding-induced gliosis is reversible, however: 4 weeks of chow diet is enough time to nearly completely reverse gliosis caused by 16 weeks of HFD feeding (Berkseth et al., 2014). Non-diet-related injuries to the hypothalamus (such as craniopharyngiomas or pituitary macroadenomas) have long been known to cause intractable weight gain. Some of these injuries (in particular craniopharyngiomas) are known to cause marked inflammatory signalling in the hypothalamus (reviewed in Roth, 2015).

Chronic CR has been shown to reduce cell proliferation in the ME. The number of ME cells labelled with proliferation marker BrdU was significantly reduced in calorie restricted mice (mice fed 70% of calorie intake of matched controls) (Lee et al., 2014) (Figure 4.1c). Importantly, close to 80% of constitutively proliferating cells in the hypothalamus are NG2+ (Robins et al., 2013b), suggesting that OPCs of the ME may able to adapt to various states of nutrient availability.
Figure 4.1 Regulation of the ME/ARC by dietary nutrients
(a) Fasting regulates TRH secretion and increases permeability of the area to blood-borne factors (b) Long-term HFD feeding in mice activates gliosis and leads to apoptosis of neurons in the hypothalamus (c) Chronic CR leads to decreased proliferation of cells in the ME
4.1.1.2 Adaptive plasticity of OL populations in the healthy adult CNS

As hypothalamic cell types change in response to nutrient availability, so too do OLs in response to stimuli such as neuronal activity or learning. Adaptive plasticity of OLs can take the form of changing rates of OPC proliferation or OPC differentiation into NFOLs, of increasing or decreasing NFOL survival, and of remodelling myelin structures².

As described in chapter 3, several subtypes of OLs of differing developmental maturity exist in the adult brain. In adult rats, OPCs make up 8-9% of cells in white matter and 2-3% of cells in grey matter (Dawson et al., 2003). OPCs are also present in a similar distribution in the adult human brain (Chang et al., 2000). In the healthy adult brain, OPCs proliferate constitutively, albeit at different rates in different ages or locations. For example, in the corpus callosum (CC), 50% of OPCs divide within 7-10 days. In the motor cortex, 40% of OPCs divide within 21d (Rivers et al., 2008). It is thought the other OPCs remain quiescent until proliferating OPCs are damaged or differentiate into NFOLs, at which time they will begin proliferating to replenish the OPC pool (this process has been shown to take 2-4 days) (Hughes et al., 2013; Xiao et al., 2016) (Figure 4.2b). Recent evidence indicates that the rate or proportion of OPCs that proliferate can change in response to stimuli such as neuronal activity. In mice, increased neuronal activity in the premotor cortex via optogenetic stimulation leads to an increase in OPC proliferation (Gibson et al., 2014).

Differentiation of OPCs into NFOLs can be an adaptive response to a learning stimulus. Learning a new motor task can cause rapid (sub-3h) differentiation of OPCs into NFOLs in the CC and motor cortex of mice as measured by an increase in EdU+/PDGFRα- cells (putative OPCs that have divided but are no longer immature) and Enpp6+ cells (5-ethyl-2'-deoxyuridine/EdU is a proliferation marker and Enpp6 is a recently-discovered NFOL marker) (McKenzie et al., 2014; Xiao et al., 2016; Zhang et al., 2014). Optogenetic stimulation of neurons in the premotor cortex can also cause an increase in OPC differentiation, as measured by an increase in the number of EdU+ cells also positive for perinuclear Olig1 expression (Olig1 is nuclear in OPCs and perinuclear in more mature OLs) (Gibson et al., 2014) (Figure 4.2a).

Another important plastic OL process is survival. While OPCs in the murine somatosensory cortex only occasionally die, close to 80% of NFOLs undergo programmed cell death in this region within 2 days of differentiating under normal conditions (Hughes et al., 2018). If the postmitotic OLs survive and begin actively myelinating (a process that occurs within 3 weeks in the mouse cortex), they are incredibly stable and have been found to last

² Mechanisms of myelin remodelling will be discussed in more detail in chapter 6.
over a year in the cortex and certain white matter tracts (Hughes et al., 2018; Tripathi et al., 2017). In development, survival and incorporation of postmitotic OLs into neuronal circuits is dependent on access of the cells to a limited amount of platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF). These growth factors likely act by suppressing constitutive death programs in the OLs (Barres et al., 1992). Study of growth factor-mediated survival of postmitotic OLs in the adult brain has not been studied, however it is known that incorporation of these cells into circuits is promoted by the presence of nearby active neuronal processes (Figure 4.2c). In the CC of young mice, axons with reduced neuronal activity (via selective overexpression of potassium channel Kir2.1) show decreased myelin protein expression (Mitew et al., 2018) (Figure 4.2d). Besides decreased neuronal activity, OPC hypoxia-inducible factor 1α/2α (HIF1/2α) signalling and neuronal expression of junction adhesion molecule 2 (JAM2) inhibit myelination, and their downregulation may encourage myelination of particular neuronal processes over others (Redmond et al., 2016; Yuen et al., 2015).
Chapter 4 – Oligodendrocytes of the median eminence are regulated by nutritional stimuli

Figure 4.2 Forms of OL plasticity in mice

(a) OPCs can rapidly differentiate into NFOLs (b) After differentiation, OPCs can increase proliferation to repopulate the OPC pool (c) Axonal activity increases survival of NFOLs (d) Neuronal activity promotes myelination
4.1.1.3 Adaptive plasticity of OLs in adults in response to dietary manipulations

In a manner similar to learning or neuronal activity, certain dietary manipulations can elicit adaptive plasticity in OLs. The effects of a HFD, a ketogenic diet, chronic CR, and intermittent fasting (IF) on OLs have been reported, and they have mainly been researched in the context of understanding myelin-related pathologies in certain diseases, or for providing complementary treatments to interventions for demyelinating diseases or CNS injuries (Table 4.1).

Chronic feeding of a HFD (usually greater than 60% calories from fat) is often used to induce obesity and/or the mouse equivalent of type 2 diabetes. Mice with diet-induced obesity (DIO) have accelerated OPC differentiation but impaired maturation of NFOLs. These mice also have decreased myelin thickness and shorter paranodes in the CC (Xiao et al., 2018). Another study found disruption of myelin (loss of compaction) around axons of the lateral hypothalamus when animals were fed a HFD for 6 months, but a return to regular myelination when animals were returned to normal chow diet (Huang et al., 2019).

Effects of ketogenic diets (high-fat, low-carbohydrate) on OLs/white matter have been studied as investigation of the diet for treatment of Pelizaeus-Merzbacher disease (a type of leukodystrophy) and inborn errors of metabolism (inability to metabolize certain nutrients) caused by aspartate-glutamate carrier deficiency. Mouse models of Pelizaeus-Merzbacher disease have very thin myelin sheaths. When fed a ketogenic diet for 10 weeks, the mice had significantly increased myelin thickness for the size of the axon (smaller g-ratio) (Stumpf et al., 2019). A ketogenic diet has also been shown to be useful for a patient with an aspartate-glutamate carrier deficiency. After 6 months of dietary treatment, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy provided evidence of increased white matter volume in the patient (Dahlin et al., 2015).

Chronic CR diets have long been touted for their purported effects of extending lifespan and protecting against age-related diseases (Spindler, 2010). As mentioned above, CR reduces proliferation of putative OPCs in the ME (Lee et al., 2014; Robins et al., 2013b). It is thought that the shift from development/growth programs to cellular maintenance programs underpin the pro-longevity effects of CR (reviewed in Nikolai et al., 2015). In addition to prolonging health, CR can also protect against disease and injury. Mice fed 60% of their normal calorie intake had significantly less demyelination after induction of experimental autoimmune encephalomyelitis (EAE, a mouse model of multiple sclerosis) than ad libitum fed controls (Piccio et al., 2008). CR can also be useful for priming recovery: mice fed 70% of their normal food intake before transient focal ischemia induced by middle cerebral artery occlusion (a...
stroke model) were protected against much of the damage caused by the ischemia and had nearly the same amount of MBP protein in the CC as sham-treated animals (*ad libitum*-fed animals had less than 50% of sham-treated animals) (Zhang et al., 2019).

IF is currently the subject of a lot of study, as it is thought to recreate many of the benefits of chronic CR without requiring a severe lifestyle change (reviewed in Johnstone, 2015). In mice with EAE fed an IF diet (repeated cycles of 3 days of very low calorie and protein intake and 4 days of *ad libitum* feeding), OPC differentiation in the spinal cord was increased (Choi et al., 2016). Another study found that 4 weeks of IF was able to increase the amount of MBP labelling in the spinal cords of mice with EAE (Cignarella et al., 2018). The fast-refeed studies described in this thesis attempt to capture the response of OLs to the rapid shift from nutrient deprivation to nutrient abundance, such as occur in IF.

### Table 4.1 Effects of dietary manipulations on OLs

<table>
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<tr>
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<th>Diet</th>
<th>Model/patient</th>
<th>OL adaptive response</th>
<th>Myelin/ white matter</th>
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<td></td>
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<td>OPC differentiation</td>
</tr>
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<td></td>
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</tr>
<tr>
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<td>accelerated</td>
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<tr>
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<td>HFD</td>
<td>mouse DIO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stumpf et al., 2019</td>
<td>ketogenic</td>
<td>mouse Pelizaeus-Merzbacher disease</td>
<td></td>
<td></td>
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<tr>
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<td>human aspartate-glutamate carrier deficiency</td>
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</tr>
<tr>
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<td>mouse EAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhang et al., 2019</td>
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<td>IF</td>
<td>mouse EAE</td>
<td></td>
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</tr>
</tbody>
</table>

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4.1.2 Hypothesis
Knowing that 1) cell types of the ME are responsive to dietary manipulations and 2) OLs can respond to stimuli including dietary manipulations, indicates that OLs of the ME may also be responsive to changes in nutrient availability. In this chapter, I tested the hypothesis that ME OLs of healthy animals respond to dietary manipulation of fasting and refeeding with altered OPC proliferation or differentiation.

4.1.3 Chapter 4 experimental objectives and design
1. I analyzed OL gene expression to find differentially expressed genes (DEGs) between cells from overnight-fasted animals and those fasted then refed 1 h. This analysis is from the scRNAseq dataset described in chapter 3.
2. I used pathway analysis of DEGs to predict pathways that are activated or inhibited by refeeding.
3. Analysis of FISH labelling was used to detect differences in OL subtype gene expression between fasting and refeeding. These are the same tissues used to describe distribution of OL subtype markers in chapter 3.
4. I used BrdU to label cells that divided during a fast-refeed experiment in order to identify if ME cells increase proliferation during refeeding.
4.2 Results

4.2.1 All cell types of the ME are regulated by fasting and refeeding

After using scRNAseq to characterize the population of ME cells (chapter 3), I used the dataset to investigate the transcriptomic changes in these populations resulting from feeding for 1 h after fasting overnight. As expected, cells from the fasted and refed samples were generally well-mixed across all clusters, indicating that nutritional state did not change clustering of cells (Figure 4.3a). The edgeR package in R (McCarthy et al., 2012; Robinson et al., 2010) was used to find genes in each of the 9 clusters that were significantly differentially expressed between fasting and refeeding ($p < 0.05$, FDR $< 0.25$) (Table 4.2, Figure 4.3b). Tanycytes, astrocytes, and VLMCs were the most nutritionally responsive cell types, whereas neurons, ependymocytes, and endothelial cells had relatively few DEGs between the fasted and refed conditions. A total of 495 genes were differentially expressed in OLs, revealing a previously unknown rapid nutritional regulation of this cell type. The rest of this thesis will focus on data from the OL populations of the ME.

Figure 4.3 Nutritional regulation of ME cell types

(a) Experimental condition of cells mapped on tSNE plot (n = 5 fasted, n = 5 refed) (b) Number of genes significantly different between fasted and refed conditions per ME cluster ($p < 0.05$, adjusted for multiple testing, FDR $< 0.25$, quasi-likelihood F-test)
Table 4.2 Top differentially expressed genes in ME cell types (fasted versus refed)

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Log2FC</th>
<th>P-value</th>
<th>FDR</th>
<th>Expression (Refed)</th>
<th>Expression (Fasted)</th>
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<td>12.0</td>
<td>6.0</td>
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<td>Tmem66a</td>
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<td>0.52</td>
<td>0.006</td>
<td>0.012</td>
<td>13.0</td>
<td>6.0</td>
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<td>Ptn</td>
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<td>0.012</td>
<td>16.0</td>
<td>6.0</td>
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</table>

Abbreviations used: logFC = log fold-change, FDR = false discovery rate, VLMC = vascular and leptomeningeal cell. All genes included here have a p value below 0.05, FDR value below 0.25 and are ranked by logFC.
4.2.2 Refeeding stimulates transcriptional changes in OLs

4.2.2.1 Genes changed in refeeding

Focusing on the reclustered OLs, I again used edgeR to identify DEGs for each of the clusters ($p < 0.05$ and FDR < 0.25). The top 30 DEGs in OPCs (based on lowest $p$ values) include genes that regulate OPC differentiation to NFOLs ($Kpna2$, $Sox4$, $Olig1$, $Olig2$, $F3$, $Id2$) (Braccioli et al., 2018; Dai et al., 2015; Havrda et al., 2014; Hu et al., 2003; Laitman et al., 2017; Liu et al., 2007), genes important for creating myelin ($Cldn11$, $Mal$, $Mobp$, $Hspa5$, $Traf4$) (Blaise et al., 2012; Holz and Schwab, 1997; Jahn et al., 2009; Maheras et al., 2018; Schaeren-Wiemers et al., 1995), and one gene involved in mTOR signalling ($Mlst8$) (Wood et al., 2013).

In NFOLs, DEGs include genes associated with OL differentiation ($Tcf7l2$, $Sox8$) (Stolt et al., 2004; Weng et al., 2017), lipid/cholesterol synthesis or transport ($Fdft1$, $Hmgcs1$, $Apoe$) (Carlin et al., 2000; Mathews et al., 2014; Voskuhl et al., 2019), or formation of myelin ($Hspa5$, $Tcf7l2$, $Jam3$, $Hspa8$, $Hsp90aa1$, $Hsp90ab1$) (Jahn et al., 2009; Weng et al., 2017).

Most top MOL DEGs are genes known to be involved in myelin formation ($Hspa1a$, $Hsp90aa1$, $Hsp90ab1$, $Hspa8$, $Cryab$, $Kcnal$, Nkx6-2) (Jahn et al., 2009; Southwood et al., 2004), although a few genes are important for OL differentiation ($Marcks$, $Neat1$) (Katsel et al., 2019; Zhang et al., 2017) and the mTOR signalling pathway ($Ddit4$, $Sgk1$) (Brugarolas et al., 2004; Laplante and Sabatini, 2009) (Figure 4.4, Table 4.3).
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Figure 4.4 Gene expression changes in refeeding
Top 30 DEGs between fasted and refed conditions for each OL cluster ($p < 0.05$ and FDR < 0.25, $-\log_{10}(0.05) = 1.3$)
In addition to examining individual DEGs, I used pathway analysis based on DEGs to predict which signalling pathways or cellular functions were changed between conditions. Consistent with the DEG analysis, top pathways included processes involved in cell cycle progression and differentiation lipid/cholesterol biosynthesis and myelination, and hormonal and growth signalling (Figure 4.5, Table 4.4).

### 4.2.2 Pathways changed in refeeding

In order to examine individual pathways, I used pathway analysis based on DEGs to predict which signalling pathways or cellular functions were changed between conditions. Consistent with the DEG analysis, top pathways included processes involved in cell cycle progression and differentiation lipid/cholesterol biosynthesis and myelination, and hormonal and growth signalling (Figure 4.5, Table 4.4).
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Figure 4.5 Signalling pathway and cellular function changes in refeeding
Radius indicates number of DEGs in current dataset that overlap with IPA gene set, yellow = IPA canonical signaling pathway, green = IPA cellular function, yellow horizontal line denotes statistical significance threshold (-log(adj p-value) of 1.3)
### Table 4. Pathways significantly changed between fasting and refeeding in OLs

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**Pathways shown are selected for correct tissue and do not include disease pathways. Pathways have adj.p_val (adjusted p-value) below 0.05.**
4.2.2.3 Upstream regulators involved in refeeding-mediated transcriptional changes
Pathway analysis also allowed identification of predicted upstream regulators of DEGs, i.e., transcriptional regulators that account for expression changes between fasting and refeeding. Top upstream regulators included Tcf7l2 and Myrf in all clusters and Mtor in OPCs and NFOLS (Figure 4.6, Table 4.5).
Chapter 4 – Oligodendrocytes of the median eminence are regulated by nutritional stimuli

Figure 4.6 Regulator genes responsible for transcriptional changes
Genes downstream from top upstream regulators Tcf7l2, Mtor, and Myrf. Figure created with IPA
Table 4.5 Upstream regulators of differentially expressed genes

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All upstream regulators listed have a $p$ value below 0.05
4.2.3 Evidence of increased OPC differentiation, not proliferation, with refeeding

4.2.3.1 NFOL/MOL gene expression is increased with refeeding

As mentioned, several genes and pathways related to OL differentiation are significantly changed with refeeding. Additionally, there is a general downregulation of genes associated with OPCs (Cspg4, Cspg5) and an upregulation of genes associated with NFOLs (Fyn, Tcf7l2) and MOLs (Mog, Plp1, Opalin, Mag) (Figure 4.7a).

To validate if the increase in OPC differentiation/OL maturation genes is found in situ, single-molecule FISH was used in tissues of animals fasted overnight or fasted and refed 1 h. Single-molecule FISH allows for detection of individual RNA molecules, or ‘spots’ (Wang et al., 2012). There was a significant increase in the number of pan-OL marker SOX10+ cells containing Bmp4 spots (7.17 cells fasted versus 13.94 refed, \( p = 0.048 \), Student’s t-test) and a not-quite significant increase in SOX10+ cells positive for Plp1 (14.00 cells fasted versus 22.79 refed, \( p = 0.09 \), Mann-Whitney U test) (Figure 4.7b-c). Additionally, there was a slight increase in the number of Bmp4, Tcf7l2, and Plp1 spots in SOX10+ cells. There were 6.53 Bmp4 spots per cell in the fasted condition versus 9.06 refed (\( p = 0.08 \), Student’s t-test); 6.57 Tcf7l2 spots fasted versus 9.06 refed (\( p = 0.15 \), Student’s t-test); and 5.98 Plp1 spots fasted versus 8.00 refed (\( p = 0.17 \), Mann-Whitney U test) (Figure 4.7b, d).
Figure 4.7 Increase in NFOL/MOL gene expression with 1 h refeed

(a) Log₂FC of genes ($p < 0.05$ and FDR < 0.25) known to be associated with specific stages of OLs. (b) Multiplex single molecule FISH labeling OL markers in the ME: red 1 = SOX10 (pan-OL marker), green 1 = Pdgfra (OPC marker), green 2 = Bmp4 (NFOL marker), yellow = Tcf7l2 (NFOL marker), red 2 = Plp1 (MOL marker). (c) Number of cells expressing markers in FISH experiment (d) Number of RNA molecules (‘spots’) per cell. (c-d) * indicates $p < 0.05$, error bars indicate mean ± SEM, p values determined by Student’s t-test, Welch’s t-test, or Mann-Whitney U test, fasted n = 6 animals, refed n = 3 animals
4.2.3.2 *Proliferation in the ME is unchanged with refeeding*

Finally, I performed pilot studies to test if cell division changed between the fasted and refeeding states. In this experiment, mice exposed to fasting/refeeding were treated with ip BrdU to label cells that divided during the paradigm. In this initial experiment, I was able to find substantial BrdU labelling in the ME within 24h (Figure 4.8a) but was not able to detect a difference in the number of cells labelled with BrdU between conditions (Figure 4.8b). To assess if OPC number changes between fasting and refeeding, I also labelled sections for PDGFRα. There was no difference in the number of PDGFRα+ cells or the number of colabelled BrdU+/PDGFRα+ cells (presumably this would label actively proliferating OPCs) (Figure 4.8b).

![Figure 4.8](image)

**Figure 4.8 No change in proliferation of cells in ME with 1 h refeed**

(a) Schematic of BrdU administration protocol (b) Cells that divided during fast/refeed experiment are labelled with BrdU (green), OPCs are labelled with PDGFRα (white) (c) Cell count of BrdU+, PDGFRα+, or colabelled cells in fasted and refed conditions. Error bars indicate mean ± SEM, p values determined by Student’s t-tests, fasted n = 8 animals, refed n = 11 animals
4.3 DISCUSSION

To recapitulate, these studies show differential expression of genes and pathways between the fasted and refed states and suggest that a rapid increase in OPC differentiation but not proliferation occurs in response to a 1 h refeeding period after a fast. In this discussion, I will highlight the usefulness of this scRNAseq dataset, explore whether fasting or refeeding acts as a stimulus to alter OPC differentiation, and note the limitations of experiments in this chapter.

4.3.1 scRNAseq dataset

The dataset produced from the scRNAseq experiment is publicly available, and represents a unique resource for those in the field of neural detection of food intake. The current scRNAseq dataset will be a useful resource for those wanting to study cells within the ME specifically, or for those interested in properties of cells that lie outside the BBB. Also, other studies have compared cells from fasted mice with those of ad libitum fed mice. Refeeding, as opposed to static ad libitum feeding, may be an important facet of increased OPC differentiation (see section 4.3.3); therefore, this dataset also represents a resource for those interested in the effects of that particular dietary manipulation.

Other scRNAseq studies examining differential gene expression in hypothalamic cells in response to dietary manipulations only reported results for neurons (although their entire datasets are publicly available). It would be useful to study OLs of their datasets to compare results and also study effects of fasting versus ad libitum feeding and HFD on the cell type. I, on the other hand, have only reported expression changes in OLs in detail, but our lab plans to analyze the data for other cell types soon. As shown in Figure 4.3b, astrocytes and tanyctyes have even greater numbers of DEGs than OLs, which was a surprising finding to our group – we expected the greatest change to occur in neurons.

4.3.2 Measuring OPC differentiation with BrdU labelling

While RNA sequencing and FISH labelling of OL marker probes suggest an increase in OL differentiation, the BrdU labelling study was only able to provide evidence about overall changes in proliferation and the number of cells labelled with an antibody to PDGFRα. Neither BrdU nor PDGFRα is a specific marker of OPCs. Robins et al. showed that 20% of BrdU+ cells were NG2+, meaning that overall changes in BrdU labelling could be due to alterations of division in other cell types. PDGFRα is known for labelling OPCs, but our dataset and the
Barres lab Brain RNA-Seq tool show that *Pdgfra* can be expressed in vascular and leptomeningeal cells and neurons (Zhang et al., 2014) (Figure 4.9a-b).

**Figure 4.9 Pdgfra expression in other cell types**
(a) *Pdgfra* expression in the scRNAseq dataset described in this thesis. Red = high expression, grey = low expression, VLMC = vascular and leptomeningeal cells (b) *Pdgfra* expression data from the Barres lab Brain RNA-Seq tool (Zhang et al., 2014).

To determine if OPC differentiation is increasing, addition of a pan-OL marker such as SOX10 is necessary to distinguish between OPCs (SOX10⁺/BrdU⁺ or / PDGFRα⁺ cells) and NFOLs (SOX10⁺/BrdU⁻/ PDGFRα⁻ cells). Our lab has since repeated the BrdU labelling fast-refeed experiment, adding SOX10 labelling to tissues. We found that the number of NFOLs is significantly increased within 1 h of refeeding (Kohnke et al., 2019) (Figure 4.10a-b). Along with finding no change in overall proliferation (Figure 4.8b), this indicates that OPC differentiation, not proliferation, is taking place during the refeed period. This is in line with previously published work that found OPC differentiation takes place rapidly (within 3 h during motor learning), and a subsequent wave of OPC proliferation takes place a few days later to repopulate the OPC pool (Xiao et al., 2016) (Figure 4.1a-b). Additionally, as there is no difference between the number of SOX10⁺ cells between conditions (Figure 4.7c), it seems the increase in NFOLs in this short time frame is not due to increased survival of cells. However, labelling tissues from fasted and refed mice with cell death markers will allow detection of decreased cell death, if present. Measuring the number of NFOLs and also cells labelled with death markers at various timepoints after refeeding will also allow us to detect if the increase in NFOLs is transient or long-lasting.
Figure 4.10 BrdU labelling shows increased OPC differentiation in the ME with fast-refeed

Images from Kohnke et al., 2019 (a) Representative images of SOX10 (red), PDGFRα (white), and BrdU (green) labelling in animals fasted overnight and those fasted then refed 1 h. White squares indicate insets to the right, arrows indicate NFOLs (b) Refed animals had an increased number of NFOLs (SOX10⁺/BrdU⁺/PDGFRα⁻ cells) with a 1 h refeed. There was also a decreased number of proliferating OPCs (SOX10⁺/BrdU⁺/PDGFRα⁺ cells). Non-proliferating OPCs are SOX10⁺/BrdU⁺/PDGFRα⁻ cells and postmitotic OLs are SOX10⁺/BrdU⁺/PDGFRα⁻ cells. * indicates $p < 0.05$, ** indicates $p < 0.01$, error bars indicate mean ± SEM. This work was conducted by Sophie Buller from the Bluuet lab

4.3.3 What is the stimulus?

It is worth questioning whether fasting or refeeding acts as the stimulus of the transcriptional changes shown in this chapter. I have presented the data to indicate that NFOL/MOL genes are upregulated in refeeding, but they could also indicate that those genes are downregulated in fasting. Indeed, other groups examining differential gene expression in hypothalamic neurons studied fasting versus ad libitum feeding and considered fasting the stimulus. Ideally, I would repeat these experiments and include ad libitum fed mice in order to judge whether fasting or refeeding has a greater effect on OLs from their normal (non-adapting/responding) state. Interestingly, in a study examining the effects of IF on OLs, cycling between states of nutrient deprivation and availability has been shown to promote OPC differentiation in an EAE model
better than a control diet (ad libitum feeding) or chronic ketogenic diet, suggesting that the combination of fasting and refeeding increases differentiation versus normal feeding (Choi et al., 2016).

I propose that fasting stalls the constitutive differentiation of OPCs in the ME and that sudden nutrient availability instigates a rebound increased differentiation of OPCs. The mechanisms by which OPCs may detect nutrient availability to initiate differentiation will be discussed in chapter 5.
Chapter 4 – Oligodendrocytes of the median eminence are regulated by nutritional stimuli
CHAPTER 5

MTOR SIGNALLING AS A CANDIDATE NUTRIENT DETECTION SYSTEM IN MEDIAN EMINENCE OLIGODENDROCYTES

5.1 INTRODUCTION

Historically, our lab has studied how the brain detects protein/amino acid (AA) intake. AAs, especially leucine, potently activate the mammalian target of rapamycin (mTOR) signalling pathway. It was through my study of this pathway that I discovered OLs use mTOR signalling in the ME, and thus began the work described in this thesis. mTOR signalling is well-known as a sensor of nutrient availability in the brain, particularly the hypothalamus. Initially surprising to me, there is also a wealth of literature about how mTOR is necessary for OPC/OL differentiation and myelination. In this introduction, I will describe what is known about the roles of mTOR signalling in both hypothalamic nutrient detection and OL maturation, then describe signalling upstream and downstream from mTOR that suggest the pathway may be involved in OPC detection of dietary nutrients leading to increased differentiation.

5.1.1 Background

5.1.1.1 The role of mTOR signalling in hypothalamic nutrient availability sensing

The mammalian target of rapamycin (mTOR) signalling pathway is mainly known for its role in cellular survival, proliferation, and growth (Laplante and Sabatini, 2009). Signalling via

1 In this chapter I use ‘OL maturation’ as a blanket term to refer to a cell’s developmental progression. The term includes OPC differentiation and initiation of myelination programs.
mTOR can occur from the mTOR Complex 1 (mTORC1, contains raptor protein and proline-rich Akt substrate of 40 kilodaltons [PRAS40]) or Complex 2 (mTORC2, contains rictor protein, protein observed with rictor [protor], and mammalian stress-activated protein kinase interacting protein 1 [mSIN1]), resulting in the activation of distinct transcriptional programmes: mTORC1 promotes ribosome synthesis and protein translation while mTORC2 enables lipid synthesis, and cytoskeletal organization (Laplante and Sabatini, 2009; Wood et al., 2013) (Figure 5.1d).

In the hypothalamus, mTORC1 signalling has been studied for its role in regulating food intake and bodyweight. Fasted animals display decreased active/phosphorylated mTOR protein (pmTOR) and decreased phosphorylation of S6 kinase 1 and S6 proteins (pS6K1 or pS6, Figure 5.1c) in the ME/ARC (Cota et al., 2006). Conversely, ingestion of a meal after fasting or intracerebroventricular (icv) administration of branched-chain AA leucine potently increases phosphorylation of S6K1 in the hypothalamus (Blouet et al., 2008; Cota et al., 2006).

Importantly, activation of mTORC1 signalling has effects on food intake and bodyweight maintenance. Injection of an adenovirus encoding constitutively active (CA) S6K1 into the ME/ARC causes reduced food intake of normal chow and HFD and reduces bodyweight gain on the two diets. Injection of an adenovirus encoding a dominant-negative (DN) form of S6K1 increases food intake and bodyweight in the period directly following the injection and increases food intake during refeeding following a fast (Blouet et al., 2008) (Figure 5.2). Sensitivity of mTORC1 activation in response to nutrient availability can seemingly decrease with chronic overactivation, for example in response to HFD. HFD feeding for a few weeks reduces hypothalamic mTORC1 signalling and may contribute to diet-induced obesity (DIO) hyperphagia and impaired glycemic control (Cota et al., 2008; Ono et al., 2008).

Antibody labelling has suggested that the cells using mTORC1 signalling to regulate food intake and bodyweight are the anorectic POMC/CART and orexigenic AgRP/NPY neurons of the ARC (Cota et al., 2006). However, another study has found that inactivation of S6K1 in either POMC/CART neurons or AgRP/NPY neurons was not sufficient to mediate changes in food intake and bodyweight (Smith et al., 2015). Thus, the cell type(s) responsible for mTOR signalling’s effects on energy homeostasis are not established.
Figure 5.1 mTOR signalling pathway
Schematic of general mTOR signalling pathway in cells (figure inspired by Figlia et al., 2018; Laplante and Sabatini, 2009; Wood et al., 2013). (a) GluRs, IGF1Rs, and transporters can enable a cell to detect nutrient availability (b) The PI3K-Akt and Erk1/2 pathways lie upstream of mTOR signalling (c) mTOR can signal via 2 different complexes (d) Signalling downstream of mTOR can initiate OPC differentiation and initiate protein and lipid synthesis and cytoskeletal organization. Proteins in red are often studied when examining mTOR signalling in the hypothalamus, proteins in blue are to genes differentially expressed in OLs between fasting and refeeding (see Figure 5.5a), and proteins in green have been perturbed in the study of mTOR signalling in OLs (Table 5.1).
5.1.1.2 The role of mTOR signalling in OPC differentiation and OL myelination

The role of mTOR signalling in OLs is well-described. Manipulation of several components of the pathway, including its upstream regulators, can alter OPC proliferation, OPC differentiation, OL survival, and myelination in vitro and in vivo. The two main upstream regulators of the mTOR signalling pathway are the PI3K-Akt pathway and the Erk1/2 pathway (Table 5.1, Figure 5.1b).

In general, inhibiting mTOR signalling (via loss-of-function of Pi3k, Mek, Erk1/2, Rheb1, or members of the mTOR complexes) leads to reduced OPC differentiation and myelin deficits (Bercury et al., 2014; Dai et al., 2014; Grier et al., 2017; Guardiola-Diaz et al., 2012; Lebrun-Julien et al., 2014; Tyler et al., 2009, 2011; Wahl et al., 2014; Zou et al., 2011, 2014) (Table 5.1). Increased mTOR signalling activity through loss-of-function of Pten or gain-of-function of Akt, Mek, or Rheb1 mainly leads to increased myelin thickness or myelin gene/protein expression (Flores et al., 2008; Goebbels et al., 2010; González-Fernández et al., 2018; Harrington et al., 2010; Ishii et al., 2016; Narayanan et al., 2009; Zou et al., 2011) (Table 5.1). Counterintuitively, even though loss-of-function of the Tsc complex proteins has the predicted effect of increasing mTOR activity, it has mainly been shown to cause decreased OPC differentiation and reduced myelination (Carson et al., 2015; González-Fernández et al., 2018; Jiang et al., 2016; Lebrun-Julien et al., 2014; McLane et al., 2017) (Table 5.1, Figure

Figure 5.2 mTOR signalling in the hypothalamus

Schematic of short-term effects of mTORC1 activation or inhibition. The cell type(s) that uses mTORC1 activity (second panel, red cells) to mediate downstream effects is unknown.
5.1b-c). Although the role of different components of mTOR signalling in OPC proliferation and differentiation and OL survival and myelination have been well-studied, none of these groups reported on the effects of these manipulations in the hypothalamus.

Downstream of mTOR, OPC differentiation is promoted via inhibition of Wnt signalling to allow activity of transcription factors Olig1 and Olig2 (both necessary for differentiation) — Wnt activation of the Frizzled receptor normally allows translocation of β-catenin to the nucleus where it inhibits Olig1 and Olig2 (Dai et al., 2015; Liu et al., 2007; MacDonald, et al., 2010; Ye et al., 2010) (Figure 5.1d). High mTOR activity is also sufficient to inhibit Wnt signalling by limiting the concentration of Frizzled receptors in the cell membrane (Zeng, et al., 2018). The involvement of the canonical Wnt pathway in preventing OPC differentiation is shown by deficits in differentiation in mice with constitutively-active β-catenin or mice lacking Wnt inhibitor Axin2 (Fancy et al., 2011; Feigenson et al., 2009). In vitro, administration of rapamycin increased Id2 and Tcf7l2 (also known as Tcf4) expression, suggesting mTOR activity normally inhibits expression of these genes/protein (Tyler et al., 2009, 2011). Additionally, deletion of Yy1 in vitro and in vivo increases Tcf4 expression and prevents OPC differentiation (He et al., 2007) (Figure 5.1d).
Table 5.1 Effects of mTOR protein perturbation on OL maturation

<table>
<thead>
<tr>
<th>Gene/protein</th>
<th>Study</th>
<th>Model</th>
<th>OPC proliferation</th>
<th>OPC differentiation</th>
<th>OL survival</th>
<th>Myelin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K</td>
<td>Dai et al., 2014</td>
<td>OPC in vitro (block PI3K with LY294002)</td>
<td></td>
<td></td>
<td></td>
<td>decreased Mbp</td>
</tr>
<tr>
<td>Pten</td>
<td>Gonzalez-Fernandez et al., 2018</td>
<td>Pdgfra-CreER; Pten&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>increased</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Harrington et al., 2010</td>
<td>Olig2-tva-Cre; Pten&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>reduced in lysolecithin</td>
<td></td>
<td>increased thickness</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Goebbels et al., 2010</td>
<td>Cnp-Cre; Pten&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>increased thickness</td>
<td></td>
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<tr>
<td>Akt</td>
<td>Narayanan et al., 2009</td>
<td>Plp-Akt-DD mice (CA Akt)</td>
<td>unchanged</td>
<td>unchanged</td>
<td>increased myelin proteins, thickness</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flores et al., 2008</td>
<td>Plp-Akt-DD9 (CA Akt)</td>
<td>unchanged</td>
<td>unchanged</td>
<td>increased myelin proteins, thickness</td>
<td></td>
</tr>
<tr>
<td>Mek</td>
<td>Ishii et al., 2016</td>
<td>Plp-CreER,Mek1DD (CA MEK)</td>
<td></td>
<td>increased thickness</td>
<td>increased Mbp, Plp1, increased thickness</td>
<td></td>
</tr>
<tr>
<td>Erk1/2</td>
<td>Guardiola-Diaz et al., 2012</td>
<td>OL culture</td>
<td>decreased</td>
<td>unchanged</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Dai et al., 2014</td>
<td>OPC in vitro (block Erk1/2 with U0126)</td>
<td></td>
<td></td>
<td></td>
<td>decreased Mbp</td>
</tr>
<tr>
<td>TSC1/2</td>
<td>Gonzalez-Fernandez et al., 2018</td>
<td>Pdgfra-CreER;Tsc1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>unchanged</td>
<td>decreased</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>McLane et al., 2017</td>
<td>Ngl2-CreER&lt;sup&gt;22&lt;/sup&gt;;Tsc1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>unchanged</td>
<td>unchanged</td>
<td>increased Mbp and Mog, thicker sheath</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jiang et al., 2016</td>
<td>Olig1-Cre or Cnp-Cre; Tsc1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>decreased</td>
<td>decreased Mbp, Plp1, thinner myelin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carson et al., 2015</td>
<td>Olig2-Tsc2 KO</td>
<td>decreased</td>
<td></td>
<td>decreased thickness, reduced Mbp protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lebrun-Julien et al., 2014</td>
<td>Cnp-Cre;Tsc1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>decreased Mog, Mag, Plp1</td>
<td></td>
</tr>
<tr>
<td>Rheb1</td>
<td>Zou et al., 2014</td>
<td>Olig1-Cre;Rheb1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>decreased</td>
<td>reduced myelinated axons, decreased Mbp, Cnp, Mog</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Olig2-Cre;Rheb1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td>increased</td>
<td>decreased</td>
<td>reduced myelinated axons, decreased Mbp, Cnp, Mog</td>
<td></td>
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<tr>
<td></td>
<td>Cnp-Cre;Rheb1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td>unchanged</td>
<td>decreased</td>
<td>decreased Plp1</td>
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<tr>
<td></td>
<td>Tmem10-Cre;Rheb1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td>unchanged</td>
<td>unchanged</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Zou et al., 2011</td>
<td>Nes-Cre;Rheb1&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>decreased Mbp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nes-Cre;Rheb1k (increased Rheb1 activity)</td>
<td></td>
<td></td>
<td></td>
<td>precocious myelination</td>
<td></td>
</tr>
<tr>
<td>mTOR - unspecified or both</td>
<td>Zou et al., 2014</td>
<td>Olig1-Cre;mTOR&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>decreased</td>
<td>decreased Mbp, Tmem10</td>
<td></td>
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<tr>
<td></td>
<td>Dai et al., 2014</td>
<td>OPC in vitro (block mTORC1 and 2 with Torin1)</td>
<td></td>
<td>decreased</td>
<td>decreased Mbp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lebrun-Julien et al., 2014</td>
<td>Cnp-Cre;Raptor&lt;sup&gt;fl/fl&lt;/sup&gt;,Rictor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>decreased</td>
<td>decreased Mbp, Mog, Mag, Plp1, reduced thickness</td>
<td></td>
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<tr>
<td></td>
<td>Wahl et al., 2014</td>
<td>Cnp-Cre;mTOR&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>unchanged</td>
<td>decreased</td>
<td>thinner sheath, greater % unmyelinated, reduced Mbp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyler et al., 2009</td>
<td>OL culture + mTOR siRNA</td>
<td></td>
<td></td>
<td>decreased Mbp, Plp1</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.1 Effects of mTOR protein perturbation on OL maturation, continued

<table>
<thead>
<tr>
<th>Gene/protein</th>
<th>Study</th>
<th>Model</th>
<th>OPC proliferation</th>
<th>OPC differentiation</th>
<th>OL survival</th>
<th>Myelin</th>
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<tr>
<td>Raptor/mTORC1</td>
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<td>OPC in vitro (block mTORC1 with rapamycin)</td>
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<td>unchanged</td>
<td></td>
<td>decreased Mbp</td>
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<td></td>
<td>Lebrun-Julien et al., 2014</td>
<td>Cnp-Cre;Raptor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>unchanged</td>
<td>unchanged</td>
<td></td>
<td>decreased Mbp, Mog, Mag, Plp1, reduced thickness</td>
</tr>
<tr>
<td></td>
<td>Plp-CreERT2;Raptor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>thinner sheath</td>
</tr>
<tr>
<td></td>
<td>Bercury et al., 2014</td>
<td>Cnp-cre; Raptor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>increased</td>
<td>decreased</td>
<td></td>
<td>reduced myelin genes/proteins, thinner sheath</td>
</tr>
<tr>
<td></td>
<td>Guardiola-Diaz et al., 2012</td>
<td>OL culture + rapamycin</td>
<td>unchanged</td>
<td></td>
<td></td>
<td>decreased Mbp</td>
</tr>
<tr>
<td>Tyler et al., 2011</td>
<td>OL culture + rapamycin</td>
<td>increased differentiation inhibitor Gpr17</td>
<td>decreased</td>
<td>increased</td>
<td>decreased Mbp, Plp1</td>
<td></td>
</tr>
<tr>
<td>Tyler et al., 2011</td>
<td>OL culture + rapamycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>decreased Mbp, Plp1</td>
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<tr>
<td>Tyler et al., 2011</td>
<td>OL culture + Raptor siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>no change Mbp, Plp1</td>
</tr>
<tr>
<td>Rictor/mTORC2</td>
<td>Grier et al., 2017</td>
<td>Olig2-Cre;Rictor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>decreased</td>
<td></td>
<td></td>
<td>Mbp reduced, delayed myelination of axons</td>
</tr>
<tr>
<td></td>
<td>Lebrun-Julien et al., 2014</td>
<td>Cnp-Cre;Rictor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td>unchanged</td>
<td>unchanged</td>
<td></td>
<td>decreased Mbp, Mog, Plp1</td>
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<tr>
<td></td>
<td>Plp-CreERT2;Rictor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>unchanged sheath thickness</td>
</tr>
<tr>
<td>Bercury et al., 2014</td>
<td>Cnp-cre;Rictor&lt;sup&gt;fl/fl&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>delayed myelination, fewer myelinated axons</td>
</tr>
<tr>
<td>Tyler et al., 2014</td>
<td>OL culture + Rictor siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>decreased Mbp, Plp1</td>
</tr>
</tbody>
</table>

List of studies perturbing mTOR signalling and the effects on OPC proliferation, OPC differentiation, OL survival, and myelin

5.1.1.3 Translating nutrient availability to OPC differentiation

Upstream even from the PI3K/Akt and Erk1/2 signalling pathways, both neurotransmitter receptor and growth factor/hormone receptor signalling has been shown to regulate OPC differentiation (Figure 5.1a). Notably, neurotransmission and the presence of growth factors and hormones are both altered by fasting and refeeding, activate mTOR, and initiate OPC differentiation.

Neurotransmission

Neuronal activity changes with fasting and refeeding have mainly been described in the ARC. Expression of cfos, a marker of neuronal activity, in the aforementioned POMC/CART and AgRP/NPY neurons is regulated by nutrient availability: cfos expression is present in nearly a quarter of NPY-expressing neurons in the fasted state and around in around 5% of neurons after a 1 h refeed. POMC neurons have barely any cfos expression in the fasted state, but over 20% are activated after refeeding (Wu et al., 2014). Additionally, fasting reduces activity of ARC TIDA neurons (Kubota et al., 2018). These neuronal populations have processes that extend into the ME (Day, 2009; Li et al., 1999; Lookingland et al., 1987). In this area, where neuronal processes have been described to have both dendritic and axonal properties (Herde et
al., 2013) and where OPCs make contact with neuronal processes (Djogo et al., 2016), it is possible electrical activity could be sensed by OLs.

It is known that OPCs express several glutamate and GABA receptors and are electrically active (reviewed in Bakiri et al., 2009; Mount and Monje, 2017). Interestingly, activation of both glutamate and GABA receptors cause depolarization of OPCs due to their high intracellular chloride concentration (Lin and Bergles, 2004). Deleting or perturbing the activity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) has been shown to decrease both OPC differentiation and survival (Chen et al., 2018; Kougioumtzidou et al., 2017). Another study has found that glutamate receptor activation via N-methyl-D-aspartate receptors (NMDARs) activates the mTOR pathway and induces OPC differentiation. In those experiments, differentiation is mediated via mTOR, as rapamycin administration blocks the process (Li et al., 2013) (Figure 5.1a).

**Growth factor/hormone signalling**

Growth factor/hormone signalling is also regulated by fasting and refeeding. Fasting can increase circulating fibroblast growth factor 21 (FGF21) and corticosterone, while CNTF, growth hormone, insulin, insulin-like growth factor 1 (IGF1), and T₃ (thyroid hormone) decrease with chronic CR or short-term fasting (Challet et al., 1995; Gälman et al., 2008; Severi et al., 2013; Tannenbaum et al., 1979; Underwood et al., 1986; Van der Geyten et al., 1999; Wilcox, 2005).

OPCs express receptors to several growth factors and hormones on their surface, and these are regularly used in vitro to elicit OPC differentiation (Goldman and Kuypers, 2015). Platelet-derived growth factor AA (PDGF-AA), CNTF, IGF1, thyroid hormone, and transforming growth factor β (TGFβ) have all been shown to induce differentiation (Cui et al., 2010; McKinnon et al., 1993; Talbott et al., 2007). Of particular interest, IGF1 and thyroid hormone activate mTOR activity in differentiating OPCs (Bibollet-Bahena and Almazan, 2009; Wood et al., 2013) (Figure 5.1a).

### 5.1.2 Hypothesis

Study of mTOR signalling in the hypothalamus led to the discovery of activity of the pathway in ME OLs. Along with knowledge about how the pathway changes in the hypothalamus in response to nutrient availability and how the pathway is responsible for OL maturation, I hypothesized that mTOR activity in OLs is regulated by nutrient availability. The experiments in this chapter tested this hypothesis.
5.1.3 Chapter 5 experimental objectives and design

1. I validated an antibody used to label cells with active mTOR signalling, then used it to find the identity of cells using the pathway in the ME.

2. I examined ME tissue from genetic mouse models that block either mTORC1 or mTORC2 activity specifically in all OLs to discover which branch of the signalling pathway is active in OLs of the ME.

3. I used rapamycin administration (inhibits mTORC1 activity in all cells) in C57BL/6 mice to confirm whether mTORC1 or mTORC2 signals in ME OLs. In this experiment, all animals were fasted overnight. Animals were given an injection of 10mg/kg rapamycin or vehicle then 30 minutes later all were refed for 1 h before sacrifice.

4. I tested how fasting versus refeeding, fat content of diet, and protein content of diet affect mTOR labelling in ME OLs. Fasting-refeeding was conducted as described in previous chapters. For dietary fat manipulation, animals maintained on a normal chow diet since weaning were changed to a LFD or HFD from 4-5 weeks of age for 6 weeks. Animals underwent fast-refeed paradigm before sacrifice. For protein manipulation, animals maintained on a normal chow diet were switched to LPD or HPD at 8 weeks of age for 3-4 days before sacrifice.

5. I used the scRNAseq dataset described in chapter 3 to examine expression of OPC plasma membrane proteins that may be involved in detecting nutrient availability and causing OPC differentiation. I averaged gene expression values in OPCs from both the fasted and refed states.
5.2 Results

5.2.1 pmTOR antibody labels OLs of the ME

5.2.1.1 Verifying specificity of antibody labelling

To assess mTOR activity in cells of the ME, I used an antibody to the mTOR protein phosphorylated at serine site 2448 (pmTOR, Cell Signaling Technology #2976). Phosphorylation at this site is correlated with high levels of mTOR activity, and mTOR phosphorylated here can bind either raptor or rictor proteins to form mTORC1 or mTORC2 (Hoeffer and Klann, 2010; Rosner et al., 2010) (Figure 5.1c). This antibody labels cells in the upper portion of the ME (Figure 5.3a), and labelling throughout the rest of the brain (the CVOs vascular organ of the lamina terminalis (VOLT), subfornical organ (SFO), and area postrema (AP); the neurogenic subventricular zone (SVZ) and subgranular zone (SGZ); the corpus callosum (CC) white matter tract; and the pituitary gland) fails to produce the striking brightness of cells labelled in the ME (Figure 5.3b). To test specificity of pmTOR labelling, I tested an antibody from another company that also labels mTOR phosphorylated at serine 2448 (Santa Cruz #sc-293133). This antibody also labels cells in the upper portion of the ME (Figure 5.3c). As the Santa Cruz antibody only worked in my hands with DAB labelling, I have used the Cell Signaling Technology antibody for the rest of the studies in this chapter. I also tested the Cell Signaling Technology antibody for specificity of labelling by combining the antibody with a peptide that blocks the serine 2448 binding site. The blocking peptide prevented antibody labelling of the tissue (Figure 5.3d).
Chapter 5 – mTOR signalling as a candidate nutrient detection system in median eminence oligodendrocytes

Figure 5.3 pmTOR antibody labelling

(a) Schematic of coronal section of the mouse brain including the ME (adapted from Paxinos and Franklin, 2001, reprinted with permission from Elsevier). Antibody labelling of mTOR protein phosphorylated at serine 2448 (antibody from Cell Signaling Technology) (pmTOR) (b) pmTOR labelling throughout the brain (adapted from Paxinos and Franklin, 2001) (c) Antibody labelling of mTOR protein phosphorylated at serine 2448 (antibody from Santa Cruz) (d) pmTOR labelling (CST) without and with a serine 2448 blocking peptide
5.2.1.2 Combining pmTOR antibody with cell-type specific markers

I next assessed cell-type specificity of labelling by combining the pmTOR antibody with antibodies to SOX10 (a pan-OL marker), APC (adenomatous polyposis coli clone CC1, an antibody shown to label postmitotic OLs [Bin et al., 2016]), and NEUN (a neuron marker). I also combined pmTOR with an antibody to GFP in Aldh111-GFP mice, which express GFP in astrocytes (Figure 5.4a). There was no co-expression of pmTOR with neuronal or astrocyte markers, but nearly all pmTOR$^+$ cells were SOX10$^+$ (89.0%). 83.2% of APC$^+$ cells are also pmTOR$^+$ (Figure 5.4a-b).

![Figure 5.4 pmTOR-labelled cells are OLs](image)

Figure 5.4 pmTOR-labelled cells are OLs

(a) pmTOR co-labelling with antibodies to pan-OL marker Sox10, postmitotic OL marker APC, neuron marker NeuN, and GFP expression (Aldh111-GFP mice). White squares indicate insets to the right

(b) Proportions of cells co-expressing pmTOR and SOX10 or APC
5.2.2 mTORC1 not mTORC2 complex is highly active in ME OLs

Signalling via mTOR can occur from the mTOR Complex 1 (mTORC1, contains raptor protein) or Complex 2 (mTORC2, contains rictor protein), resulting in the activation of distinct transcriptional programmes (Figure 5.1c-d). I took advantage of exclusive expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) in OLs and used Cnp-Cre:raptor^0/fl (raptor KO) and Cnp-Cre:rictor^0/fl (rictor KO) mice to interrogate which signalling complex is most active in OLs of the ME. When I labelled tissues of raptor or rictor KO mice with the antibody to pmTOR, I was only able to detect an absence of labelling in raptor KO mice (Figure 5.5a). This indicates that signalling through mTOR is inhibited in this region when mTORC1 is unable to form.

Additionally, pmTOR expression is sensitive to rapamycin (an mTORC1 inhibitor) administration. Intraperitoneal rapamycin significantly decreases the number of cells labelled with pmTOR in the ME (15.92 cells with vehicle injection, 8.34 cells with rapamycin, p = 0.0059, Student’s t-test) (Figure 5.5b).

Figure 5.5 mTORC1 is active in ME OLs
(a) mTOR labelling in tissue with absence of mTORC1 signaling (raptor KO) or absence of mTORC2 signaling (rictor KO). Scale bars = 100µm. (b) Cell count of pmTOR⁺ cells in MEs of animals treated with vehicle or rapamycin. ** indicates p < 0.01, error bars indicate mean ± SEM, p values determined by Student’s t-test, vehicle n = 3 animals, refed n = 4 animals.
5.2.3 Dietary manipulation affects mTOR activity in ME OLs

5.2.3.1 Fast-refeed

Pathway analysis indicated that the mTOR signaling pathway is regulated by fasting and refeeding in NFOLs and MOLs (Table 4.4). Indeed, several genes in the mTOR signaling pathway are differentially expressed in OLs between conditions (p < 0.05, FDR < 0.25) (Figure 5.1a, Figure 5.6a). When I examined pmTOR expression in tissue from fasted and refed animals, I was unable to detect a change in the number of pmTOR+ cells per section, but the proportion of APC+ cells that had active mTOR was significantly increased (36.7% fasted versus 72.5% refed, p = 0.0069, Student’s t-test) (Figure 5.6b-d). A possible interpretation of these data is that in the fasted state OPCs are using mTOR for survival, but in refeeding there is a shift to its use in postmitotic OLs in preparation for myelination.

Figure 5.6 mTOR activity in fasting-refeeding

(a) Heatmap of genes involved in mTOR signaling that are differentially expressed in scRNAseq dataset (p < 0.5, FDR < 0.25). Pik3r produces the p85 protein. See also Figure 5.1 (b) pmTOR (red) and APC (green) labelling in fasted and refed animals. Arrowheads indicate cells that express both markers (c) Number of pmTOR+ cells per section and (d) percentage of APC+ cells that also express pmTOR in fasted and refed animals. ** indicates p < 0.01, error bars indicate mean ± SEM, p values determined by Student’s t-tests, fasted n = 7 animals, refed n = 4 animals.
5.2.3.2 Fast-refeed after LFD/HFD

As mentioned in chapter 4, diet-induced obesity from chronic HFD feeding can prevent OPC differentiation in a stroke model in mice (Xiao et al., 2018). To test if HFD can also alter pmTOR expression in the ME, I examined tissue from fasted and refed animals that had been fed either a LFD or HFD for 6 weeks prior to the terminal fast-refeed. After 5 and 6 weeks of feeding on LFD or HFD, mice fed a HFD were significantly heavier ($p = 4.1e-03$ at 5 weeks and $p = 3.0e-04$ at 6 weeks, two-way ANOVA with Sidak’s multiple comparisons test) (Figure 5.7a). While the number of pmTOR$^+$ cells was unchanged between fasting or refeeding or between diets (Figure 5.7b), the percentage of APC$^+$ cells with pmTOR expression is significantly decreased with a HFD compared with a LFD (48.5% of cells in LFD versus 40.0% in HFD, $p = 0.03$, two-way ANOVA) (Figure 5.7c).

![Graphs showing mTOR activity in fasting-refeeding after chronic LFD or HFD](image)

**Figure 5.7 mTOR activity in fasting-refeeding after chronic LFD or HFD**

(a) Animal weights after chronic LFD or HFD for 5 or 6 weeks (b) Density of pmTOR$^+$ cells in the ME of fasted and refed mice on LFD and HFD (c) Percentage of APC$^+$ cells that also express pmTOR in fasted and refed animals fed on LFD and HFD. * indicates $p < 0.05$, *** indicates $p < 0.001$, error bars indicate mean ± SEM, p values determined by two-way ANOVA, LFD fasted n = 4, LFD refed n = 7, HFD fasted n = 6, HFD refed n= 3
5.2.3.3 LPD/HPD

As AAs are potent activators of hypothalamic mTOR activity (Blouet et al., 2009; Cota et al., 2008), I used diets of different protein content to test effects on signalling in OLs. Mice did not eat a significantly different amount of food per day, but HPD mice did weigh significantly less than LPD mice for the first few days of the diet ($p = 7.3 \times 10^{-03}$ at Day 1 and $p = 2.9 \times 10^{-4}$ at Day 2, two-way ANOVA with Sidak’s multiple comparisons test) (Figure 5.8a-b). When I labelled tissues from mice fed a low protein diet (LPD) or high protein diet (HPD) diet for 3-4 days, I found an increase in both the number and size of pmTOR+ cells in the ME. There was an average of 10.7 pmTOR+ cells in animals fed a LPD and an average of 20.64 labelled cells in animals fed a HPD ($p = 0.039$, Student’s t-test) (Figure 5.8c-d). pmTOR+ cells from LPD-fed animals were an average of 54.8 µm² versus 62.3 µm² in HPD-fed animals ($p = 0.051$, Student’s t-test) (Figure 5.8e).

![Figure 5.8 mTOR activity after 3-4d LPD or HPD](image)

(a) Food intake per cage of animals fed a LPD or HPD for 3-4d (b) Individual animal weights on LPD or HPD (c) pmTOR labelling (red) in tissues of animals fed a low or high protein diet. White squares indicate insets, scale bars = 25µm (d) Number of pmTOR+ cells in the ME of mice fed LPD or HPD (e) Size of pmTOR+ cells in the ME of LPD- or HPD-fed mice. * indicates $p < 0.05$, *** indicates $p < 0.001$, error bars indicate mean ± SEM, p values determined by two-way ANOVA or Student’s t-tests, LPD n = 13, HPD n = 6
5.2.4 ME OPCs express genes to plasma membrane proteins involved in differentiation and mTOR signalling

As mentioned in the introduction to this chapter, activation of certain neurotransmitter and growth hormone receptors can initiate mTOR signalling and/or OPC differentiation. In order to determine proteins that link the extracellular space (and nutrients/molecules found there) with intracellular mTOR signalling or differentiation, I examined genes encoding plasma membrane proteins in OPCs in the scRNAseq dataset.

Of 11,147 genes expressed in OPCs, 13.79% are genes of proteins expressed on the plasma membrane (Figure 5.9a). Of those proteins, ion channels, G-protein-coupled receptors (GPCRs), transmembrane receptors, and transporters may allow OPCs to sense external stimuli.

Genes of several ionotropic glutamate receptor subunits are expressed in OPCs, including relatively high expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subunit 2 (Gria2). Additionally, the glutamate transporter excitatory AA transporter 2 (Slc1a2) and γ-aminobutyric acid (GABA) transporter 1 (Slc6a1) genes are expressed in these cells (Figure 5.9b). This indicates OPCs are poised to sense any changes in neuronal transmission in the ME upon refeeding.

Receptors to several growth factors are also expressed on OPCs. Within the 30 most-expressed transmembrane receptors in OPCs are receptors to platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), bone morphogenetic protein (BMP), insulin-like growth factor 1 (IGF1), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), and insulin (Figure 5.9b).

Finally, ability of OPCs to directly detect nutrients from ingested food might allow initiation of differentiation. Signalling cascades, such as mTOR signalling, can occur in response to detection of dietary substrates (Figure 5.1a, c) rather than ligand binding, so transporters may also represent a mechanism of stimuli detection in OLs. Slc22a7 and Slc50a1 are genes encoding sugar transporters, while Slc7a3 and Slc7a10 are genes encoding AA transporters (Figure 5.9b).
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Figure 5.9 OPC plasma membrane genes
(a) Location of proteins formed by OPC-expressed genes in the scRNAseq dataset (b) Gene expression of plasma membrane proteins in OPCs.
5.3 DISCUSSION

In summary, experiments from this chapter show that the antibody to pmTOR specifically labels OLs of the ME. Deletion of raptor and rapamycin administration inhibit pmTOR labelling, suggesting mTORC1 is signalling in the OLs. Refeeding significantly increases the proportion of APC+ OLs that have active mTOR, HFD significantly decreases that proportion compared to LFD-fed animals, and HPD increases the number and size of pmTOR+ cells in the ME. Finally, ME OPCs express several plasma membrane proteins that would enable them to detect nutrient availability. Here, I will examine my use of a pmTOR antibody to study mTOR signalling, discuss further experiments to definitively determine if mTOR is involved in OPC differentiation, detail how HPD might be able to exaggerate effects seen in refeeding, and suggest how ME OPC plasma membrane proteins might be involved in detecting dietary nutrient availability.

5.3.1 Tools to study mTOR activity

I have used an antibody to the phosphorylated mTOR protein to act as a proxy for mTOR activity in these experiments. While the studies show that mTORC1 is likely to be the main mTOR signalling complex in ME OLs, mTORC2 may also be active in these cells. There is still some pmTOR labelling in raptor KO tissues and after rapamycin administration (Figure 5.5). Therefore, mTORC2 activity might be active in a few cells, or mTORC1 may still be signalling despite the inhibition. An additional experiment that examines colocalization of pmTOR and downstream indicators of mTORC1 versus mTORC2 activity (such as pS6K or PKCa, respectively) will provide more certainty about the active complex in these cells.

In previous studies of mTORC1 activity in the hypothalamus, antibodies to pS6 have been used to detect mTORC1 activity in situ in tissue sections. pS6 has been used to show mTORC1 activity in AgRP neurons, for example (Villanueva et al., 2009) (Figure 5.10a). However, phosphorylation of the S6 protein lies downstream of several other signalling cascades and is even used as a proxy for neuronal activation (Knight et al., 2012) (Figure 5.10b). I propose that using an antibody to phosphorylated mTOR protein, therefore, is a more specific marker of the activity, and will enable detection of the cell types responsible for the pathway’s effects on food intake and bodyweight.
5.3.2 Does mTOR mediate OPC differentiation?

Differential expression analysis from the scRNAseq data shows that mTOR signalling is regulated by fasting-refeeding in NFOLs and MOLs but not OPCs (Table 4.4). Additionally, pmTOR expression colocalizes with postmitotic OL marker APC (Figure 5.4a-b). These findings do not exclude mTOR from being involved in OPC differentiation, but it is also possible that mTOR may play more of a role in promoting (future) myelination (discussed in more detail in chapter 6). Indeed, most of the studies examining effects of mTOR activity perturbation show effects in myelin gene or protein expression or changes in myelin structure (Table 5.1). In order to precisely identify the pathway’s role, I could use BrdU labelling during rapamycin treatment to investigate whether increased OPC differentiation normally seen during refeeding (Figure 4.10) is inhibited.

Additionally, while several model systems have found mTOR signalling to be necessary for OPC differentiation (Lebrun-Julien et al., 2014; Tyler et al., 2009; Wahl et al., 2014; Zou et al., 2014), it is possible the mechanism of increased OPC differentiation in the ME may be mTOR-independent. A recent study has found that mTOR is not required for increased OL development and myelination found in OPCs lacking Pten (González-Fernández et al., 2018) (Figure 5.1b).

Figure 5.10 pS6 is downstream of mTORC1 and neuronal activity
(a) Image from Villanueva et al., 2009 showing pS6 expression in AgRP neurons, reprinted with permission from Oxford University Press (b) Image from Knight et al., 2012 showing pS6 expression in neurons activated by certain stimuli (cfos is another marker of neuronal activity), reprinted with permission from Elsevier
As a HPD had a large effect on increasing pmTOR labelling and size of cells, it would be worthwhile introducing BrdU labelling into the LPD/HPD experiment as well in order to study whether OPC differentiation is increased with high protein intake.

5.3.4 mTOR activity in OLs

As mTOR signalling is ubiquitous (Takei and Nawa, 2014), it is interesting that OLs (especially those of the ME) seem to have very high activity of the pathway. When myelin programmes are most active in an OL, the cell is producing three times its weight in myelin membrane per day (reviewed in Bradl and Lassmann, 2010). Considering that some of the main downstream effects of mTOR signalling are protein synthesis and lipid synthesis (reviewed in Wood et al., 2013), it makes sense that mTOR activity would be high in these cells. High mTOR activity could indicate OLs that are actively myelinating.

Additionally, it was notable that compared with fasting/refeeding and LFD/HFD feeding, LPD/HPD feeding produced a more pronounced effect on increasing the number of cells with pmTOR labelling in the ME (Figures 5.5, 5.8). It is possible that protein content determines the level of pmTOR activity. However, all three dietary manipulations took place over different time frames and the diets did not control for macronutrient proportions. These factors would need to be controlled to determine the role of protein content, specifically, in activating mTOR.

In the LPD/HPD experiment, animals ate less, weighed less, and had more mTOR activity in the hypothalamus when fed a HPD, as predicted (Sørensen et al., 2008). Additionally, there was an increase in the size of pmTOR+ cells in animals fed a HPD (Figure 5.8c, e). As pmTOR labelling is mainly in the cell body of OLs rather than the processes, the increase in size is not a measure of increased process arborization/length. The significance of this is yet to be determined, as other studies examining OL growth (in response to growth factors) only found an increase in process length and did not find a change in cell body size (Furusho et al., 2012). However, deficit of TOR signalling in other animals models is known to decrease cell size throughout the body (Montagne et al., 1999).
5.3.5 Plasma membrane proteins potentially involved in ME OPC differentiation

As shown in this chapter, ME OPCs have the ability to detect both neurotransmitters and growth factors/hormones. If neurotransmitter receptor signalling is involved in translating nutrient availability into increased OPC differentiation, it would be useful to know which neuronal population was mediating the response. ARC POMC/CART, AgRP/NPY and TIDA neurons all have altered neuronal activity in fasting and refeeding (Kubota et al., 2018; Wu et al., 2014) and all have processes that extend into the ME (Day, 2009; Li et al., 1999; Lookingland et al., 1987). AgRP/NPY and TIDA neurons both release GABA upon stimulation (Horvath et al., 1997; Ovesjö et al., 2001; Zhang and van den Pol, 2015), while POMC neurons have subpopulations that release glutamate or GABA (Collin et al., 2003; Hentges et al., 2004). Any/all of these neuronal types could form synapses with ME OPCs to signal changes in nutrient availability.

ME OPCs express glutamate receptor AMPAR subunit 2 (Gria2) (Figure 5.9b). While activation of AMPARs has been shown to block OPC differentiation (Gallo et al., 1996; Yuan et al., 1998), activation has also been shown to increase myelination (Gautier et al., 2015). Additionally, specific dysfunction of AMPAR subunit 2 is also shown to block differentiation, indicating different subunits may have varied roles in downstream OL signalling (Chen et al., 2018).

Effects of GABA have mainly been studied through its action on GABARs, where it acts to promote OPC differentiation and myelination (Hamilton et al., 2017; Serrano-Regal et al., 2019). ME OPCs express GABA transporter 1 (Slc6a1) which may mediate some of the effects of GABA signalling. Our lab is currently analyzing the proportion of glutamatergic versus GABAergic synapses on ME OPCs, and we are forming a collaboration with the Káradóttir lab to assess electrophysiological changes in ME OPCs in the fasted versus refeeding state.

Growth factor/hormone receptors whose genes are expressed in ME OPCs and which are also known to regulate OPC differentiation include PDGFRA, CNTF, TGFβ, and IGF1. Notably, Igf1r is also an upregulated DEG in OPCs (Table 4.3), indicating signalling through the protein is responsive to refeeding. Additionally, IGF1 signalling is a potent activator of mTOR signalling. Blocking or activating receptors to growth factor/hormone receptors in the ME (possibly during electrophysiological experiments mentioned above) would be a good way to interrogate their effect on OLs.
In conclusion, experiments from this chapter show that ME OPCs have the capability to act as sensors of nutrient availability, and this may underlie the increased differentiation response found in refeeding. Future studies are needed, though, to show with certainty the mechanism(s) that translates energy availability signals into OPC differentiation. Further, the effects of OPC differentiation on food intake behavior are yet to be determined. Experiments in chapter 6 aim to discover the functional consequences of increased differentiation in refeeding.
CHAPTER 6

FUNCTIONAL CONSEQUENCES OF INCREASED OPC DIFFERENTIATION IN THE MEDIAN EMINENCE

6.1 INTRODUCTION

Our lab has shown that OPCs of the ME rapidly differentiate with a 1 h refeeding period after a fast. In this introduction, I will describe two possible downstream effects of this differentiation: myelin remodelling and changes in food intake behavior. Additionally, I will describe a tool that has been used to study the effects of OPC differentiation on behavior.

6.1.1 Background

6.1.1.1 Potential outcome of increased OPC differentiation: myelin remodelling

As the canonical role of OLs is to myelinate, a natural supposition is that increased ME OPC differentiation leads to increased myelination in the area (Figure 6.1a). Constitutive or adaptive myelin remodelling can occur when NFOLs form new myelin internodes on unmyelinated sections of axons (Hughes et al., 2018). Many regions of the brain have partially-myelinated axons, and these regions accumulate OLs and myelin segments throughout life (Hughes et al., 2018; Tripathi et al., 2017). The extent of myelination of the magnocellular vasopressin and oxytocin neurons that pass through the ME (Figure 3.1a-b) has not been studied (neither here nor in currently available literature).

If myelin remodelling was to occur in response to the fast-refeed paradigm, it is important to consider the timepoint where changes in myelin could be detected. In mice,
heightened sensory experience (being housed in cages fitted with strings of hanging beads) increased the number of myelinating OLs in the barrel cortex and also increased the number of myelin sheaths in the area. However, the researchers measured this change 20-22 days after the stimulus had been initiated (Hughes et al., 2018) (Figure 4.2d). Other model systems have exhibited a quicker myelin/white matter response: young zebrafish are able to produce myelin sheaths within 5 hours (Czopka et al., 2013), and humans learning how to play a computer game for just 2 h show structural changes in white matter as measured with diffusion tensor imaging of the fornix (a tract connecting the hippocampus and the hypothalamus) (Hofstetter et al., 2013).

Of note, while increased myelin sheath thickness from pre-existing OLs has been demonstrated through non-physiological means such as inducible genetic overexpression of the ERK1/2 signalling in adult mice (Jeffries et al., 2016), any physiological increases in myelin production are thought to come from NFOLs and not from pre-existing myelinating OLs (reviewed in Kaller et al., 2017).

In addition to adding myelin to unmyelinated portions of axons, it is possible that NFOLs are replacing myelin that could have been lost during fasting (Figure 6.1b). It is possible that ME myelin sheaths, which are exposed to potentially toxic blood-borne factors, are regularly lost this region. Myelin pruning has indeed been shown to take place in healthy animals. In young zebrafish, myelin sheaths are normally pruned via microglia engulfment during formation of neural circuits. Silencing neuronal activity increased myelin pruning, indicating activity of neural circuits plays a role in maintaining myelin sheaths in this model (Hughes and Appel, 2019).
Figure 6.1 Possible outcomes of increased OPC differentiation
(a) Refeeding could increase myelination of vasopressin and oxytocin neurons that pass through the ME on their way to the posterior pituitary gland (b) Fasting may cause myelin sheath pruning. Refeeding may increase OPC differentiation to replace lost sheaths

6.1.1.2 Potential outcome of increased OPC differentiation: changes in food intake and bodyweight

Previous studies have postulated that an increase in OPC differentiation leading to increased myelination causes increased conduction efficiency to reinforce a newly-utilized neuronal circuit (Gibson et al., 2014; Hughes et al., 2018; McKenzie et al., 2014; Xiao et al., 2016). Decreased OPC differentiation in the CC and motor cortex can cause reduced performance on a learning task while increased myelination in the premotor cortex correlates with altered motor function (Gibson et al., 2014; McKenzie et al., 2014; Xiao et al., 2016). In humans, the degree of white matter alteration from learning a new task correlates with the participants’ performance (Lakhani et al., 2016).

The myelinated population of axons that pass through the ME are vasopressin and oxytocin neurons. Chemogenetic activation of vasopressin but not oxytocin neurons has anorectic effects, although optogenetic activation of oxytocin neurons along with anorectic AgRP neurons significantly decreases food intake versus AgRP stimulation alone (Atasoy et
al., 2012; Sutton et al., 2014; Yoshimura et al., 2017). Increased myelination of these neurons in the ME may facilitate more efficient neural transduction in a food intake behavior circuit.

In chapter 3, I discussed the non-insulating role of OPCs to provide trophic support for lepR neurons in the ME. When ME/ARC OPCs were ablated via third ventricular delivery of mitotic blocker AraC, animals ate significantly more food and gained fat mass (Djogo et al., 2016) (Figure 6.2). These effects show the importance of the presence of OPCs for maintaining bodyweight. However, the studies conducted did not allow for distinction between the total absence of OPCs or the blockade of OPC differentiation into myelinating OLs for the observed effects on food intake behavior. The creation of a transgenic mouse line that enables blockade of OPC differentiation without depleting the OPC pool has been used to test the importance of OPC differentiation, specifically (McKenzie et al., 2014; Xiao et al., 2016).

**Figure 6.2 Hypothalamic OPC ablation causes increased food intake and bodyweight gain**

Image from Djogo et al., 2016 showing that hypothalamic OPC ablation via 3V delivery of AraC leads to increased food intake and bodyweight gain (gain of fat mass, specifically). The red bar in E indicates the period where AraC was delivered via an implanted pump. Reprinted with permission from Elsevier
6.1.1.3 Tools to study effects of OPC differentiation

Myelin regulatory factor (Myrf) is a transcription factor necessary for OPC maturation into myelinating OLs (Bujalka et al., 2013; Emery et al., 2009). The Pdgfra-CreERT2;Rosa-YFP;Myrf$^{fl/fl}$ (Myrf KO) mouse line enables tamoxifen-inducible deletion of Myrf in OPCs, thus blocking OPC differentiation (Figure 6.3a). The mouse has been used previously to study the effects of blocking OPC differentiation on learning a new task. Myrf KO mice learning how to run on a wheel with irregularly spaced rungs were not able to learn the task as well as their control littermates, showing the necessity of NFOLs for learning (McKenzie et al., 2014; Xiao et al., 2016) (Figure 6.3b). Importantly, while the number of NFOLs formed from differentiation ($YFP^+/CC1^+[APC]^+$ cells) is inhibited in the CC of Myrf KO mice, the overall OPC pool (PDGFRα$^+$ cells) remains the same. The number of pre-existing postmitotic OLs (CC1$^+$ cells) also remains the same in this region (Figure 6.3c).

In order to specify that ME OPC differentiation, specifically, is important for any downstream effects, we will need to limit Myrf deletion to the ME only. One way this could be accomplished is via injection of a tamoxifen derivative directly into the ME. Injection of tamoxifen derivative endoxifen has been used previously to induce Cre expression in specific brain sites (Benedykcinska et al., 2016). To my knowledge, CNS injections targeting the ME of mice have not been described before.
Figure 6.3 Use of Pdgfra-CreERT2;Rosa-YFP;Myrf<sup>fl/fl</sup> (Myrf KO) mice to block OPC differentiation
(a) Schematic of cross between Pdgfra-CreERT2 mice (will express Cre upon tamoxifen administration), Rosa26-YFP mice (will express YFP when Cre is present to remove the STOP sequence through genomic recombination), and Myrf<sup>fl/fl</sup> mice (will delete a portion of the Myrf allele when Cre is present, inhibiting Myrf protein formation). The crossed Pdgfra-CreERT2;Rosa-YFP;Myrf<sup>fl/fl</sup> mice have complete inhibition of Myrf production, while Myrf<sup>+/+</sup> mice are used as control animals (b) Image from McKenzie et al., 2014 showing that Myrf KO mice (P-Myrf<sup>−/−</sup> mice) have decreased average and maximum running speeds compared to littermate controls (P-Myrf<sup>+/−</sup>) while learning to run on a wheel with irregularly-spaced rungs (complex wheel, CW), reprinted with permission from The American Association for the Advancement of Science (c) Image from McKenzie et al., 2014 showing that production of NFOLs (YFP<sup>+</sup>/CC1<sup>+</sup> cells) is inhibited in Myrf KO mice while the overall OPC pool (PDGFRα<sup>+</sup> cells) and the number of pre-existing myelinating OLs (CC1<sup>+</sup> cells) remain unchanged. CC1 is called APC in the results described in this chapter.
6.1.2 Hypothesis
Based on knowledge from previous studies about how myelination/white matter volume can be altered in response to physiological stimuli such as learning (within 2 h in humans), I hypothesized that the increased OPC differentiation that occurs after refeeding would lead to increased myelination. While I was unable to test hypotheses about the behavioral or phenotypic effects of increased OPC differentiation during my PhD, in this chapter I describe development of tools to explore them in future experiments.

6.1.3 Chapter 6 experimental objectives and design
1. In fasted and refed mice, tissues were processed for myelin analysis. Toluidine blue labelling was used to measure density of myelinated axons and transmission electron microscopy (TEM) was used to measure thickness/g-ratio of individual myelin sheaths. Unlike other fast-refeed experiments, refeeding lasted for 2 h, as this is a timeframe shown to allow white matter changes after learning a new task (Hofstetter et al., 2013).
2. To test if Pdgfra-CreERT2;Rosa-YFP;Myrffl/fl (Myrf KO) mice will be useful for study of ME blockade of OPC differentiation, Myrf KO mice and littermate controls were given tamoxifen as previously described at P60 (McKenzie et al., 2014; Xiao et al., 2016). Three weeks after tamoxifen administration, all animals were fasted overnight and refed 1 h before sacrifice.
3. To test targeting the ME with brain injections, animals were given injections of a Cre-independent virus encoding tdTomato into the parenchyma adjacent to the ME. After 2 weeks animals were sacrificed to study tdTomato expression.
6.2 RESULTS

6.2.1 Molecular/cellular effects of OPC differentiation

6.2.1.1 Genes encoding myelin proteins are upregulated by refeeding but myelin itself does not change in the short term

In the scRNAseq dataset, genes encoding proteins found in the myelin proteome are upregulated in all clusters. This includes well-known genes such as Mal, Mbp, and Plp1, and also several heat shock proteins (Cryab, Hspa1a, Hspa5, etc.) (Jahn et al., 2009). Genes related to synthesis of cholesterol, phospholipids and sphingomyelin that make up the myelin membrane (Pmvk, Fa2h, Ugt8a, Elovl1) and also a gene related to transport of lipids to the membrane (Stard3) are also upregulated in all clusters, in accordance with pathway analysis (Table 4.4, Figure 6.4a).

To study if the increase in lipid and myelin gene transcription translates to changes in myelin, animals were fasted overnight and refeed for 2 h – a timepoint that can allow for changes in white matter after learning a new task (Hofstetter et al., 2013). Toluidine blue labelling of thin sections allowed viewing of the distribution of myelinated axons in the ME. As supported by MBP labelling in thick cleared sections (Video 3.1), myelinated axons are seen in the transverse plane, indicating their passage in a rostral-to-caudal direction through the ME (Figure 6.4b-c). In line with MBP labelling and Plp1 expression, myelin is located in the upper third of the ME (Figure 6.4b, Figure 3.6a-b). TEM provides extremely high magnification of tissue and enables precise measurement of myelin sheaths around axons (Figure 6.4d). Within 2 h of refeeding, neither the density of myelinated axons in the ME nor the thickness/g-ratio of myelin sheaths around axons changes (Figure 6.4e-g).
Figure 6.4 Myelin-related genes but not myelin structure change with refeeding

(a) Log2FC of genes related genes significantly changed between fasted and refed conditions ($p < 0.05$ and FDR $< 0.25$ for at least one cluster) include those of proteins found in the myelin proteome and those related to lipid synthesis and transport. An ‘X’ indicates the gene is not expressed in one or both conditions in that OL cluster.

(b) Toluidine blue-labelled thin ME sections show transverse profiles of myelinated axons with a clear distribution in the upper ME. Scale bar = 10 µm, box indicates inset in c. (c) Dark circles are transverse cross sections of myelin ensheathing an axon. (d) Transmission electron micrograph of myelin in the ME. Scale bar = 500 nm, M = myelin, MA = myelinated axon, UM = unmyelinated axon. (e) Measurements of density of myelinated axons of animals fasted ($n = 5$) and those fasted then refed 2 h ($n = 3$) (f) Measurements of g-ratio of myelinated axons in fasted ($n = 5$ mice, 481 axons) and refed ($n = 3$ mice, 308 axons) conditions. Error bars indicate mean ± SEM (g) Measurement of slopes of linear regression lines to fit individual axon g-ratios plotted against axon diameter.
6.2.2 Developing tools for study of behavioral/phenotypic effects of OPC differentiation

6.2.2.1 Myrf KO successfully blocks OPC differentiation in the ME and reveals NFOL/MOL turnover in this region

To test if the Pdgfra-CreERT2; Rosa-YFP; Myrffl/fl (Myrf KO) mouse model would also work to block OPC differentiation in the ME, Myrf KO and control littermate mice were given tamoxifen at P60 as previously described (McKenzie et al., 2014; Xiao et al., 2016). At P81, animals were fasted overnight and refed for 1 h. There was no difference in the percentage of bodyweight lost between control littermate and Myrf KO animals during the fast (Figure 6.5a). OPC differentiation was successfully blocked in the ME, as evidenced by a significant decrease in the number of YFP+ cells also positive for APC (Pdgfra-expressing cells that have matured since tamoxifen administration) (control animals had 3.25 cells per section while Myrf KO animals had 1.00 cell per section, p = 0.017, Mann-Whitney U test) (Figure 6.5b-c). Unexpectedly, the number of APC+/YFP- cells (pre-existing postmitotic OLs) was also significantly decreased in Myrf KO animals (control = 11.37 cells per section, Myrf KO = 3.94 cells per section, p = 0.0020, Student’s t-test) (Figure 6.5b, d). This indicates constitutive OPC differentiation is needed to maintain the postmitotic OL population.
Chapter 6 – Functional consequences of increased OPC differentiation in the median eminence

6.2.2.2 Viral delivery to the ME is inhibited by ME-ARC diffusion barrier

The Myrf KO mouse is extremely useful for studying the specific effects of blocking OPC differentiation into NFOLs without affecting other processes. However, tamoxifen delivery-mediated induction of Cre expression is not specific to a certain location. In order to determine if targeting of the ME is possible (for delivery of a tamoxifen derivative for example), I piloted parenchymal injections with a Cre-independent tdTomato-expressing virus to label the injection site. The ME is approximately 700 µm long, 500 µm wide, and 150µm tall and is extremely delicate (Figure 6.6a). To avoid damage to the structure, I targeted injections to the parenchyma adjacent to the ME rather than attempting to introduce the injector into it (Figure

Figure 6.5 OL labelling in Myrf KO mice
(a) Percentage bodyweight loss in littermate control and Myrf KO mice after OVN fast (control n = 9 animals, Myrf KO n = 4 animals) (b) APC (red) and YFP (green) labelling in control and Myrf KO mice (c) Number of NFOLs (APC+/YFP+ cells) per section in control and Myrf KO tissues (d) Number of pre-existing postmitotic OLs (APC+/YFP− cells) per section in control and Myrf KO tissues. * indicates p < 0.05, ** indicates p < 0.01, error bars indicate mean ± SEM, p values determined by Student’s t-tests and Mann-Whitney U test, control tissue n = 5 animals, Myrf KO tissue n = 4 animals, scale bars = 100 µm

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6.6b). When injecting into the parenchyma, a small amount of virus was able to pass into the ME, but the majority of transfection took place in the lateral hypothalamus (Figure 6.6c). This indicates the diffusion barrier between the ME and ARC limits passage of substances in both directions, and means that targeting of the ME will have to bypass the barrier.

Figure 6.6 AAV injection into the hypothalamic parenchyma
(a) Images from Paxinos and Franklin, 2001 showing the rostro-caudal, dorsoventral, and lateral dimensions of the ME, reprinted with permission from Elsevier (b) Injection strategy for viral delivery to the ME (c) Spread of tdTomato-AAV (red) after injection into the parenchyma.
6.3 DISCUSSION
To summarize, I found that neither density of myelinated axons nor myelin thickness changes between fasting and 2 h of refeeding. Myrf KO successfully prevents OPC differentiation in the ME, but pre-existing postmitotic OLs are lost as well, showing OPC differentiation is required to maintain this population. Finally, trials of viral injections targeting the ME show that the diffusion barrier is bidirectional – future targeting of the region will require circumvention of the barrier.

6.3.1 Measurement of myelin
Although gene expression indicated that myelin formation might be increased during the 1 h refeeding period, I decided to allow 2 h for refeeding as that was the shortest time I had seen published for a stimulus to cause an effect in white matter (Hofstetter et al., 2013), and I wanted to ensure any changes that occurred were directly related to the refeeding stimulus. Some studies have found that white matter structural changes measured with MRI are highly correlated with actual changes in myelin structure (Mollink et al., 2017). However, Hofstetter et al. mention that white matter diffusivity changes found in their study after task learning could indicate swelling of glia or other cells or changes in extracellular volume in addition to changes in myelin structure, so this study may not have measured white matter changes in that time frame per se (Hofstetter et al., 2013). I was unable to detect a change in myelin thickness or density of myelinated axons between fasted animals and those refed for 2 h – this could either be due to myelin being unresponsive to nutritional changes, or to using a timeframe that would be too short to measure a change.

Additionally, I may not have examined all parameters required to check for changes in myelination if they occur. As previous literature suggests that physiological changes in myelin remodelling occur as a result of NFOLs maturing and adding myelin to an unmyelinated axon segment (reviewed in Kaller et al., 2017), a better measurement of myelin changes in this region could be comparison of myelinated versus unmyelinated axons. Although I measured myelinated axon density with toluidine blue labelling, the method is not sensitive enough to detect the thin myelin that occurs from initial myelin contact with an axon. By re-imaging fasted and refed samples with TEM at greater resolution, I should be able to assess the proportion of myelinated versus unmyelinated axons.

It is worth mentioning here that OLs do not have to actively produce myelin in order to facilitate axonal conduction. Pre-myelinating OLs are able to cluster sodium channels at
regular intervals along an axon, facilitating conduction even in the absence of myelin segments (Freeman et al., 2015). Thus, it is possible that neuronal activity is being altered even in the absence of detectable myelin changes.

### 6.3.2 Possible NFOL/MOL turnover

An unexpected finding was that the number of pre-existing postmitotic OLs (APC+/YFP- cells) is decreased in Myrf KO mice (Figure 6.5b, d). Notably, the number of APC+/YFP- cells is unchanged in the CC of Myrf KO mice (Figure 6.3c), showing this phenomenon is different in the ME. This indicates that OPC differentiation is necessary to maintain the postmitotic OL population in the ME. A possible explanation for this finding is that postmitotic OLs normally turnover in this region.

APC (clone CC1) labels postmitotic OLs (Bin et al., 2016), so should label MOLs. If NFOLs are producing myelin genes or proteins, it is possible they could be labelled by this antibody as well. Constitutive NFOL turnover has described before. In the mouse cortex, around 80% of NFOLs die shortly after differentiating unless they incorporate into a circuit (Hughes et al., 2018). Turnover of MOLs, however, is counterintuitive to previously published studies. MOLs are known to be incredibly stable once integrated into circuits (Hughes et al., 2018), and in certain murine white matter tracts MOLs have been shown to survive for over 1.5 years (Tripathi et al., 2017). That MOLs turn over in this region would be a substantial finding. Our lab is currently conducting fate-mapping studies in mice that inducibly express fluorescent proteins in mature OLs to test this possibility – a decrease in labelled cells over time could indicate turnover. Additionally, direct measurement of cell death (via labelling with terminal deoxynucleotide dUTP nick end labelling [TUNEL] or caspases, for example) could be used to show if MOL turnover occurs in this region.

### 6.3.3 Targeting the ME

The presence of a bidirectional diffusion barrier between the ME and ARC suggests that injection into the parenchyma adjacent to the ME may not be suitable for targeting the ME. The injector likely would have to be introduced into the ME itself, possibly causing damage to the region. An angled injection approaching the ME from either (or both) side(s) is better than an injection at the midline, as midline injection would break the ME-3V barrier, causing diffusion of injected substance into the ventricular system.
In order to maintain the OPC-specific expression of YFP and deletion of Myrf, injection of a tamoxifen derivative is preferable to injection of a Cre-expressing virus, which would theoretically cause YFP expression and Myrf deletion in every cell transfected by the virus. Specificity of Myrf deletion in both cell type and region is necessary for understanding the importance of OPC differentiation in the ME for any potential effects on food intake or body weight. One could envision animals altering food intake behavior if global brain Myrf KO caused problems with memory or learning. Also, while previous studies showed global Myrf deletion caused no gross motor defects (McKenzie et al., 2014), blockade of OPC differentiation in extra-ME locations might cause subtle changes in energy expenditure (e.g. from altered locomotion during light or dark periods) which could, in turn, influence bodyweight.

Besides targeting the ME via brain injections, another option is to exploit the position of this structure outside the BBB. Pegylation has been used to prolong the half-life of tamoxifen in the blood for breast cancer treatment (Majd et al., 2017). It is possible a similar substance could be used to cause Cre-lox recombination in Pdgfra-expressing cells of the ME. Pdgfra is expressed in tissues throughout the body in adult mice (Andrae et al., 2014) and, thus, peripheral administration of tamoxifen in Myrf KO mice would cause YFP expression outside the CNS. However, Myrf is only expressed in OLs so effects of its deletion would be specific. No matter the method of administration, timing of Cre-lox recombination needs to be optimized to study effects of OPC differentiation blockade.

6.3.4 Aligning Myrf deletion and OPC differentiation studies
The unexpected finding that postmitotic OLs may constitutively turnover in the ME means that studies of food intake effects of OPC differentiation blockade need to occur after substantial Myrf deletion but before depletion of the postmitotic OL population. Otherwise, potential food intake or bodyweight effects could be due to absence of the OL population rather than prevention of an increased number of NFOLs.

We know from our experiment that a large proportion of the postmitotic OL population was depleted within 3 weeks. A previous study reported maximal Cre-lox recombination in certain lines with 5 once-daily ip tamoxifen doses followed by 21 days of rest before sacrifice (Jahn et al., 2018). However, this group also showed close to 40% recombination occurred with one dose of ip tamoxifen in particular mouse strains. Our lab is currently testing various tamoxifen administration strategies in Pdgfra-CreER$^{T2}$;Rosa-YFP;Myrf$^{fl/fl}$ mice to ensure a
balance between preventing pre-existing postmitotic OL depletion and achieving the maximum possible genomic recombination.

While studies from this chapter were not able to link OPC differentiation and food intake behavior through a possible mechanism of increased myelination at a 2 h timepoint, they did allow for additional characterization of ME OLs and the ME-ARC border.
CHAPTER 7

GENERAL DISCUSSION

Experiments described in this thesis provide evidence that: 1) there are three subtypes of OLs in the ME and a unique enrichment of MOLs/myelin in the dorsal portion of the ME; 2) genes related to NFOLs and OPC differentiation pathways are increased with 1 h refeeding after a fast; 3) mTOR activity (from mTORC1) is highly active in ME OLs and is increased with refeeding and HPD; and 4) although OPC differentiation increases with refeeding, neither myelin thickness nor density increases in the short term. Additionally, transgenic mouse models that globally block differentiation of OPCs in the brain cause a loss of postmitotic OLs in the ME, suggesting postmitotic OLs normally require NFOL production to maintain the population. Finally, prevention of viral transfection of the ME from an ARC injection suggests that the ME-ARC diffusion barrier is bidirectional. These findings have helped to address my overarching goal of the thesis: to explore the role that OLs of the ME may play in maintaining energy homeostasis.

7.1 THE NEURON+GLIA HOMEOSTAT MODEL

The data from experiments described in this thesis provide clues about the ways in which OLs might be involved in maintaining energy homeostasis. First, NFOLs may alter diffusion of energy availability signals into the ARC, thus affecting the ability of neurons, OLs, or other cell types to detect the signals. Second, OPCs may act as energy availability sensors, as they express receptors to several nutrient availability signals. Third, OPCs respond to nutrient availability by rapidly differentiating. The functional relevance of this differentiation is still unknown, but NFOLs could be involved in reinforcing the ME-ARC diffusion barrier or
facilitating myelination of axons in the region (at a timepoint greater than two hours). mTOR signalling is highly active in ME OLs – the pathway may play a role in OPCs’ detection of energy availability signals to facilitate differentiation, or in promoting myelination in more mature OLs. When combined with what is known about how other glia influence energy homeostasis mechanisms, the data from this thesis allow a much fuller picture of how energy homeostasis is maintained via neural control of food intake (Figure 7.1 versus Figure 1.2a).

Figure 7.1 Neuron+glia homeostat model
Schematic of how increased energy/nutrient availability may affect different cell types in the body’s attempt to return to homeostasis.
7.2 STUDIES NEEDED TO CONFIRM THE ROLES OF ME OLS IN ENERGY HOMEOSTASIS MAINTENANCE

The findings described in this thesis leave several questions unanswered. Do NFOLs actually reinforce the ME-ARC diffusion barrier, and is reinforcement dependent on nutrient availability? Which signalling molecule is sensed by OPCs to elicit differentiation? Does OPC differentiation regulate food intake and bodyweight? Here I will describe experiments our lab has planned to address these questions.

7.2.1 NFOLs as diffusion barriers

The idea that NFOLs may form or reinforce the diffusion barrier between the ME and the ARC is compelling, as this was previously thought to be mediated by tanycytes. As mentioned in chapter 3, tenascin-R (Tnr) is expressed in NFOLs more than any other cell type of the ME (Figure 3.12). In order to confirm that OLs act as a diffusion barrier, we will need to analyze TNR and MBP expression in animals peripherally injected with a dye (such as Evans blue) to visualize the extent of diffusion. As we know the diffusion barrier ‘lifts’ in fasting (Figure 4.1), it will be interesting to investigate if myelin also changes in some way. Our lab has recently published findings that TNR expression is increased in refeeding (Kohnke et al., 2019) (Figure 7.2) – it is possible these barrier proteins are more diffuse in the fasted state, allowing greater diffusion of blood-borne molecules into the ARC. Another way to determine whether myelin itself plays a role in regulating diffusion (or indeed food intake or bodyweight), would be to use an inducible mouse model that ablates myelin while keeping other OL proliferation/maturation processes intact; for example, an Olig2-CreER$^{T2}$;Mobp$^{0/0}$ line. Again, we would try to target Cre expression to the ME only.
7.2.2 IGF1 as candidate OPC differentiation signal

Due to its nutritional regulation (Table 4.3), its relatively high expression in OPCs (Figure 5.9), and knowledge of its ability to stimulate both mTOR activity and OPC differentiation, IGF1R is our top candidate for the protein allowing OPCs to sense nutrient availability signals. In order to test if it is involved in OPC differentiation, we will administer IGF1 in ad libitum fed mice in a BrdU labelling experiment. Additionally, administration of synthetic IGF1R blockers has been shown to prevent differentiation in other cell types (Gong et al., 2017). If IGF1 administration increases differentiation and IGF1R blockade prevents ME OPC differentiation in refeeding, we could presume that its actions normally facilitate increased OPC differentiation in refeeding.
7.2.3 Food intake and bodyweight studies in Myrf KO mice

The finding that the presence of ME OPCs regulates bodyweight and food intake (Djogo et al., 2016) was exciting for our group. In order to interrogate whether OPC differentiation, specifically, is important for food intake/bodyweight maintenance, we will perform a fast-refeed experiment in Myrf KO mice.\(^1\) In the energy homeostasis model, OPC differentiation appears to promote food intake cessation in refeeding (Figure 7.1):

- OLs may limit diffusion of energy availability signals to the ARC. When diffusion is increased in fasted animals via tanyctyic VEGFA secretion (Langlet et al., 2013), it causes animals to eat more, thus the opposite may be true for refeeding.
- Stimulation of vasopressin or oxytocin + AgRP neurons causes anorectic effects (Atasoy et al., 2012; Sutton et al., 2014; Yoshimura et al., 2017). If NFOL production facilitates their activity (via channel clustering or myelination after the 2 h timepoint), the phenomenon could lead to food intake cessation.

Therefore, I hypothesize that Myrf KO animals will eat more than their control counterparts during refeeding. Over time, this increased food intake could lead to increased bodyweight.

7.3 Impaired maintenance of energy homeostasis in disease

The majority of this thesis has focused on how OLs of healthy animals respond to nutrient availability. In order to widen the impact of this work, it will be necessary to examine how these cells respond in pathological conditions where energy homeostasis or OL maturation is known to be impaired.

7.3.1 Obesity

Obesity is an archetypal condition of impaired energy homeostasis. Obesity is often characterized by insulin and leptin resistance, a phenomenon where the body’s cells are either unable to sense or respond to these particular energy availability signals. This causes increased

\(^1\) Of course, bearing in mind the necessary optimization of tamoxifen administration mentioned in the last chapter. The experiment will need to take place when substantial Myrf deletion has occurred, but postmitotic OLs are still present.
food intake and weight gain as the body is not able to adapt behavior to changes in energy abundance with decreased food intake (Gruzdeva et al., 2019).

In my experiment examining mTOR signalling in LFD versus HFD, I found significantly decreased mTOR activity in ME OLs of HFD-fed mice in both the fasted and refeed conditions (Figure 5.7c). As mTOR is important for myelination in postmitotic OLs (Lebrun-Julien et al., 2014; Wahl et al., 2014; Zou et al., 2014), this could indicate reduced capacity of the cells to produce myelin. Interestingly, in mice and humans, obesity is associated with reduced myelin or decreased white matter volume, respectively (Karlsson et al., 2013; Sena et al., 1985).

### 7.3.2 Demyelinating disease

The prevailing view about the pathogenesis of demyelinating diseases like multiple sclerosis (MS) is that myelin damage is caused by an autoimmune response. The ‘autoimmune hypothesis’ posits that the disease starts when peripheral immune cells infiltrate the central nervous system, secreting cytokines that damage OLs (reviewed in Nakahara et al., 2011). However, there is evidence that OL damage in MS can occur in absence of an immune response (Barnett and Prineas, 2004). The ‘oligodendrogliopathy hypothesis’ suggests that inflammation is secondary to oligodendrocyte apoptosis and functions to clear myelin debris (reviewed in Nakahara et al., 2011). As MS is notoriously heterogeneous, it is possible that both of these conjectures are true – for different patients. The difference in cause of the disease is important when considering which treatment will be more efficacious for patients. Most current treatments for MS act by suppressing immune responses (Ghasemi et al., 2017). More recently, many studies are focussing on reigniting the ability of existing OPCs to mature and produce myelin (Hooijmans, et al., 2019).

Here, study of OLs of the ME could be very useful. These cells represent a population of cells that lie outside the blood-brain barrier (BBB) and could potentially be used as a model of OLs within lesions. Permeability of the barrier is found in all clinical subtypes of MS (van Horssen et al., 2007; Shimizu et al., 2018). Not having to induce an injury in the animal would allow for study of OLs in absence of microglia activation or astrocytosis as occurs in the cuprizone and experimental autoimmune encephalomyelitis (EAE) models of MS (van der Star et al., 2012).

Additionally, later studies in our lab showed that rapid OL differentiation in response to refeeding occurs in the ME but not the corpus callosum, indicating region-specific
susceptibility to this stimulus (Kohnke et al., 2019). When researching therapies for conditions with limited OPC differentiation, it could be useful to study: 1) what makes ME OLs, in particular, able to differentiate so rapidly, and 2) how the fast-refeed stimulus enables rapid maturation of these cells.

### 7.3.3 IF as a potential therapy for obesity and demyelinating disease

IF, a time-restricted pattern of eating with rapid shifts between energy deficit and availability (similar to the fast-refeed paradigm), is currently being studied as a treatment for both obesity and demyelinating diseases (Choi et al., 2016; Harris et al., 2018). Choi et al. found that switching between states of energy deficit and abundance after EAE induction was able to increase OPC differentiation and enhance myelination more than a control diet.

In clinical studies, IF has been shown to benefit circulating insulin levels in obese participants, and to improve self-reported Health-Related Quality of Life score in patients with relapse-remitting MS (Choi et al., 2016; Harris et al., 2018). However, much more research is needed to determine if these effects are due in part to increased OPC differentiation.

### 7.4 Conclusion

This thesis constitutes the first in-depth analysis of oligodendrocytes of the median eminence of the hypothalamus and details their response to dietary nutrient availability. Future studies will reveal downstream effects of increased OPC differentiation on food intake behavior and body weight, and may prove useful in identifying therapies for diseases of impaired energy homeostasis or OL function.
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References


APPENDIX

Kohnke_et_al_2019

July 19, 2019

1 Code to analyze and visualize scRNAseq data - R

1.1 Cluster cells and find defining genes per cluster

Packages required: cellrangerRkit, NbClust, edgeR, GOplot, devtools

References:

Locate file path that contains files produced from 10x sequencing (including gene BC matrix and mouse genome info), set as working directory

```r
setwd("~/your/file/path")
```

Load gene BC matrix (contains info about sample barcodes, expression counts per cell) and analysis results (10X performs some preliminary analysis)

```r
[.] gbm<-load_cellranger_matrix("./")
analysis_results<-load_cellranger_analysis_results
```

1.1.1 Find optimal number of clusters for the dataset

Use coordinates for all cells in tSNE plot: `tsne_kmeans.csv` (in Github repository)

```r
[.] tsne_kmeans<-read.csv("tsne_kmeans.csv")
fviz_nbclust(tsne_kmeans,kmeans,method="silhouette",k.max=15)
```

See Supplementary Figure 1d for output

1.1.2 Find top defining genes per cluster

Use 9 K-means clusters based on silhouette analysis. Can change ‘n’ to the number of defining genes required. Output file will be in the working directory

```r
[.] cells_to_plot<-order_cell_by_clusters(gbm,
--analysis_results&Clustering$kmeans_9_clusters$Cluster)
prioritized_genes<-prioritize_top_genes(gbm,
--analysis_results&Clustering$kmeans_9_clusters$Cluster, "sseq", min_mean=0.5)
write_cluster_specific_genes(prioritized_genes, output_folder, n_genes=50)
```
1.1.3 Find genes differentially expressed between conditions

Use new .csv file that only contains experimental condition and gene expression data from a single cluster. See "Cluster1_edgeR.txt" (in Github repository). The stringency set in rowSums is based on trial and error for similar experiments in our lab. Can change ‘n’ to the number of DEGs required.

```r
x <- read.delim("Cluster1_edgeR.txt", row.names="symbol")
group <- factor(c(...)

y <- DGEList(counts=x, group=group)
keep <- rowSums(cpm(y)>5) >= 8
y <- y[keep, , keep.lib.sizes=FALSE]
y <- calcNormFactors(y)
design <- model.matrix(~group)
y <- estimateDisp(y, design)
fit <- glmQLFit(y, design)
qlf1vs2 <- glmQLFTest(fit, coef=2)
topgenesQLFtest <- topTags(qlf1vs2, m=2000)
write.csv(topgenesQLFtest, "topgenesQLFtest.csv")
```

1.2 Produce figures for scRNAseq data

Packages required: cellrangerRkit, ggplot2, tidyr
1.2.1 UMI counts mapped to tSNE plot

```r
[.] tsne_proj<-analysis_results\$tsne
visualize_umis_counts(gbm.tsne_proj[c("TSNE.1","TSNE.2"],limits=c(5,4),marker_size=0.05)
```

See Supplementary Figure 1c for output

1.2.2 Visualize cluster assignments on tSNE plot

Based on 9 clusters determined by silhouette analysis. Cluster names were assigned based on
defining genes.

```r
[.] visualize_clusters(analysis_results\$clustering\$kmeans_9\_clusters\_Cluster,tsne_proj[c("TSNE.1","TSNE.2"],
=colour=c("green","blue","red","purple","yellow","orange","pink","gray","black"),
legend anno = c("tanyocyte","astrocyte","oligodendrocyte",
=1","microglia","neuron",
"oligodendrocyte 2","VLMC","ependymocyte","endothelial"))
```

See Figure 1b for output

1.2.3 Create heatmap of top gene expression per cluster

Can change n.genes to the number of top genes required

```r
[.] gbm_heatmap(log.gene_bc_matrix(gbm,base=2),prioritized_genes,cells.to.plot,n.genes=5,colour=c("green","blue","red","purple","yellow","orange","pink","gray","black"),limits=c(-1,2))
```

See Figure 1c for output

1.2.4 Create violin plot of defining genes per cluster

Genes chosen based on defining genes per cluster. Use .csv file with expression values of
the chosen genes for all cells. See `violingenes2.csv` (in Github repository).

```r
[.] nineclusters<-read.csv("violingenes.csv")
nineclusters\$Cluster<-as.factor(nineclusters\$cluster)
nineclusterslong<-gather(nineclusters, key="measure", value="value", c("Rax","Agt","Ermm","Clqc","Shhl1","OS9","Dcm","Elof1","Itx2a"))
nineclusterslong\$measure.f<-factor(nineclusterslong\$measure,levels=c("Rax","Agt","Ermm","Clqc","Shhl1","OS9","Dcm","Elof1","Itx2a"))
ggplot(nineclusterslong, aes(x=cluster, y=value, fill=cluster))+
geom_violin(scale='width')+facet_wrap(~measure.f, scales="free_y", nrow=1)+
coord_flip()+theme_classic(base.size=7)+scale_y_discrete(limits=unique(rev(nineclusterslong\$cluster)))+
scale_fill_manual(values=c("green","blue","red","purple","yellow",
"orange","pink","gray","black"))
```
 labs(y="UMI")

See Figure 1d for output

1.2.5 Create bubble plot of pathways changed between experimental conditions


```r
opc_ipa<-read.csv('IPAOPBubble.csv')
opcgenelist<-read.csv('opcgenelist.csv')
ocirc<-circle_dat(opc_ipa,opcgenelist)
GOBabble(opccirc, labels=3)
opcreduced_circ<-reduce_overlap(opccirc,overlap=0.8)
GOBabble(opcreduced_circ,colour=c("yellow","light green"),labels=2.05)

nfol_ipa<-read.csv('NFULIPABubble.csv')
nfolgenelist<-read.csv('nfolgenelist.csv')
nfolcirc<-circle_dat(nfol_ipa,nfolgenelist)
GOBabble(nfolcirc,labels=5)
nfolreduced_circ<-reduce_overlap(nfolcirc,overlap=0.8)
GOBabble(nfolreduced_circ,colour=c("yellow","light green"),labels=4.8)

mol_ipa<-read.csv('MOLIPABubble.csv')
molgenelist<-read.csv('molgenelist.csv')
molcirc<-circle_dat(mol_ipa,molgenelist)
GOBabble(molcirc,labels=5)
molreduced_circ<-reduce_overlap(molcirc,overlap=0.8)
GOBabble(molreduced_circ,colour=c("yellow","light green"),labels=5.2)
```

See Figure 4b for output