

**Characterization of murine HRASLS-1: transcript variant analysis, metabolic regulation,  
developmental expression, and a role in inflammation.**

by

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## **AUTHOR'S DECLARATION**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## Abstract

N-acyl ethanolamides (NAE) are involved in a variety of biological processes ranging from fat oxidation to inflammation mediation. Recently, the HRASLS family, a group of class II tumour suppressor proteins, have been identified and shown to have in vitro phospholipase as well as N-acyltransferase activity. HRASLS-1 functions predominantly as an N-acyltransferase to generate NAPE in animal cells that can be further metabolized into bioactive NAEs. Although this enzyme has been characterized with regards to its molecular function, no physiological role has been identified. In order to gain some insight into possible cellular or tissue functions for this enzyme, studies were conducted to analyze *Hrasls-1* gene expression under changing nutritional states, during inflammation, and at different embryonic developmental time points. Two distinct *Hrasls-1* transcripts that code for the same protein, a 0.8 kb and a 2.9 kb variant, were initially identified. Stability of the transcripts was tested by treating cultured BV2 microglial immunocytes with actinomycin D to arrest transcription, followed by qPCR analysis of *Hrasls-1* mRNA levels. It was found that the *Hrasls-1* 0.8kb transcript variant has a shorter half-life than the 2.9 kb variant. Next, expression of the *Hrasls-1* transcript variants was analyzed within mice that were non-fasted, fasted or re-fed. *Hrasls-1* expression was determined in liver, kidney, white adipose tissue, heart, and brain. In the liver, kidney and white adipose, *Hrasls-1* was induced by fasting, while in the heart and brain there was no significant induction during any of the different nutritional states. This work suggests a possible role for HRASLS-1 in regulation of the substrate oxidation between fasting and re-feeding. The regulation of *Hrasls-1* and the generation of NAPE upon induction of inflammation in BV2 microglial immunocytes was tested. *Hrasls-1* total levels were induced 24 h after activation of BV2 cells with lipopolysaccharide (LPS), and this was largely due to an increase in the 0.8 kb transcript variant. In line with these findings, there was an increase in the incorporation of [<sup>14</sup>C]palmitate into NAPE. Whole embryos were analyzed at

different developmental stages (E10.5, 14.5 and 18.5), in order to characterize the regulation of *Hrasls-1* in embryogenesis. It was found that the 2.9kb transcript variant showed significant modulation of expression throughout the measured time points. These results show regulation of *Hrasls-1* gene expression under differing nutritional, developmental and inflammatory conditions, and are consistent with a role for modulation of NAPE synthesis in conditions where NAEs play a signaling role.

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## List of Abbreviations

ACOX1	Acyl-Coenzyme A oxidase 1
AEA	N-arachidonoylethanolamine
ARE	Adenylate/uridylate-rich element
Ca-NAT	Calcium-dependant n-acyltransferase
CPT1	Carnitinepalmityltransferase 1
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FAT/CD36	Fatty acid translocase
GLP-1	Glucagon-like peptide-1
GRP119	G protein-coupled receptor 119
iNAT	Calcium-independent n-acyltransferase
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LRAT	Lecithin retinol acyltransferase
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NAE	N-acylethanolamide
NAPE	N-acylphosphatidylethanolamide
NAPE-PLD	N-acylphosphatidylethanolamide phospholipase-D
NAT	N-acyltransferase
NEFA	Non esterified fatty acid
NF- $\kappa$ B	Nuclear factor-beta kappa
NOPE	N-oleoylphosphatidylethanolamide
NPPE	N-palmitoylphosphatidylethanolamide
NSPE	N-stearoylphosphatidylethanolamide
OEA	N-oleylethanolamide
PE	Phosphatidylethanolamidene
PEA	N-palmitoylethanolamide
PC	Phosphatidylcholine
PLA	Phospholipase activity
PPAR- $\alpha$	Peroxisome proliferator activated receptor $\alpha$
PPRE	Peroxisome proliferator response elements
SEA	N-stearoylethanolamide
TAG	Triacylglycerol
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TRPV1	Transient receptor potential cation channel subfamily V member 1
UCP2	Uncoupling protein 2
UCP3	Uncoupling protein 3
UTR	Untranslated region
WAT	White adipose tissue

## Chapter 1

### Introduction

N -acylethanolamides (NAEs) are “ethanolamides of long chain fatty acids”<sup>1</sup> that are naturally occurring biological signalling molecules and endogenous lipid mediators in various organisms including animals and plants<sup>21, 94</sup>. NAEs are synthesized from precursor N-acylphosphatidylethanolamides (NAPEs). Depending on the acyl chain present on the NAE, which in turn is dependant on the fatty acyl chain found on the NAPE precursor, the NAE species generated may have a variety of physiological functions as well as a unique affinity to bind and activate specific receptors<sup>97</sup>. The most abundant NAEs found in animal tissue include N-palmitoylethanolamide (PEA), N-oleoylethanolamide (OEA), and N-steroylethanolamide (SEA)<sup>26,98</sup>. These lipid molecules influence a variety of physiological functions, ranging from anti-inflammatory and neuroprotective responses<sup>107, 108</sup> to anorexiant roles that regulate feeding and body weight<sup>116,117</sup>. Understanding the regulation of NAPEs/NAEs is therefore important for understanding normal physiology, and has implications for treating myriad disease conditions from obesity to cancer, diabetes, and heart disease.

NAEs are synthesized from glycerophospholipids via a “two-step enzymatic pathway”<sup>23, 24</sup>. The first reaction transfers a fatty acyl chain from a donor glycerophospholipid to the amino group of an ethanolamine phospholipid<sup>25</sup>, catalyzed by an N-acyltransferase enzyme<sup>2,3,25</sup>. A family of class II tumour suppressor proteins has been identified and characterized with this activity<sup>4</sup>. This family is known as the Harvey-Ras-like tumour suppressor enzymes (HRASLS), and consists of five similarly structured enzymes (HRASLS-1-5)<sup>37-39</sup>. Limited molecular analysis of HRASLS-1 has identified it as having relatively high N-acylation activity, both in vitro and in vivo<sup>5</sup>. This suggests that HRASLS-1 may play a role in the regulation of some physiological and disease processes, by modulating the in vivo generation of NAPE molecules and their downstream signaling products,

NAEs. However, a role for HRASLS-1 in normal or aberrant physiology has not yet been investigated. Identifying conditions under which *Hrasls-1* expression is modulated will provide insight into possible sites of action and physiological roles for this enzyme. Therefore, the primary goal of this work was to assess the expression of *Hrasls-1* under changing physiological conditions known to be regulated, at least in part, by NAE signaling activity. Studies in this thesis were centered on the expression of *Hrasls-1* in inflammation, embryological development, and changes in different dietary states.

## Chapter 2

### Biochemical Foundations

#### 2.1 Overview of N-acylphosphatidylethanolamide synthesis

The HRASLS-1 pathway directly synthesizes NAEs. Understanding the regulation of HRASLS-1 is therefore important for understanding the regulation of NAE signaling, and the physiological and disease processes that are controlled by NAE-regulated pathways.

NAEs are bioactive molecules generated in response to a variety of stimuli <sup>6</sup>. They tend to be rapidly produced and then rapidly hydrolyzed, and therefore are short-lived signaling intermediates, making the amount and chemical composition of precursor NAEs an important regulatory element in NAE biosynthesis and action. In the classical “N-acylation/phospholipase D” (NAPE-PLD) pathway<sup>7</sup>, the first step forms a NAPE, by the transfer of the *sn-1* fatty acyl chain from a donor glycerophospholipid to the primary amino group of an ethanolamine phospholipid such as PE <sup>8</sup> (**Figure 1**). A wide variety of fatty acyl species can be attached to the nitrogen of phosphatidylethanolamine, and the availability of different fatty acids at the *sn-1* position of donor glycerophospholipids is an important determinant of the specific NAPE species that are produced in any given tissue <sup>9</sup>. In the second step of NAE synthesis, phospholipase D-mediated cleavage releases an NAE, leaving phosphatidic acid. Given the role of NAEs in signaling events, the initial step of NAPE biosynthesis has considerable biological relevance, since the specific nature of the acyl species attached to the ethanolamine group will determine the corresponding NAE that can be synthesized in the second enzymatic step, and hence the specific biological signaling role that it can play<sup>3</sup>. In mammalian cells, two groups of NATs – calcium-dependent NAT (Ca-NAT) and calcium independent NAT (iNATs) - are responsible for the synthesis of NAEs, and both utilize glycerophospholipids as acyl donors. HRASLS-1 belongs to the second group, the

iNATs, which were discovered after Ca-NAT. An acyl-CoA dependent N-acyltransferase called NAPE synthase has also been identified, but is found only in plants.

## **2.2 Ca-dependent NAT activity, expression and signaling role**

Ca-NAT activity has been studied in various animal tissues<sup>3</sup>. All information on this enzyme is based on studies of activity, since it has not yet been identified and cloned. As a result, it is unknown whether Ca-NAT activity in cells is a result of one enzyme or a group of enzymes, and it is not possible to determine mRNA expression regulation of Ca-NAT(s) with changing physiological conditions. Activity assays have found this enzyme to be catalytically active at neutral and alkaline pH<sup>10,11</sup>. Cellular Ca-NAT activity results in the formation of NAPE using phospholipids as acyl donors, with release of an acyl chain occurring entirely from the *sn-1*, and not the *sn-2* position<sup>3</sup>. Ca-NAT activity is significantly activated by Ca<sup>2+</sup><sup>3</sup>. Phospholipids that act as acyl donors for Ca-NAT activity include phosphatidylcholine (PC), 1-acyl-lyso PC, PE and cardiolipin<sup>3</sup>. The fatty acyl-chain acceptors include phospholipids such as PEs, alkyl acyl-type, and alkenyl-acyl type PEs, as well as lyso PE<sup>3</sup>. Ca-NAT does not show preference for a specific length or unsaturation of the acyl chain chosen for transfer<sup>10</sup>.

Inflammation is associated with calcium release from tissues and immunocytes. Accumulation of NAPE and NAE have been found in inflammation and cellular degeneration<sup>12</sup>. NAPE and NAE generation has been associated with the activation of the Ca-NAT enzyme by Ca<sup>2+</sup> stimulation<sup>3</sup>. Whether calcium-independent N-acyltransferases, such as HRASLS1, could also be activated by inflammation alongside Ca-NAT(s) has not been tested.

Similar to HRASLS-1 expression patterns, the highest activity for Ca-NAT activity in rat was found in brain, with lower levels in testis and muscle<sup>13-15</sup>. The highest activity in brain was observed in the brainstem, with intermediate activity in the cortex, striatum, hippocampus,

medulla, and cerebellum, and the lowest activity in thalamus, hypothalamus, and olfactory bulbs<sup>13-15</sup>. Taken together, these data suggest that NAPE synthesis is critical in brain signalling, and that both Ca-NATs and iNATs may play important roles in mediating these processes.

### **2.3 NAPE Synthase N-acylation Activity and Acyl Donor Specificity**

In addition to the mammalian acyltransferases, the enzyme NAPE synthase has been cloned from the plant *Arabidopsis thaliana*<sup>16</sup>. The enzyme is of 284 amino acids long, and is characteristically similar to glycerophospholipid acyltransferases such as lyso PC acyltransferase<sup>3</sup>. Like Ca-NAT, NAPE synthase catalyzes the N-acylation of PE, although it uses acyl-CoA instead of glycerophospholipid as an acyl donor<sup>3</sup>. Since purified recombinant NAPE synthase uses only activated acyl-CoA, but not free fatty acids, this rules out the possibility that it incorporates free fatty acid directly into PE<sup>3</sup>. Currently, it is not known which enzymes would be responsible for this catalysis within animal tissues<sup>3</sup>.

### **2.4 Identification of iNAT Family Members, Catalytic Activity and Enzymatic Role**

iNATs were initially enzymatically characterized for a conserved motif, shared with lecithin retinol acyltransferase (LRAT). This motif contains a reactive cysteine residue involved in the family's catalytic activity<sup>16,17</sup>. LRAT catalyzes the transfer of a palmitoyl chain from the *sn-1* position of phosphatidyl choline to all-*trans*-retinol, forming retinyl ester<sup>18</sup>. LRAT has also shown activity in acylating the amino group of retinylamine<sup>19</sup>. This catalytic resemblance between LRAT and Ca-NAT encouraged the examination of LRAT-homologous proteins for similar PE N-acylation activity<sup>3</sup>.

The Harvey-Ras-like tumor suppressor (HRASLS) enzymes consist of five, similarly structured class II tumor suppressor proteins (HRASLSL1-5)<sup>20-22</sup> that make up the iNAT enzymes.

While all five proteins are encoded in the human genome, murine studies have only identified the presence of HRASLS-1, HRASLS-3, and HRASLS-5<sup>23</sup>. HRASLS-1-4 range from 162 to 168 amino acids, with a molecular weight of ~18 kDa<sup>4</sup>. HRASLS-5 has 279 amino acids and is larger than the others, at ~31 kDa in size<sup>4</sup>. In mice, HRASLS-1 is located on chromosome 16, while HRASLS-3 and -5 are located on chromosome 19<sup>4</sup>.

All five members of the HRASLS family have been shown to have in vitro phospholipase A1/2 (PLA1/2) activity, which releases a fatty acid from the *sn*-1 or *sn*-2 position of a glycerophospholipid. All five members have also been shown to have in vitro O-acyltransferase activity, catalyzing the transfer of an acyl group from a glycerophospholipid donor to the hydroxyl group of a lysophospholipid, as well as in vitro N-acyltransferase activity, catalyzing the formation of NAPE<sup>5,24-26</sup>. In vivo, however, these enzymes do not always recapitulate their in vitro functions, tending to favor only one of these specific catalytic functions. When considering the iNATs, it is important to note that their catalytic properties differ from those of the Ca-NAT enzyme in several ways, most predominantly by the fact that the activity of these proteins is only marginally increased by Ca<sup>2+</sup> addition, indicating Ca<sup>2+</sup>-independent activity<sup>10</sup>.

Despite the conservation of their catalytic sites, the HRASLS proteins show distinctive tissue specificity and unique catalytic preference for different reactions.

#### **2.4.1 HRASLS-1**

HRASLS-1 was “originally cloned by differential display between two mouse cell lines, the embryonic fibroblast line C3H10T1/2, and the chondrogenic ATDC5 line”<sup>27</sup>. HRASLS-1 is highly expressed in mouse heart, brain and testis<sup>28-30</sup>. HRASLS-1 shows predominant PLA1 activity over PLA2 activity, as well as a preference for O-acylation of the *sn*-1 position of lyso PC<sup>5</sup>. Relative to other HRASLS family members, HRASLS-1 shows in vitro PE N-acylation activity that is as high as

murine/human HRASLS-5 and human HRASLS-2<sup>5</sup>. However, it is clear that in vivo HRASLS-1 functions predominantly as an N-acyltransferase to generate NAPE in animal cells, which can be further metabolized into bioactive NAEs<sup>5,31</sup>.

#### **2.4.2 HRASLS-2**

Purified recombinant human HRASLS-2 shows the highest in vitro acyltransferase activity among the HRASLS family members<sup>31</sup>, with higher PE N-acyltransferase and lysophospholipid O-acyltransferase activities than either HRASLS-3 or HRASLS-4<sup>26</sup>. Constitutive expression of HRASLS-2 in stably transfected human embryonic kidney 293 cells (HEK 293) increases endogenous levels of NAPE and NAE<sup>31</sup>, indicating that HRASLS-2 acts as a NAPE-forming acyltransferase in vivo, and the enzyme activity of HRASLS-2 has been found to increase production of downstream NAE containing 16:0, 18:0, and 18:1<sup>32</sup>. However, unlike HRASLS-1, HRASLS-2 shows a different tissue expression profile, with highest levels in the small intestine, kidney and trachea<sup>4</sup>. Additionally, HRASLS-2 is absent from the murine genome, and therefore unlike HRASLS-1, cannot regulate NAPE/NAE-mediated pathways in mouse cells or tissues<sup>23</sup>.

#### **2.4.3 HRASLS- 3**

HRASLS-3 was the first HRASLS family member to be characterized enzymatically<sup>33</sup>. In vitro, a weak PE N-acyltransferase activity was detected<sup>34</sup>, although cells overexpressing HRASLS-3 do not show a significant increase in NAPE synthesis in vivo<sup>23</sup>. Rather, recombinant murine and human HRASLS-3 show a PLA<sub>1/2</sub>-like hydrolyzing activity toward various PCs and PEs<sup>25,33</sup>. As a result, HRASLS-3 is best considered a PLA rather than an iNAT, despite homology with the HRASLS family, and likely does not modulate the same pathways as HRASLS-1.

#### 2.4.4 HRASLS-4

HRASLS-4 has in vitro enzymatic activity that is similar to HRASLS-3, with significant PLA<sub>1/2</sub> activity and relatively low PE N-acylation activity<sup>26,35</sup>. In contrast, however, transient expression of the protein caused intracellular accumulation of NAPE, indicating that N-acyltransferase activity predominates over PLA activity of HRASLS-4 in vivo<sup>23</sup>. However, unlike HRASLS-1, HRASLS-4 is primarily expressed in skin, colon, lung and liver tissues<sup>36-39</sup> and, furthermore, HRASLS-4 is one of two HRASLS family members found in humans but not mice<sup>23</sup>, where my studies were conducted.

#### 2.4.5 HRASLS-5

HRASLS-5 shares a high degree of homology with both HRASLS-1 and HRASLS-3<sup>40</sup>, and has shown conservation through human, mouse and rat species<sup>24</sup>. Unlike other HRASLS family members, this enzyme is detected primarily in the cytosol, instead of the membrane region of cells<sup>24</sup>. Enzymatically, HRASLS-5 has N-acyltransferase activity and is able to abstract an acyl group from not only the *sn*-1 position, but also the *sn*-2 position of PC<sup>24</sup>. Although HRASLS-5 is most likely to play a similar role to HRASLS-1 in mouse tissues and cells, the tissue distribution of this enzyme is different, with HRASLS-5 showing predominant expression in the pancreas<sup>40</sup>.

### 2.5 NAE Synthesis via NAPE-PLD-Dependent and Independent Pathways

As previously mentioned, the second enzymatic reaction involves the hydrolysis of NAPE to NAE by NAPE-hydrolyzing phospholipase D (NAPE-PLD)<sup>41</sup>. Recombinant NAPE-PLD produces several long-chain NAEs<sup>3</sup>, including N-arachidonylethanolamine (AEA), PEA and OEA from corresponding NAEs<sup>41</sup>. NAEs with medium-chain N-acyl species (C4:0 and longer) have also been shown to be active substrates, while N-acetyl-PE and N-formyl-PE are much less

active substrates for NAPE-PLD<sup>42</sup>. Ca<sup>2+</sup> stimulation has shown to have a very minimal effect on membrane-associated NAPE-PLD<sup>3</sup>.

Alternatively, recent evidence has identified NAPE-PLD independent pathways for NAE formation<sup>3</sup>. N-acyl-lyso PE (Lyso NAPE), glycerophospho(N-acyl)ethanolamine (GP-NAE), and NAE phosphate can all act as “intermediate metabolites” in these unique NAE-synthesizing pathways<sup>43</sup> (**Figure 1**). Considerable additional work will be required to clearly understand the role of HRASLS-1 and other NAPE/NAE pathway enzymes in NAE biosynthesis.

## 2.6 NAE Degradation Pathways and a Possible Mechanism for Elevating Levels of NAE

Degradation of NAE is achieved by hydrolysis of the molecule to a fatty acid and an ethanolamine. Fatty acid amide hydrolase (FAAH) is the most common enzyme used to employ this degradation<sup>44</sup> (**Figure 1**). FAAH is broadly spread through mammalian organs, and has high sensitivity to serine hydrolase inhibitors<sup>45</sup>. Anandamide is the most reactive substrate for FAAH, and PEA is the least<sup>45</sup>. In addition to FAAH, another “NAE-hydrolyzing amidohydrolase” exists<sup>46</sup>. N-acylethanolamine-hydrolyzing acid amidase (NAAA) differs from FAAH by its preference for PEA to other NAEs, and the ability of some ester and amide compounds to inhibit NAAA activity without having any inhibitory effects on FAAH<sup>46,47</sup>. Thus, although N-acyltransferases such as HRASLS-1 primarily control the species of NAE that are synthesized in cells through regulation of the synthesis of precursor NAPEs, the substrate selectivity of the NAE-hydrolyzing enzymes may also contribute another level of regulatory control to the concentrations of specific NAEs found in cells. Understanding of the regulation of enzymes in both of these pathways will therefore be required to fully understand NAE regulation in cells.

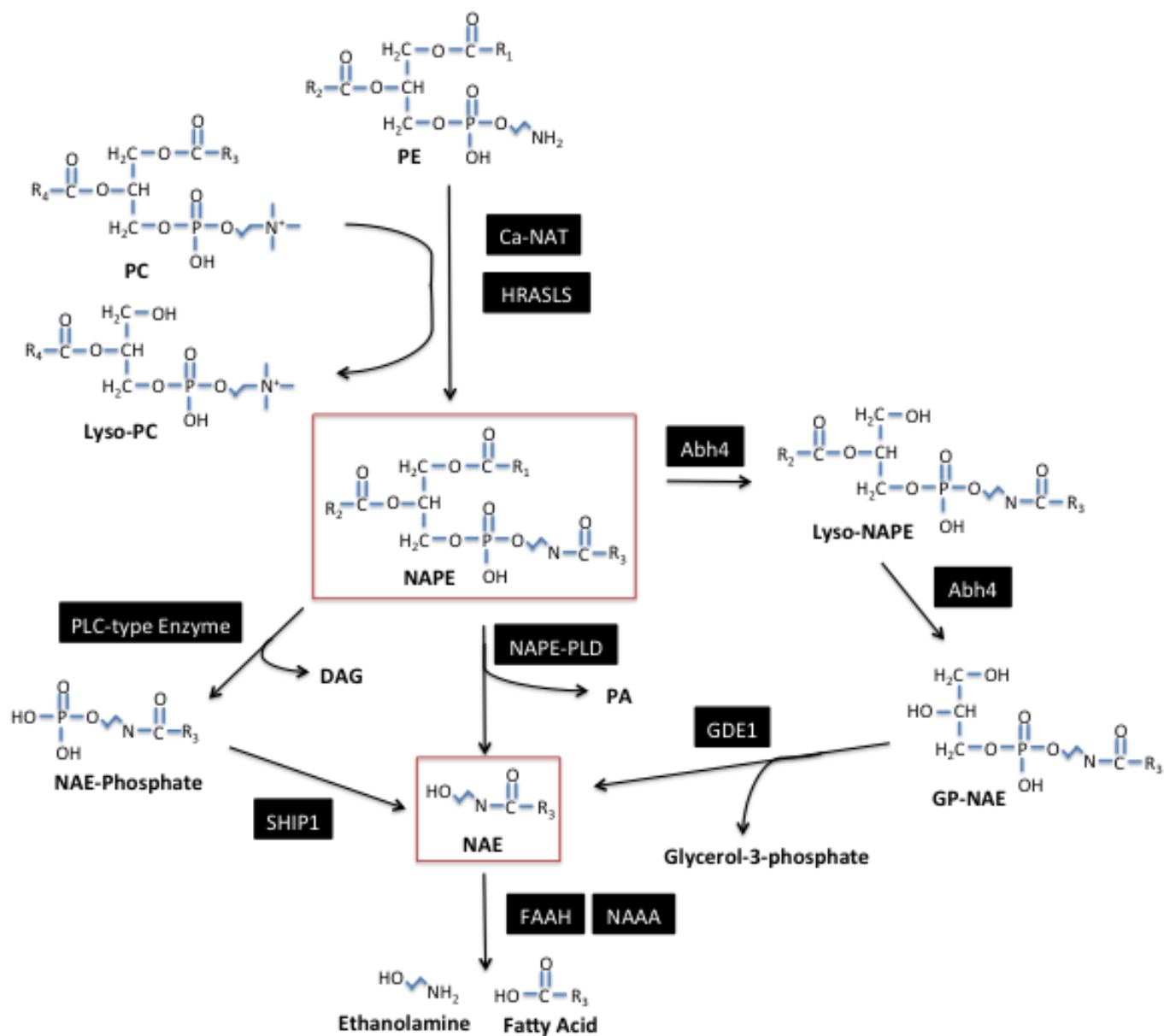


Figure 1. Major biosynthetic and degradative pathways of NAPE. PC is a representative acyl donor phospholipid. Abh4,  $\alpha/\beta$ -hydrolase 4; GDE1, glycerophosphodiesterase 1; PA, phosphatidic acid; SHIP1, Src homology 2 domain-containing inositol-5-phosphatase <sup>143</sup>.

## 2.7 NAE-Activated Signaling Pathways

NAEs are potent signalling molecules, involved in a host of biological processes. Important in regulating the biological activity of NAEs is the presence and nature of the signaling mediators through which these signalling lipids act. These include cell surface receptors, G-protein coupled receptors, and transcription factors. Specifically, peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), transient receptor potential cation channel subfamily V member 1 (TRPV1), and G-protein coupled receptor 119 (GRP119) have shown activation by NAEs. I will discuss each receptor individually, in order to offer a better understanding of the possible cellular and physiological effects that HRASLS-1 may mediate through control of NAPE/NAE biosynthesis.

### 2.7.1 PPAR- $\alpha$ activation by NAEs Can Regulate Substrate Oxidation, Inflammation and Embryogenesis

Peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors<sup>48</sup>. They control gene expression by binding to retinoid x receptor (RXR) to specific DNA sequence elements termed peroxisome proliferator response elements (PPRE)<sup>48</sup>. In following, this complex binds to related sequences in promoter regions of target genes, many involved in the catabolism of fatty acids<sup>49</sup>. There are three main isotypes of PPARs: PPAR- $\alpha$ , PPAR- $\delta$ , and PPAR- $\gamma$ . Once PPARs are activated, they bind to DNA and regulate gene transcription. They are distinctive by individual patterns of expression, and also by their ligands ability to activate each receptor<sup>50</sup>.

The “multifunctional ligand-binding pocket of PPAR- $\alpha$  allows this protein to recognize 3 distinct classes of natural agonists:”<sup>51</sup> NEFAs, oxygenated fatty acids, and NAEs such as PEA and OEA<sup>51</sup>. The presence of a role for NAEs in regulation of PPAR- $\alpha$ , therefore also suggests a role for their upstream regulators - the N-acyltransferases like HRASLS-1, in regulating PPAR- $\alpha$  and its

various functions. This includes mediating the switch to increased  $\beta$ -oxidation in fasting. PPAR- $\alpha$  is highly expressed in tissues with high rates of mitochondrial fatty acid oxidation<sup>52</sup>, such as liver, heart, muscle, kidney, brain<sup>53</sup>. PPAR- $\alpha$  regulates the expression of genes involved in the peroxisomal and mitochondrial  $\beta$ -oxidation pathways, such as acyl-Coenzyme A oxidase 1 (Acox1), the first enzyme of the fatty acid  $\beta$ -oxidation pathway<sup>54</sup>, as well as carnitine palmitoyltransferase 1 (CPT1), an enzyme that is essential for the  $\beta$ -oxidation of longer fatty acids<sup>55</sup>. PPAR- $\alpha$  also regulates fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), liver cytosolic fatty-acid binding protein (L-FABP), and uncoupling proteins 2 and 3 (UCP2 and UCP3)<sup>56</sup>. By altering the transcription of these genes, activated PPAR- $\alpha$  can increase hydrolysis of TAG and oxidation of fatty acids, and increase cellular fatty acid uptake<sup>57</sup> - effects that are all activated with fasting. Investigation of ligands activating PPAR- $\alpha$ -mediated fat metabolism is necessary to gain greater insight into mechanisms responsible for substrate oxidation regulation. Analysis of the expression of genes that are regulated by PPAR- $\alpha$  provides a surrogate measure of PPAR- $\alpha$  activation.

PPAR- $\alpha$  can regulate the development of inflammation by inhibition of proinflammatory transcription factors NF- $\kappa$ B and AP1<sup>58</sup>. Negative regulation of acute inflammation by PPAR- $\alpha$  has been confirmed in PPAR- $\alpha$ -deficient mice seem, which more sensitive to inflammatory stimuli<sup>59</sup>. Since NAEs such as palmitoylethanolamide (PEA) have been found to have anti-inflammatory effects, it is also possible that N-acyltransferases such as HRASLS-1 may be involved in inflammation resolution, also through activation of the NAPE/NAE/ PPAR- $\alpha$  signalling pathway. To date this has not yet been studied.

Finally, PPAR- $\alpha$  is differentially expressed during embryonic development. Reports of the expression of PPAR- $\alpha$  indicate detection of mRNA primarily only in later stages of development<sup>60,61</sup>. Northern blot analysis of total RNA from homogenized whole mouse embryos

failed to detect PPAR- $\alpha$  from E9.5 to E12.5, which was followed by weak mRNA expression on E13.5 that increased from E14.5 through E18.5<sup>61</sup>. Thus, the expression of PPAR- $\alpha$  is expected to play a critical role in mediating developmental signaling events during later stages of gestation, and may influence the responses of an embryo to PPAR- $\alpha$  agonists including NAEs. The expression of NAEs within embryogenesis has gained very little attention, and even less for the enzymes responsible for their synthesis.

### **2.7.2 TRPV1 Activation by OEA and AEA Weakens Inflammatory Response**

The transient receptor potential (TRP) family is a family of ion channels that can “act as molecular transducers to depolarize neurons”<sup>62</sup> that can set off impulses within pain pathways<sup>63,64</sup>. Among these ion channels, are the members of the transient receptor potential (TRP) family. TRPV1 receptor has gained the most attention due to its unique ability to be activated by capsaicin<sup>65</sup>. Activation of TRPV1 allows for the activation of many signalling pathways, including those of the inflammatory response<sup>66</sup>. TRPV1 activation is “dynamic”, meaning that through prolonged or consistent activation via agonist agents, such as anandamide (AEA) and OEA<sup>66, 67</sup>, therefractoriness of the TRPV1-expressing nerve fibers can be desensitized. Consequentially, this will weaken the inflammatory response observed<sup>67</sup>. Thus, N-acyltransferases such as HRASLS-1 may also regulate inflammation resolution through the NAPE/NAE/TRPV1 pathway.

### **2.7.3 GPR119 is Activated by NAEs to Enhance Nutrient Absorption**

Gut hormones such as glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), and glucagon-like peptide-1 (GLP-1), are peptides activated with the income of nutrients<sup>68</sup>. They regulate glucose and lipid metabolism by regulating insulin and glucagon secretion,

accordingly<sup>69,70</sup>. GPR119 can secrete both GLP-1 and GIP<sup>71</sup>, thus GPR119 agonists control glucose levels by augmenting insulin secretion in pancreatic  $\beta$ -cells and by stimulating the secretion of gut hormones<sup>72,73</sup>. In turn, these hormones can promote further insulin secretion and improve overall glucose metabolism<sup>72,73</sup>. NAEs are effective agonists of GPR119, specifically OEA and PEA<sup>68</sup>. Since GPR119-mediated events are typically activated during a nutrient replete, or fed state, evidence that *Hrasls-1* gene expression is induced postprandially, would provide preliminary evidence suggesting a role for this enzyme in regulation of satiety and insulin responsiveness. Unfortunately, this data has not yet been examined.

It is important to recall that the relationship between HRASLS-1 and the discussed receptors is not direct. The HRASLS-1 pathway must synthesize the precursor NAPE molecules, in order to generate the lipid mediator NAEs. Thus, in the following section I will introduce the NAE molecules most relevant to the HRASLS-1 pathway, along with their individual physiological relevance.

## **2.8 Physiological Signalling Activities of Anandamide and the HRASLS-1- Relevant NAEs**

NAEs are part of a group known as endocannabinoid-like compounds that share common biosynthetic and degradation pathways with endocannabinoids, but lack cannabinoid receptor affinity<sup>74,75</sup>. PEA, OEA, and N- stearoylethanolamide (SEA) are quantitatively major NAE's in animal tissue, where they actively undergo biosynthesis and breakdown<sup>9,76</sup>.

### **2.8.1 AEA is a Multifunctional Signalling Molecule, Although Not Likely a Major Downstream Product of the HRASLS-1 Pathway**

N-arachidonylethanolamide, also known as anandamide, is an “endogenous cannabinoid neurotransmitter”<sup>77</sup>. Anandamide's effects can either be mediated centrally in the brain, or

peripherally, and are transduced primarily by CB1 cannabinoid receptors, although it can also be a ligand for CB2<sup>78</sup> and TRPV1<sup>79</sup>. Cannabinoid receptors were originally discovered for their sensitivity to  $\Delta^9$ -tetrahydrocannabinol, which is the primary cannabinoid found in cannabis<sup>80</sup>. AEA affects multiple physiological processes, having distinct effects on the immune system by employing an anti-inflammatory response during increased inflammation<sup>81</sup>. Anandamide is important in pre-implantation of the embryo, specifically, the development of the blastocyte from a 2-cell embryo<sup>82</sup>. Anandamide also plays a role in the regulation of feeding behavior mainly by increasing food intake<sup>81</sup>. Thus, AEA may play a role in mediating each of the physiological processes investigated in this thesis. However, this NAE is unlikely to be a primary mediator of any pathway controlled by HRASLS-1/NAPE/NAE signaling, since arachidonic acid is typically located in the sn-2 position of glycerophospholipids, and HRASLS-1 predominantly utilizes donor fatty acids in the sn-1 position of glycerophospholipids. Additionally, although anandamide has gained the most attention as an NAE signaling molecule, it is much less abundant in animal tissues, accounting for less than 5% of total NAEs<sup>83,84</sup>, and therefore suggesting that other NAEs likely play roles in HRASLS-1 mediated effects.

### **2.8.2 The Anti-inflammatory Role of PEA and Related Mechanisms**

PEA is an “endogenous fatty acid amide”, acting as an anti-inflammatory protein since the 1950’s<sup>85</sup>. Today, AEA is known to be “an important analgesic, anti-inflammatory and neuroprotective mediator”<sup>86</sup>. PEA has been proposed to act “on demand” as an anti-inflammatory mediator<sup>86</sup>. Unfortunately, due to the lack resources able to deliberately alter the tissue levels of PEA specifically, the protective functions of endogenous PEA have not yet been discerned<sup>86</sup>

Presently, three mechanisms explain the anti-inflammatory effects of PEA. The first proposes that PEA acts by locally “down-regulating mast-cell degranulation”<sup>87</sup>. The second, the

“entourage effect”<sup>88</sup> proposes that PEA is able to inhibit the degradation of AEA, and enhance its anti-inflammatory properties<sup>89-91</sup>. The final mechanism, the “receptor mechanism”<sup>92</sup> suggests PEA directly stimulates an unknown cannabinoid CB2 receptor-like target<sup>92</sup>, or the nuclear hormone receptor PPAR- $\alpha$ <sup>53</sup>. PEA may eventually lead to a new therapeutic approach for the treatment of pathological conditions, most attractively, those associated with pain and inflammation.

### **2.8.3 OEA Can Have Opposing Roles During Different Feeding States**

OEA is an anorexiant that controls feeding and body weight by prolonging the latency period between meals<sup>93,94</sup>. As OEA was initially characterized as a primary ligand for PPAR- $\alpha$ , it suggested that OEA stimulates satiety and reduces bodyweight gain through PPAR- $\alpha$  activation<sup>93</sup>. It was also suggested that through the activation of PPAR- $\alpha$ , OEA lowered lipid levels in liver and blood<sup>93</sup>, implicating OEA with anti-obesity outcomes, such as increased lipid oxidation<sup>93</sup>.

Further to this, the anorexic actions of OEA were hypothesized to be facilitated by stimulation of vagal sensory nerves<sup>95</sup>. OEA is reported to be an agonist of TRPV1<sup>95</sup> that caused reduced food intake in wild-type, but not in TRPV1<sup>-/-</sup> mice<sup>96</sup>. Although it has not yet been examined, OEA may also be regulating other metabolic responses involving TRPV1, such as reduction in weight gain, adiposity and triglycerides.

In addition to actions mediated through TRPV1, OEA may also stimulate GLP-1 secretion from the intestinal L-cells through GPR119 stimulation<sup>97</sup>. Administration of OEA into the intestinal lumen in rats, increased bioactive GLP-1 levels, supporting a role for OEA as a GLP-1 producer *in vivo*<sup>97</sup>.

#### **2.8.4 SEA Regulates Feeding as well as Inflammation**

Very little is known about the physiological significance of SEA. SEA has been shown to decrease food intake with an associated decrease in liver stearoyl-CoA desaturase-1 (SCD-1) mRNA expression<sup>98</sup>. SCD-1 is “the rate-limiting enzyme in the biosynthesis of monounsaturated fats”<sup>98</sup>. Thus, its reduction decreases the rate of lipogenesis, and promotes fatty acid oxidation<sup>99</sup>. Although this appears to suggest possible involvement of PPAR- $\alpha$ , these effects have been found to be independent of PPAR- $\alpha$  activation, and the pathway of SEA-mediated regulation of these effects has not yet been elucidated. SEA is also capable of down-regulating inflammation in inflamed or damaged tissue, and this effect likely involves TRPV1<sup>67</sup>.

## Chapter 3

### Rationale and Objectives

#### 3.1 Rationale

HRASLS-1 predominantly functions as an *N*-acyltransferase in vivo to generate NAPes that are further metabolized to bioactive NAEs<sup>31</sup>. In contrast to the other iNATs, HRASLS-1 is the only murine-characterized HRASLS family member to show high *N*-acylation activity, and to be expressed in a wide variety of animal tissues<sup>40</sup>. Evidence suggests that the type and level of NAE found in tissues corresponds to the type and amount of NAPE, which is determined by the respective donor fatty acid species available in the *sn*-1 position of PC in cells<sup>9</sup>. If donor PC *sn*-1 fatty acid species availability primarily regulates HRASLS-1 product type, then the most abundant downstream products of increased HRASLS-1 activity in cultured cells are likely to be PEA, SEA and OEA. These NAEs have been shown to activate PPAR- $\alpha$  or TRPV1, both of which may signal to regulate nutrient metabolism<sup>93</sup>, as well as mediate the inflammatory response<sup>100</sup>, and function in embryological development<sup>101</sup>. Regulation of these processes is critical for normal physiological function, and deregulation of these processes may lead to a variety of diseases. Understanding the regulation of NAE synthesis, therefore, is of critical importance to health. Enzyme mediators such as HRASLS-1 play important roles in controlling NAE biosynthesis. To date, however, a role for HRASLS-1 in any physiological process has not yet been investigated. It is therefore likely that *N*-acyltransferases, such as HRASLS-1, that help to control NAE levels, similarly undergo changes in gene expression in order to modulate production of NAPes/NAEs.

Modulation of *Hrasls-1* by changes in nutritional feeding state would strongly suggest a role for this enzyme in regulating the substrate switch between fasted and fed states as a

regulator of the NAPE/NAE/ PPAR- $\alpha$ , TRPV1, or GPR119 signalling pathways, thus regulation of *Hrasls-1* gene expression under these varying conditions needs to be investigated. One way to determine if HRASLS-1 may play a role during the fed/fasted switch is to analyze the expression of the receptors that may be activated downstream of HRASLS-1-regulated NAEs, and the expression of the genes that they, in turn, control. Additionally, determination of whether *Hrasls-1* gene expression is regulated during inflammation is a critical first step in the identification of a role for this enzyme in this physiological process. Finally, neither the concentration of various NAEs, nor the expression of *Hrasls-1*, have been investigated during embryogenesis. Thus investigating expression of *Hrasls-1* in mouse embryos at varying time points would suggest a role for this enzyme during embryological development.

The aim of the current study is to generate an initial understanding of the physiological relevance of the HRASLS-1 enzyme by determining *Hrasls-1* expression in different nutritional states, during inflammation, and during embryonic development. Since this area of research is extremely novel, the work done here is exploratory but essential in forming the foundation for future work in this research domain.

### **3.2 Objectives**

1. Since I am focused on studies of murine HRASLS-1 gene expression, my initial objective was to identify the mouse transcripts that encode for this gene, and to investigate differences in their stability using the transcription inhibitor actinomycin D.
2. To investigate whether this enzyme may play a role in substrate oxidation regulation by measuring mRNA expression of *Hrasls-1* transcript variants, and genes potentially regulated by Hrasls-1-derived NAEs in liver, kidney, white adipose tissue, heart, and brain from fed, fasted and re-fed mice.

3. To analyze the change in level of NAPE during acute activation of cultured BV2 microglial immunocytes, and to analyze whether changes have occurred in levels of *Hrasls-1* transcript variants, or any of the other known murine iNATs (*Hrasls-3* and *Hrasls-5*) that may mediate this effect.
4. To determine whether changes in *Hrasls-1* may play a role in regulating NAPE/NAE in mid to late embryonic development by measuring expression of the *Hrasls-1* transcript variants in differing stages of embryonic development (E10.5, E14.5, E18.5).

### 3.3 Hypotheses

1. The *Hrasls-1* transcript variants have unique 3' and 5' UTRs that play a role in stability. Due to the significantly longer AU-rich 3' UTR tail, the 2.9kB transcript will be more unstable and rapidly degraded upon transcription inhibition.
2. The different transcript variants of *Hrasls-1* will be differentially expressed during non-fasted, fasted, and re-fed states. Due to the greater predicted instability of the 2.9 kb transcript variant, I predict that it will show greater variability as a result of its rapid degradation.
3. *Hrasls-1* transcript variant mRNA expression will be increased in fasting
4. As a result of increased *Hrasls-1* expression in the fasted state, there will be increased mRNA expression of receptors activated downstream of the HRASLS-1 pathway, including *Ppar- $\alpha$* , its target genes *Acox1* and *Cpt1*, and *Scd-1*, and *Trpv1* mRNA expression will decrease in the fasted state.
5. Expression of the *Hrasls-1* transcript variants will be induced upon activation of BV2 cells with LPS. As a result, NAPE content will increase in microglial BV2 cells as a result of acute activation with LPS.

6. The different transcript variants of *Hrasls-1* will be differentially expressed during embryonic development, and the 2.9 kb transcript variant will show greater variability in expression.

## Chapter 4

### Study Design and Analytical Techniques

#### 4.1 Study Design: Overview

In order to target each objective, unique experiments were required; therefore objectives were divided into unique studies. In order to characterize the different transcript variants identified for HRASLS-1, in study #1, two different primer pairs were designed, that would measure the mRNA expression of each transcript variant exclusively. BV2 cells were grown and treated with a transcription inhibitor for various time periods, and then mRNA expression for each transcript variant was quantified. Study #2 looked at energy substrate regulation of *Hrasls-1*, and was performed on C57Bl/6J female mice that were either: fed, fasted 16 h overnight, or fasted overnight followed by a 4 h period of re-feeding. Tissues were excised, and cDNA was extracted in order to measure *Hrasls-1* mRNA expression. In study #3, the expression of HRASLS-1 under an acute inflammatory response was examined in cultured BV2 cells. Expression of other murine HRASLS family members was also determined for comparison with *Hrasls-1* expression, and specificity of the *Hrasls-1* response. Additional BV2 cells were activated and simultaneously treated with radiolabeled [ $C^{14}$ ] palmitate, for quantification of NAPE. For study #4, *Hrasls-1* mRNA expression was also measured in whole embryos at three mid- to late-stage time points, when embryos were visible. Details regarding specific experiments for each study are found within the respective section, while commonly used methods are discussed below.

#### 4.2 BV2 Cell Culture

Microglia are the “resident macrophage-like cells of the central nervous system (CNS)”<sup>102</sup>, have the ability to react within a wide variety of inflammatory neuropathologies<sup>102</sup>. As their proliferation capacity is limited, they have to be isolated fresh for each experiment<sup>103</sup>. This has a

large impact on overall animal consumption in biomedical research and imposes a significant restriction on the use of these cells in research. BV2 cells are a cell line alternative derived from raf/myc-immortalized murine neonatal microglia and are the most common used substitute<sup>104</sup>. BV2 microglial cells were maintained and routinely subcultured at 37°C and 5% CO<sub>2</sub> in DMEM (high glucose with L-glutamine and sodium pyruvate) containing 10% FBS and penicillin/streptomycin.

### **4.3 RNA Extraction**

With reference to the ambion Life Technologies procedure<sup>141</sup>, previously described within the MSc thesis of Ryan Bradley<sup>140</sup>, RNA was isolated from tissue and embryonic samples using 1mL TRIzol® reagent, followed by complete homogenization using a Polytron® homogenizer. Samples were incubated for 5 minutes at room temperature, in order to allow for dissociation of the nucleoprotein complex. Once this was done, 0.2mL of chloroform were added and “shaken vigorously by hand” for 15 seconds. Samples were incubated at room temperature once again, for 2-3 minutes, followed by centrifugation of the samples at 12,000 x g for 15 minutes at 4°C. Next, the aqueous phase of each sample was removed, and placed into a new 1.5mL Eppendorf tube. 0.5 mL of 100% isopropanol was added to the new tube, and incubated at room temperature for 10 minutes. Following this, samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was then removed from the tube, leaving only the RNA pellet, which was washed with 1 mL of 100% ethanol. Before a final centrifugation at 7500 x g for 5 minutes at 4°C, the sample was vortexed briefly. Once centrifugation was complete, samples were air-dried for approximately 5-10 minutes, making sure to not dry the pellet. Finally, the pellet was resuspended in 30-50uL DEPC-treated water at 58°C for 12 minutes. RNA concentrations and purity were immediately determined using a Nanodrop-2000, and samples were stored at -80°C.

#### 4.4 Reverse-Transcriptase (RT) –quantitative PCR

RNA sample concentrations were adjusted to 2 µg/µl, and complementary DNA (cDNA) was produced by reverse-transcription of 1 µg of RNA. RNA was mixed with 1 µL of 10 mM dNTPs and 1 µL of oligo(dTs)<sub>12-18</sub> and incubated at 65°C for 5 minutes. Once complete, the samples were cooled on ice for 1-2 minutes. This was followed by the addition of 6 µL of reaction mix (per sample) containing, 4 µL of 5 X first strand buffer, 2µL of 0.1 M DTT. Samples were then immediately incubated at 42°C for 2 minutes in order for primers to anneal. The final step required the addition of 1µL of Superscript II mix, which consisted of 2 uL of 5X first strand buffer, 3 uL of ddH<sub>2</sub>O, and 5 uL of Superscript II, to each sample. At last, samples were incubated in a 42°C water bath for 50 minutes. Once completed, samples were transferred to a prepared, elevated water bath at 70°C for 15 minutes. cDNA was stored at -20°C.

Quantitative real-time PCR was performed using SsoFastEvaGreen PCR Super Mix (Bio-Rad Laboratories, Mississauga, Canada) with targeted primers (**Table 1**) or PerfeCTaqPCRToughmixLowRox with Taqman predesigned gene expression primers with FAM-labeled probes (Applied Biosystems) (**Table 2**). cDNA generated by RT was amplified at a reaction volume of 10 µl, by 2-step thermal cycling in a CFX96 Touch™ Real-Time PCR Detection System. The initial incubation was 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 55°C for 30 seconds. Following 40 cycles, a dissociation curve was produced by increasing the temperature from 60°C to 95°C at a rate of 1°C per minute. Quantitation was performed by assessment of relative fluorescence after each cycle. The relative quantity of each *Hrasls-1* transcript ( $C_t$ ) was normalized to a group of reference genes by calculating  $\Delta C_t = [C_t (\text{tissue}) - C_t (\text{average of three reference genes})]$ . The expression of *Hrasls-1* transcripts in different tissues was calculated relative to the expression of that tissue in the group with the

lowest abundance ( $\Delta\Delta C_t$ ). *Hrasls-1* gene expression was normalized to an average of 18S,  $\beta$  actin, and RPII expression within tissues.

#### 4.5 Primer Design

The 2.9 kb *Hrasls-1* transcript variant was amplified with sense primer (5'-TGGAAGTGTGAGCTGTGAGCAG-3') and antisense primer (5'-CCAAGGCCTTTGTGCTGAAC-3'). The sense primer was located within the 5'-UTR, while the antisense primer was located within the coding region. The 0.8 kb *Hrasls-1* transcript variant was amplified with sense primer (5'-ATGGAGTCACCCAGGGTCTG-3') and antisense primer (5'-CCTGCCCAATAGCGAACTCT-3') that were located within the 5'-UTR and coding regions, respectively. Since the 5'UTRs differed between the 2.9 kb and 0.8 kb transcripts, this design led to specific amplification of only one of the two transcripts. Primers for mouse sequence of *Hrasls-3*, *Hrasls-5*, *Hrasls-1* (coding region),  $\beta$ -actin, 18S, and RPII, *Trpv1* and *Cpt1 $\alpha$*  were designed with Primer-BLAST program on the NCBI website and ordered from Sigma-Aldrich. Primers for the murine sequence of *Ppar- $\alpha$* , *Scd-1* and *Acox1* were graciously provided by Kristen Marks and the Stark Lab.

#### 4.6 Statistical Analyses

All statistical analyses were done using Prism6 for Mac. Independent samples t-test was used for comparison of NPPE content within BV2 cells, and within the embryonic development data between transcript variants. One-way ANOVA was used for comparison between *Hrasls-1* transcript variants as well as between *Ppar- $\alpha$* , *Acox1*, *Cpt1- $\alpha$* , *Scd-1*, and *Trpv1* within non-fasted, fasted, and re-fed states. One-way ANOVA was also used for comparisons between mRNA expression of HRASLS family members, and between *Hrasls-1* transcript variants upon activation with LPS. Newman-Keul's Multiple Comparison Test was used following a significant one-way ANOVA result. Significance was inferred when  $p < 0.05$ .

Gene	Genbank Accession Number	Forward Sequence	Reverse Sequence
HRASLS-1 2.9kB Transcript	NM_013751	TGGAAGTCTGAGCTGTGAGCAG	CCAAGGCCTTTGTGCTGAAC
HRASLS-1 0.8kB Transcript	BC048482	ATGGAGTCACCCAGGGTCTG	CCTGCCCAATAGCGAACTCT
HRASLS-1- coding	NM_013751/ BC048482	CACAACCCACACCCAGGAGA	CACCAAGGCCTTTGTGCTGA
HRASLS-3	NM_139269	TGTCCTTCCCGGCCACATGG	CCCAAGCCTGGAGACCTGAT
HRASLS-5	NM_025731	GGGTCTTGGATTGCCCTCAA	CCGATTAGAACAGGCCGAA
18S	M11188	GATCCATTGGAGGGCAAGTCT	AACTGGAGCAAGTTTAATATACGCTATT
Scd1	NM_009127.4	TGCGATACACTCTGGTGCTCA	CTCAGAAGCCCAAAGCTCAGC
Acox1	NM_015729.3	GCTGCGGAGACAGGTTGTCATCG	GCTCCTTGCGCAGATCGGGATTC
Ppar- $\alpha$	NM_011144.6	CCGAACATTGGTGTTCGCAGCTGT	CAGGGGACAACCAGAGGACCCAG
Trpv1		AGCCCCACATCTTTGCTACC	GACAACAGAGCTGACGGTGA
Cpt1 $\alpha$	NM_013495.2	CCCCGCGAGTCCCTCC	AGTCATGATGATCGCCACCC

**Table 1** Mouse SYBR Green-based primer sequences for RT-PCR

HRASLS-1 2.9kB Transcript: HRAS-like suppressor 2.9kB, HRASLS-1 0.8kB transcript variant: HRAS-like suppressor 0.8kB transcript variant, HRASLS-1- coding: HRAS-like suppressor coding region only, HRASLS-3: phospholipase A2, group XVI, HRASLS-5: HRAS-like suppressor family, member 5, 18S: 18S ribosomal RNA, Scd1: stearyl-Coenzyme A desaturase 1, Acox1: acyl-Coenzyme A oxidase 1, Ppar- $\alpha$ : peroxisome proliferator activated receptor alpha. Trpv1: transient receptor potential cation channel, subfamily V, member 1. Cpt1 $\alpha$ : carnitinepalmitoyltransferase 1a, liver.

**Table 2** Mouse TaqMan-based primer sequences for RT-PCR

Gene	Primer Reference Number
Actb	Mm00607939_s1
RPII	Mm00839493_m1

Actb: beta actin, RPII: polymerase (RNA) II polypeptide A.

## Chapter 5

### Identification of Two Unique Transcript Variants for HRASLS-1 with Unique Stability

#### 5.1 Introduction

In order to explore *Hrasls-1* mRNA expression within various physiological changes, it is important that we first investigate the *Hrasls-1* sequence for different transcript variants. Transcript variants are generated by alternative splicing during gene expression<sup>105</sup>. Within this process, exons may be included or excluded from the mRNA, creating transcripts that possibly hold opposing roles if significant differences are found<sup>106</sup>. Differences can implicate a range of effects, from biological roles to transcript stability. The purpose of this work was to gain insight on possible factors that could be altering the total *Hrasls-1* mRNA expression observed. This included transcript variant identification and analysis of transcript stability.

Actinomycin D is a commonly used inhibitor of transcription. Actinomycin D acts by interfering with mRNA synthesis, by preventing elongation of the RNA chain by RNA polymerases<sup>107</sup>. For cell culture applications, actinomycin D is used at a concentration of 1 µg/ml, and is initially dissolved at 1000x concentration in DMSO. Once cells are treated with actinomycin D, transcription stops. Essentially what we are left with in each treated plate is the RNA that existed prior to actinomycin D treatment, minus the RNA that has been degraded, without the complication of continuous input from the nucleus. In this experiment we compared the level of each of the *Hrasls-1* transcript variants at different time points, which is a function of the initial content when actinomycin D was added, and the relative degradation rate for a particular transcript. If one transcript is more rapidly degraded, the relative level will disappear at a faster rate.

## 5.2 Methods

BV2 cells were used in this experiment. Cells were grown at 37°C to 80% confluency in 60 mm dishes containing DMEM with 10% fetal bovine serum and penicillin/streptomycin. Actinomycin D (1 µg/ml) was added to each plate for 0 h (prior to addition of actinomycin D) 0.5 h, 1h, or 2 h. Media was then carefully pipetted off, and Trizol ® was added directly to cells for extraction of RNA. Complementary DNA (cDNA) was produced by reverse-transcription of 1 µg of RNA. Quantification of expression by qPCR provides a relative indication of how much RNA is left after the actinomycin D incubation.

## 5.3 Results

### 5.3.1 HRASLS-1 has unique transcripts with identical coding regions but different 5' and 3' UTR

We searched the National Center for Biotechnology (NCBI) Genbank database and identified two different HRASLS-1 transcript variants with identical coding regions but highly dissimilar 5' and 3' untranslated regions (UTRs) (**Figure 2**). Both transcripts had 5'UTRs of a similar length (116bp vs. 150 bp), although the nucleotide sequence through this region differed entirely. Conversely, the 3'UTR of these transcripts shared sequence identity for the first 139 bp after the stop codon, but the longer transcript contained an additional 2,157 bp beyond this sequence, creating a large difference in relative size of the transcripts (0.8 kb versus 2.9 kb). Differences in transcript size, particularly in the untranslated regions, can affect transcript stability and rates of translation. The presence of two highly dissimilar transcripts encoding the same protein suggests that regulation during different conditions may also vary, and understanding this regulation may give insight into the physiological role of this enzyme. Analysis was done on the relative stability of

these transcripts, and then the relative expression of these transcripts during changing metabolic states, during inflammation activation, and during different periods of embryonic development.

### **5.3.2 The Half-life of the *Hrasls-1* 0.8 kb transcript is shorter than that of *Hrasls-1* 2.9 kb transcript variant**

We treated BV2 cells with actinomycin D, and harvested cells for RNA analysis at 0, 0.5, 1, and 2 h time points. Our data indicates that the 0.8 kb transcript was degraded faster than the 2.9 kb transcript, since the half life of this transcript was reached approximately twice as fast as that of *Hrasls-1* 2.9kB transcript (time point 0.5 h vs 1 h)(**Figure 3**). Interestingly, the 2.9 kb transcript showed a slight initial elevation in mRNA expression from time point 0 h to time point 0.5 h. Similarly, the 0.8 kb transcript variant also demonstrated a slight elevation from time point 1 h to 2 h.

## **5.4 Discussion**

Although both transcripts share identical sequences in the proximal portion of the 3' UTR, the longer transcript is defined by an additional 2 kb of mRNA continuing downstream of the coding region. This extended UTR is adenylate-uridylylate-rich. Adenylate/uridylylate-rich elements (AREs) are “found in the 3' untranslated region (UTR) of many messenger RNAs (mRNAs) that code for proto-oncogenes, nuclear transcription factors and cytokines”<sup>108</sup>. They commonly have several copies of the pentanucleotide, AUUUA, and a high concentration of 'U' and 'A' residues<sup>108</sup>. They are associated with RNA destabilizing ability in mammalian cells<sup>108</sup>. It is important to note that “ARE-

directed mRNA degradation is influenced by many exogenous factors”<sup>108</sup>, such as transcription inhibitors.

Due to the AREs found in the 3' UTR of the 2.9 kb transcript variant, this *Hrasls-1* transcript should show reduced stability in comparison to the 0.8 kb transcript variant. Contrary to my hypothesis, cells incubated with actinomycin D showed a more rapid drop in the percentage of remaining *Hrasls-1* transcript variant 0.8 kb in comparison to the 2.9 kb transcript. Although the general consensus in the literature<sup>108</sup> states that these AREs found in the 3' UTR of mRNAs would target the transcript variant for rapid degradation, various factors can counter the ARE function. Depending on the specific class and sequence of ARE present, the destabilizing ability may be different, as it is portrayed in a “cell-type specific” manner<sup>108</sup>. One example illustrates how IL-3 AREs are required in a tumour cell line for the destabilization of IL-3 mRNA, while the stability of IL-4 mRNAs, containing a different class of AREs, remains unchanged in the same cells<sup>108</sup>. This could imply that AREs present at the 3' UTR of the 2.9 kb transcript variant may not be acting to destabilize mRNA in the BV2 cell line. Therefore, replicating the experiment on several cell lines would be beneficial in gaining a greater understanding of the role of the AREs present. Similarly, cloning of the different 3' UTRs, and performing mutagenesis of some of the AREs, would provide greater insight into the role of some of the individual sequence elements.

Further analysis of mRNA levels during the time course of actinomycin D exposure indicated an interesting phenomenon. We observed a small initial increase in mRNA level of the 2.9 kb transcript at 0.5 h after transcription arrest. Theoretically, when transcription is inhibited by

actinomycin D, there should not be any novel formation of mRNAs. Therefore mRNA levels after actinomycin D treatment should not be higher than mRNA levels seen at time point 0h. Evidence from other experimental reports suggests, however, that this is not always the case, and that there are some limitations to the use of transcriptional inhibitors such as actinomycin D for the study of mRNA stability. Due to “feedback loops”, during transcript inhibition, transcription of a small group of genes may be induced for protection against genotoxic stress<sup>109</sup>. This may lead to the accumulation of certain genes when inhibitors are introduced at moderate concentrations<sup>110</sup>. Secondly, the binding activity of the ARE-binding proteins can “inversely or proportionally” relate to the stability of ARE-containing mRNAs<sup>108</sup>. One theory discusses how during transcription inhibition, ARE-binding proteins can travel between the nucleus and cytoplasm, causing a disturbance in the cytoplasmic concentration of these proteins<sup>108</sup>. Consequentially, ARE-protein complexes, necessary for ARE-directed mRNA decay, may be interrupted<sup>108</sup>.

Taken together, our results indicate unique 5'- and 3'-UTRs and a unique stability for each transcript variant, suggesting that differential regulation of alternate transcripts may allow cells to ‘fine-tune’ control of this gene, in order to respond to rapidly changing conditions



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558 GCAGCTGGTATTGATATCTTCACATTCCTCGGCTTGTTTCCCAAAGACA 607
      |||
554 GCAGCTGGTATTGATATCTTCACATTCCTCGGCTTGTTTCCCAAAGACA 603
      |||
608 AAGAACGAAATATTAGCAGTTTATTGAAGAGATTGGAATGAAGAAATTTG 657
      |||
604 AAGAACGAAATATTAGCAGTTTATTGAAGAGATTGGAATGAAGAAATTTG 653
      |||
658 TGAGGAGAAAAAAAAATCCTAGGGTGAATACTTATTTTGAATGCATCATT 707
      |||
654 TGAGGAGAAAAAAAAATCCTAGGGTGAATACTTATTTTGAATGCATCATT 703
      |||
708 ATTGCTCATGGTCCCATGATGGATGGCAGACTCTGTAATAAATTGCTTG 757
      |||
704 ATTGCTCATGGTCCCATGATGGATGGCAGACTCTGTAATAAATTGCTTG 753
      |||
758 CTGA---TAA-----AAAAAAAA-----AAAAAAAA 780
      |||  |||  ||.||||  |||.|.|.
754 CTGATTTTAATCTTATCATTGAGCCAAGAAAAGTTTTTCCCAACTAGCAG 803
      |||
781 AAA-----AAAAAAAA---AA----- 792
      |.  ||.||||  ||
804 AGATTTGCCGTGGCAGCTTGAACAAAATGCAATTGCCTTTTGATCGAGCC 853
      |||
793 ----- 792
854 AGCTGAGGATCTTAACAGAACCAAGACCACATTTTATCTTCTGCTGTAAA 903
      |||
793 ----- 792
904 TATTGTTTTCTTTTCCCTAAGGACAGCTGTTTTGCCAGAGGTGTGGAGA 953
      |||
793 ----- 792
954 ACCATTTGCATACACTGCTGAGAGACAGTTGTTAGGGCCAACATCTAAAT 1003
      |||
793 ----- 792
1004 TCCTTTTGCTTTCTTTGTCAGAAAAGGAGCGTGAACATATCCAGTAGTT 1053
      |||
793 ----- 792
1054 TGGATACATGGATATATATCCATGATATATCCAAGTACTTAAGGATCTCT 1103
      |||
793 ----- 792
1104 GCATGACAAAGCATTGAGGTGGTACCCCATATTGCTGCACCTGTGTGG 1153
      |||

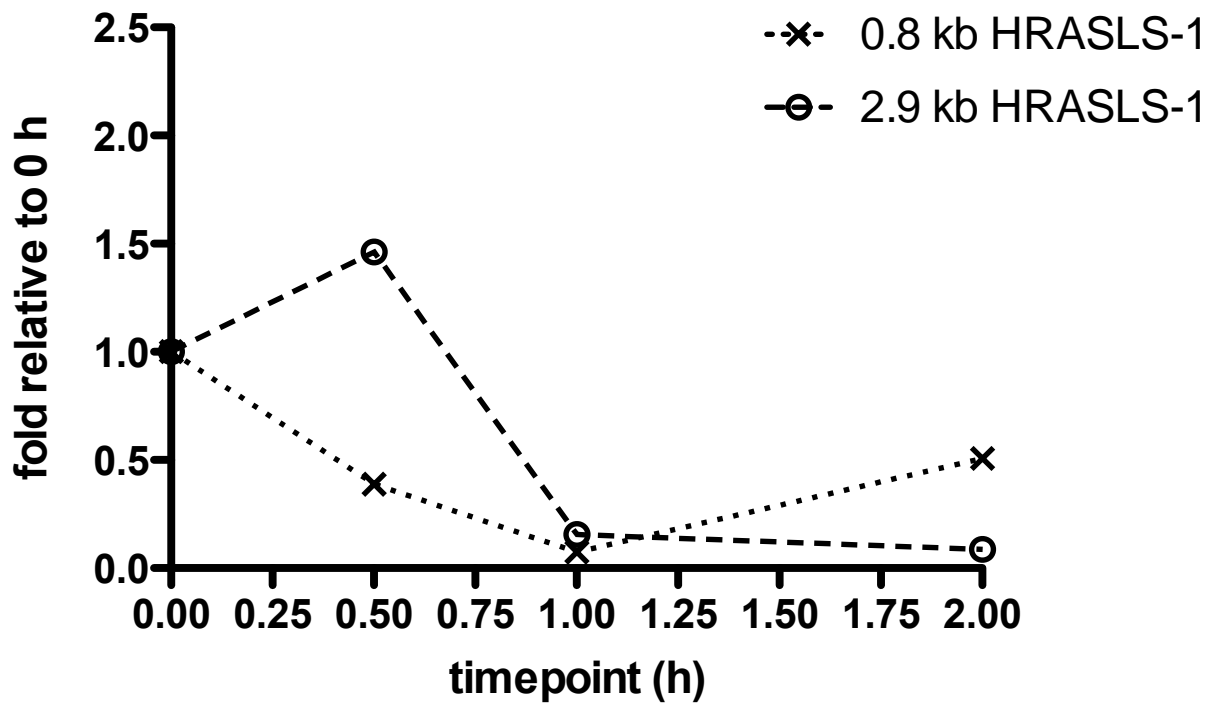
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793	-----	792
1204	AATTCTTATGAATGGTGTGCATGTATAGGGACGGTCTTGGTCTCACCTTT	1253
793	-----	792
1254	TTTATTCTTATTTTATTCTTATTCTTATTTTATTTTATAATTTATAAAAG	1303
793	-----	792
1304	GAGACAGGGTATGCTGTCCTTTATAGCTTATGGCAAACCTAGCTAGCCTGT	1353
793	-----	792
1354	GGGCATCCAGAGAGTCTCCTGTCTCTGCCTCCATTTACGGTAGGAGTTC	1403
793	-----	792
1404	TGGAGTTACCGAAGTGAAC TAACAACCCAAGCTTCTGTGGACTCTGGGG	1453
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1454	ATCTGAGCTCAAGACATCATGCTTATGTTTAAGATGCTTTACCCACTGAG	1503
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1504	CCCCTCTCCAATCTCTCTCTCTCTTTCTTTCTCTCTCTCTCTCCCTTG	1553
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1554	TACGTT CAGACCCAGTGTTCAAGACTCAGTGATGAACAATGGGCTGTCGT	1603
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1604	TTTATTGATTTAAATGGATCCCTCCACATTGTGTAGTAGCTCAGTGGTTT	1653
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1654	GACCTTTGGGGCTTGGCAAGCAATCATTAAATATTAATGCAAAGAGCTATA	1703
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1704	TTTAAAACACAAAATTCCTCATTCCCTTTAAAGATGTGAACTGTAAATCAA	1753
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1754	GGAATCCGCTTATTTTATTCTTTTATTTTACTGATTATCCGTTGAATTAA	1803
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1804	ACTCCTTCTTAATGCCATTTCTGCAGAATTCTTGATAGCAGGTCAGGAGA	1853
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1854	GGTGGGACGTTAGTCTTTCTACATGAAGGTAGTATAAGGGCATCATGACA	1903
793	-----	792
1904	TTACCTCATAAACCACACAGCTAGCCCGGCATCCTTTTGTGCCATTATCA	1953
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1954	AAAGCATGTCAATCAATAAGGAAAGAGTGCCAGTGACCATGGGGATAGAA	2003
793	-----	792
2004	CGTGATGGAGTATGGTAAAGTCAAGTGCACAAGACTCGGTGGAAAGCTCA	2053
793	-----	792
2054	GACCTACTTAACTGATTTGACCTTGGTGCTGGAAACTCACACTGATGA	2103
793	-----	792
2104	GGGTAACAGTAATGTGAACAGAACTATGTGCTGCCACTCCCATCCAGACA	2153
793	-----	792
2154	GAGTCCCCTCTGTCCCTTCTTTACTTTTCTAACAGGCCCTTCTTGTC CGA	2203
793	-----	792
2204	ACCTCCAGACTCATTCTTTAGTTGTCTCAGAAGGCACGAGGTTACTTTTA	2253
793	-----	792
2254	TCCTGAATGCAGGTCTCTGTTCCCAAATAGTAAAGAATATTCAGTTTTCT	2303
793	-----	792

2354	CCTGGTGTGCCACCTTCTCAATAAACTATATTTGCAGGGCATTGTCTCT	2403
793	-----	792
2404	GGCAGAATTACACCGCCTGTCAGGAAAGGGACTAGGACTGAGAACCAAAT	2453
793	-----	792
2454	GCCTCTTAGGGATTCAAATGAAACCTTTCTAGAGGACTCTGACATCTCCA	2503
793	-----	792
2504	CTCCAGAAGAAAAGCAAAATGTTAAAAGGAAAACGTCAGAAATGCACTTT	2553
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2554	TTAATCCATGGGTTTATTTCCCTTATCAGTTATGTGAAGCTTGGCAGCCT	2603
793	-----	792
2604	TGGTTTGCTGTAAACATGGCCAACCTTAAAATGGGCAGCCCTAAATAGCT	2653
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2654	GTGGTTGTTTTCCCTGCAGGTACTCAATACCCACACGTTCAATGCAACTTA	2703
793	-----	792
2704	TACTTGGATCAGCTATTTCCCCAACACTATGAAATATTGTCTGGGCATAT	2753
793	-----	792
2754	TTTCAGCAGTCAGGAATGGAGTCCACTTCTATCATGTTTCAAAAAATGGA	2803
793	-----	792
2804	ATGTAAAGATCACCTGCATCAAGTGATAGGTTTTGGTAATACAAGGAAA	2853
793	-----	792
2854	GAAATGTCTGTATTTTTATTGAATATGTACATATAACAAATAAAATATGC	2903
793	-----	792
2904	TATCTAAATGTC	2915

**Figure 2.** Nucleotide alignment of both *Hrasls-1* Transcripts. 792bp: *Hrasls-1* 0.8kB transcript variants, 2915bp: *Hrasls-1* 2.9kB transcript variant. Coding region denoted by shaded area.



**Figure 3.** mRNA content of *Hrasls-1* transcript variants in BV2 cells treated with actinomycin D for increasing periods of time. Data were normalized to beta-actin and expressed relative to the mRNA expression at time point 0 (n = 4). 2.9kB: HRASLS-1 2.9kb transcript variant, 0.8kb: HRASLS-1 0.8kb transcript variant

## Chapter 6

### Differences in HRASLS-1 Transcript Variant mRNA Expression in Various Tissues During Non-Fasted, Fasted, and Re-fed States

#### 6.1 Introduction

Energy metabolism impacts all cellular functions. The human body goes between a fasted and non-fasted state happens several times throughout the day, depending on the incoming supply of nutrients, and requires a major shift in substrate regulation involving hundreds, if not thousands of different cellular processes. Extracellular factors communicate signals between tissues to regulate substrate handling and energy balance<sup>111,112</sup>. These signals are often produced at the cellular level through the action of enzymes. Modulation of the expression of genes encoding regulatory enzymes allows rapid tissue-specific responses. Through regulation of mRNA transcription, splicing, stability, and translation the amount of protein formed can be controlled, at least in part<sup>113</sup>. If these regulating factors are incorrectly controlled, it may lead to an abnormal metabolic state, with health implications including obesity development and associated co-morbidities. Therefore, identifying genes that mediate signalling of energy balance is important for understanding both normal and dysregulated physiology. Many of the genes that function in signalling pathways pertaining to energy homeostasis will undergo expression regulation in order to respond to changing substrate and energy availability. A role for NAEs in regulation of food intake<sup>9,114</sup> and substrate oxidation<sup>115</sup> has previously been identified, although enzymes regulating their synthesis have not yet been investigated during the different nutritional states.

## 6.2 Methods

### 6.2.1 Mouse Care and Tissue Harvesting

C57Bl/6J mice were bred and housed at the central animal facility according to standards of the Canadian Council on Animal Care and in accordance with an approved animal use protocol (AUPP 12-15). Female mice age 15-18 weeks of age were subjected to changing feeding conditions prior to euthanasia in accordance with animal use protocol (AUPP 12-08) that received ethical approval by the University of Waterloo Animal Ethics Committee. Mice were fed a standard chow diet with ad libitum access to food (non-fasted) or were fasted overnight for 16 h (fasted), or were fasted overnight for 16 h followed by a 4 h period of (re-feeding). Following euthanasia by cervical dislocation, mice were rapidly dissected and excised organs, specifically liver, WAT, kidney, heart and brain, were snap-frozen in liquid nitrogen, and then stored at -80°C prior to RNA isolation.

## 6.3 Results

### 6.3.1 Both transcript variants of *Hrasls-1* in the liver exhibit up-regulation in the fasted state

The relative expression of *Hrasls-1* transcript variants (0.8 kb and 2.9 kb) was determined in cDNA prepared from liver. Samples were analyzed by the deltaCt method, and were normalized to an average of three different housekeeping genes used as loading controls ( $\beta$ -actin, 18S, and RPII), in order to minimize variation. Transcript levels were expressed as fold-difference relative to the mRNA expression of the 2.9kb transcript variant in the non-fasted state. Results showed a significant up-regulation of both transcript variants in the fasted state in comparison to the non-fasted and re-fed state (**Figure 4**).

The 2.9kb *Hrasls-1* transcript variant was 15-fold higher in the fasted state when compared to the non-fasted state. After 4 h of re-feeding, we see a significant 6-fold down-regulation of expression relative to fasting. Indeed, in the re-fed state, the 2.9kb *Hrasls-1* transcript variant shows

an almost complete quantitative return to mRNA levels similar to those of the non-fasted state. This is evidenced by the fact that there is no significant difference in expression level between *Hrasls-1* transcript variant 2.9kb in the non-fasted and re-fed state.

When analyzing the expression of *Hrasls-1* 0.8kb transcript variant in the liver, results indicate a similar pattern of regulation, but an even greater magnitude of effect. The *Hrasls-1* 0.8kb transcript variant showed nearly a 30-fold increase in expression from the non-fasted to the fasted state. Similar to the 2.9kb transcript variant, in the re-fed state the 0.8kb transcript variant shows a decrease in mRNA expression, adjusting back to levels similar to those seen in the non-fasted state. The *Hrasls-1* 0.8kb transcript variant was down-regulated 8-fold from the fasted to the re-fed state. There was no significant difference between *Hrasls-1* 0.8kb transcript variant mRNA expression between the non-fasted and re-fed state, indicating that a 4 h period of re-feeding was sufficient to return *Hrasls-1* 0.8 kb transcript expression to the non-fasted baseline levels.

Our results show no significant difference in the relative expression of *Hrasls-1* 0.8kb and 2.9 kb transcript variants in either the non-fasted or re-fed states. However, in the fasted state, where both transcripts showed highest mRNA expression, there was a significant 3.7-fold difference between transcript variants.

### **6.3.2 The 0.8 kb *Hrasls-1* transcript is the predominant variant in kidney in all measured states, although both variants are up-regulated by fasting**

Expression of the 2.9 kb and 0.8 kb transcript variants of *Hrasls-1* was analyzed in kidney cDNA from non-fasted, fasted, and re-fed mice and normalized to an average of loading controls ( $\beta$ -actin, 18S, and RPII), then expressed relative to the level of the *Hrasls-1* 2.9kb transcript variant in the non-fasted state (**Figure 5**). In the kidney, both transcript variants were up-regulated in the fasted state.

The *Hrasls-1* 2.9 kb transcript variant showed an 18-fold increase from the non-fasted to the fasted state. This was followed by a significant 6-fold decrease in expression from the fasted to the re-fed state. This decrease was not as extensive as the up-regulation seen between the non-fasted and fasted state, but it was enough of a decrease that there was no significant difference between the levels of expression of the 2.9kb transcripts in the re-fed and non-fasted states. Thus in kidney, similar to liver, a 4 h period of re-feeding appears to be sufficient to return levels of the 2.9 kb transcript to baseline non-fasted levels.

The *Hrasls-1* 0.8kb transcript variant increased 11-fold from the non-fasted to fasted state. From the fasted to re-fed state, there was a significant down-regulation of transcript variant expression, by 4-fold. This resulted in an intermediate level of expression of the *Hrasls-1* 0.8kb transcript variant that fell between the non-fasted and fasted states, and was significantly different from both. A 4 h period of re-feeding, therefore, was not sufficient to completely return expression levels of the 0.8 kb transcript variant to baseline levels seen in the non-fasted state, with mRNA levels remaining approximately 2-fold higher in re-fed kidney than in non-fasted kidney.

Within the kidney, results indicate a significant difference in mRNA expression levels between *Hrasls-1* 0.8kb and 2.9kb transcript variants in the non-fasted, fasted, and re-fed states. Interestingly, the relative difference (~4-fold) between levels of the transcript variants was least in the fasted state when levels of both transcripts were most elevated, but greatest in the non-fasted and re-fed states, when differences of approximately 7-fold were evident between 0.8 kb and 2.9 kb transcripts.

### **6.3.3 *Hrasls-1* transcript variants are induced in the fasted state in white adipose tissue**

*Hrasls-1* transcript variant expression was analyzed in adipose tissue, and values were normalized to an average of three loading controls,  $\beta$ -actin, 18S, and RPII. Values are expressed relative to expression of the *Hrasls-1* 0.8 kb transcript variant in the non-fasted state (**Figure 6**).

Both *Hrasls-1* transcript variants were up-regulated in the nutrient deprived, fasted state. This is in agreement with effects observed in kidney and liver.

The *Hrasls-1* 2.9kb transcript showed a moderate up-regulation of 6-fold from the non-fasted to the fasted state. The 0.8kb transcript variant, however, showed a sizeable increase in expression of approximately 85-fold from the non-fasted to the fasted state. This result suggests that modulation of the *Hrasls-1* 0.8 kb transcript variant during feeding is the principle transcript variant for up-regulation of HRASLS-1 levels. In the re-fed state, the 0.8 kb transcript variant was able to return to levels similar to those seen in the non-fasted state. *Hrasls-1* 2.9 kb transcript variant did not normalize to values seen in the fasted state, and showed a moderate level of expression with a 3-fold difference from the non-fasted state, and a 2-fold difference from the fasted state.

Expression levels of the 2.9 kb and 0.8 kb transcript variants were significantly different in the up-regulated fasted state. Results indicate that expression of the *Hrasls-1* 0.8kb transcript variant was 4-fold higher than that of the 2.9 kb transcript in fasted white adipose tissue. In the non-fasted state the levels of the transcript variants were not significantly different, indicating that baseline level expression of both transcript variants is similar.

#### **6.3.4 *Hrasls-1* transcript variants within the heart show similar expression levels between different measured states**

Expression of heart *Hrasls-1* transcript variants was normalized to an average of 18S and RPII expression as loading controls, and levels are given as fold-difference relative to the *Hrasls-1* 2.9kb transcript variant in the non-fasted (ad libitum fed) state.

Our results agree with the concept of preserving constant energy and metabolite levels (**Figure 7**). They indicate that there is no significant effect on heart *Hrasls-1* mRNA expression during changes between nutritional states.

Furthermore, there was no significant difference between the *Hrasls-1* transcript variants within the non-fasted, fasted state, or re-fed state.

### **6.3.5 Expression of *Hrasls-1* transcripts showed no significant variation in the brain**

Analysis of brain *Hrasls-1* transcript variant expression (normalized to  $\beta$ -actin, 18S, and RPII), expressed in relation to the 0.8 kb transcript in the non-fasted state, illustrated that there were no significant differences between changing nutritional states, or between levels of the individual transcripts within any given state (**Figure 8**). The high degree of conservation of regulation of the *Hrasls-1* transcript variants differs from any other tissue tested except heart, and may be related to the relative isolation and homeostatic independence of brain tissue.

### **6.3.6 PPAR- $\alpha$ , *Acox1*, and TRPV1 mRNA expression were significantly increased in the fasted state within the liver**

The relative expression of *Ppar- $\alpha$* , *Acox1*, *Cpt1a*, *Trpv1* and *Scd-1*, were also determined in cDNA prepared from liver, and normalized to  $\beta$ -actin, 18S, and RPII. Transcript levels were expressed as fold-difference relative to control mRNA expression. Results showed a significant up-regulation of *Ppar- $\alpha$* , *Acox1*, and *Trpv1* mRNA within the fasted state (**Figures 9,10,13**), while *Scd-1* and *Cpt1a* expression did not show significant differences (**Figures 11, 12**).

*Ppar- $\alpha$*  mRNA expression was significantly increased 3-fold from the non-fasted to the fasted state, and a significant 31-fold decrease was observed from the fasted to re-fed state. This resulted in no significant difference in *Ppar- $\alpha$*  mRNA expression between the non-fasted and re-fed states.

Expression of *Acox1* showed a similar pattern of regulation within the non-fasted, fasted, and re-fed states. *Acox1* mRNA expression resulted in a significant 33-fold increase from the non-fasted

to the fasted state, and a significant 5-fold reduction in expression from the fasted to the re-fed state. Again, no significant difference was observed between the non-fasted and re-fed states.

mRNA expression of *Trpv1* was significantly different in all three feeding states analyzed. mRNA expression was lowest in the non-fasted state, followed by an intermediate 26-fold induction in the re-fed state. The fasted state displayed the greatest levels of *Trpv1* mRNA expression, resulting in a 69-fold induction, relative to the non-fasted state.

### **6.3.7 mRNA expression of receptors known to be activated by PEA, OEA, and SEA are not significantly changed in different nutritional states within the kidney**

Expression of *Ppar- $\alpha$* , *Acox1*, *Cpt1a*, *Trpv1* and *Scd-1* were analyzed in kidney cDNA from non-fasted, fasted, and re-fed mice and normalized to an average of loading controls ( $\beta$ -actin, 18S, and RPII), then expressed relative to either the non-fasted or fasted state (**Figures 14-17**). mRNA expression of *Ppar- $\alpha$* , *Acox1*, *Trpv-1*, and *Scd-1* showed no significant difference within the kidney at either the non-fasted, fasted, and re-fed state.

### **6.3.8 *Ppar- $\alpha$* and target gene *Acox1* are both significantly up-regulated in the fasted state within white adipose tissue**

Within white adipose tissue, *Ppar- $\alpha$* , *Acox1*, *Trpv1* and *Scd-1* mRNA expression was quantified and normalized to an average of three loading controls,  $\beta$ -actin, 18S, and RPII. Values are expressed relative to the expression of each gene of interest, in the lowest nutritional state. Analysis indicates that both *Ppar- $\alpha$*  and *Acox1* were significantly up-regulated in the fasted state (**Figures 18,19**), while there was no significant change in mRNA expression of *Trpv1* and *Scd-1* (**Figures 20,21**).

Similar to the liver and kidney, *Ppar- $\alpha$*  mRNA expression was significantly different between the non-fasted and fasted states, and between the fasted and re-fed states, showing a 6-fold increase, and a 10-fold decrease respectively.

*Acox1* mRNA expression increased 4-fold from the non-fasted to the fasted state. *Acox1* decreased 4-fold from the fasted to re-fed state. Both *Ppar- $\alpha$*  and *Acox1* showed no significant difference in mRNA expression between the non-fasted and re-fed states within WAT.

As mentioned, *Trpv1* and *Scd-1* mRNA expression within WAT showed no significant difference, although *Trpv1* expression was completely undetected within the fasted state.

## 6.4 Discussion

With respect to energy supply during a deficit state, the TAG stored in adipose tissue are an enormous reservoir of metabolic fuel. Adipocytes have an ongoing ability to synthesize and break down these TAGs, depending on the body's current energy demands<sup>116</sup>. They provide a fuel reserve that can be mobilized by lipolysis during food scarcity, releasing fatty acids for oxidation and energy in other organs<sup>116</sup>. In the present work, fasting significantly induced both *Hrasls-1* transcript variants, strongly suggesting a role for this enzyme in adipocyte biology. Although the functional significance of this induction must still be determined in future studies, possible effects are suggested by knowledge of the adipose-specific action of the NAEs that would likely be produced downstream of HRASLS-1. In adipocytes, OEA stimulates lipolysis and increases the release of NEFAs in a "dose-dependent" manner<sup>117</sup>. OEA also increases  $\beta$ -oxidation at least in part through activation of PPAR- $\alpha$ <sup>8</sup>. Additionally, OEA is an agonist of the capsaicin receptor TRPV1, which has been found in preadipocytes where its activation inhibits preadipocyte differentiation and adipogenesis<sup>118</sup>. Thus, induction of *Hrasls-1* may help to reduce adiposity through regulation of NOPE/OEA synthesis. Induction of *Hrasls-1* may also help to reduce adiposity through regulation of

the NPPE/PEA/ PPAR- $\alpha$  pathway that activates  $\beta$ -oxidation in adipocytes<sup>114</sup>(**Appendix B (A.)**). A potential role for HRASLS-1 in adipocyte differentiation, lipolysis regulation, and  $\beta$ -oxidation, is therefore suggested by the present study, indicating that future work on the role of HRASLS-1 in this tissue is merited.

Analysis of genes involved in fat metabolism indicated that within WAT, *Ppar- $\alpha$*  and *Acox1* mRNA expression, increased within the fasted state. Since both of these proteins play a role within  $\beta$ -oxidation and lipolysis, these results further confirm the prospective role of HRASLS-1 synthesizing PPAR- $\alpha$ -activating NAEs, to enhance  $\beta$ -oxidation and lipolysis within WAT. *Trpv1* mRNA expression was not increased during the fasted state, although further analysis such as activity assays, and protein concentrations under changing nutritional states must be done in order to make any conclusive decisions with regards to its role downstream of HRASLS1.

Under changing metabolic conditions, the *Hrasls-1* transcript variants shared a similar relative pattern of expression within the liver and kidney. In the re-fed state, liver and kidney mRNA levels of both *Hrasls-1* transcript variants showed a significant down-regulation from the fasted state. Within the liver, a 4 hour re-feeding period following an overnight 16 hour fast, was sufficient in bringing the expression of both *Hrasls-1* 0.8 kb and 2.9 kb transcripts back to levels seen within the non-fasted state. In the kidney, both transcripts were significantly down-regulated upon the reintroduction of nutrients, although only the 2.9 kb transcript returned to non-fasted levels by 4 hours. What this indicates is a rapid metabolic adaptation once nutrients are re-introduced. In both tissues there was also a significant induction of both *Hrasls-1* transcripts from the non-fasted state to the fasted state. The liver is a major regulator of energy metabolism within the body<sup>119</sup>. One of its primary roles is the synthesis and repackaging of fuel components for use by other organs. The transition from a non-fasted to fasted state changes the equilibrium of hepatic lipid metabolism. In a non-fasted state, circulating glucose and insulin are high leading to an increase in hepatic plasma

glucose and the de novo synthesis of fatty acids<sup>119</sup>. Consequentially, hepatic TAG synthesis is increased, and  $\beta$ -oxidation of fatty acid is inhibited<sup>119</sup>. At this time, the liver is in a “lipogenic” state<sup>119</sup>. Alternatively, in a fasted state, the glucose and insulin concentrations fall, driving TAG breakdown, for fatty acid release from adipose tissue and increasing plasma fatty acid levels<sup>119</sup>. In peripheral tissues, such as the liver, fatty acids are then hydrolyzed as needed to supply fuel substrates through  $\beta$ -oxidation to relieve energy shortages<sup>119</sup>. At this time, the liver is in a “lipolytic” state<sup>119</sup>. Similar regulation has been observed in the kidney<sup>120</sup>. Although the kidney is also a site for lipoprotein synthesis and secretion<sup>121</sup>, it was reported that NEFA and esterified FA are largely assimilated by the kidney in the state of starvation<sup>120</sup>. Along with the liver, in the fasted state the kidney is also in a lipolytic state, with increased dependency on  $\beta$ -oxidation of NEFAs as a fuel source.

Our data indicates that *Hrasls-1* is induced in response to fasting within the liver as well as within the kidney. A possible implication of this effect is that HRASLS-1 may play a role in stimulating NAPE/NAE signalling to increase  $\beta$ -oxidation, in a state where glucose stores have been depleted, and NEFA are more abundant. Several NAEs, including PEA and OEA, are known to activate PPAR- $\alpha$  that is a potent activator of  $\beta$ -oxidation<sup>114</sup>. OEA has been shown to enhance fatty acid oxidation in dissociated hepatocytes, through activation of PPAR- $\alpha$ <sup>122</sup>. OEA is also able to induce the expression of PPAR- $\alpha$ -regulated genes that participate in fatty acid utilization, including FAT/CD36 and FABP that mediate uptake of fatty acids, and UCP-2 and UCP-3 that mediate the dissipation of excess energy following fatty acid oxidation. Induction of HRASLS-1 may also increase the downstream synthesis of SEA. SEA is able to reduce liver SCD-1 mRNA expression<sup>98</sup>, and in the fasted state may be doing so in order to facilitate increased liver fatty acid oxidation and decreased lipogenesis. Therefore, since *Hrasls-1* is clearly regulated in liver and kidney, it suggests that this enzyme and its associated signalling pathways may play an essential role in mediating the metabolic

switch between fasting and re-feeding that is necessary to ensure that energy demands continue to be fulfilled upon nutrient withdrawal (**Appendix B (B.)**). It is important to mention that although the potential relevance of GPR119 was introduced as a possible mediator of the HRASLS-1 pathway, our results have suggested otherwise. GLP-1, activated by GPR119, stimulates insulin secretion and promotes nutrient metabolism, essentially delaying further nutrient intake<sup>69,70</sup>, a response that is related to the non-fasted state. Since our results show *Hrasls-1* to be down-regulated in the non-fasted state, it suggests that it does not mediate the NOPE/OEA/GRP119 pathway. Thus, GPR119 may not be a significant mediator of HRASLS-1-associated signalling of nutritional regulation.

Since HRASLS-1 may act through regulation of PPAR- $\alpha$  or TRPV1, we tested expression of these genes and others that they regulate in liver and kidney as well. It is important to note that CPT1 $\alpha$  is the liver isoform of the enzyme, and its expression is limited within other tissues<sup>123</sup>. If key enzymes and receptors involved in  $\beta$ -oxidation and fat metabolism were up-regulated, in the same manner as *Hrasls-1*, this would confirm the possibility that the HRASLS-1 pathways could be activating these proteins downstream. Results indicated that within the liver, mRNA expression of *Ppar- $\alpha$* , *Acox1*, and *Trpv1* were significantly up-regulated within the fasted state, relative to both the non-fasted and re-fed states. Thus, HRASLS-1 can be increasing the synthesis of OEA and PEA to activate both TRPV1 and PPAR- $\alpha$ . As mentioned earlier, *Acox1* is a classic target gene of PPAR- $\alpha$ <sup>124</sup>, therefore its up-regulation is expected to follow that of PPAR- $\alpha$ . Furthermore, the ACOX1 protein is the first enzyme in the fatty acid  $\beta$ -oxidation pathway, catalyzing the desaturation of acyl-CoAs<sup>119</sup>. Thus, it would be essential in the oxidation of incoming NEFAs during the fasted state. Unfortunately we saw no significant difference in expression of *Scd-1*, and *Cpt1 $\alpha$*  mRNA. This does not rule out the possibility of SEA activation via HRASLS-1, but simply imposes for further investigation. Within the kidney, we did not see any significant change in mRNA expression of all genes investigated, thus we are unable to make further speculations about the involvement of

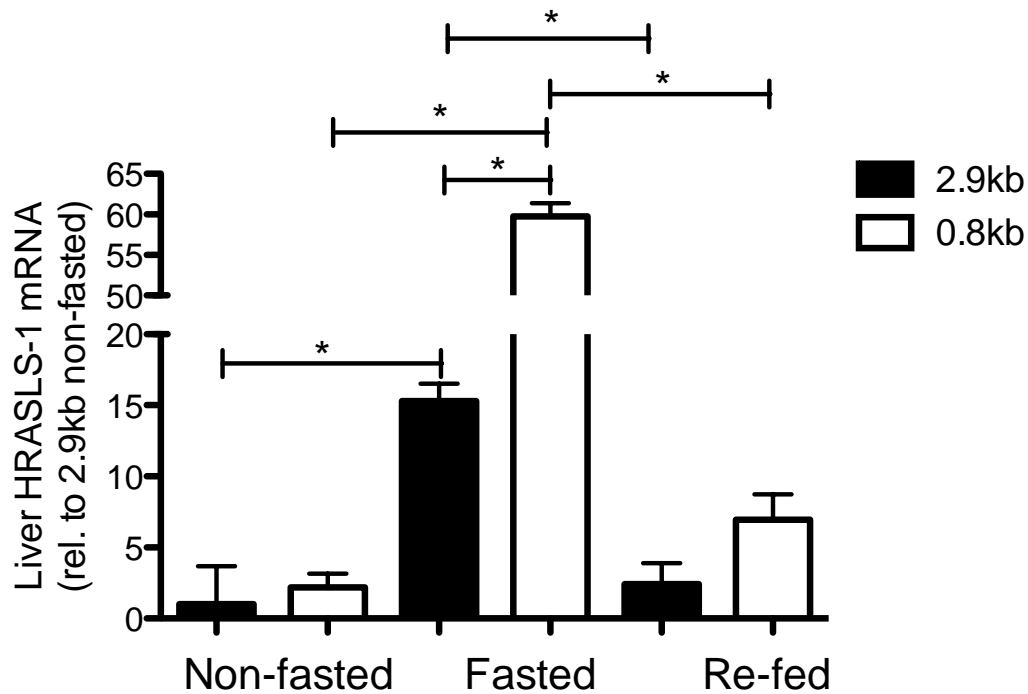
HRASLS-1 through these receptor-mediated pathways.

Our results suggest that HRASLS-1 may play a significant role in activating extra-adipose fatty acid oxidation, and that the increase in activation may be due, at least in part, to its role in helping to elevate levels of circulating NEFAs from adipose tissue during the fasted state. There is a widespread acceptance in the literature that NEFAs can mediate many adverse metabolic affects<sup>125</sup>. In obesity a significant elevation of NEFA concentrations are found due to the increase in adipose tissue mass<sup>125</sup>. An increase in circulating NEFA molecules will force an increased uptake of NEFAs into peripheral tissues. This will aggravate lipid accumulation in other tissues, leading to a progression of obesity-linked diseases. It would be of great interest to investigate whether HRASLS-1 overexpression could decrease this lipid accumulation by enhancing fatty acid oxidation. It is possible that HRASLS-1 pathway may also up-regulate the expression of uncoupling protein UCP-3 through increased production of OEA. UCP-3 has been shown to play a role in fuel source management and energy metabolism, leading to improved body weight regulation<sup>126</sup>. Over expression of HRASLS-1 in obese model would allow us to examine this theory and further analyze the role of HRASLS-1 in fatty acid oxidation. These findings could eventually hold therapeutic relevance.

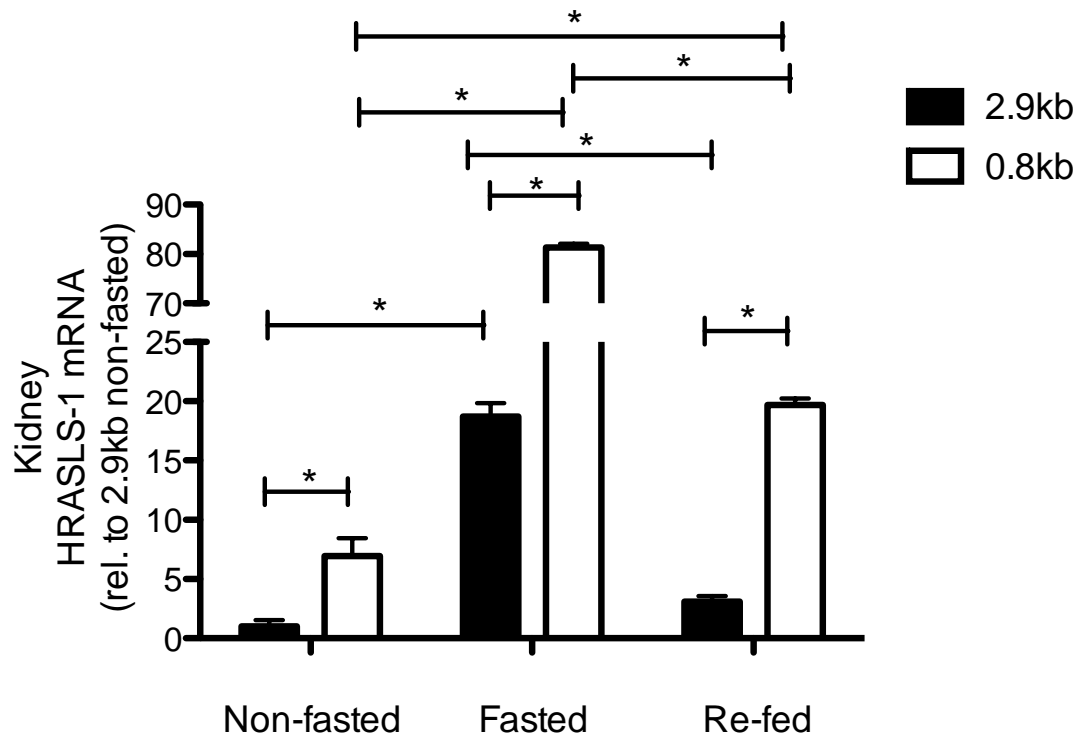
Both the heart and brain are energy-demanding tissues, which require a continuous supply of energy, regardless of non-fasted, fasted, or re-fed state. The heart has a high rate of energy production and turnover that is required to maintain its continuous mechanical work, and any perturbations in energy-generating processes may therefore affect contractile function directly<sup>127</sup>. In contrast, the brain lacks lipid fuel stores and hence requires a continuous supply of glucose for energy production<sup>128</sup>. Even the slightest perturbations in energy supply to the brain can be damaging to its overall function<sup>138</sup>. Thus, maintaining glucose/energy metabolism in an unchanged manner is critical for brain health. With that said, in tissues that are highly reliant on a constant

supply of substrate, such as the heart and brain, energy homeostasis is relatively constant. It is possible that the role of HRASLS-1 in these tissues does not show significant difference between the changing metabolic states, because energy metabolism does not need further activation.

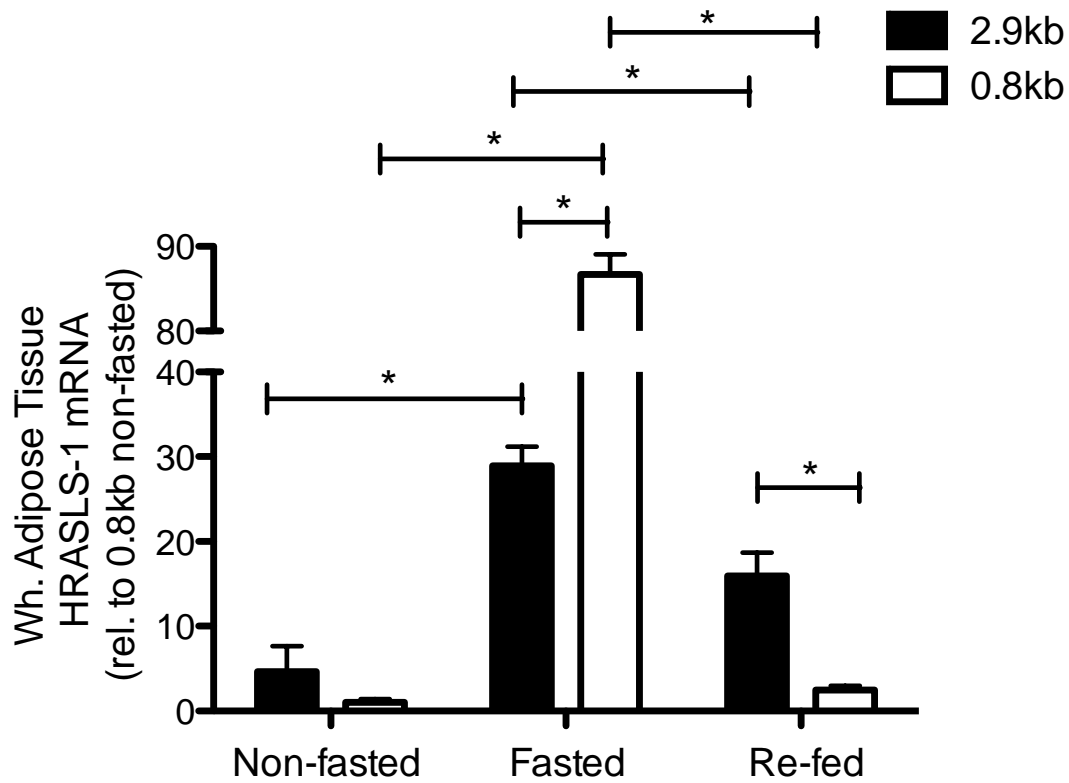
Our results indicated no difference in the expression of either *Hrasls-1* transcript variant in the brain, nor the heart, in different measured states. In addition, the heart and the brain showed similar effects, where no significant change was seen with either transcript at either state. Interestingly, this suggests the possibility that induction of *Hrasls-1* could be mediated by changes in glucose, since we see less of a change in HRASLS-1 in environments where glucose supply is typically unchanged. Demand for glucose supply in the brain and heart is relatively stable. Hence, this may help to mediate the lack of *Hrasls-1* flux during transition between the different states. Additional studies will clearly be required to test this idea.



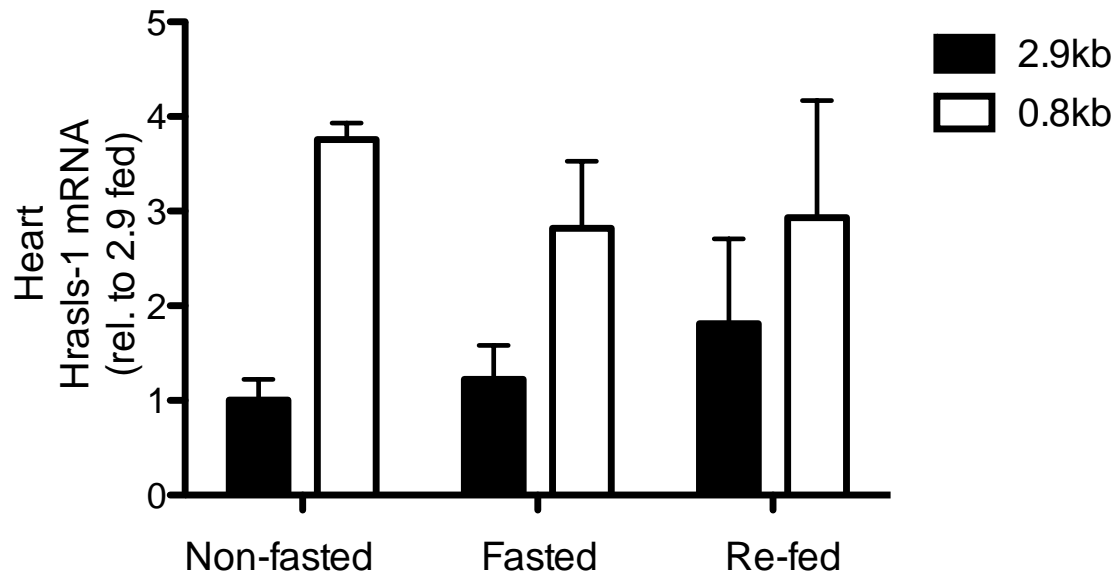
**Figure 4.** Liver mRNA content of *Hrasls-1* transcript variants under different nutritional states. Data are expressed relative to the mRNA expression of the *Hrasls-1* 2.9 kb transcript variant in the non-fasted state (n = 6) and have been normalized to an average of housekeeping genes ( $\beta$ -actin, 18S, and RPII). Significant expression differences are denoted by  $*P < 0.05$ . 2.9 kb: *Hrasls-1* 2.9 kb transcript variant, 0.8 kb: *Hrasls-1* 0.8 kb transcript variant.



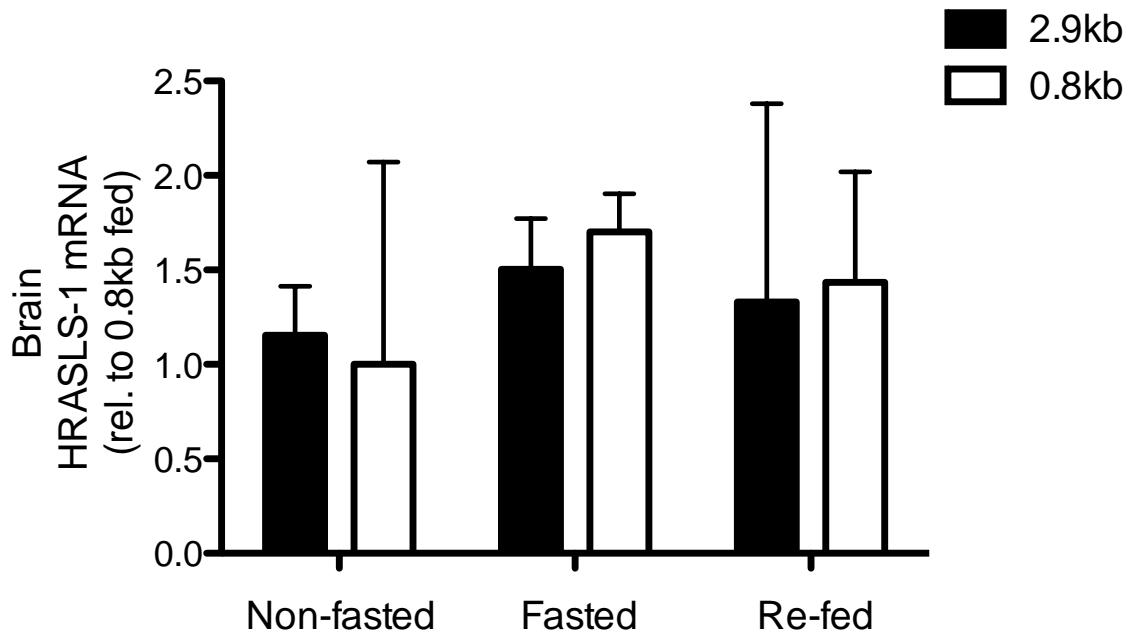
**Figure 5.** Kidney mRNA content of *Hrasls-1* transcript variants in different nutritional states. Data are expressed relative to the mRNA expression of the *Hrasls-1* 2.9kB transcript variant in the non-fasted state (n = 3-5), and normalized to the average expression of three housekeeping genes ( $\beta$ -actin, 18S, and RPII). Significant expression differences are denoted by  $*P < 0.05$ . 2.9kB: *Hrasls-1* 2.9kB transcript variant, 0.8kB: *Hrasls-1* 0.8kB transcript variant.



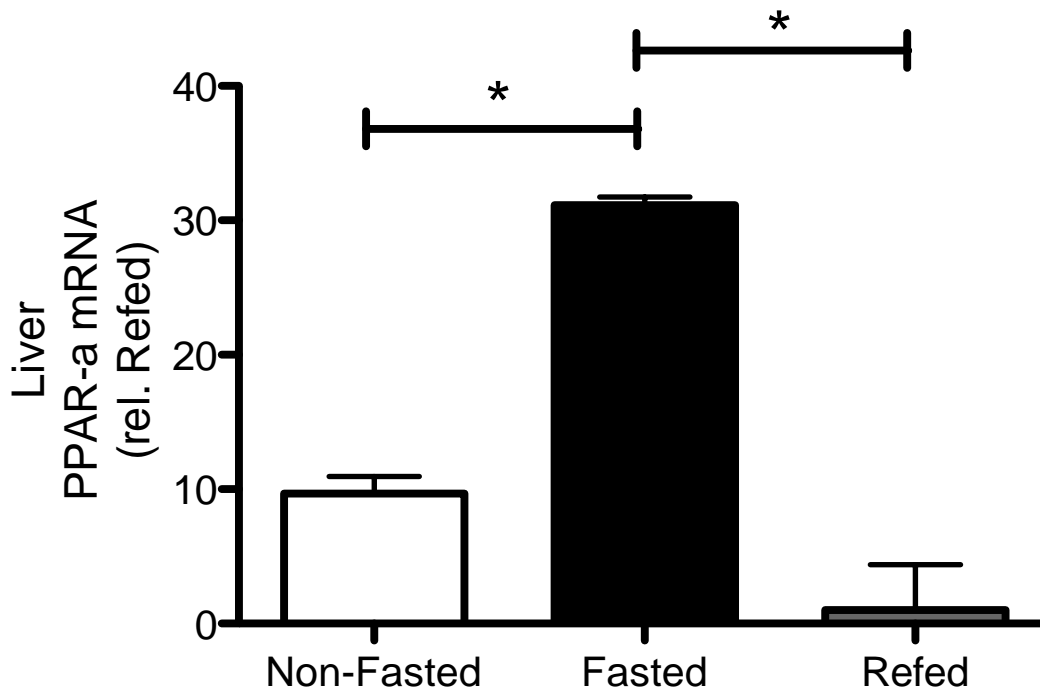
**Figure 6.** White adipose tissue mRNA content of *Hrasls-1* transcript variants in different nutritional states. Data are expressed relative to the mRNA expression of the *Hrasls-1* 0.8 kb transcript variant in the non-fasted state, and normalized to the average expression of three housekeeping genes ( $\beta$ -actin, 18S, and RPII) (n=3). Significant expression differences are denoted by \* $P < 0.05$ . 2.9 kb: *Hrasls-1* 2.9 kb transcript variant; 0.8 kb: *Hrasls-1* 0.8 kb transcript variant.



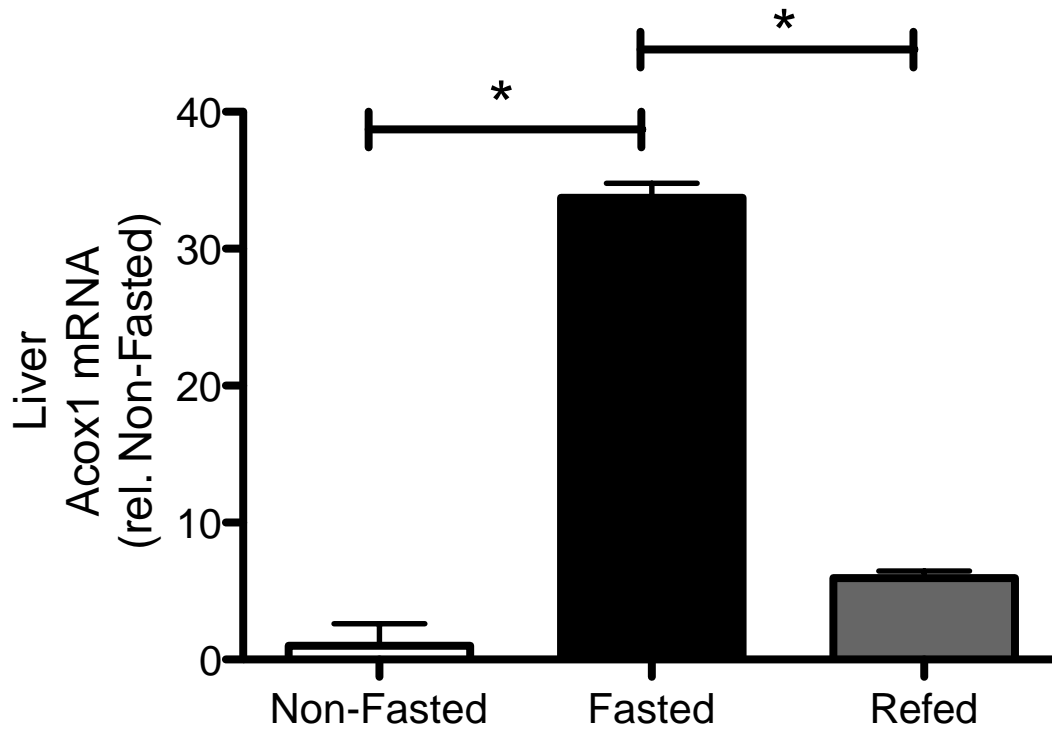
**Figure 7.** Heart mRNA content of *Hrasls-1* transcript variants under different nutritional states. Data are expressed relative to the mRNA level of the *Hrasls-1* 2.9kB transcript variant in the non-fasted state (n = 4-6), and normalized to housekeeping genes 18S and RPII. 2.9kb: *Hrasls-1* 2.9kB transcript variant; 0.8 kb: *Hrasls-1* 0.8kB transcript variant.



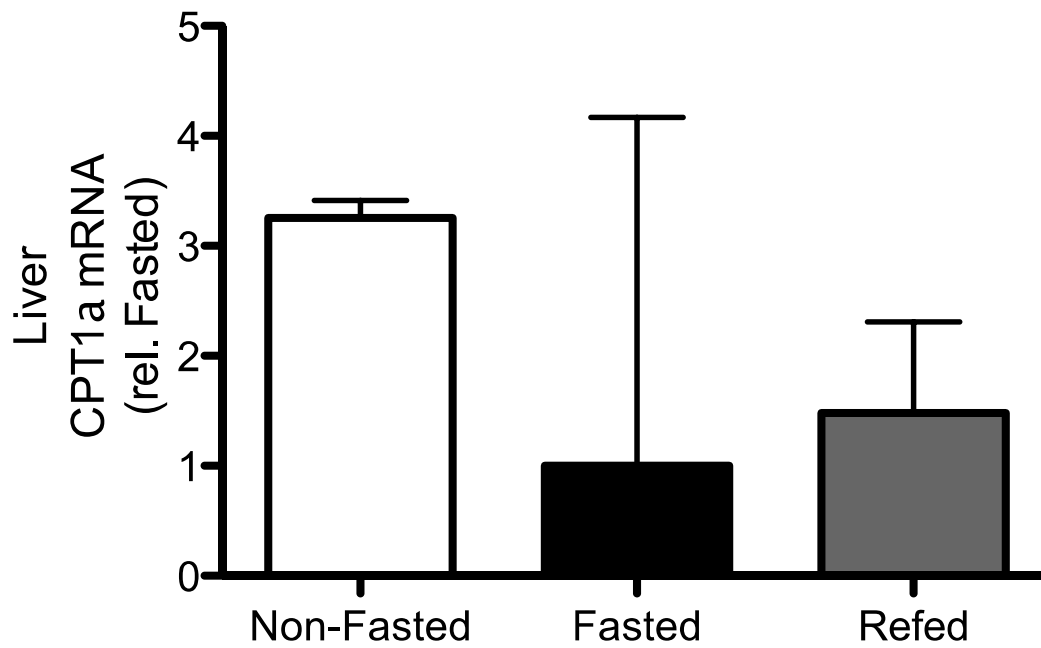
**Figure 8.** Brain mRNA content of *Hrasls-1* transcript variants in different nutritional states. Data are expressed relative to the mRNA expression of the *Hrasls-1* 0.8 kb transcript variant in the non-fasted state (n = 3-4), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII). No significant differences were observed between transcript levels or among groups. *Hrasls-1* 2.9 kb transcript variant, 0.8 kb *Hrasls-1* 0.8kb transcript variant.



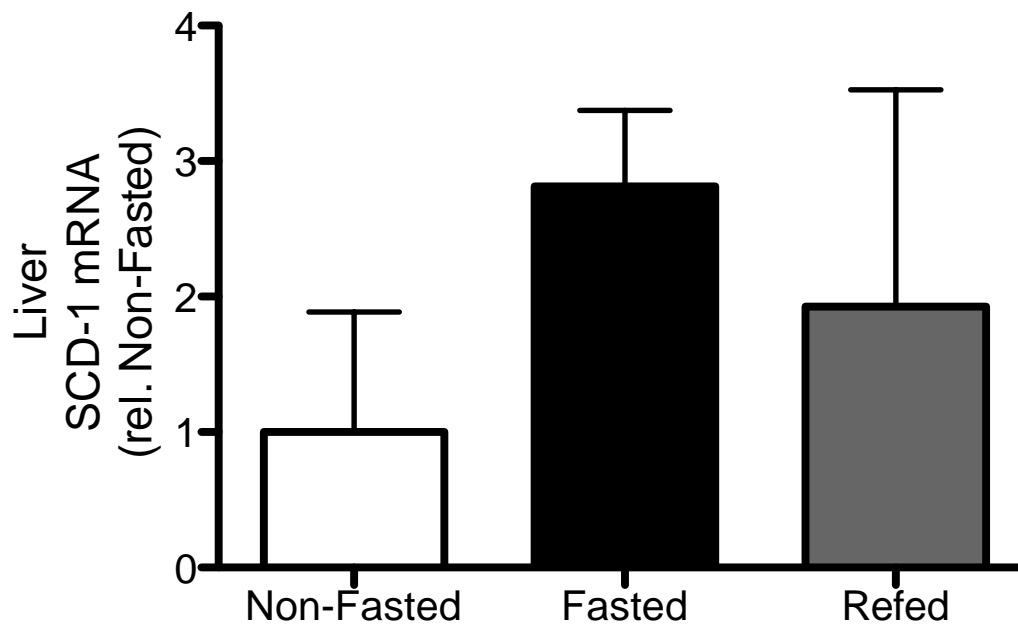
**Figure 9.** Liver mRNA content of *Ppar- $\alpha$*  under different nutritional states. Data are expressed relative to the mRNA levels in the re-fed state (n = 2-3), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



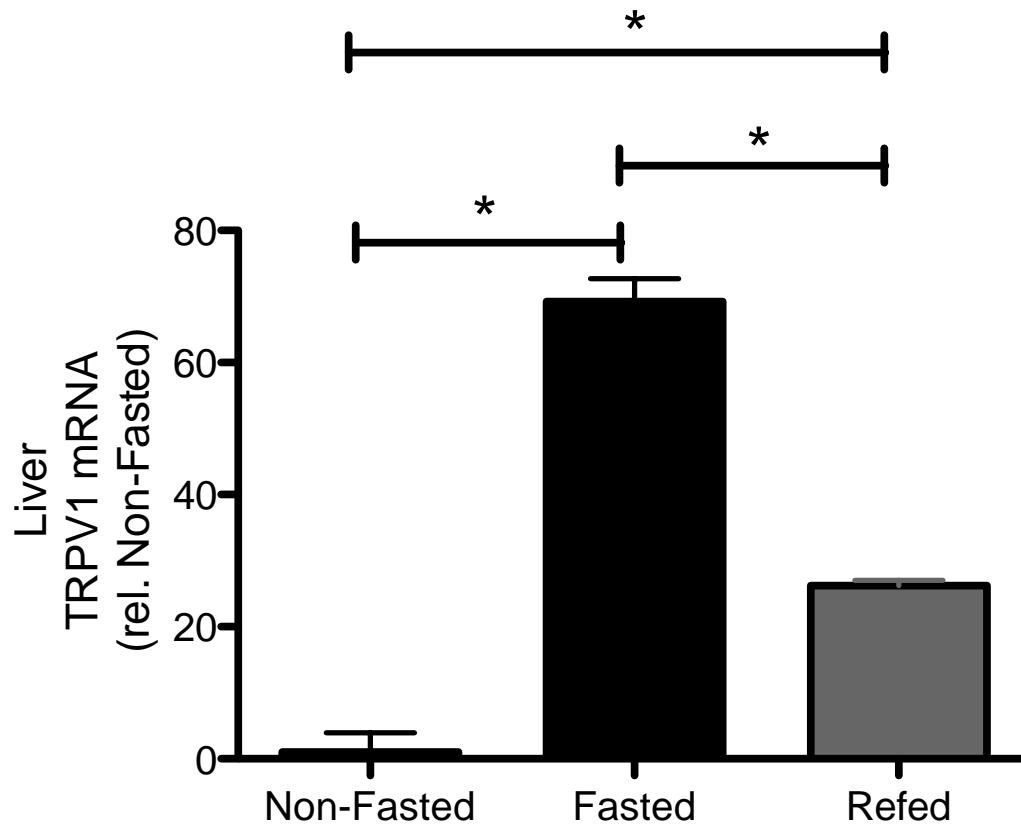
**Figure 10.** Liver mRNA content of *Acox1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 2-3), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII). \* $P < 0.05$ .



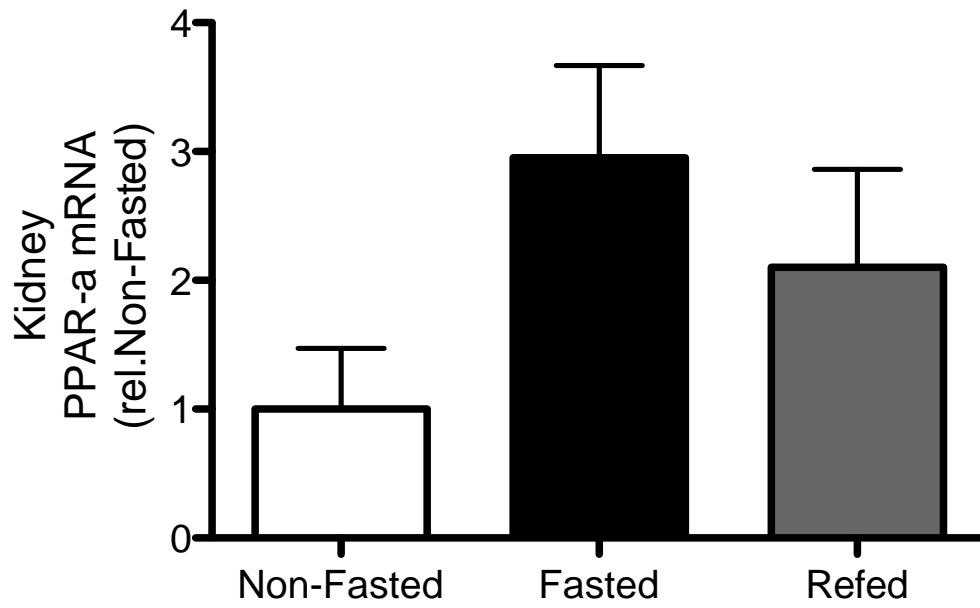
**Figure 11.** Liver mRNA content of *Cpt1α* under different nutritional states. Data are expressed relative to the mRNA levels in the fasted state (n = 3), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



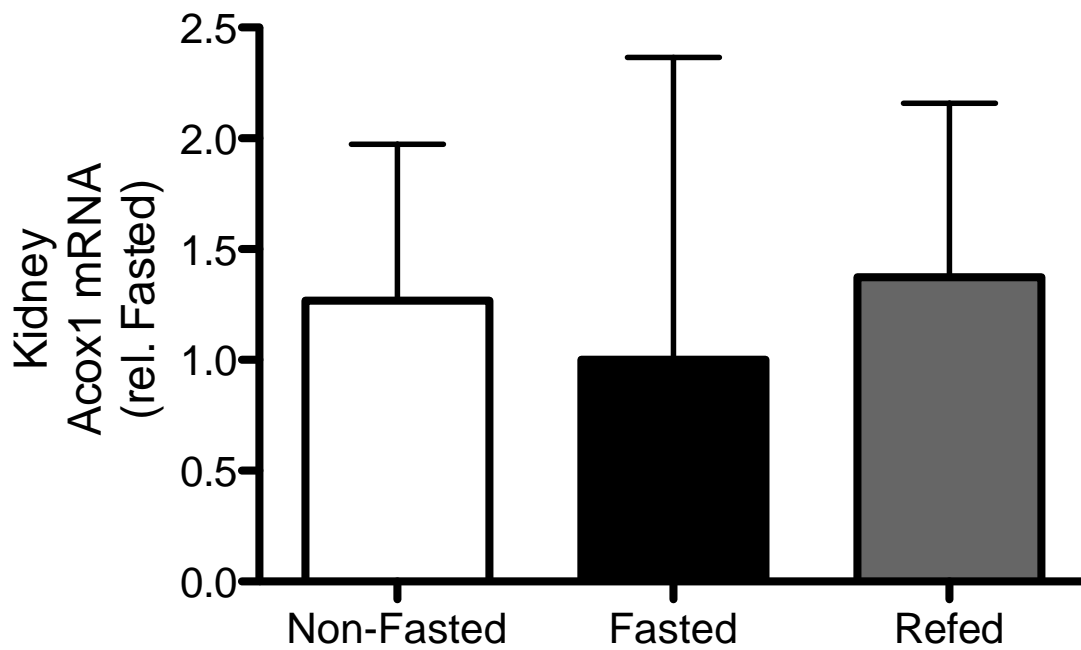
**Figure 12.** Liver mRNA content of *Scd-1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 2-4), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



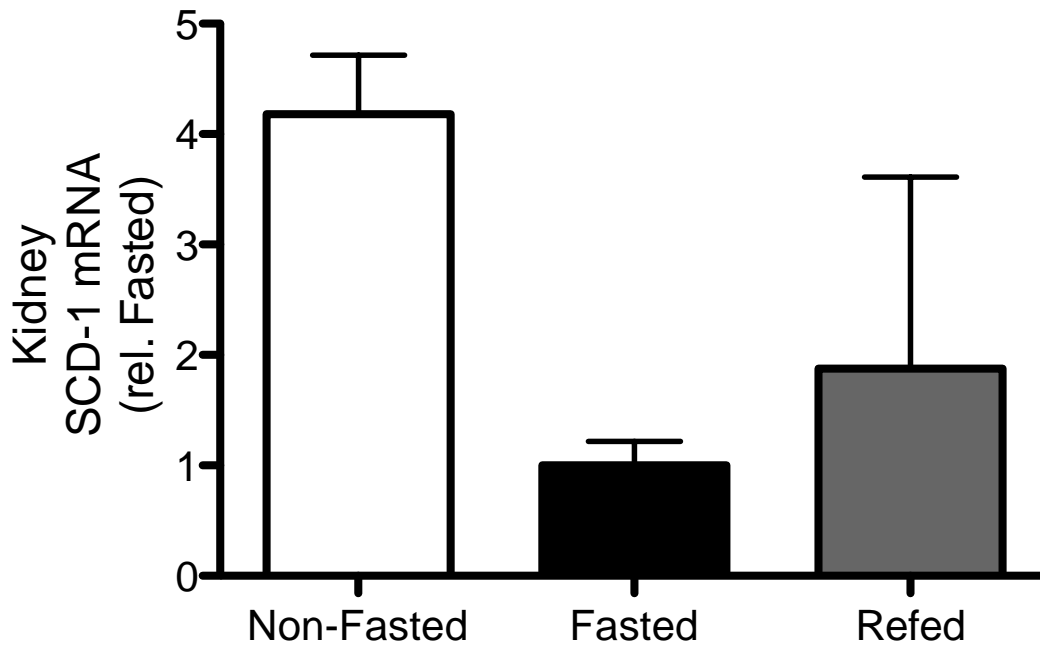
**Figure 13.** Liver mRNA content of *Trpv1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 3-5), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII). \* $P < 0.05$ .



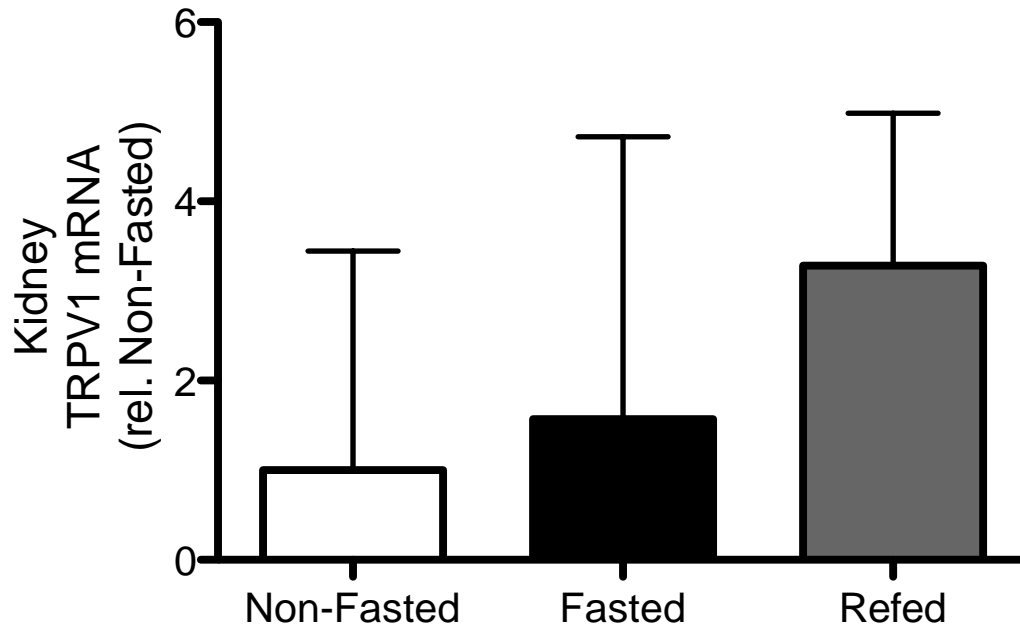
**Figure 14.** Kidney mRNA content of *Ppar- $\alpha$*  under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 3-5), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



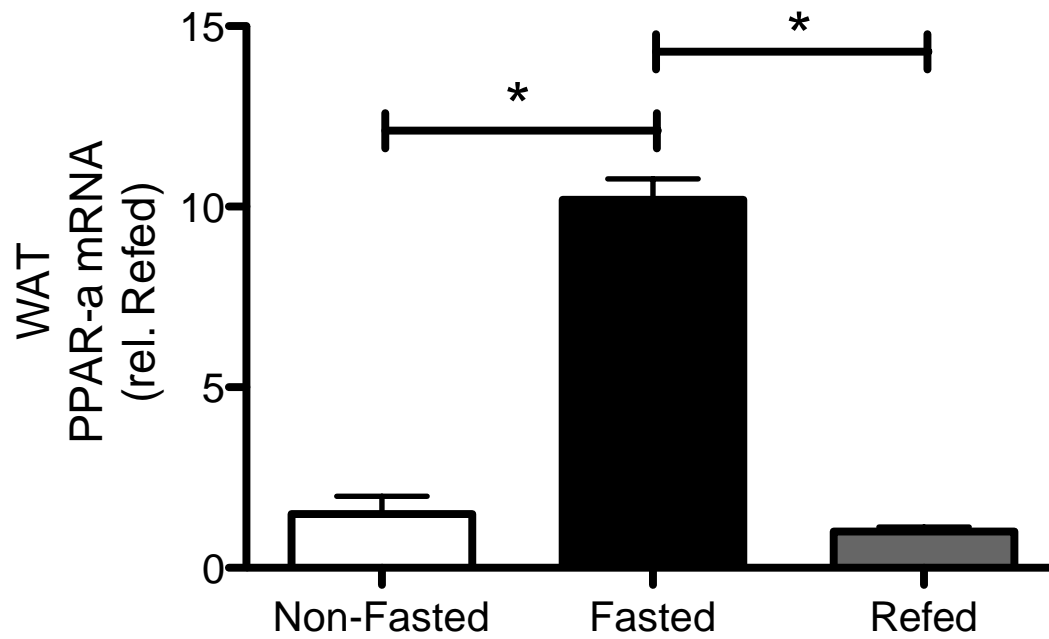
**Figure 15.** Kidney mRNA content of *Acox1* under different nutritional states. Data are expressed relative to the mRNA levels in the fasted state (n = 6), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



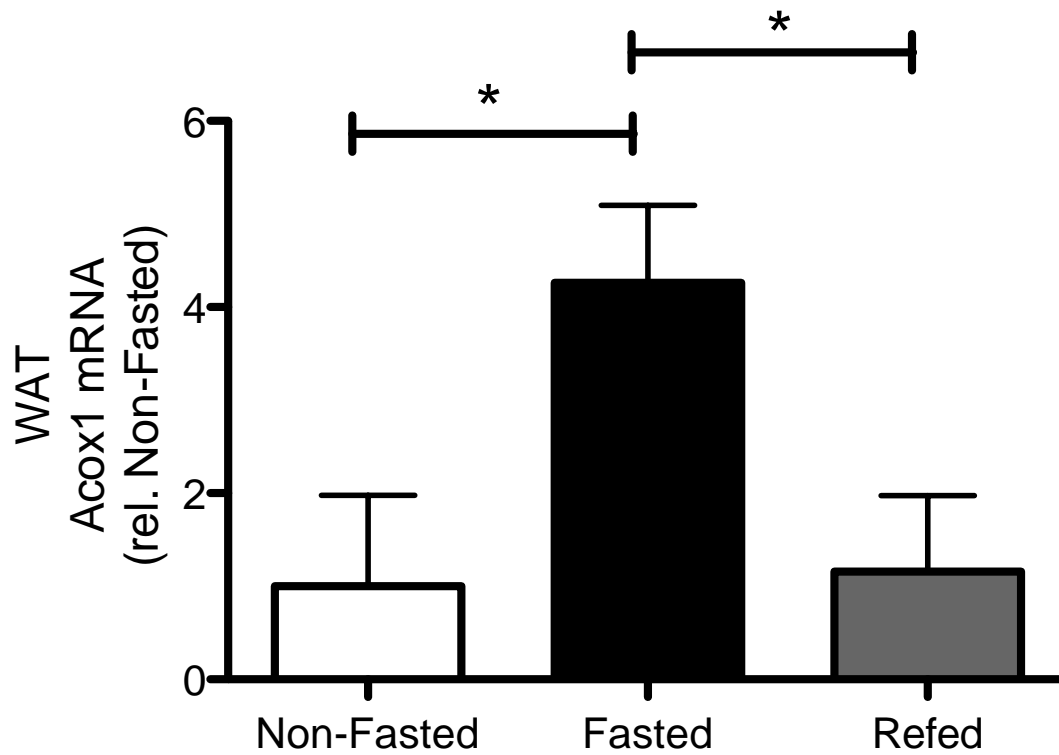
**Figure 16.** Kidney mRNA content of *Scd-1* under different nutritional states. Data are expressed relative to the mRNA levels in the fasted state ( $n = 3$ ), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



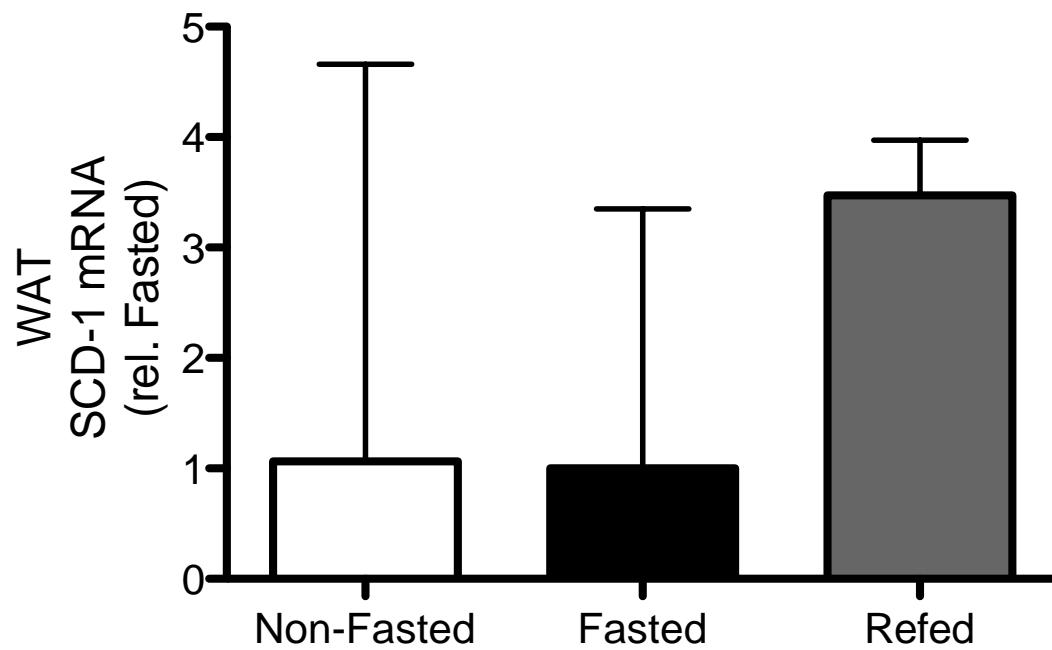
**Figure 17.** Kidney mRNA content of *Trpv1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 3-4), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



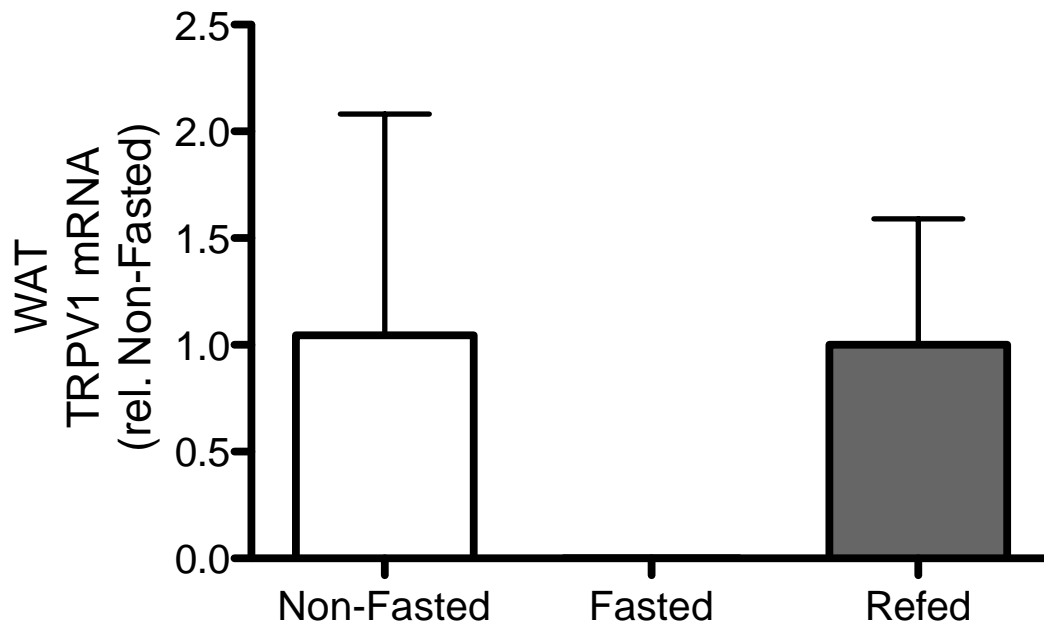
**Figure 18.** WAT mRNA content of *Ppar-α* under different nutritional states. Data are expressed relative to the mRNA levels in the re-fed state (n = 3-6), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII). \* $P < 0.05$ .



**Figure 19.** WAT mRNA content of *Acox1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 2-4), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII). \* $P < 0.05$ .



**Figure 20.** WAT mRNA content of *Scd-1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 3-5), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).



**Figure 21.** WAT mRNA content of *Trpv1* under different nutritional states. Data are expressed relative to the mRNA levels in the non-fasted state (n = 2-4), and normalized to housekeeping genes ( $\beta$ -actin, 18S, and RPII).

## Chapter 7

### iNAT mRNA Expression and [ $C^{14}$ ]NAPE Content in BV2 Microglia Treated with LPS

#### 7.1 Introduction

Microglia are immunocytes found in the central nervous system that function similarly to macrophages in the periphery<sup>129</sup>. They are activated by a variety of stimuli and function as the primary cellular mediator of neuroinflammation<sup>129</sup>. As the “macrophages of the central nervous system”, microglia are responsible for clearing foreign invading pathogens as well as clearing damaged cells resulting from mechanical, chemical, or biological trauma. In response to direct activation by foreign antigens, such as lipopolysaccharide (LPS), or to proinflammatory signalling by cytokines, microglia undergo a series of major changes to an activated phenotype that includes increased formation of fine processes (filopodia)<sup>130</sup>, increased lytic capabilities<sup>131</sup>, and increased secretion of cytokines. Lipids may play roles as structural and bioenergetic elements, as well as signalling elements in these processes. In particular, NAEs are known to be involved in the resolution of inflammation, by activating receptors that inhibit proinflammatory cytokines and transcription factors<sup>67,88</sup>. Identification of enzymes involved in inflammation, and in particular in signalling inflammation resolution, has major implications for the treatment of multiple chronic diseases with inflammatory components, ranging from rheumatoid arthritis to Crohn’s disease to Alzheimer’s disease, cardiovascular disease, and diabetes<sup>53,132,133</sup>.

#### 7.2 Methods

##### 7.2.1 BV2 Cell Culture

BV2 cells express toll-like receptor 4 and can be activated by treatment with LPS<sup>134</sup>. LPS (Sigma Aldrich, Mississauga, Canada, #L6529-1MG) was dissolved in DMEM at 1 mg/ml, then diluted in DMEM at 1000× concentration (1 µg/ml) prior to addition to cells.

### **7.2.2 Incorporation of [<sup>14</sup>C]palmitate into cellular NPPE**

BV2 cells were grown at 37°C to 80% confluency in 60-mm dishes containing DMEM with 10% fetal bovine serum and penicillin/streptomycin. Labeling medium (6 ml per plate) was prepared by mixing 2.0  $\mu$ Ci of [<sup>14</sup>C]palmitic acid per dish with 0.6 ml FBS per dish to allow the fatty acid to complex with bovine serum albumin (BSA). The fatty acid-BSA-FBS mixture was then diluted 1:10 with DMEM, and apportioned equally into two tubes. LPS (dissolved in DMEM) was added to one tube, to a final concentration of 1  $\mu$ g/ml, so that half of the plates were treated with radioactive fatty acid plus LPS, and half of the plates were treated with radioactive fatty acid without LPS (control medium). At time point 0, medium was removed from BV2 cells, and replaced with labeling medium for a period of 18 h. Media was then carefully pipetted off, and cells were washed and harvested by trypsinization, with the resulting mixture transferred into glass tubes. Any remaining cellular material was collected by washing plates with an additional 100  $\mu$ L PBS, and this was added to the harvested cell suspension for total lipid extraction.

### **7.2.3 Lipid Extraction**

Total lipids were extracted from harvested cells by the method of Bligh and Dyer<sup>135</sup>. Briefly, cells harvested in trypsin/PBS were mixed with methanol: chloroform (2:1) (1.9 ml /0.5 ml of sample) and vortexed vigorously for at least 30 seconds. Chloroform was added at 0.625 ml/0.5 ml of initial sample volume, and samples were vortexed again vigorously for >30 seconds. Deionized H<sub>2</sub>O was added next (0.625 ml/0.5 ml of sample), and samples were mixed thoroughly by vortexing. Samples were centrifuged for 5 minutes at 1500  $\times$  g at room temperature in order to separate aqueous (top) and organic (bottom) phases. The organic phase was recovered into a new glass tube, and evaporated under a stream of N<sub>2</sub> gas prior to separation by thin layer chromatography.

#### 7.2.4 Thin Layer Chromatography – Resolution of Polar Lipids

Organic samples dried under nitrogen gas were carefully dissolved in 50  $\mu$ L of chloroform and spotted onto a silica-coated glass chromatoplate. The chromatoplate was then developed in a tank containing a solvent mixture of chloroform:methanol:28% ammonium hydroxide (80:20:2) to separate polar lipids including N-acylphosphatidylethanolamide (NAPE)<sup>31</sup>. Sample components move upwards through capillary action at different rates, resulting in resolution based on polarity. Samples are visualized under UV light or by reaction with iodine vapors, allowing for easy detection of known standards that are run in separate columns on the same plate. Authentic standards (50  $\mu$ g each) utilized included PC, PE, palmitate, PEA, TAG, and NPPE. Radiolabelled lipid samples were overlaid with NPPE (25  $\mu$ g each) to facilitate visualization when incubated with iodine crystals prior to scraping and quantification by scintillation counting.

### 7.3 Results

#### 7.3.1 Total *Hrasls-1* mRNA is Induced in BV2 Cells Treated with LPS

Activation of BV2 microglial cells with LPS can activate pro- and anti-inflammatory mediators<sup>136</sup>. NAEs are acute anti-inflammatory signalling lipids derived from NAPEs that are regulated by HRASLS family members. Effects of inflammation activation on HRASLS family member expression has not, however, been reported. Both HRASLS-5 and HRASLS-3 are present in the genome of murine species, while HRASLS-2 and HRASLS-4 are not. We analyzed the mRNA expression of *Hrasls-5* and *Hrasls-3* through qPCR analysis of cDNA of BV2 cells that had either been treated with control media or media containing LPS. The mRNA expression of total *Hrasls-1* was also analyzed under these conditions, by performing qPCR with primers specific for the *Hrasls-1* coding region that would recognize both transcript variants.

Our results indicated a significant increase in *Hrasls-1* mRNA expression upon activation with LPS. In this experiment, *Hrasls-1* expression was increased 11-fold when cells were activated with LPS (**Figure 22**). Showing a similar pattern, the expression of *Hrasls-3* was significantly induced 15-fold upon LPS activation. *Hrasls-1* levels were below the level of detection in either control or LPS-treated cells, suggesting that levels of this N-acyltransferase are insignificant in this cell type.

Comparison of *Hrasls-3* and *Hrasls-1* levels in control cells illustrates a greater baseline level of expression of *Hrasls-1* in BV2 cells. There was a significant difference of 8-fold between *Hrasls-1* and *Hrasls-3* mRNA within the control treated BV2 cells. Once activated with LPS this difference was largely maintained, showing a difference of approximately 6-fold. In both conditions, *Hrasls-1* expression was highest.

### **7.3.2 The *Hrasls-1* 0.8 kb transcript, but not the 2.9 kb transcript, is significantly induced by LPS-activation of cells**

In order to determine whether a change in one or both transcripts was responsible for the induction of total *Hrasls-1* observed in LPS-treated cells, we measured the expression by qPCR of both transcript variants in BV2 microglial cells that were treated with either 1  $\mu$ g/mL of LPS or control medium. We normalized our values to those of the control gene 18S, and then expressed these values relative to expression of the *Hrasls-1* 2.9 kb transcript variant from cells incubated in control media. Our results show that activation of BV2 cells with LPS caused a significant induction of the expression of the *Hrasls-1* 0.8 kb transcript variant (**Figure 23**). We observed a 38-fold increase in the expression of the *Hrasls-1* 0.8 kb transcript variant when comparing cells treated with control media, to those activated with LPS. The longer, 2.9kb transcript variant showed no

significant change in expression between control or LPS activation, indicating no change in the expression of this transcript when BV2 cells are stimulated by a pro-inflammatory molecule.

When comparing the 0.8 kb transcript variant to the 2.9 kb transcript variant, we observed much lower expression of the 2.9 kb transcript variants in both groups (control treated and LPS treated). This indicates greater baseline levels of the *Hrasls-1* 0.8kb transcript in BV2 microglial cells.

### 7.3.3 NAPE content is increased in BV2 cells treated with LPS

The band corresponding to NAPE was detected and was scraped for quantification by liquid scintillation counting. Relative radioactive counts (cpm) were normalized to cell numbers (**Figure 24**), so that NAPE levels could be expressed relative to control-treated plates. Results indicated that acute activation of BV2 microglial cells with LPS resulted in a significant 2-fold increase in [<sup>14</sup>C]NAPE content compared to control cells ( $P < 0.05$ ) (**Figure 25**).

## 7.4 Discussion

With respect to HRASLS-1, there is a predominant preference by this enzyme for N-acylation using fatty acids transferred from the sn-1 position of glycerophospholipids<sup>18</sup>. In general, the fatty acids located at the sn-1 position are saturated, and are primarily the saturated species palmitate and stearate that would generate NPPE/PEA and NSPE/SEA through the *Hrasls-1*/PLD pathway. PEA and SEA are among the major NAE species in animal tissue<sup>9,76</sup> and, in injured tissues, high levels of saturated, such as SEA and PEA, rather than unsaturated, AEA, NAEs tend to accumulate<sup>67</sup>. The C16:0 NAE, PEA is able to directly activate PPAR- $\alpha$  and employ an anti-inflammatory role, by altering the transcription of proinflammatory transcription factors, NF- $\kappa$ B and AP-1<sup>58</sup>. In addition to PEA, C18:0 NAE, SEA is also an important regulator of the inflammatory response. In inflamed or

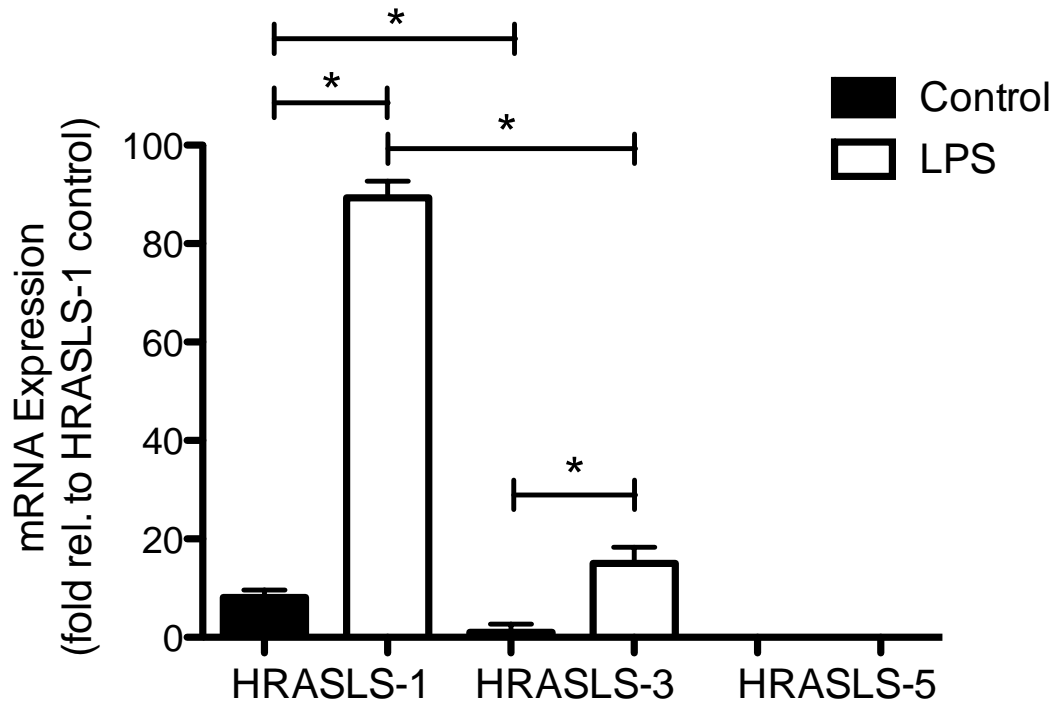
damaged tissues, SEA has proved to be a signaling lipid capable of down-regulating inflammation, mediated by the activation of the TRPV1 receptor <sup>67</sup>. Instead of acting directly, SEA utilizes a mechanism similar to the “entourage effect”, where it increases AEA concentrations, allowing AEA to gate TRPV1 receptors, favoring their desensitization and dampening inflammation <sup>67</sup>. It therefore seems plausible to hypothesize that during an inflammatory response, *Hrasls-1* expression and activity may increase due to its catalytic role in synthesizing PEA and SEA, which are both anti-inflammatory mediators (**Appendix B, (C.)**).

In order to test this hypothesis, we compared the NAPE content formed in cells undergoing an inflammatory response versus those that did not. BV2 microglial cells were treated with the TLR4 agonist LPS in order to illicit a pro-inflammatory response. Our results indicate a significant increase in NAPE content, within LPS-treated cells. Alongside this experiment, we determined the change in mRNA expression of *Hrasls-1*, *Hrasls-3* and *Hrasls-5* family members during an acute inflammatory response. All three are conserved throughout human, mouse and rat species, and all exhibit N-acyltransferase activity in vitro<sup>19</sup>. Results indicated a significant increase in the expression of *Hrasls-3* and total *Hrasls-1*. For *Hrasls-1*, we first used primers directed towards the coding region, which therefore represented the sum of both transcript variants. *Hrasls-1* was up-regulated to a greater extent than *Hrasls-3* upon LPS activation. However, HRASLS-3 is only reported to show N-acyltransferase activity in vitro, not in vivo<sup>20</sup>. Thus, its contribution to the synthesis of NAE in cells is likely negligible. Similar to HRASLS-1, HRASLS-5 shows in vivo N-acyltransferase activity which results in NAPE generation, and eventual downstream NAE synthesis<sup>19</sup>. However, *Hrasls-5* expression was undetectable in either group, and is therefore also unlikely to play a significant role in NAPE synthesis in BV2 cells, either at rest or during inflammation. *Hrasls-1* therefore appears to be the predominant mediator of iNAT-regulated NAPE synthesis in this cell line.

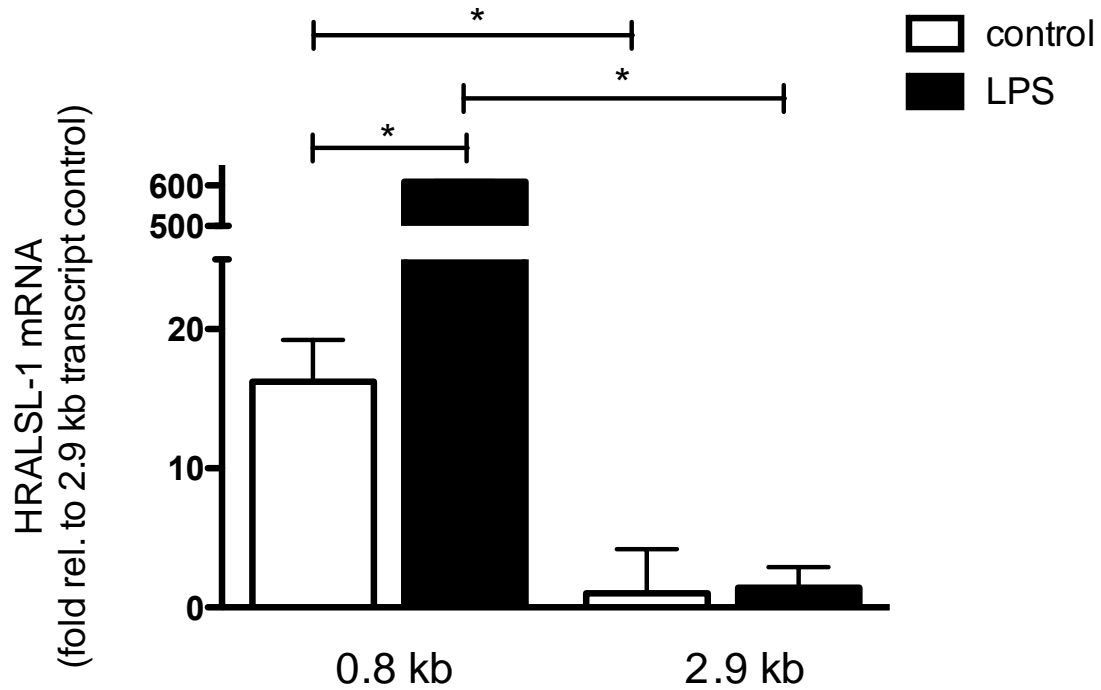
Although, these results suggest that NAE levels would also be up-regulated under the same conditions, it is difficult to make any conclusive statements from our study, however, since the parameters of the experiment were not ideal. BV2 microglial cells were treated simultaneously with [<sup>14</sup>C]palmitate and LPS, which may increase the uptake and incorporation of fatty acids into all complex lipids through a general mechanism such as increased expression of fatty acid transport proteins. In agreement with this possibility, the radioactivity incorporated into NEFA, PC, and PE, was also increased in the LPS treated BV2 cells (**Appendix A**). It is therefore difficult to conclude whether increased NAPE was a result of the elevation in HRASLS-1 expression that we found, or an increase in the production and availability of the substrates for NAPE synthesis (namely PC and PE), or both. In order to determine this, it is critical that this experiment is repeated, but with radiolabeling of both plates prior to activation with LPS, so that uptake and incorporation of [<sup>14</sup>C]palmitate into complex lipids is initially identical. Although it would still be possible that differences in PC and PE synthesis would occur after addition of LPS, and therefore affect NAPE formed, it would remove confounding from differences in rates of fatty acid transport into cells that might be affected by LPS treatment. Although radiolabeling experiments provide a rapid means of determining whether treatment effects exist, this work would also benefit from other methodological studies such as mass spectrometry (MS) analysis of NAPE content in order to determine both the quantity and identity of the specific fatty acyl species attached. Finally, *in vitro* assay of cellular lysates, measuring the incorporation of radiolabeled fatty acid from the sn-1 position of PC into PE<sup>23</sup> or, more specifically, into NAE following PLD-mediated hydrolysis of PE, would also provide direct evidence of increased cellular N-acyltransferase activity with LPS treatment.

When taken together, these results suggest that up-regulated *Hrasls-1* expression plays a role in the increased LPS-induced synthesis of NAPE that would support an enhanced production of anti-

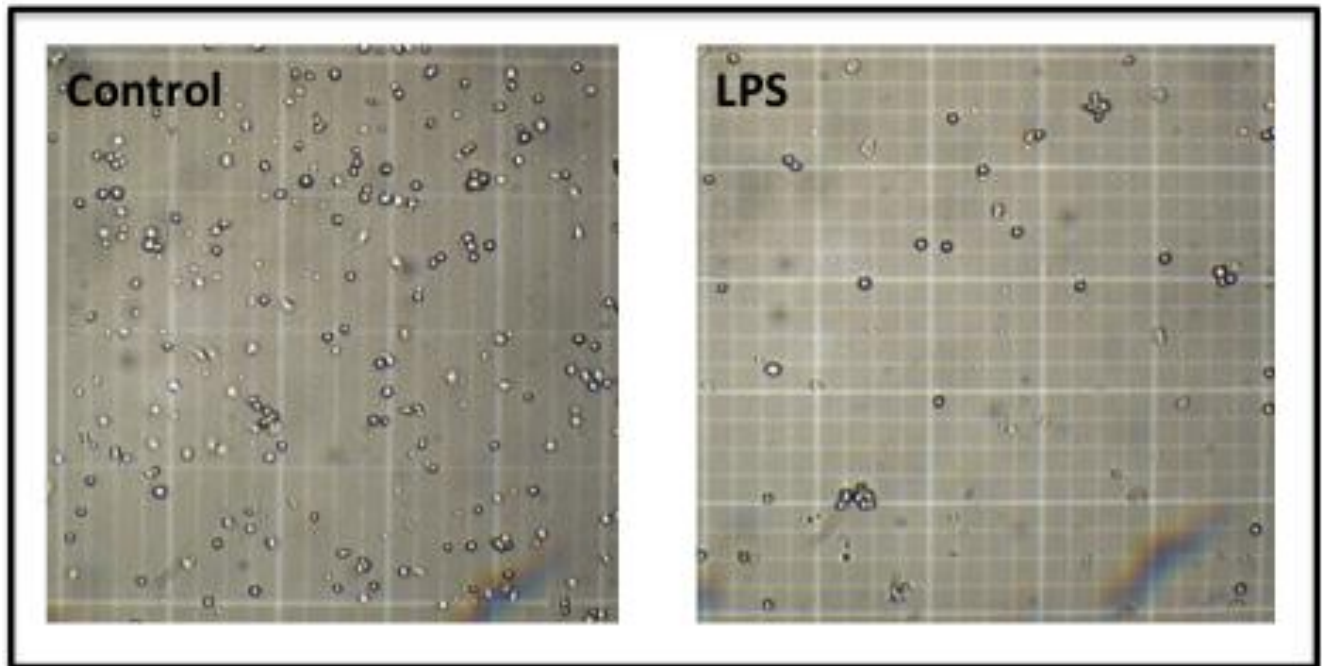
inflammatory saturated-type NAEs<sup>67</sup>. Clearly, however, mechanistic studies examining the direct role of HRASLS-1 in inflammation will be required to confirm this notion but, nevertheless, this suggests HRASLS-1 may have important health implications.



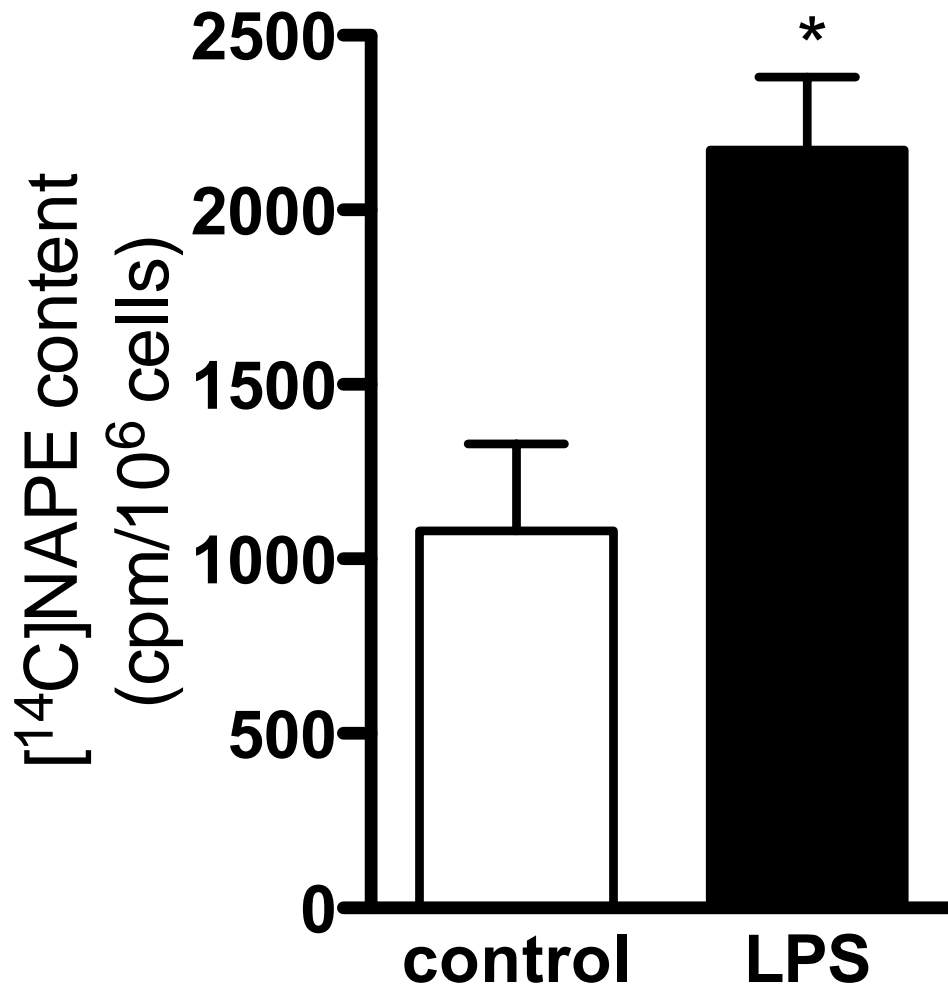
**Figure 22.** mRNA content of *Hrasls-1*, *Hrasls-3*, and *Hrasls-5* in BV2 microglial immunocytes treated with control medium or LPS. Data are expressed relative to the mRNA expression of HRASLS-1 2.9kB control group. Normalized to housekeeping gene,  $\beta$ -actin. Significant expression differences are denoted by \*  $p < 0.05$ .



**Figure 23.** mRNA content of *Hrasls-1* transcript variants in BV2 microglial immunocytes treated with control medium or medium containing LPS. Data are expressed relative to the mRNA expression of the *Hrasls-1* 2.9kB transcript variant control group and have been normalized to the housekeeping gene  $\beta$ -actin. Significant expression differences are denoted by  $*P < 0.05$ . 2.9 kb: *Hrasls-1* 2.9 kb transcript variant, 0.8 kb *Hrasls-1* 0.8 kb transcript variant.



**Figure 24.** Image of BV2 microglial cells on hemacytometer. Replicate plates were treated with non-radioactive control media or non-radioactive media containing LPS in parallel with radiolabeled cells in order to estimate the number of cells per plate after 18 h of treatment.



**Figure 25.** [<sup>14</sup>C]N-acylphosphatidylethanolamide content in BV2 microglial cells treated with control medium or medium containing LPS. Quantification was performed by liquid scintillation counting and is expressed in cpm normalized per 10<sup>6</sup> cells analyzed (n=4), \**P*<0.05.

## Chapter 8

### **Analysis of *Hrasls-1* Transcript Variant Expression During Mid- to Late-Stage Embryogenesis**

#### **8.1 Introduction**

Embryogenesis is the process by which the embryo forms and develops into a multicellular organism from a single cell. Due to its rapid (21 days) gestational period, the mouse is frequently used as an embryological model<sup>101</sup>. The intricate processes that result in formation of the tissues and organs “require the activation and inactivation of specific genes at specific times, highly integrated cell-cell interactions, and interactions between cells and their non-cellular environment”<sup>137</sup>.

NAEs have been shown to be constituents of human amniotic fluid <sup>101</sup>. Previous studies also suggest roles for NAE receptors in placental physiology, embryogenesis, and fetal development, and increasing evidence suggests that NAE signaling is critical to early pregnancy events<sup>101</sup>. In the present work, studies report the expression of HRASLS-1 expression in mouse embryos at varying developmental time points.

#### **8.2 Methods**

##### **8.2.1 Harvest of Mouse Embryos**

For harvest of embryos (AUP 12-09), timed mated mice were plug checked by animal health technicians to determine embryonic day 0.5, then pregnant dams were euthanized 10, 14, and 18 days later. Embryos were harvested and snap frozen in liquid nitrogen, then stored at -80C until RNA isolation.

## 8.3 Results

### 8.3.1 *Hrasls-1* transcript variants are regulated during embryogenesis.

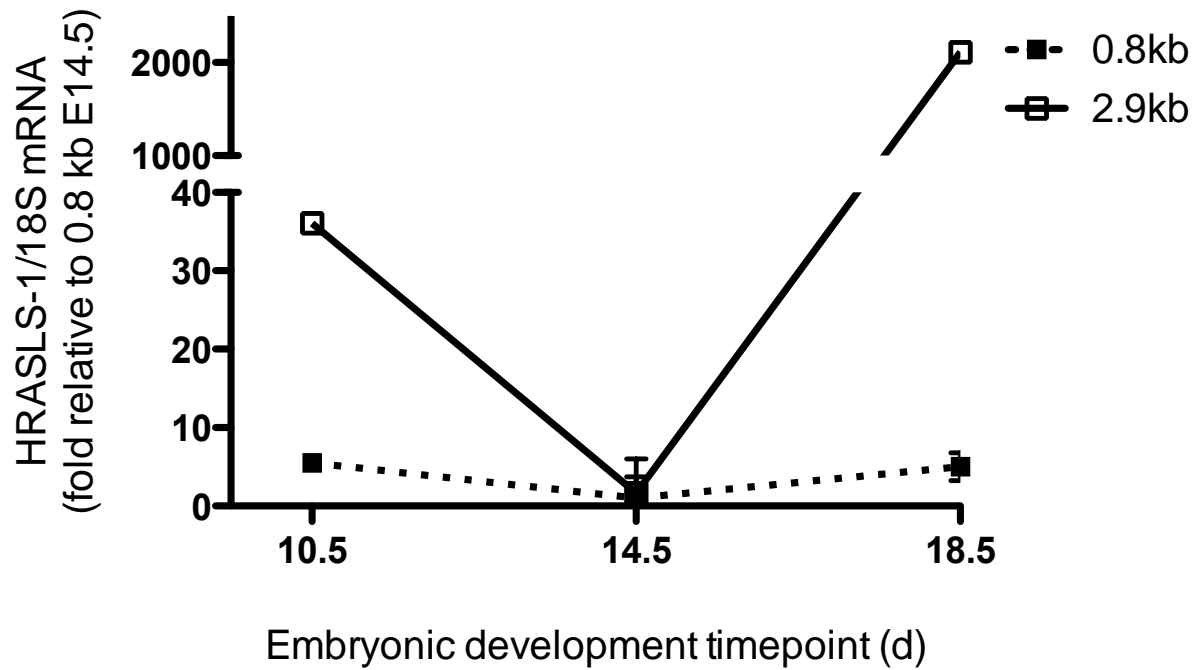
We analyzed the expression of both *Hrasls-1* 0.8kb and 2.9kb transcript variants during mid- to late-stage embryonic development by homogenizing whole mouse embryos at time points E10.5, E14.5 and E18.5. RNA from embryos was extracted and reverse transcribed to cDNA for quantitative analysis. Expression levels were analyzed by the  $\Delta C_T$  method, with transcript levels normalized to 18S loading control, and then compared to the 0.8 kb transcript variant at embryonic development day 14.5 (**Figure 26**).

The *Hrasls-1* 0.8 kb transcript variant showed stable expression throughout development. We saw no significant difference in levels of expression between day 10.5, 14.5 or 18.5. However, expression of the *Hrasls-1* 2.9 kb transcript variant was variable between all three time points. The 2.9 kb transcript showed an approximate 40-fold decrease in expression from embryonic development day 10.5 to day 14.5. Even more pronounced was the amplification seen from embryonic day 14.5 to day 18.5. Over the course of 4 days, during the last two time points measured (embryonic day 14.5 to 18.5), the *Hrasls-1* 2.9kb transcript showed a 2000-fold increase. These results suggest a varied role for each HRASLS-1 transcript variant in embryonic development, since one shows a significantly greater degree of regulation than the other.

When comparing the two transcript variants during embryonic development, results indicate higher expression of the 2.9 kb transcript than the 0.8 kb transcript at both day 10.5 and 18.5. At embryonic development day 14.5, both transcript variants show relatively low expression. As a result, we see no significant difference in expression levels between the two transcripts at this time point.

## 8.4 Discussion

Enzymes involved in brain signaling become stimulated as early as embryogenesis. Furthermore, lipid-signaling molecules such as AEA, OEA, and PEA have been shown to be elements of human amniotic fluid <sup>101</sup>, although their biological role within these fluids is still unknown. Previous studies also focus on the expression and role of cannabinoid receptors in placental physiology, embryogenesis, and fetal development<sup>101</sup>. It is known that a tonic physiological level of anandamide is essential for proper preimplantation to occur in mice, where either too much or too little can delay development<sup>138</sup>. Whether other NAEs have any effects during pregnancy or embryogenesis remains unknown. Due to its role in synthesizing NAE-precursor NAEs, we chose to investigate the expression of both mRNA transcript variants of *Hrasls1* at embryonic development day 10.5, 14.5 and 18.5. Results indicate that the 0.8 kb transcript variant remained largely unchanged throughout these developmental time points, while the 2.9 kb transcript variant underwent a profound, two thousand-fold induction in the days immediately prior to parturition. This massive induction of *Hrasls-1* strongly suggests a role in embryonic development that merits further study. It is important to note that the first time point analyzed (E10.5) commences the second half of embryonic development. In order to achieve a better understanding of the regulation of *Hrasls-1* during embryonic development, it would be useful to analyze embryos at an early stage, particularly during the first week of development when organogenesis is occurring.



**Figure 26.** mRNA content of *Hrasls-1* transcript variants at different stages of embryonic development. Data are given relative to the mRNA expression of the *Hrasls-1* 0.8 kb transcript variant at embryonic development day 14.5 (n = 3 for all groups) and have been normalized to the housekeeping gene 18S.

## Chapter 9

### An Integrated Discussion

While each study presented was unique, and directed at specific objectives, the results obtained may be discussed cohesively in order to address future directions within this field.

When analyzing the metabolic data, a result that was consistent throughout the tissues measured was the relative level of expression between each transcript variant in each measured state. We found that, in general, a higher level of the 0.8 kb transcript variant was present in all states and tissues, in comparison to the 2.9 kb transcript variant. What this initially indicates is that modulation of the *Hrasls-1* 0.8 kb transcript variant may be primarily responsible for the up-regulation of overall *Hrasls-1* expression with fasting in most tissues, except heart and brain.

Observation of these results, with the results of variable transcript variant stability, can implicate both findings and permit for further discussion. In the previous chapter, it was proposed that due to various confounding factors, the 2.9 kb transcript variants was not less stable, and alternatively the 0.8 kb transcript variant showed quicker mRNA degradation. This imposes questioning as to what is truly happening upon increased activation of the 0.8kb transcript variant within the fasted state. The 0.8 kb transcript variant may not be as stable, suggesting that its increased activation may be short-lived and sufficient in mediating the necessary response for a short-term substrate depletion, but unable to maintain high mRNA expression as the fast persists. Nevertheless, the 2.9 kb transcript variant is also up-regulated during the fasted state, and due to its observably slower degradation, may be responsible in maintaining substrate oxidation via fatty acid metabolism. Interestingly, within study #3, the 0.8 kb transcript variant was alone at showing significant mRNA expression induction upon activation with LPS.

Due to the coinciding increase in NAPE content, it is suggested that HRASLS-1 may play a role in regulating NAPES, although the durability of this effect is unknown, as the 0.8 kb transcript

variant mRNA may be degraded shortly after. Furthermore, during embryogenesis, only the 2.9 kb transcript variant showed a significant change in mRNA expression. Therefore, future work would largely benefit from integrating the potential transcript stability of both transcript variants under each physiological demand investigated, as this would give further insight to the significance in activating either transcript variant for either a restricted or resilient response.

As presented within the discussion of HRASLS-1 in substrate oxidation and inflammatory regulation, there is evidence to suggest that future work should look at the role of HRASLS-1 in obese models that exhibit both high levels of circulating fatty acids as well as an overactive inflammatory response. Control of inflammation represents an essential approach in the clinical management wide range of diseases. For example, obesity is described as a “chronic, systemic low-grade state of inflammation”<sup>139</sup>. Furthermore, macrophages, which play a critical role during inflammation, are increased in adipose tissue of obese<sup>139</sup>. It is possible that HRASLS-1 may function to increase fatty acid oxidation and decrease adipogenesis, thereby decreasing adiposity and offering hope for treatment of obesity. It is also possible that HRASLS-1 may mediate further protective effects in obesity, by attenuation of inflammation. A study looking at the possible beneficial roles of HRASLS-1 in obese mice would be highly valuable in elucidating a physiological role for this enzyme. Data, showing that HRASLS-1 is activated during an inflammatory response to potentially increase the release of anti-inflammatory lipid mediators, raises the possibility that pharmacological treatments that increase activation of this enzyme might be of promising therapeutic benefit, especially for obese individuals.

## Chapter 10

### Conclusion

There are significant changes in the mRNA expression of HRASLS-1 during different nutritional states in diverse tissues. This is important since very little is known about the physiological role of HRASLS-1. Due to the lack of substrate specificity shown by HRASLS-1, it is difficult to suggest one specific role for the enzyme. Indication of tissue specific activation of fat metabolism-associated genes, positively associated with the up-regulation of HRASLS-1, give further insight on the possible mechanism of action. The data we have generated on the differential expression of this enzyme provides a starting point from which we can infer a unique role within each tissue. In order to truly elucidate the physiological relevance of HRASLS-1, further work clearly must be done. Protein expression analysis via Western blotting would be useful for further characterization of HRASLS-1 expression, but unfortunately an acceptable antibody that recognizes the murine isoform has not been available during my studies.

From my thesis work it is evident that these transcript variants are differentially regulated. Although we predicted that the 2.9kb transcript variant would show greater instability due its 3' UTR AU-rich elements<sup>108</sup>, results indicated the opposite. Further work must be done in order to understand the different mechanisms that regulate the two transcript variants, and to make concise implications about the transcript variants in varying physiological conditions.

My thesis work suggests a potential role for HRASLS-1 in the regulation of NAPE during an acute inflammatory response, since a coinciding increase between *Hrasls-1* mRNA expression and NAPE content was seen. *Hreals-1* showed a significantly greater induction than both *Hrasls-3* and *Hrasls-5* under an acute activation by LPS and may play a predominant role in NAPE synthesis during the inflammatory response in BV2 cells, although gene knockdown studies would be required to confirm this. A role for HRASLS-1 in neuroinflammation has clinical implications for the

treatment of disease, although the significance of HRASLS-1, as well as HRASLS-2 and HRASLS-4 within the human system must first be understood.

To date, very little is known about the role of NAE's during embryogenesis. AEA has been identified during early stage implantation in the embryo<sup>82</sup>, although the role of additional NAEs on early pregnancy and embryogenesis remains unknown. These results illustrate that there are significant changes in the mRNA expression of *Hrasls-1* during embryonic development, suggesting a unique role for NAEs that act as downstream products of the HRASLS-1 pathway. Further investigation of this role seems likely to yield important information on the nature of lipid-mediated signalling in development, with implications for both fetal and maternal health, and reproductive success.

In conclusion, this work presents an initial insight into the physiological role(s) of HRASLS-1, and suggests that HRASLS-1 may be valuable in multiple physiological processes ranging from substrate oxidation regulation to embryonic development to inflammation.

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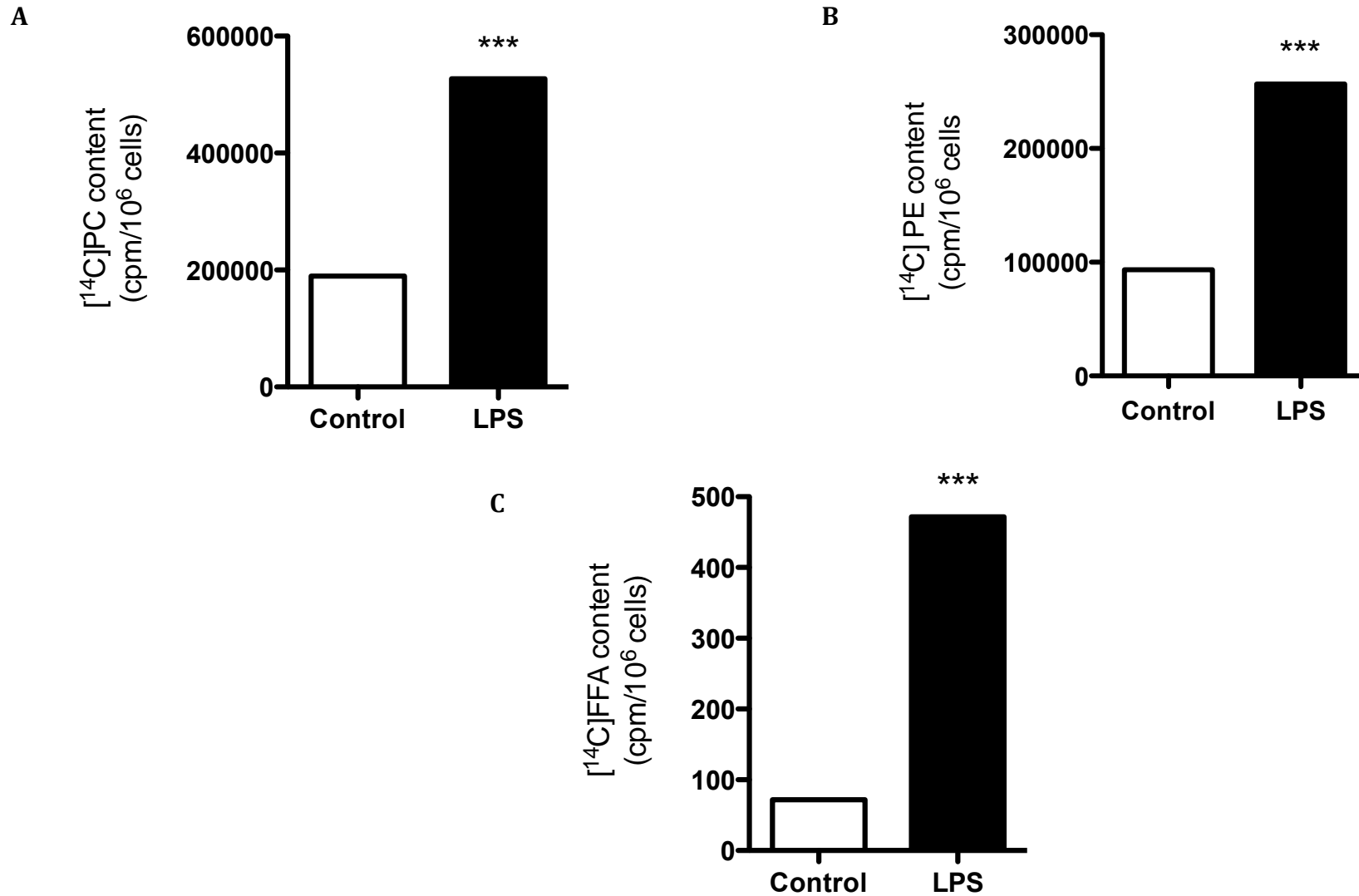
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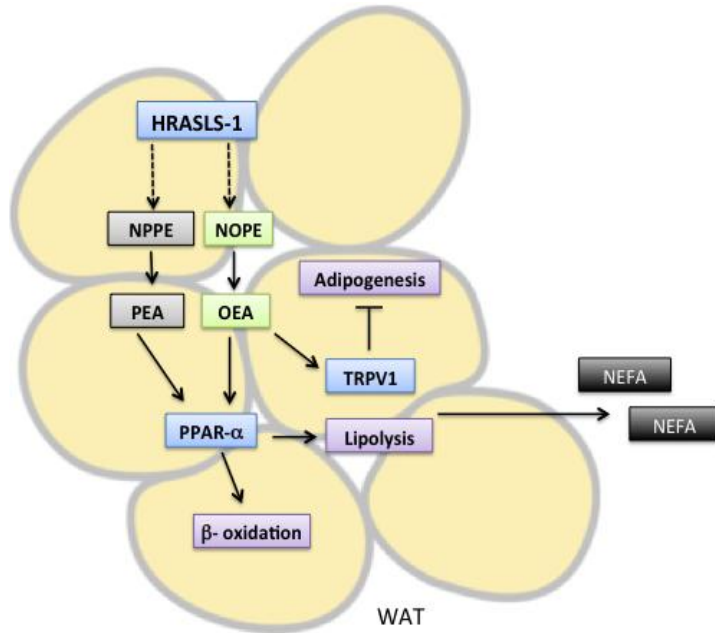
Appendix A: Supplementary figures of TLC- resolved lipids.



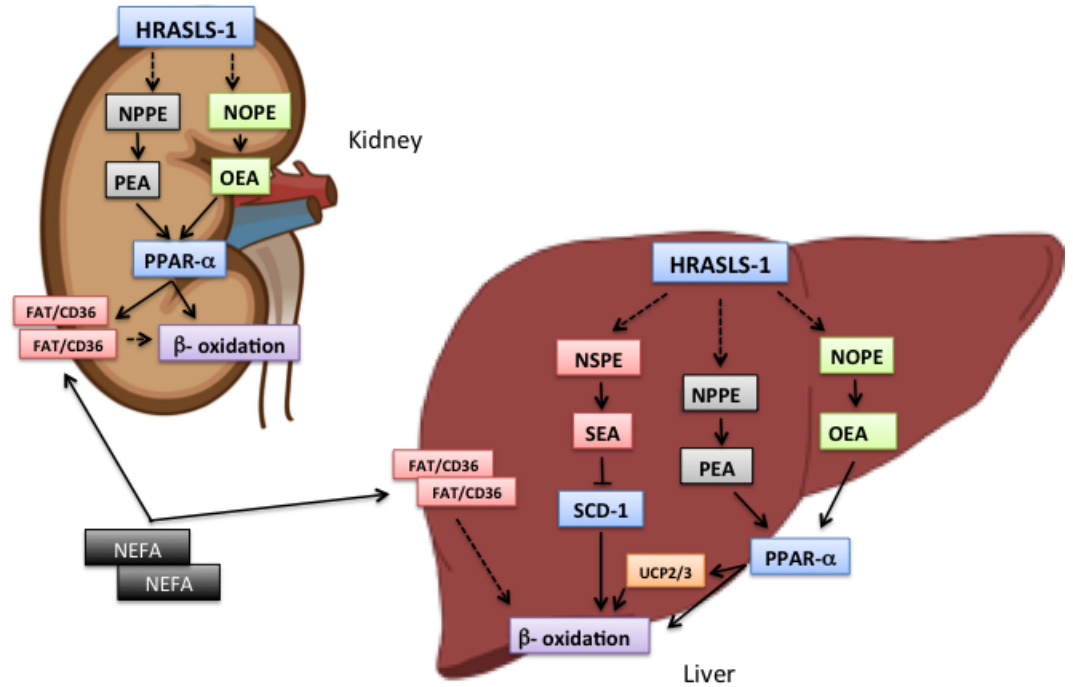
Suppl Fig. 1. [ <sup>14</sup>C]PC (A), [ <sup>14</sup>C]PE (B), and [ <sup>14</sup>C]NEFA (C) content in BV2 microglial cells treated with control medium or medium containing LPS for 18 h. Quantification was performed by liquid scintillation counting and is expressed in cpm normalized per 10<sup>6</sup> cells analyzed (n=4), \**P*<0.05.

## Appendix B: Illustrations of proposed physiological roles of HRASLS-1

A.

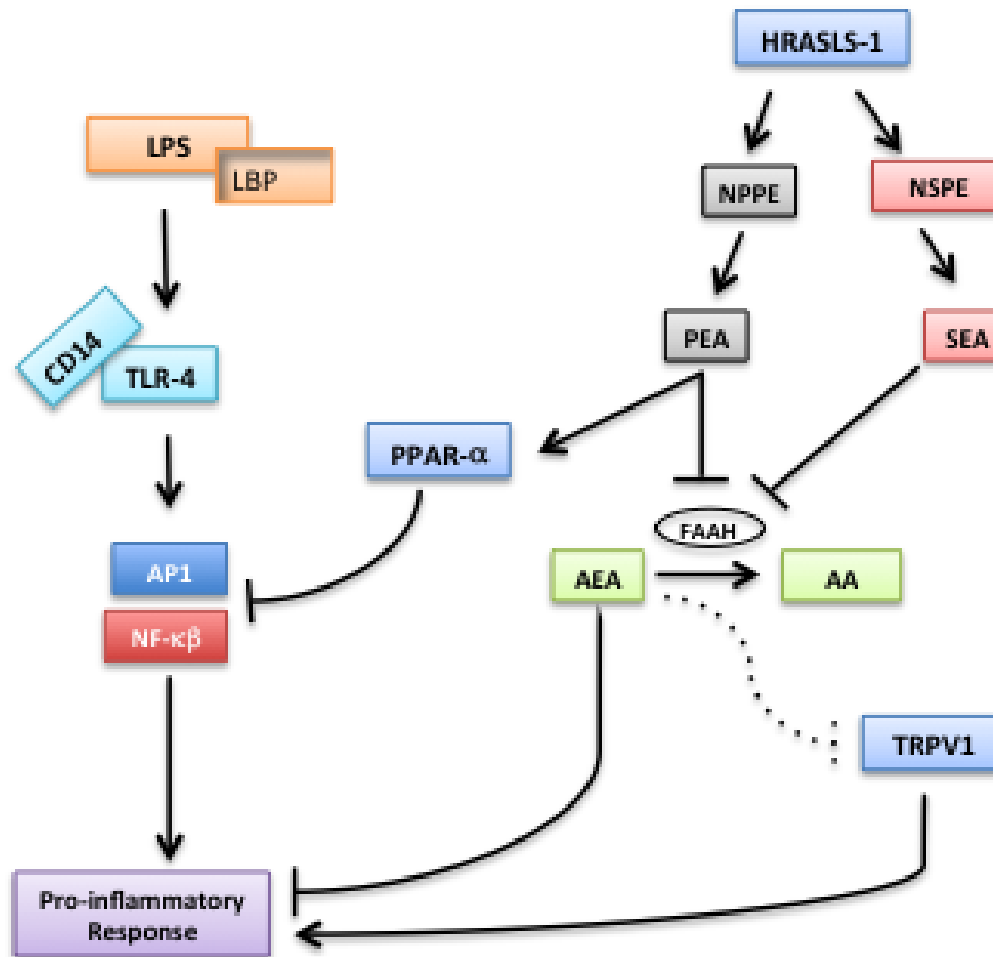


B.



Proposed HRASLS-1 mediated pathway within the fasted state of white adipose tissue (WAT), liver, and kidney. Due to the continued requirement for energy by peripheral tissues during the fasted state, HRASLS-1 of WAT mediates signalling of lipolysis activation and leads to a release of NEFA into the circulation (A). HRASLS-1 of peripheral tissues synthesizes NAE-precursors in order to signal to increase the uptake of NEFA via FAT/CD36, and the utilization of NEFA in  $\beta$ -oxidation, generating a switch to the use of NEFA as the primary substrate for energy metabolism (B).

C.



Proposed HRASLS-1 mediated pathway during an LPS-induced inflammatory response. HRASLS-1 synthesizes precursor NAE molecules that will induce PPAR- $\alpha$  activation and, in turn, inhibit transcription of specific pro-inflammatory genes. The NAEs, PEA and SEA, are also able to inhibit the degradation of AEA (through FAAH), which can elicit an anti-inflammatory response independently, and well as desensitise TRPV1 receptors to pro-inflammatory stimuli (C).