Mechanisms of action of satiating gut peptides in the regulation of food intake through vagal afferent pathways

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Ecole Doctorale ABIES
Agriculture
Agriculture
Alimentation
Food
Biologie
Biology
Environnement
Environment
Santé
Health

Doctorat ParisTech

T H È S E

pour obtenir le grade de docteur délivré par

L'Institut des Sciences et Industries du Vivant et de l'Environnement

(AgroParisTech)

Spécialité : Science de vie et santé

présentée et soutenue publiquement par

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le 6 Janvier 2014

Mechanisms of action of satiating gut peptides in the regulation of food intake through vagal afferent pathways

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Acknowledgements

The completion of my dissertation would not have been possible without the guidance of my mentors, help from lab members, and support from my family and friends.

I would like to express my deepest gratitude to my advisor, Helen Raybould, for her excellent guidance, patience, and encouragement through the past three years. Helen you are a wonderful mentor and I am grateful to be continuing on in your lab.

Thank you to Daniel Tomé for your help and guidance through my dissertation, and for the encouragement to do my PhD in Helen's lab.

I would like to thank Will de Lartigue for being an amazing and caring mentor. Thank you for your time, patience and guidance through my dissertation. I am very grateful to have worked so closely with you, I have learned so much.

Thank you Kristi Hamilton for being such a great friend and amazing coworker. I greatly appreciate the times you let me talk through ideas, vent when things weren't working and always putting a positive spin on things.

Thank you to all the past and present members of the lab who have been here to help in more ways than one; Jim, Gaelle, Shirley, Liz, Sofia, Myrta, Analise, Ricky and Mathilde.

I would like to express my gratitude towards my family and friends for all their love and support. To my parents, thank you for your unconditional love and encourgement and most importantly, thank you for teaching me to never give up.

I would like to especially thank Tim for being there during my good days and bad. Your kindness, humor and unconditional support have made everything easier. I wouldn't know what to do without you.

Thesis Summary

As the initial interface for nutrient sensing, digestion and absorption, the gastrointestinal (GI) tract plays a critical role in the regulation of energy homeostasis. Information that arises from the GI tract is key to normal physiological responses controlling gut function and regulating food intake. Vagal afferent neurons (VAN) are a major pathway by which information about ingested nutrients reaches the central nervous system to influence GI function and food intake behavior. VAN express receptors for many of the regulatory peptides released from the gut that are involved in regulation of food intake and body weight.

This dissertation addresses the role of two gut peptides, leptin and glucagon-like peptide-1, acting at the level of VAN, to inhibit food intake. First, the mechanism of action of glucagon-like peptide-1 (GLP-1) on VAN is addressed. GLP-1-induced satiation requires a postprandial state; the data support that feeding changes the localization of GLP-1Rs from the cytoplasm to the neuronal cell membrane. Further, ghrelin and its receptor GHSR1 expressed by VAN is involved in regulating GLP-1 receptor translocation. Second, the importance of leptin receptor expression by VAN in the development of hyperphagia and obesity was demonstrated by selective knockout of the leptin receptor (LepR) in VAN; mice express an obesogenic phenotype.

Obesity and its resultant health consequences are a major worldwide health problem. Effective or preventative treatments for obesity are limited. Our findings have filled the gap in our knowledge of the mechanism of GLP-1 and leptin signaling on VAN. Understanding the physiology regulating feeding behavior is imperative in developing non-invasive anti-obesity treatments.

Resumé

Le tractus gastro-intestinal, interface initiale pour la détection, la digestion et l'absorption des nutriments, joue un rôle critique dans la régulation de l'homéostasie énergétique. Les signaux qui proviennent du tractus gastro-intestinal sont nécessaires au contrôle de la fonction intestinale et de la régulation de la prise alimentaire. Les neurones afférents vagaux (NAV) sont une voie importante via laquelle les informations sur les nutriments ingérés atteignent le système nerveux central pour influencer ces deux fonctions. Les NAVs expriment les récepteurs pour la plupart des peptides régulateurs libérés par l'intestin impliqués dans la régulation de la prise alimentaire et du poids corporel.

Cette thèse porte sur le rôle de deux peptides de l'intestin, la leptine et le glucagon-like peptide-1 (GLP-1), qui agissent au niveau des NAVs pour inhiber la prise alimentaire. Tout d'abord, nous expliquons le mécanisme d'action du GLP-1 sur les NAVs. La satiété induite par le GLP-1 nécessite un état post-prandial ; les données confirment que le statut nutritionnel régule la localisation du GLP-1R du cytoplasme vers la membrane des cellules neuronales. De plus, la ghréline et son récepteur GHSR1, exprimés par les NAVs, sont impliqués dans la régulation de la translocation du GLP-1R. Deuxièmement, ’utilisation de souris knockout pour le recepteur a la leptine sur les NAVs nous a permis de montrer l’importance de ce recepteur dans la physiopathologie de l’obésité et de l’hyperhagie. En effet, ces souris KO présentent un phénotype obésogène.

L'obésité et ses conséquences sur la santé sont des problèmes majeurs de santé dans le monde entier. Les traitements efficaces de prévention ou de l'obésité sont

limités. Nos résultats ont apporté des connaissances sur le mécanisme du GLP-1 et sur la signalisation de la leptine au niveau es NAVs. Comprendre la physiologie de la régulation de la prise alimentation est impératif dans le développement des traitements non-invasifs contre l'obésité.

Resumé substantiel de thèse

L'obésité est devenue une pandémie mondiale dont la prévalence a augmenté au cours des dernières décennies. L'obésité est associée à des comorbidités graves telles que le diabète de type 2, l'hypertension, et les maladies cardiovasculaires. C'est un problème de santé publique pour lequel on observe un manque de traitements simples et efficaces. Le contrôle de la prise alimentaire et du poids corporel est un mécanisme complexe qui implique l'intégration de nombreux signaux périphériques au niveau du système nerveux central. Ainsi les signaux humoraux d'un certain nombre de tissus comme le pancréas, le foie ou le tube digestif influencent l'homéostasie énergétique. En raison des données récentes montrant une grande efficacité de la chirurgie bariatrique contre l'obésité, une grande attention est actuellement portée sur le rôle de l'intestin dans cette pathologie.

Suite à l'absorption de nutriments lors d'un repas, des signaux nerveux et hormonaux, témoin de la quantité et de la qualité des aliments ingérés, sont émis le long du tractus digestif. Les hormones sécrétées dans la muqueuse intestinale peuvent agir soit directement par voie humorale sur l'hypothalamus, soit via les récepteurs présents sur les terminaisons vagales. L'activation de cette voie afférente vagale va relayer des signaux efférents jusqu'au tissus cibles pour contrôler la fonction intestinale et le comportement alimentaire. Les neurones afférents vagaux (NAV) sont la principale voie de communication entre le tube digestif et le cerveau. Le nerf vague est le lien entre les signaux périphériques de l'intestin et le système nerveux central dans le contrôle de la fonction digestive et du comportement alimentaire. Une vagotomie chirurgicale ou la destruction de neurones par la capsaïcine chez les rongeurs entraîne une augmentation

de la prise alimentaire et inhibe les signaux de satiété. La signalisation vagale contribue donc à la régulation de la prise alimentaire.

Cette thèse vise à comprendre le mécanisme d'action de deux hormones anorexigènes, le Glucagon like peptide-1 (GLP-1) et la leptine, dans le contrôle de la prise alimentaire par les NAVs. Nous avons divisé cette présentation en deux parties. Tout d'abord, nous identifierons le mécanisme d'action du GLP-1 sur le nerf vague. Nous avons démontré que, contrairement à tout autre récepteur couplé à la protéine G qui augmente le niveau d'expression de sa protéine, le récepteur du GLP-1 modifie sa localisation cellulaire en fonction du statut nutritionnel. De plus, nous étudierons les médiateurs impliqués dans cette translocation en réponse à la prise alimentaire. Dans une deuxième partie, nous montrerons l'importance de la résistance à la leptine au niveau des NAVs en invalidant spécifiquement les récepteurs de la leptine sur le nerf vague. Nous avons également comparé la différence entre les souris mâles et femelles KO au niveau de la composition corporelle et de la prise alimentaire. Ce travail vise à approfondir nos connaissances sur les mécanismes physiologiques de la régulation de l'homéostasie énergétique.

Première Partie: GLP-1

GLP-1 et son récepteur se trouvent au niveau périphérique et central. Au niveau périphérique, le GLP-1 est dérivé du gène pré-proglucagon présent dans les cellules L intestinales et les cellules A du pancréas. Le GLP-1 est sécrété par les cellules L en réponse à la prise alimentaire. En condition de jeun, les concentrations du GLP-1 sont très faibles et augmentent avec l'ingestion de nutriments. De nombreuses études ont

montré que le GLP-1 est rapidement dégradé dans sa forme inactive par la dipeptidyl peptidase IV (DDP-IV).

Le GLP-1 peut traverser la barrière hémato-encéphalique mais étant dégradé très rapidement il est peu probable qu'une quantité importante de GLP-1 périphérique actif puisse atteindre le cerveau. Dans des conditions physiologiques et en raison de la courte demi-vie plasmatique du GLP-1, il agit plus probablement localement dans la paroi intestinale pour influencer la prise alimentaire. Les NAVs sont un site principal d'action pour les signaux anorexigène dérivés de l'intestin. L'administration systémique de GLP-1 augmente l'activité électrophysiologique des neurones du ganglion noueux. De plus, une désafférentation sous-diaphragmatique atténue les effets rassasiant du GLP-1 chez l'homme. En revanche, les souris GLP-1R knockout (KO) présentent un poids corporel et une prise alimentaire normale, ce qui remet en question le rôle physiologique du GLP-1 endogène dans le contrôle de la prise alimentaire.

En effet, il existe des incohérences dans la littérature en ce qui concerne le rôle du GLP-1 natif périphérique dans ce contrôle. La plupart des méthodes utilisées dans ces études n'utilisent pas le GLP-1 dérivé de l'intestin mais utilise soit des analogues du GLP-1 qui échappent à la dégradation et peuvent ainsi facilement atteindre les récepteurs centraux soit administrent de façon prolongée le GLP-1 natif en perfusion intraveineuse. Dans notre étude, nous avons cherché à imiter les actions du GLP-1 natif par l'administration d'une injection aiguë de GLP-1 périphérique chez les rats soit à jeun soit à jeun puis re-nourris avant l'injection. Nous avons ainsi démontré que le GLP-1 périphérique nécessite un état postprandial pour induire la satiété.

Des travaux antérieurs ont montré que les NAVs changent leur phénotype neurochimique en fonction de l'état nutritionnel. Les NAV présentent deux phénotypes : orexigène ou anorexigène. Dans un état à jeun, l'expression des récepteurs anorexigènes est diminuée tandis que l'expression des récepteurs orexigènes est augmentée. A l'opposé, ces changements sont inversés par la prise alimentaire par un mécanisme dépendant de la CCK. Étant donné que le GLP-1 ne diminue pas la prise alimentaire dans un état réalimenté, nous avons supposé que l'expression de la protéine du GLP-1R changeait selon le statut nourris ou à jeun.

Nous avons montré que ni l'expression d'ARNm ni la protéine du GLP-1R ne sont altérées sur les NAVs par l'alimentation, mais que la localisation du GLP-1R sur les corps cellulaires des NAVs est modifiée. La localisation du GLP-1R sur la membrane plasmique des neurones des ganglions noueux augmente de manière significative en fonction de la consommation énergétique par rapport à l'état à jeun. Ces résultats signifient que le GLP-1R exprimé sur les neurones afférents vagaux se déplace vers la membrane plasmatique dans la période postprandiale. Le déplacement du récepteur vers la membrane fournit une explication possible au fait que l'administration exogène de GLP-1 inhibe la prise alimentaire seulement après un repas.

Mécanisme de la translocation du GLP-1R

Afin de comprendre le mécanisme de GLP-1 sur les NAVs, nous avons voulu comprendre les facteurs qui sont impliqués dans la translocation du GLP-1R. Il a été montré que les hormones dérivées de l'intestin agissent en synergie pour réguler la prise alimentaire, en particulier au niveau du nerf vague. Mais le mécanisme de

translocation du GLP1-R vers la membrane plasmatique n'est pas encore connu. Deux hypothèses sont possibles : soit un signal est libéré suite à la consommation alimentaire qui amène le récepteur à la surface, soit la diminution d'un signal en réponse à la mise à jeun maintient le récepteur dans le cytoplasme. Nous avons décidé d'étudier les interactions entre le GLP-1 et la CCK et entre le GLP-1 et la ghréline.

Nous avons démontré *in vivo* que la ghréline, plutôt que la CCK, module les effets rassasiant du GLP-1 natif systémique. Lorsque la ghréline endogène est bloquée, le GLP-1 diminue de façon significative la consommation énergétique dans un état de jeûne chez le rat. Dans un état de jeûne, le GLP-1 augmente l'activité des NAVs en présence d'un antagoniste du récepteur de la ghréline. Par ailleurs, nous avons confirmé, en culture, que la translocation du GLP-1R varie en fonction de la composition nutritionnelle du milieu. Le blocage de la ghréline en condition nourrie restaure le GLP-1R au niveau de la membrane plasmique. *In vitro*, la ghréline inhibe la translocation du GLP-1R vers la membrane plasmatique, mécanisme atténué en présence d'un antagoniste du récepteur de la ghréline. Les données indiquent que la ghréline conserve les récepteurs dans le cytoplasme par l'intermédiaire de l'AMPc et de la voie de la MAPK p38 dans un état à jeun.

Nous avons ainsi approfondi notre connaissance sur le mécanisme de régulation de la translocation du GLP-1 sur le NAV. La ghréline est un médiateur majeur dans la signalisation du pouvoir rassasiant du GLP-1 sur les NAVs.

Deuxième Partie: Leptine

Récemment, beaucoup d'attention a été portée sur la leptine, une hormone dérivée du tissu adipeux et de l'intestin, qui contrôle l'apport énergétique, la graisse corporelle, la reproduction et l'homéostasie glucidique. La leptine est un régulateur clé de l'homéostasie énergétique; elle diminue la prise alimentaire et le poids corporel chez les rongeurs et chez l'homme. De plus, les taux circulants de leptine sont corrélés avec l'adiposité. Les récepteurs de la leptine sont exprimés dans des sites centraux et périphériques, et l'expression la plus élevée se trouve dans le noyau arqué (ARC) de l'hypothalamus. Un déficit de la leptine et de son récepteur induit une augmentation de la prise alimentaire et de l'adiposité et entraine une obésité morbide chez les rongeurs et l'homme. L'hyperphagie et l'obésité sont associées à la résistance à la leptine caractérisée par l'incapacité de répondre à la leptine exogène et endogène. Il a été démontré que la résistance à la leptine dans l'ARC de l'hypothalamus est un facteur clé de l'obésité.

Résistance à la leptine sur les NAVs

La signalisation de la leptine sur les NAVs joue un rôle important dans la régulation de l'homéostasie énergétique et le développement d'une résistance à la leptine sur les NAVs conduit à un phénotype obèse. Dans des modèles d'obésité induite par un régime riche en lipide, la résistance à la leptine sur les NAVs entraine une augmentation du poids corporel et de la prise alimentaire. En parallèle, en réponse à la leptine, la phosphorylation de STAT-3, marqueur de la signalisation de la leptine, est diminuée sur les NAVs. La résistance à la leptine ne se produisant qu'après l'augmentation de la prise alimentaire, du poids corporel et de l'adiposité, nous avons

supposé que la résistance à la leptine spécifiquement sur les NAVs jouait un rôle important dans la physiopathologie de l'obésité.

Notre hypothèse était que la résistance à la leptine sur les NAVs induit l'hyperphagie et conduit à un phénotype obèse. En utilisant un système Nav1.8Cre-LoxP, nous avons développé une souris KO conditionnel qui invalide le récepteur de la leptine seulement dans les neurones afférents primaires. Les souris mâles KO présentent une augmentation du poids corporel, de la prise alimentaire et de l'adiposité par rapport à un témoin sauvage. Les souris KO ont une sensibilité réduite à la CCK et ont été incapables de répondre à la leptine. La CCK est un médiateur prédominant du "switch" de phénotype des NAVs, et l'absence du récepteur de la leptine exprimée par NAV compromet l'activation de NAV par la CCK. Nous avons étudié si le phénotype de sensibilité à l'obèsité chez les souris KO était dû à la perte de plasticité des NAVs. En effet, le phénotype des NAVs des souris KO est bloqué dans un état orexigénique : les récepteurs orexigènes sont constamment exprimés indépendamment de l'état de l'alimentation.

Pour déterminer si la résistance à la leptine sur les NAVs est nécessaire pour le développement de l'obésité, nous avons nourris les souris KO et les souris sauvages (WT) avec un régime riche en lipide pendant trois mois. Comme attendu, la consommation d'un régime hyperlipidique a augmenté le poids corporel et la grasse masse des souris WT comparées aux souris WT nourries avec un régime contrôle. En ce qui concerne les souris KO, après 21 semaines, elles présentent un poids corporel et une masse grasse plus importante que les souris WT nourris avec régime témoin. En

revanche, avec un régime riche en lipide, le poids corporel et la masse grasse des souris KO étaient moins importants des souris WT.

Nous avons démontré que la résistance à la leptine sur les NAVs est suffisante pour induire une augmentation du poids même nourri avec un régime contrôle. En revanche, la consommation d'un régime hyperlipidique n'augmente pas le poids corporel chez les souris KO. Nous en avons donc conclu que d'autres facteurs sont impliqués dans l'augmentation du poids corporel induit par un régime riche en lipide et qu'il y a d'autres facteurs qui augmentent la prise alimentaire avant la résistance à la leptine sur les NAV.

Nous présumons que les souris KO acquièrent des mécanismes compensatoires pour corriger la perte des récepteurs de la leptine sur les NAVs au cours du développement les empêchant ainsi de prendre plus de poids lors d'une consommation d'un régime hyperlipidique.

Différences des sexes dans la régulation de la prise alimentaire

Les fluctuations des concentrations d'hormones gonadiques influencent le comportement alimentaire et la composition corporelle. L'oestrogène influence l'homéostasie métabolique en régulant l'appétit et la prise alimentaire. Les rattes ovariectomisées (OVX) sont hyperphagiques et présentent une augmentation du poids corporel et de l'adiposité. En outre, les rattes OVX n'ont pas le même comportement alimentaire que les témoins. Le traitement à l'oestrogène normalise ce comportement et rétablit la consommation alimentaire et la composition corporelle.

La perte du récepteur de la leptine spécifiquement sur les NAVs conduit à un phénotype obésogène quel que soit le sexe: les souris KO femelles et mâles voient leur poids corporel et leur adiposité augmentés par rapport aux témoins. De plus, les souris KO augmentent leur apport alimentaire pendant la phase de nuit par rapport aux WT. De plus, l'absence du récepteur à la leptine sur les NAV atténue l'effet rassasiant de la CCK et de la leptine.

Nous avons démontré que la perturbation de la signalisation de la leptine induit des différences de composition corporelle et de comportement alimentaire entre les souris femelles et mâles. Le poids corporel des souris est significativement augmenté chez les males par rapport aux femelles. En revanche, la masse grasse est significativement plus importante chez les femelles que chez les mâles. Les souris KO femelles et mâles mangent plus que les souris WT, mais leur comportement alimentaire est très différent. Les souris KO femelles diminuent la quantité du repas mais augmentent leur fréquence par rapport aux souris WT. A l'opposé, les souris KO mâles mangent des repas plus copieux à une plus faible fréquence.

Nous avons mis en évidence que la résistance à la leptine sur les NAV mène à l'obésité par différents comportements alimentaires chez les souris mâles et femelles. Des travaux supplémentaires sont nécessaires pour comprendre les mécanismes conduisant à ces différences entre mâles et femelles. Comprendre le rôle des peptides de l'intestin et la façon dont leur signalisation module la prise alimentaire dans les deux sexes est nécessaire afin de développer un traitement non-invasif contre l'obésité.

Conclusion

Nous avons démontré que le GLP-1 périphérique joue un rôle important dans le contrôle de la prise alimentaire et que la signalisation du GLP-1 sur les NAVs est fonctionnelle et est régulée par la ghréline. Il apparaît aussi clairement que la résistance à la leptine sur les NAVs est nécessaire et suffisante pour induire l'obésité. L'absence de signalisation de la leptine sur les NAVs induit l'obésité d'une manière différente chez les femelles et les mâles. Il est nécessaire de comprendre la physiopathologie de maladies métaboliques pour lutter contre les symptômes et développer des traitements efficaces et des mesures préventives. Comprendre les dysfonctionnements des voies de détection des nutriments par les afférences vagales est essentiel pour le développement de thérapies contre l'obésité. Les études présentées dans cette thèse soulignent l'importance de la voie afférente vagale dans la réponse aux signaux hormonaux dérivés de l'intestin.

List of Publications and Scientific Communications

Publications

Ronveaux CC, De Lartigue G., Raybould HE (2014) Ability of GLP-1 to decrease food intake is dependant on Nutrient Status. *Physiology and Behavior*. 135(222-9).

De Lartigue G. **Ronveaux CC**, Raybould HE (2014) Leptin receptor knockout in vagal afferent neurons drives hyperphagia and weight gain. *Molecular Metabolism*. 3(6):595-607 (Featured on the cover).

Ronveaux CC, Tome D, Raybould HE (2015) Glucagon-like-peptide-1 interacts with ghrelin and leptin to regulate glucose metabolism and food intake through vagal afferent neuron signaling. Journal of Nutrition (Ahead of Print).

In Preparation

Ronveaux CC, De Lartigue G., Raybould HE. Ghrelin inhibits translocation of GLP-1R to the plasma membrane of vagal afferent neurons*. In preparation* (2014).

M. Arnold, **Ronveaux CC**, de Lartigue G., Langhans W., Raybould HE. (2014) Vagal afferent neurons in rats and mice express the monocarboxylate transporter-2 (MCT2). *In preparation* (2014).

Prebiotic bovine milk oligosaccharides improves gut barrier function in high-fat diet induced obesity (2014). Hamilton MK, **Ronveaux CC**, Barile D, Mills D, and Raybould HE. *In preparation* (2014).

Presentations

*(*denotes presenter)*

Oral Presentations

Ghrelin inhibits translocation of GLP-1Rs to the plasma membrane on vagal afferent neurons**. Ronveaux CC**.*, de Lartigue G., Raybould HE. Society for the Study of Ingestive Behavior. Seattle Washington. August 2014. (Received ABIES Internataional Travel Award and New Investigator Travel Award).

Deletion of leptin signaling in vagal afferent neurons results in an obese phenotype. **Ronveaux CC**.*, de Lartigue G., Raybould HE. Interdisciplinary Graduate and Professional Student Symposium. Davis, California. April 2014.

Ability of GLP-1 to decrease food intake is nutrient dependent. **Ronveaux CC**.*, de Lartigue G., Raybould HE. Society for the Study of Ingestive Behavior. New Orleans, Louisana. August 2013.

GLP-1R expression on vagal afferent neurons change according to nutritional status. **Ronveaux CC**.*, de Lartigue G., Raybould HE. Interdisciplinary Graduate and Professional Student Symposium. Davis California. April 2013. (Received Dean's Prize for Best Oral Presentation in Veterinary Medicine Award).

Posters

Progressive Increase in Large Intestine Transcellular but Not Paracellular Permeability Correlates with Plasma Endotoxemia in Diet-Induced Obese Rats Digestive. Boudry G*, Hamilton MK, de Lartigue G, **Ronveaux CC**, Raybould HE. Disease Week (DDW), Orlando, May 2013.

Prebiotic bovine milk oligosaccharides improves gut barrier function in high-fat diet induced obesity. Hamilton MK*, **Ronveaux CC**, Barile D, Mills D, and Raybould HE. October 2014.

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Abbreviations

α-MSH: alpha-melanocyte-stimulating hormone

AgRP: agouti gene-related protein

ARC: arcuate nucleus

cAMP: cyclic adenosine monophosphate

CART: cocaine amphetamine regulated transcript

CB1: cannabinoid receptor type 1

CB1R: cannabinoid receptor type 1 receptor

CCK: cholecystokinin

CCK-1R: CCK-1 receptor

CCK-2R: CCK-2 receptor

CLAMS: comprehensive lab animal monitoring system

CNS: central nervous system

CREB: cAMP responsive element binding protein

DEXA: dual-energy X-ray absorptiometry

DIO: diet-induced obesity

DMEM: Dulbecco's modified Eagle's medium

DS: Donkey serum

DPP-IV: dipeptidyl peptidase-4

EEC: enteroendocrine cell

EGR-1: early response gene-1

ER: estrogen receptor

Ex-4: exendin 4

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPCR: G protein coupled receptor

GFP: green fluorescent protein

GHS-R: growth hormone secretagogue receptor

GI: gastrointestinal

GIP: glucose-dependent insulinotropic peptide

GIPR: glucose-dependent insulinotropic peptide receptor

GLP-1: glucagon like peptide 1

GLP-1R: GLP-1 receptor

GOAT: ghrelin O-acyltransferase

HBSS: Hank's Balanced Salt Solution

HF: high fat

ICV: intracerebroventricular

IP: intraperitoneal

KO: knockout

LepR: leptin receptor

MAPK: mitogen-activated protein kinase

MCH: melanin concentrating hormone

MCH1R: MCH receptor

MIN6: mouse insulinoma 6

NPY: neuropeptide Y

NTS: nucleus of solitary tract

OVX: ovariectomy

PBS: phosphate buffered saline

PM: plasma membrane

POMC: pro-opiomelanocortin

PYY: peptide tyrosine tyrosine

SDA: Subdiaphragmatic vagal deafferentation

STAT-3: Signal transducer and activator of transcription 3

TBST: Tris-Buffered Saline and Tween 20

VAN: vagal afferent neurons

WT: wildtype

Y2: peptide tyrosine tyrosine receptor

Chapter 1: Literature Review on gut-derived peptides and their mechanism of action on vagal afferent neurons

1.1 Introduction

Obesity is one of the most significant global public health issues. 2/3 of Americans are either overweight or obese. In France, in 2012, the percentage of the population being obese was at 14.5%. Its prevalence is increasing worldwide; in the last 30 years the obese population has more than doubled in the United States, France, and United Kingdom (1). Obesity has been linked to numerous health complications such as diabetes and heart disease. The causes of obesity are likely to be complex and multifactorial, but can be characterized as the overconsumption of high-calorie foods and reductions in energy expenditure. In the last 30 years, the food environment has changed in that the availability of energy dense and palatable foods have increased significantly. In parallel, energy expenditure has decreased due to advancements in transportation modes. In addition, there are genetic mutations that result in an obese phenotype.

Although obesity is influenced by a complex interaction between diet, physical exercise and the environment, the scope of this dissertation will primarily focus on diet-induced physiological changes. Chronic high fat (HF) diet leads to hyperphagia, obesity and metabolic diseases such as insulin resistance (2). The mechanism by which HF diet alters energy homeostasis is not well understood. Diet-induced obesity (DIO) results in modulations in neuronal and tissue responsiveness to nutrients that are essential for regulating food intake and energy homeostasis. Obese individuals have elevated circulating leptin suggesting that individuals do not respond to endogenous leptin. Indeed, chronic high fat feeding leads to leptin resistance in mice (3). Diet-induced obese models have blunted responses to satiating signals such as cholecystokinin (CCK) (4). Altogether, evidence suggests that diet is one of the factors responsible for inducing obesity through physiological changes. Although balancing food intake and

energy expenditure would lead to weight loss, DIO leads to physiological changes that drive obese subjects to overeat.

Control of food intake involves many organs interacting in both the peripheral and central system that respond to nutrients with pleiotropic biological actions including gastric motility, food intake inhibition, and body weight regulation. Among them are the gastrointestinal tract and the vagus nerve, which will be the main focus of this dissertation. The vagus nerve is the main conduit by which gut-derived signals is communicated to the central nervous system (CNS) via the Nucleus of the solitary tract (NTS). Afferents in the NTS can communicate to higher order neurons such as the hypothalamus to influence food intake. Vagotomies or destruction of vagal afferent neurons via capsaicin treatment in rodents leads to hyperphagia and impaired responses to gut peptides involved in feeding compared to sham operated animals (5).

The vagus nerve is unique in that it innervates all tissues that are involved in digestion and absorption of nutrients, where activation of vagal afferents is necessary for the preparation and the onset of a meal. Furthermore, vagal afferent neurons (VAN) express receptors for many of the regulatory peptides and molecules released by enteroendocrine (EEC) cells that line the mucosal epithelia of the gut. EEC cells release humoral peptides that activate vagal afferent fibers, which communicate signals from the periphery to the brainstem activating the vago-vagal pathway. Gut-derived hormones, such as CCK and leptin, have been implicated in the regulation of long-term energy homeostasis.

Diet-induced obese models have altered gut-brain signaling present on the vagus nerve, which coincides with hyperphagia. Although much progress has been done using diet-induced obese rodent models, the origins of overnutrition are still unclear and need to be elucidated. Therefore we have focused on investigating the mechanism of action of

anorexigenic hormones on vagal afferent neurons. Understanding the underlying drivers of hyperphagia is important in developing effective non-invasive treatments against obesity.

1.2 Nutrient sensing in the GI tract

The gastrointestinal (GI) tract plays an important role in the regulation of energy homeostasis. It is the initial interface of the body at which nutrients are digested, absorbed and assimilated. EECs are specialized chemosensing epithelial cells dispersed along the mucosa of the GI tract. EECs are the first level of integration of information from the gut lumen that respond to the presence or absence of nutrients and secrete a variety of gut-derived hormones such as CCK. They are strategically located in the mucosa of the GI tract to taste and sense luminal contents and release hormones basolaterally to enter into the circulation (Figure 1.1). Gut-derived hormones either act directly on central receptors or they bind to their receptors found on vagus nerve fiber terminals to activate vago vagal pathway. In turn, efferent signals will target peripheral targets to control gut functions and ingestive responses.

1.2.1 Recent advances in understanding nutrient sensing

ECCs are chemosensing cells that finely coordinate nutrient sensing with metabolic and behavioral functions such as regulating energy homeostasis and glucose metabolism. ECCs release hormones such as CCK, glucagon-like-peptide 1 (GLP-1) and peptide YY (PYY) in response to nutrient ingestion (6). These signals are important in regulating appetite and body weight; in a prepandial state, circulating levels of orexigenic peptides are high to stimulate food intake and conversely, in postprandial states, anorexigenic peptides are secreted to inhibit food intake.

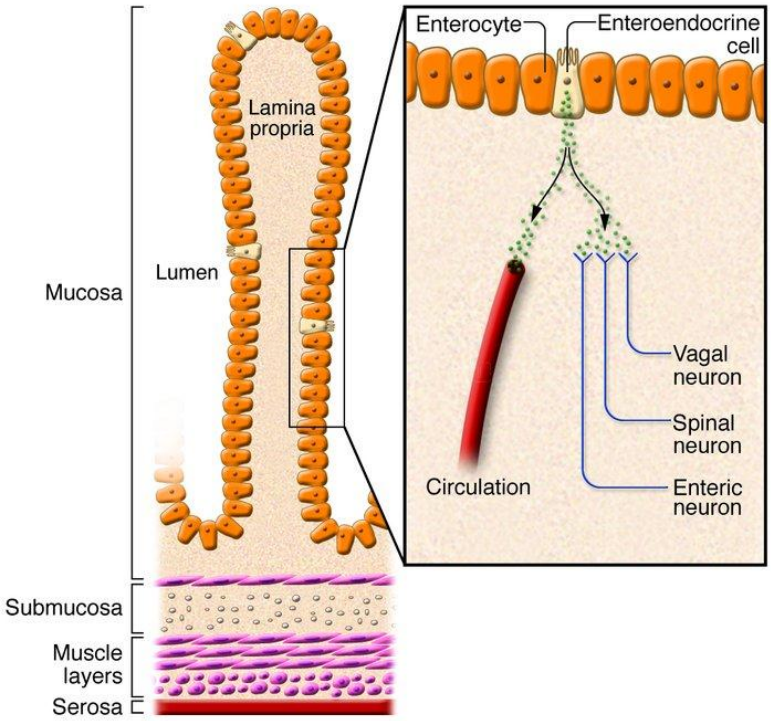

Figure 1.1 Schematic of the topography of enteroendocrine cells

EECs are located in the mucosa of the gastrointestinal epithelium to taste and sense nutrients passing through the lumen. Peptides known to regulate food intake are released on the basolateral side and act either on nearby neural pathways or can enter directly into the circulation. Schematic taken from Cummings D.E and Overduin J, 2007 (44).

EECs are scattered throughout the epithelium of the GI tract and represent less than 1% of the epithelial cell population making the study of native EECs difficult. However, the development of transgenic mice expressing green fluorescent protein (GFP) under the control of specific EECs gene promotors has greatly enhanced the ability to study gut EECs in cell culture. GFP is used as a marker of gene expression and protein localization in living organisms without the use of an exogenous substrate. GFP with enteroendocrine promoters label hormone precursors, chemosensor receptors and granular proteins.

Given that EECs are difficult to study, much of what we know about EECs and nutrient sensing stems from cell lines such as STC-1 and GLUTag cells. These cells have been helpful in understanding intracellular signaling, however, they are transformed cells whereby their exact protein content or responses to stimuli may not replicate *in vivo* EEC function. Physiological and anatomical characterization of ECCs has been greatly enhanced through GFP-tagged mice. Traditionally, ECCs have been classified according to hormonal content, morphology and localization in the GI tract leading to the concept of “one cell type, one hormone”. However, results from transgenic mice with tagged EECs have questioned this concept. GFP-tagged mice with enteroendocrine promoters have demonstrated that multiple hormones are synthesized and released from a single ECC revealing the pluritrophic actions of EECs (7, 8). For example, studies using CCK-GFP mice have demonstrated that CCK is coexpressed with GLP-1, glucose-dependent insulinotrophic peptide (GIP), PYY, secretin and neurotensin (9, 10). Surprisingly, Egeord *et al.* found that promoters for CCK and GLP-1 were scattered throughout the gut rather than segregated in either the proximal or distal gut (8). The hormone content of individual cells differs according to their localization. For example, EECs from the proximal gut contain high levels of CCK and low levels of GLP-

1. Conversely, EECs cell from the distal gut have high levels of GLP-1 and low levels of CCK (9). An extensive study on CCK-containing cells demonstrated that the coexpression of hormones, such as CCK and ghrelin, was consistent between immature and fully differentiated cells (10).

The anatomical arrangement within each cell has been extensively studied in the last few years; PYY and GLP-1 secretory granules have been demonstrated to be contained at the base of L-cells. Furthermore L cells contain a prominent basal cytoplasmic process (7, 11). GLP-1 and PYY granules are contained in separate storage organelles within the same cell and these organelles can be selectively mobilized according to different stimuli (12).

Gastrointestinal functions are modulated by changes in luminal contents to protect against harmful substances. EECs act as primary chemoreceptors by releasing signaling peptides in response to changes in the luminal environment. Transgenic GFP-mice has been possible to look at the mechanism of nutrient sensing directly on native EECs. For example, using duodenal extract from CCK-GFP mice, it has been demonstrated that amino acid-induced CCK release acts through calcium sensing receptor (12). There are several molecular responses stimulated by nutrient sensing such as protein transport, glucose sensors and G–protein coupled receptors (GPCR). Convincing evidence suggests that EECs in the GI tract express several G-protein coupled receptors that are involved in chemosensing (13). These receptors are stimulated by glucose, calcium and short and long chain fatty acids. Many gut-derived proteins have been shown to act specifically through GPCR. It has been established that GPCR are involved in nutrient sensing of fatty acid (12, 13). In mouse intestine, GPR120 is colocalized with GLP-1(13). Furthermore, fatty acid induced secretion of GLP-1 is attenuated in GPR120 knockout mice (13). GPR40 gene and protein expression was

identified in CCK-expressing ECCs. GPR40 was shown to directly mediate fatty acid-induced CCK secretion, which was further attenuated by the deletion of GPR40 (14).

1.3 Importance of gut-derived hormones on regulation of food intake

Gut-derived hormones are secreted in the absence or presence of nutrients to control food intake. They are localized in the epithelium of the GI tract in an ideal location to respond to luminal contents. Their patterns of release alter according to nutrient availability in ways that could affect short and long-term feeding behavior. Studies using exogenous administrations of gut peptides have revealed the functional importance of signals that arise from the GI tract in regulating energy homeostasis.

1.3.1 Glucagon like peptide-1

GLP-1 is derived from the expression of the transcriptional product of the pre-proglucagon gene in intestinal L-cells and pancreatic α-cells. GLP-1 is released by L-cells predominately found in the distal gut in response to nutrient ingestion. Glucose and fat are the most potent stimulators of GLP-1 stimulation (15). GLP-1 receptors (GLP-1R) are found throughout the peripheral and CNS, where its highest expression is found in the lung, distal gut and brain (16, 17). GLP-1 was considered until recently for its role as an incretin hormone and ability to restore glucose homeostasis in type 2 diabetic patients. In fact, GLP-1 analogs and DPP-IV inhibitors are on the market for the treatment of type 2 diabetes. Emerging evidence suggest a possible physiological role for GLP-1 in regulating food intake. Exogenous administration of GLP-1 or its long-acting analogs dose-dependently inhibit food consumption and the satiating effects of GLP-1 are attenuated by prior administration of GLP-1R antagonist (18, 19). Given that GLP-1Rs are found in the peripheral and CNS, the site of action of native GLP-1 in regulating

food intake is unclear and this is discussed in further detail in the *GLP-1 and control of food intake* section.

GLP-1 has specifically been implicated in the pathogenesis of obesity. DIO leads to blunted GLP-1 release; decreased postprandial plasma concentrations and blunted anorexigenic response to GLP-1R activation (20, 21). Duca *et al.* have demonstrated that DIO results in impaired GLP-1 satiation signaling; DIO rats have an attenuated response to GLP-1R antagonist exendin 4 (Ex-4) compared to controls. Furthermore, DIO rats have decreased GLP-1 protein expression in the intestinal epithelium indicating a reduction in intestinal nutrient response from L-cells (22). Evidence suggests that GLP-1 would be an effective treatment for obesity. Plasma concentrations of GLP-1 dramatically increase in patients following bariatric surgery (23). Infusions of GLP-1 will induce satiation in lean adults by suppressing food intake and meal size. Interestingly, this effect is preserved in obese subjects implying GLP-1 as a prime candidate for anti-obesity treatments (24).

1.3.2 Ghrelin

The discovery of ghrelin opened a new frontier to research in energy homeostasis in that ghrelin is currently the only circulating hormone that can stimulate food intake and adiposity in humans and rodents in comparison to the large family of identified anorexigenic gut hormones. Ghrelin is a 28-amino acid polypeptide produced mainly by endocrine X/A-like cells in rodents and P/D1 in humans located in the gastric epithelium (25). Although the stomach is the main site of secretion, ghrelin is also secreted by the pituitary, hypothalamus, lung, heart and pancreas. Acetylated ghrelin, which is converted by ghrelin O-acyltransferase (GOAT) enzyme, acts on the growth hormone secretatgogue receptor (GHS-R) which is constitutively expressed (26, 27).

GHS-R is predominately found in the pituitary and the hypothalamus (28). The biological functions of ghrelin are widespread; it plays a role in lipid metabolism, glucose homeostasis and growth hormone release (28, 29). Additionally, ghrelin stimulates appetite, body weight and adiposity.

Interestingly, circulating ghrelin concentrations are decreased in obese individuals compared to their lean controls (30). Given that ghrelin plasma levels are negatively correlated with insulin and leptin plasma levels, the downregulation in ghrelin may be a consequence of elevated insulin or leptin. However, antagonizing ghrelin receptor can decrease food intake in obese Zucker rats (31) possibly blocking the constitutive receptor activity (27). Given that ghrelin is involved in meal initiation makes it an attractive target in understanding feeding and energy homeostasis.

1.3.3 Leptin

Leptin is a 167-amino acid peptide that is mainly expressed in white adipose tissue but also found in a variety of tissues such as placenta, stomach, skeletal muscle and pituitary glands. Leptin is produced and secreted predominately by adipose tissue and the stomach (32). Circulating leptin levels positively correlate with body adiposity which reflects long term energy stores (33). Leptin is a gut and adipose tissue-derived hormone that regulates a range of biological functions and processes; including energy intake and expenditure, body fat, neuroendocrine systems, autonomic function, and insulin and glucose balance (34) (Figure 1.2). It exerts its effect by binding to its receptor, LepR, which is located throughout the CNS (35). The highest expression of the long isoform of LepR is found in the

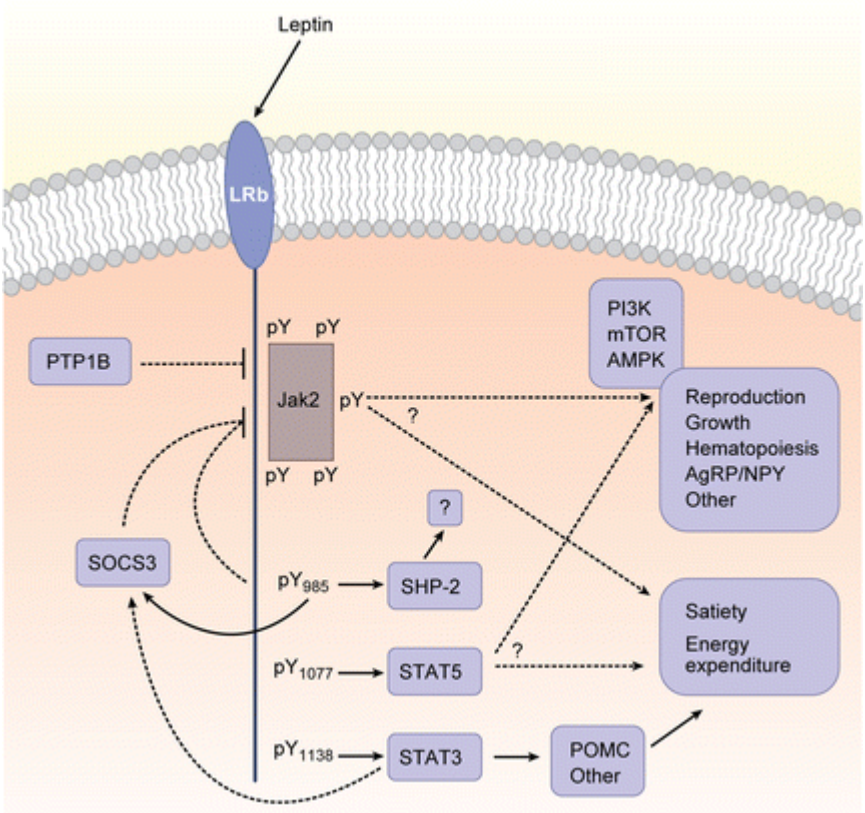

Figure 1.2 Leptin signaling leads to various biological activites

Leptin binds to its receptor to regulate energy intake and energy expenditure, glucose homeostasis as well as reproduction and growth. Schematic taken from Myers *et al.* 2008 (33).

hypothalamus, an area known to regulate energy homeostasis. Six isoforms of LepR have been identified in rats; the long form has been implicated in the suppression of food intake and stimulation of energy expenditure (34).

The presence of leptin was predicted before it was cloned in 1994. Coleman *et al.* performed parabiosis studies in which they joined ob/ob mice, those lacking leptin, and db/db mice, those lacking leptin receptor. They concluded that ob/ob mice were missing a circulating factor that was abundant in db/db mice. The factor could cure obesity in ob/ob mice but not in db/db mice (36). The discovery of the leptin was initially thought as a cure for human obesity however, daily injections of recombinant leptin only fully corrected obesity in few cases of leptin deficient patients. It was discovered that a majority of the obese population have high circulating leptin levels and are unresponsive to leptin thereby defining them as leptin resistant (36). Evidence has undoubtedly highlighted the importance of leptin signaling for the maintenance of energy homeostasis.

Obese individuals have high circulating leptin levels which fail to reduce energy intake (33). Moreover, the response to central administrations of leptin is attenuated in mice in part due to the development of leptin resistance in the hypothalamus (37). The importance of leptin in energy homeostasis is most evident in leptin deficient models. Ob/ob mice with global leptin deficiency exhibit hyperphagia, low metabolic rate and rapid onset of obesity, associated with high expression of orexigenic neuropeptide Y (NPY) and melanin-concentrating hormone (MCH) and low expression of anorexigenic pro-opiomelanocortin (POMC) in the hypothalamus (34, 38).

1.3.4 Cholecystokinin

CCK was the first identified gut-derived hormone shown to have satiating effects on ingestive behavior. CCK is secreted predominately from I cells found in the upper

small intestine and is released in response to fat. Plasma levels markedly increase in response to a meal. There are two subtypes of CCK receptors, CCK1R and CCK2R. CCK binds to its receptors to induce gallbladder contraction, delayed gastric emptying and release of pancreatic enzyme to induce digestion (Figure 1.3). CCK1Rs are predominately responsible for mediating CCK-induced effects on short-term food intake (39, 40). CCK is involved in the regulation of food intake and GI function.

CCK inhibits food intake in a dose dependent manner in many species including humans (41, 42). Central and peripheral administrations induce inhibitory effects on energy intake. Specifically, exogenous CCK inhibits cumulative food intake by reducing meal size and increasing intermeal interval in rodents and humans (39, 43). Perfusions of fat or protein into the small intestine inhibit feeding and the administration of CCK1R antagonists will reverse these effects (41). The satiating actions of CCK regulate short-term feeding; CCK1R knockout (KO) mice have no difference in body weight and cumulative food intake compared to wildtype (WT) mice, however, their early meal events are altered. For example, CCK1R KO mice ingest larger, longer first compared to their control littermates (44).

The major site of action of CCK is the vague nerve; damage to the vagus nerve attenuates the satiating effects of CCK (41). Exogenous administrations of CCK to rats will rapidly induce vagal electrophysiological activity (45). CCK binds to CCK1R present on the vagus nerve to terminate feeding and induce satiety integrated by the NTS, the area where vagal afferents terminate (39). CCK has been demonstrated as the master regulator of VAN phenotype switch, which alters sensitivity of VAN to fasting and refeeding conditions. This mechanism is further elucidated in the *Phenotypic Changes of VAN According to Feeding Status* section.

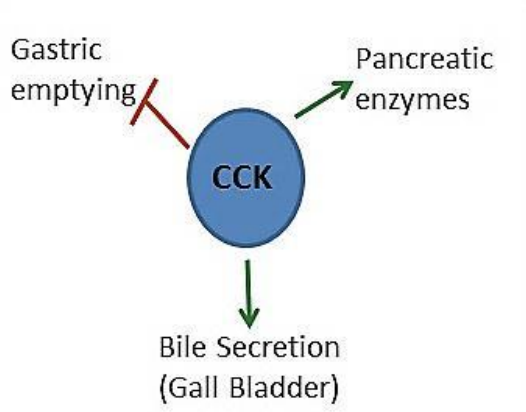

Figure 1.3 Biological activities of cholecystokinin

Schematic representing that CCK has various biological activities; it delays gastric emptying, increases pancreatic enzymes to facilitate digestion and induces bile secretion from gall bladder.

Studies using diet-induced obesity models have highlighted the importance of CCK in regulating energy homeostasis. Animals maintained on a chronic HF diet leads to hyperphagia and obesity in part due to a decreased sensitivity to exogenous CCK (2, 46). HF fed rats are less sensitive to the satiating effects of CCK through reductions in neural activation of the hindbrain where vagal afferent fibers terminate (46). Interestingly, Diet-induce obese rats have reduced lipid-induced satiation compared to diet resistant rats, which is associated with the decrease in intestinal protein expression of CCK (47). Indeed, obese humans have decreased circulating CCK (42).

The satiating effect of CCK is the most established compared to other gut-derived peptides. Many studies have highlighted that endogenous CCK acts on VAN and plays an important role in regulating food intake. The ability of CCK to change the sensitivity of VAN provides a great interest in understanding the mechanism of action of other gut-derived peptides and how they may interact with each other to regulate energy homeostasis.

1.4 The Vagus Nerve

The vagus nerve is the major neuronal link between the GI tract and CNS relaying gut-derived signals to the NTS. Afferent neurons in the NTS project to second order neurons in the hypothalamus to communicate peripheral signals influencing food intake behavior. The vagus nerve densely innervates organs involved in digestion and is necessary for meal ingestion and digestion. Vagal afferent fibers provide the primary neural site in which gut-derived hormones released from ECCs to relay sensory input of the periphery via the nodose ganglion to the NTS in the brain stem (6, 48).

Lack of vagal function will result in impaired responses to feeding; lesions to vagal afferents by vagotomies and capsacin destruction in rodents result in hyperphagia

and rapid weight gain (49). Specifically, subdiaphragmic deafferenation (SDA), which target all vagal afferent fibers, results in attenuated responses to anorexigenic gut peptides in lean rats (50-52). For example, the satiating effect of CCK is completely abolished in animals without intact afferents (49). Activation of the vagus nerve mediates external stimuli of the food bolus and activates internal stimuli from the gut to communicate the information to the brain. These signals activate neurons in the NTS and in turn control peripheral tissues through vagal efferents.

Vagal signaling is important in regulating ingestive behavior and food intake regulation. Peripheral satiety signals, such as CCK, will increase proto-oncogene protein c-fos expression, an indicator of neural activity, in the NTS, which in turn signals for meal termination in higher ordered neurons. In vagotomized animals, CCK induced c-fos expression in the hindbrain is attenuated (53). Lesions to ascending projections from the NTS to the hypothalamus block the satiating effect of systemic CCK and inhibit CCK induced c-fos expression in the NTS (54). Satiety signals from the periphery will activate c-fos expression in the NTS, which will project to vagal efferent in the dorsal motor nuclei (55). In turn, vagal efferents project to pre and post ganglic neurons to target peripheral tissues influencing gastric and insulinotrophic functions. This is known as the vago-vagal loop (Figure 1.4).

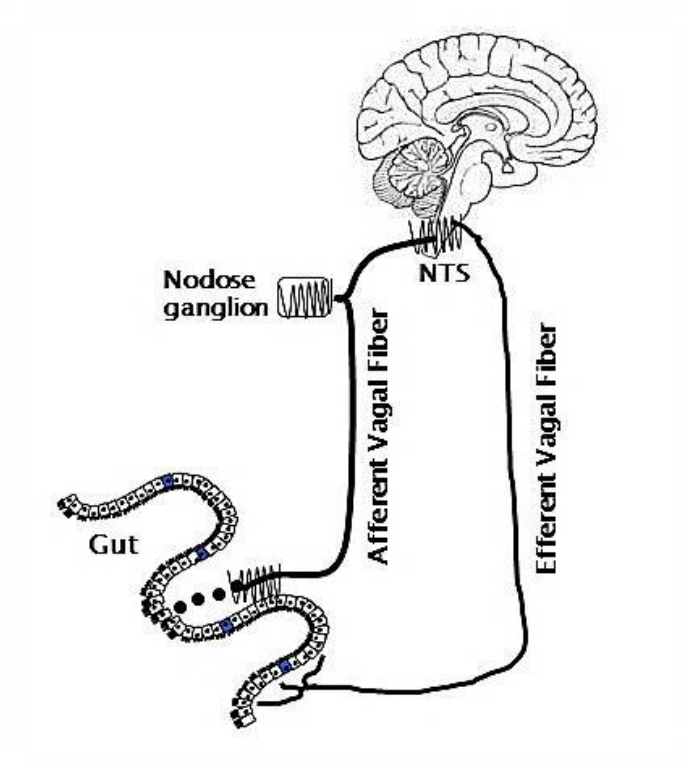

Figure 1.4 The vago-vagal loop

Schematic representation of vagally-mediated nutrient sensing along the gut-brain axis. The presence of nutrients in the lumen are sensed by enteroendocrine cells in the gut to induce the release of hormones that can then enter circulation and bind to receptors found on terminals of the vagus nerve. These signals are processed in the nodose ganglia and transduced to the NTS of the hindbrain.

1.4.1 Phenotypic Changes of VAN According to Feeding Status

It is well established that VANs exhibit plasticity according to physiological changes. Nutrient status induces neurochemical changes in VAN by regulating the switch between states that promote orexigenic and anorexigenic phenotypes (56). Anorexigenic receptor expression will decrease and orexigenic receptor expression will increase in VAN in a food-deprived condition. Conversely, anorexigenic receptor expression is upregulated and orexigenic receptor expression is downregulated postprandially. For example, the expression of anorexigenic PYY receptor (Y2) receptor expression is downregulated after a fast between 6 and 12 hours (57). Retrograde labeling revealed that the changes in fasting-induced Y2R expression in VAN occur in neurons innervating the upper GI tract. Refeeding of fasted rats will restore Y2R expression (57). Orexigenic receptors have been demonstrated to cannabinoid-1 (CB-1) and MCH-1 receptors increase significantly in a fasted state whereas Y2, a receptor associated with inhibition of food intake, is decreased (56).

CCK induces phenotypic changes of VAN by regulating the expression of peptides and receptors according to nutritional status (56). In a fasted state, when CCK levels are low, mRNA levels and protein expression of orexigenic MCH, MCH1R and CB1 receptor (CB1-R) are increased. In a fed state, when CCK levels are high, the expression of anorexigenic PYY, cocaine amphetamine transcript (CART) and Y2R are decreased (Figure 1.5). Furthermore, administrations of exogenous CCK will act on CCK1Rs to decrease expression of orexigenic MCH, MCH-R and CB1-R and increase PYY, Y2R and CART expression (57, 58). The mechanism by which CCK alters neuropeptide CART expression according to nutritional status has been shown to work through phosphorylation of CREB and translocation of EGR-1 (59). In culture, CCK-induced CART expression is decreased by ghrelin (59). Although exogenous ghrelin

does not increase the abundance of orexigenic peptides in refed rats, it does inhibit the decrease in expression of MCH and CB-1 in fasted then refed rats (60). CCK is the master regulator of the VAN neurochemical switch involved in regulating food intake to potentate the actions of gut-derived hormones.

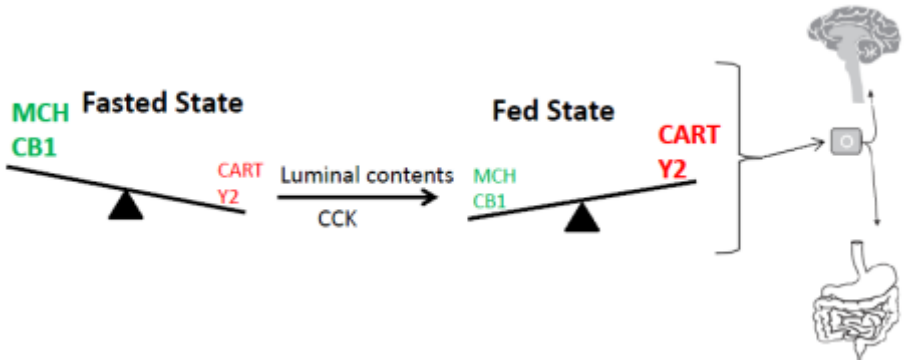

Figure 1.5 Neurochemical phenotype of VAN changes according to feeding status

In a fasted state, when CCK levels are low, mRNA levels and protein expression of orexigenic melanin-concentrating hormone (MCH), MCH receptor (MCH-R) and CB1 receptor (CB1-R) are increased. In a fed state, when CCK levels are high, the expression of anorexigenic PYY, CART and PYY receptor (Y2R) are decreased.

1.4.2 VAN phenotype in diet induced obesity

The inability to respond to satiety signals in obese models may be due to the disruption in VAN signaling. VANs DIO have decreased sensitivity to VAN stimulation. Daly *et al.* showed reduced VAN sensitivity to CCK, 5HT and distension in DIO mice (61). Furthermore, de Lartigue *et al.* have demonstrated that chronic exposure to a HF diet leads to the development of leptin resistance on VAN of obese diet-induced rats compared to lean low fat-fed rats (2). VAN sensitivity to anorexigenic peptides is significantly reduced in high fat feeding. For example, CCK fails to inhibit food intake in DIO rats compared to lean chow-fed rats. NTS neuronal activation is decreased in DIO animals in response to exogenous peripheral CCK compared to control (46).

The exact mechanism by which VAN sensitivity is attenuated is unclear. However, a possible mechanism that may play a role in development of an obese phenotype is the inability of VAN to change their expression of orexigenic and anorexigenic peptides and receptors. Rats maintained on a high fat diet for 8 weeks became hyperphagic, increased body weight and adiposity compared to rats on a control diet (2). Studies have demonstrated that orexigenic receptors such as CB1R and GHS-R are increased and anorexigenic Y2R and CART are decreased in obese models (27). Leptin resistance in VAN of DIO rats leads to a change in neurochemical phenotype of VAN and hyperphagia. Altogether, studies have indicated that the normal vagal afferent phenotypic switch in response to feeding is lost in obesity where VANs are locked in a fasting phenotype (Figure 1.6).

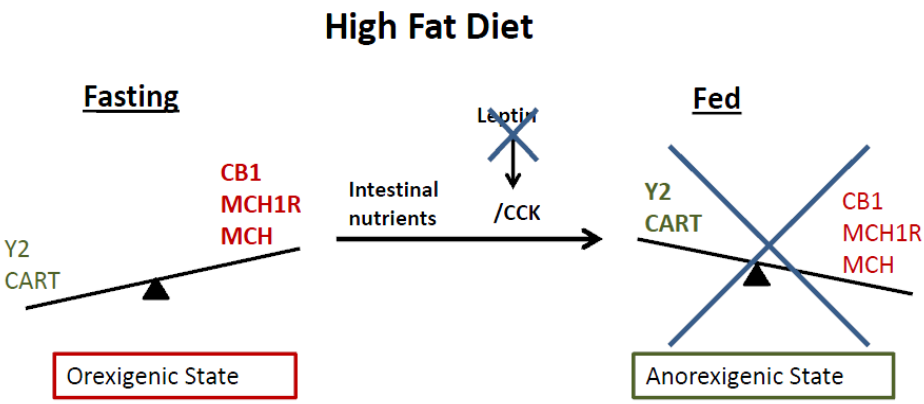

Figure 1.6 Chronic high fat diet leads to a dysregulation in VAN phenotype

Long-term ingestion of a high fat diet renders the ability of vagal afferent neuronal phenotypic switch between fasting and fed conditions. Chronic high fat diet leads a consititutive fasting phenotype in nodose ganglia.

1.4.3 Summary

CCK plays an important role in regulating the phenotype switch of VAN to regulate food intake. CCK has been shown to potentiate the satiating response of other gut-derived hormones. For example, Y2 receptor expression is increased in response to CCK on VAN. There is much less information on other peptides involved in the control of food intake and their actions at the level of the vagus. Emerging evidence suggests that GLP-1 acts on VAN to induce its satiating effects, therefore we have focused on the actions of GLP-1 at the level of the vagus and it's role in regulating energy homeostasis.

1.5 GLP-1 secretion in the GI tract

GLP-1 is derived from the expression of the transcriptional product of the pre-proglucagon gene in intestinal L-cells and pancreatic A cells. Pre-proglucagon is cleaved into several fragments; the main translational products are glucagon-containing glicentin, GLP-1 and glucagon like peptide-2 (GLP-2) in the GI tract, and glucagon and glicentin-related polypeptide in the pancreas (62, 63) (Figure 1.7).

GLP-1 is released by L-cells in response to nutrient ingestion (15). L-cells are an open type of endocrine cells where their base lies on the basement membrane and their cytoplasmic processes project into the gut lumen. These processes have microvilli, and it is hypothesized that these microvilli are part of the nutrient-sensing machinery in these cells resulting in sensing of luminal contents discharge of granules on the basolateral side. Once stimulated, L-cells secrete peptides into the interstitial space. They are found in close proximity to both neurons and systemic circulation in the intestine, which allows them to be influenced by both neural and humoral signals (64, 65). L-cells are found throughout the gut; the highest density is found in the ileum and distal colon, with fewer cells in the proximal gut (66). GLP-1 is co-localized in intestinal L-cells with PYY and GIP (67, 68).

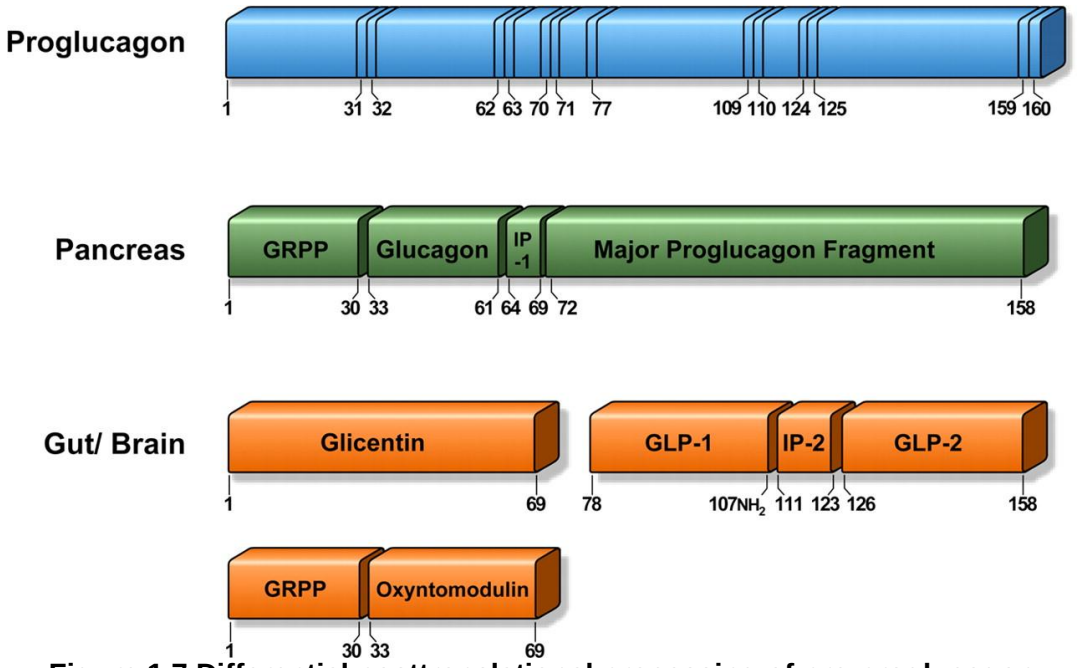

Figure 1.7 Differential posttranslational processing of pre-proglucagon

Pre-proglucaon is cleaved into several segments in the pancreas, the gut and the brain. The numbers indicate amino acid positions in the 160-amino acid proglucagon sequence. The vertical lines represent positions of basic amino acid residues, typical cleavage sites. Glicentin-related pancreatic polypeptide (GRPP); Intervening peptide-1 (IP-1); Intervening peptide-2 (IP-2). Schematic taken from Holst *et al.* 2007 (62).

Once secreted, GLP-1 is released into the lamina propria and enters the capillary bed or lymphatics. Preprandial plasma concentrations of GLP-1 are very low and increase with nutrient ingestion (69). Multiple studies have demonstrated that GLP-1 is quickly degraded into its inactive form by dipeptidyl peptidase IV (DDP-IV) (70). DDP-IV is abundant in the brush border and endothelial cells that line the capillaries (71, 72). It is estimated that around 50% of GLP-1 released into the capillaries *in vivo* is transformed into its inactive form, N-terminally truncated GLP-1 9-36 amide, before it reaches the hepatic portal vein. Further degradation takes place in the liver leaving only 10-15% of intact GLP-1 by the time it reaches the systemic circulation. In the circulation, GLP-1 has a 2-3min half-life due to the presence of DDP-IV (70). Inhibiting DDP-IV prevents GLP-1 degradation in porcine ileum by 46% at baseline (73, 74). GLP-1 can cross the blood brain barrier (75) but given that it is degraded so quickly, it is unlikely that a significant amount of active GLP-1 released from the periphery can reach the brain. In addition, GLP-1 concentrations are higher in intestinal lymphatics than in the hepatic portal vein likely because lymph flow is lower than portal blood flow and there is less DDP-IV in lymphatics than in blood vessels (76). The concentration of GLP-1 in the intestinal lymphatics reflects interstitial concentrations and is increased after meal ingestion (76). This evidence supports the hypothesis that GLP-1 acts in a paracrine way on VANs (69). Indeed, Punjabi *et al.* demonstrated that systemic active GLP-1 concentrations do not increase in response to a regular unpurified diet meal in rats (77).

GLP-1 and its receptor are found at central and peripheral sites. Currently there is only one known GLP-1R, which has high single binding affinity for GLP-1 (78). GLP-1R was originally cloned from pancreatic islet cells (79). It is a GPCR that is distributed in various tissues, both centrally and peripherally (16, 80). It is most abundant in the lung, brain and distal GI tract. There are two different signaling pathways downstream of

the GLP-1R. In the hindbrain and the pancreas, GLP-1 binds to its receptor and activates adenylyl cyclase to induce cyclic adenosine monophosphate (c-AMP) pathway (Figure 1.8) (81, 82). In muscle and liver, GLP-1R may activate a c-AMP independent pathway (83, 84). Thus although there is evidence for a single receptor for GLP-1, there are differences in signal transduction in different tissues. The biological activities of GLP-1 include maintaining glucose homeostasis and regulating gastric motility and food intake. The insulinotrophic effect of GLP-1 is mainly mediated through the pancreas whereas the satiating effect of GLP-1 is mainly mediated through the vagus nerve.

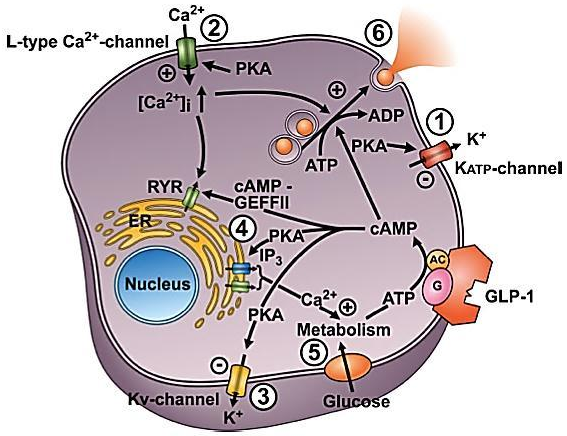

Figure 1.8 GLP-1 signaling pathway

GLP-1 binds to its receptor to activate adenylate cyclase; intracellular cAMP levels elevate leading to increased PKA and cAMP-regulated guanine nucleotide exchange factor II. In the pancreas, GLP-1 acts with glucose to close ATP-sensitive K+ channels and facilitate membrane depolarization. Schematic taken from Holst (62).

1.6 The insulinotropic activity of GLP-1

GLP-1 is a major player in regulating glucose homeostasis. It is partly responsible for inducing the incretin effect where the amplification of insulin secretion that is observed when glucose is taken orally as opposed to infused intravenously to provide identical plasma glucose concentrations (85, 86). The incretin effect is induced by both GLP-1 and GIP. GIP is released from K cells in the duodenum in response to nutrients and activates insulin secretion in a glucose-dependent manner (87). The release of GLP-1 and GIP from the gut after an oral glucose load accounts for 60% of insulin secretion (88). They are both released in response to nutrient stimuli and degraded by DDP-IV in the circulation. These two peptides work synergistically to potentiate glucose-stimulated insulin secretion (Figure 1.9). This is confirmed through GLP-1R and GIPR knockout (KO) mice. GLP-1R KO mice exhibit rather modest perturbations on glucose homoeostasis; they have mild hyperglycemia, glucose intolerance and abnormal glycemic excursions in response to glucose (89). Isolated pancreatic β-cells from GLP-1R KO mice preserve insulin storage and glucose-dependent insulin secretion (90). GLP-1R KO mice exhibit a compensatory mechanism by which glucose homeostasis is maintained. GIP and GLP-1 signaling is significantly upregulated in pancreatic β-cells of KO mice possibly explaining why GLP-1R KO mice only have a mild change in phenotype (86). Likewise, GIPR KO mice display a mild change in phenotype with reduced glucose tolerance and glucose induced insulin secretion in response to glucose. In contrast to GLP-1R KO mice, GIPR KO mice have normal fasting glycemia and normal glucose excursion (86, 89, 91). Together these studies demonstrate the compensatory mechanisms that exist between GLP-1 and GIP *in vivo*. To date, GLP-1 and GIP are the only hormones that fulfill the definition of an incretin hormone in rodents and humans.

GLP-1R is expressed in β-pancreatic islet cells; this has been demonstrated through immunohistochemical evidence (92, 93). Pancreatic specific GLP-1R KO mice have normal glucose tolerance following oral and intraperitoneal (IP) glucose tolerance test. Pancreatic GLP-1R signaling was restored in pancreatic-specific-GLP-1R *ex vivo* islet extracts compared to whole-body GLP-1R knockout islet extracts (94). GLP-1 regulates glucose homeostasis by inhibiting glucagon, stimulating insulin release, increasing insulin biosynthesis, increasing β-cell proliferation and decreasing β-cell apoptosis (95) (Figure 1.9). In β-cells, GLP-1 binds to its receptor to stimulate adenylate cyclase and cAMP. Subsequently, cAMP activation leads to protein kinase A and cAMP-regulated guanine nucleotide exchange factor II which elevates intracellular calcium levels leading to exocytosis of insulin-containing granules (62, 96). Like other GPCRs, the GLP-1R undergoes ligand-induced internalization by complex and numerous mechanisms. *In vitro* studies have demonstrated that GLP-1R in mouse insulinoma 6 (MIN6) cells, a pancreatic cell line, is endocytosed upon activation via both a clathrin-coated-dependent and caveolin-1-dependent mechanisms (97). In resting MIN6 cells, the receptor is constitutively cycled between the plasma membrane and the cytoplasm (97).

The idea that the incretin effect of GLP-1 is predominately mediated by its effect on pancreatic β -cells has been debated. Given that GLP-1 is rapidly metabolized and its postprandial concentrations are considerably less compared to GIP brings into question as to how much intact peptide actually reaches the pancreas. Studies demonstrate that the activation of extrapancreatic GLP-1Rs may be necessary in maintaining glucose homeostasis. For example, activation or attenuation of GLP-1R in the CNS exerts profound effects on glucose-dependent insulin secretion. Of interest, GLP-1Rs have been localized in the portal vein and blockade of these GLP-1Rs significantly impairs

glucose tolerance in rodents (98). Peripheral GLP-1 administration potently stimulates insulin secretion and improves glucose tolerance in rodents and humans (99, 100). *In vitro* studies indicate that GLP-1 and its agonist can act directly on pancreatic β-cells (101, 102). *In vivo*, GLP-1 and its receptor agonist also modulate glucose metabolism (103, 104). In addition, GLP-1 acts through sensory nerves to regulate glucose homeostasis. Infusions of the active form of GLP-1 into the hepatic vein stimulate vagal afferent and efferent fibers innervating the pancreas; this effect is attenuated by ganglion blockade (105). Furthermore, infusions of a low dose of GLP-1 in mice with intact vagal fibers induces insulin secretion; this effect is attenuated in capsaicin-treated mice (106). Together, these studies provide evidence that the peripheral insulinotrophic effect of GLP-1 is at least partly mediated through VAN.

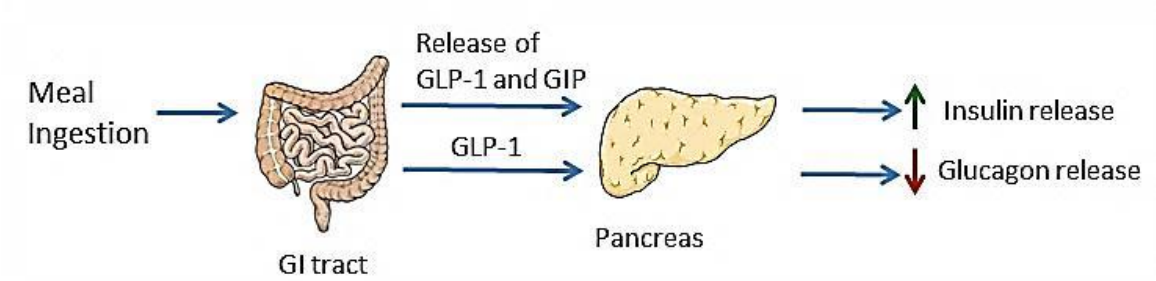

Figure 1.9 GLP-1 and GIP effects on glucose homeostasis

After ingestion of a meal, GLP-1 and GIP are released and act on β cells. They work together to potentiate glucose-stimulated insulin release. Futhermore, GLP-1 will inhibit glucagon release and subsequently suppress glucose production.

1.7 GLP-1 and the control of food intake

Plasma GLP-1 concentrations are low in fasting conditions and rapidly increase postprandially, especially in the presence of carbohydrates and fat (69). There is evidence that exogenous GLP-1 inhibits food intake. Acute peripheral GLP-1R activation by exendin-4 (Ex-4) and native GLP-1 inhibits food intake in a dose-dependent manner in rodents and humans (107, 108). Results from studies using GLP-1 analogs, such as Ex-4 and liraglutide, may be enhanced by their longer half-lives and their ability to act on additional central receptors by crossing the blood brain barrier. Given that GLP-1R KO mice exhibit normal body weight and food intake calls into question whether endogenous GLP-1 plays a physiological role. Studies in rodents indicate that peripheral administrations of native GLP-1 induces satiation but require higher doses than GLP-1R agonists (109, 110). Several lines of evidence also support the notion that native gut-derived GLP-1 plays a physiological role in satiety. Peripheral administrations of native GLP-1 that mimic its release from the GI tract under physiological conditions decrease food intake in a dose-dependent manner (111-113). Blockade of peripheral GLP-1Rs attenuates satiation following a nutrient preload or a peripheral GLP-1 administration (18). However, there are discrepancies in the literature regarding whether endogenous gut-derived GLP-1 plays a functional role as no effect of various doses of peripheral native GLP-1 on food intake was observed in some studies (114) whereas others show a significant decrease in food intake at a lower dose in rats (115). Studies have demonstrated that peripheral, native GLP-1 requires a postprandial state to induce satiation (113, 116, 117). Prolonged fasting attenuates the satiating effects of GLP-1. This concept could explain the discrepancies in the literature regarding the satiating effects of peripheral GLP-1. Consequently, rodents in a postprandial phase will respond to GLP-1 whereas 24h and 48h-fasted rodents do not response to various doses of

native acute GLP-1 (110, 111). GLP-1 inhibits food intake in mice fed *ad libitum* up to 30mins prior to a GLP-1 administration (118) and a short bout of food allows GLP-1 to induce satiation and decrease food intake.

The site of action of GLP-1 in relation with its effect on food intake remains to be discussed. Central mechanisms are important in regulating the anorexigenic effects of GLP-1 and activation of central pathways that affect behavior is necessary to mediate the downstream responses irrespective of the site of primary action of GLP-1. Peripheral native GLP-1 administration activates c-fos expression in the hindbrain and the hypothalamus in rodents (118-120) indicating that peripheral GLP-1 actions are activating central circuits. Blockade of either central or peripheral GLP-1R attenuates GLP-1-induced satiation (18, 121, 122). Likewise, central administration of native GLP-1 and its agonist, Ex-4, significantly reduces food intake in rodents (19, 81). Activation of central GLP-1Rs play a role in mediating food intake; intracerebroventicular (ICV) administrations of Ex-4 into the third ventricle induces satiation and activates c-fos expression in hypothalamic regions (123). Lesions to the brainstem-hypothalamic pathway attenuate GLP-1-induced satiation in rats indicating the importance of central regions mediating systemic GLP-1 (52). GLP-1Rs are colocalized with POMC neurons located in the hypothalamus (123). Central administrations of GLP-1 prevent fasting-induced upregulation of hypothalamic NPY and agouti-related peptide (AgRP) and fasting-induced downregulation of POMC and CART (124, 125). Altogether, these studies highlight the important role in which central pathways are necessary to mediate the inhibitory effects of GLP-1.

It is likely that endogenous gut-derived GLP-1 suppresses food intake by acting in a paracrine manner on adjacent GLP-1Rs expressed on vagal afferents (Figure 1.10). Evidence to support this hypothesis is 1) active GLP-1 is rapidly degraded resulting in an

extremely short half-life (126), and 2) subdiaphragmatic vagal deafferentation prevents GLP-1 from inhibiting food intake (52). Ruttimann *et al.* demonstrated that intraperitoneal rather than intravenous administration, which more accurately mimics the endogenous route of action of GLP-1, requires intact vagal afferent fibers to induce satiation (127). In addition, administrations of GLP-1 will increase electrophysiological activity of VAN *in vitro* and *in vivo* (128, 129). In addition, several studies indicate that GLP-1R gene expression is preset on VAN (129). The question remains as to whether GLP-1Rs are functional on VAN.

Gut-derived hormones interact with each other at the level of VAN in order to regulate energy homeostasis. Emerging evidence suggests that GLP-1 acts on VAN to induce its satiating effects, specifically; several studies indicate that GLP-1 interacts with several gut peptides to regulate energy homeostasis and glucose homeostasis.

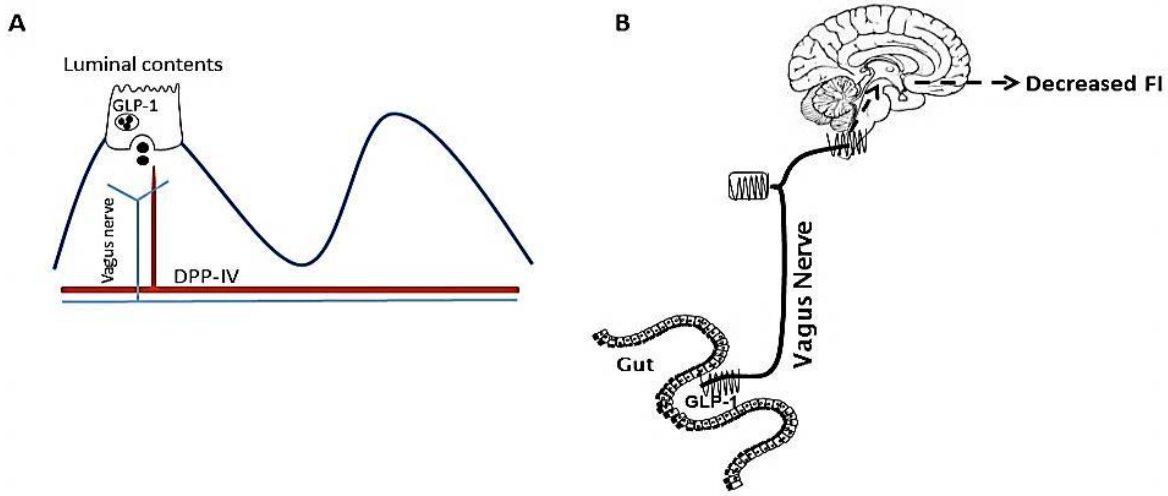

Figure 1.10 Gut-derived GLP-1 communication to the CNS

(A) In response to a meal, GLP-1 is released from L-cells into the lamina propria and enters the capillaries where it is quickly degraded into its inactive form. GLP-1 can in a paracrine manner on nearby vagal afferent fibers expressing GLP-1Rs. (B) Endogenous gut-derived GLP-1 suppresses food intake by acting in a paracrine manner on adjacent GLP-1Rs expressed on vagal afferents.

1.7.1 Evidence that Ghrelin Modulates GLP-1-induced Actions

Native ghrelin undergoes a unique post-translational acylation of the third serine residue converting it into its active form. The enzyme responsible for the acylation of ghrelin is called GOAT enzyme. Acetylated ghrelin is an endogenous ligand for GHS-R which is constitutively expressed. GHS-R is principally found in the pituitary and hypothalamus. The highest density of GOAT mRNA expression is found in gastric gastrin cells indicating a high association between ghrelin and GOAT (130). The biological functions of ghrelin are widespread; it plays a role in lipid metabolism, glucose homeostasis, and growth hormone release. Additionally, ghrelin stimulates appetite, body weight gain, and adiposity. The acylated form of ghrelin has been recognized as the major active orexigenic molecule. Circulating concentrations of acylated ghrelin do not increase with prolonged fasting, whereas deacylated ghrelin accounts for up to 90% of the majority of circulating ghrelin (131). Endogenous acylated ghrelin serves as a gastric sensor and increases appetite and food intake, which indicates that ghrelin acts as a physiologic hunger signal (132).

Plasma concentrations of ghrelin are high during fasting and robustly decrease in a postprandial state suggesting that ghrelin may be a main player in meal initiation. Exogenous ghrelin is known to stimulate food intake; central and peripheral administrations of ghrelin will increase energy consumption and body weight in rodents (133). Intravenous injections of ghrelin will stimulate appetite and food consumption in humans (134). In rats, ghrelin enhances weight gain by decreasing energy expenditure. The regulation of food intake by ghrelin is dependent on feeding status; exogenous ghrelin will stimulate food intake in *ad libitum* fed rats but not in fasted animals (135). Ghrelin acts centrally in the arcuate nucleus (ARC) of the hypothalamus, a region known to regulate feeding behavior. ICV administrations of ghrelin in the third ventricle will

increase food intake and activate c-fos expression in the hypothalamus (136). Immunohistochemical evidence has found ghrelin-expressing neurons in multiple regions of the hypothalamus. Evidence supports the notion that mRNA levels of AgRP and NPY are increased in response to an injection of ghrelin into the third ventricle (133). Given that there are central and peripheral distributions of ghrelin, several mechanisms have been proposed in which ghrelin will activate its receptor in the hypothalamus; crossing the blood brain barrier, activating vagal afferent neurons or synthesized locally. The rate at which peripheral ghrelin crosses the blood brain barrier is very low (137) suggesting that its actions are vagal mediated. Electrophysiological studies reveal that as CCK increases vagal activity, ghrelin attenuates it (45).

GHS-R is expressed in 40% of vagal afferent neurons and is colocalized with orexigenic receptors MCH-1R and CB-1R, possibly involved in food initiation (60). Lesions to vagal afferent fibers abolish ghrelin-induced feeding and c-fos expression in the ARC (45). The administration of ghrelin to fasted rats prior to refeeding prevents the downregulation of MCH and CB-1 receptors in vagal afferent neurons suggesting ghrelin mediates the expression of orexigenic receptors to induce food intake (60). Furthermore, exogenous ghrelin inhibits CCK-stimulated upregulation of CART by inhibiting phosphorylation of CREB in the nucleus of VAN (59). These studies support the idea that the stimulatory effect of ghrelin on food intake is mediated by vagal afferent neurons by decreasing CCK signaling. Studies have demonstrated that ghrelin interacts with other gut peptides to control energy balance as well as glucose homeostasis. GHS-Rs are expressed on VAN and co-express with other gut peptides such as CCK. Ghrelin interacts with numerous gut-derived peptides on VAN. For example, CB-1 and MCH expression levels decrease in a refed state; ghrelin will attenuate the decrease of expression in refed rats (58, 60). Electrophysiological studies reveal that as CCK

increases vagal activity, ghrelin attenuates it (45). Systemic infusions of ghrelin dose dependently attenuate the anorexigenic effects of GLP-1 in rats (138). Conversely, native GLP-1 infusions in humans inhibited postprandial increase in ghrelin plasma concentrations (139). In 72h fasted rats, GLP-1R activation potently reduces ghrelin plasma concentrations (110). Together, these studies indicate that there is a clear interaction between ghrelin and GLP-1 to regulate food intake, however, the exact mechanism of action is unknown.

Insulin secretion from pancreatic cells is modulated by gut peptides such as ghrelin and GLP-1. GLP-1 induces insulin secretion and ghrelin attenuates the release and increases blood glucose levels. There is evidence that there is an interaction between ghrelin and GLP-1 to regulate the insulinotrophic effects. In the pancreas, GLP-1 has been shown to counteract the endogenous and exogenous actions of ghrelin. GLP-1 stimulated glucose-induced insulin release and cAMP production in β-cells is attenuated by ghrelin. Furthermore, the presence of D-Ly^3-GHRP-6, a ghrelin receptor antagonist, markedly enhances the insulinotrophic effects of GLP-1 (140). It is well established that ghrelin has the ability to block VAN from responding to anorexigenic signals. For example, CCK increases electrophysiological activity of the vagus, whereas, ghrelin attenuates the neuronal excitation.

1.7.2 Evidence that Leptin Interacts with GLP-1 actions

Leptin is a 167-amino acid peptide mainly secreted by adipocytes and to a lesser degree from the stomach (141). Leptin is known to suppress appetite, energy intake, body weight and adiposity in humans, rodents and monkeys (131, 142, 143). Circulating levels of leptin are correlated with body adiposity. In rodents and humans, leptin signaling in the brain results in decreased energy intake and increased energy

expenditure to maintain the body fat store (131, 144). Leptin acts on leptin receptors (LepR), which are abundantly found in the hypothalamus. Leptin easily crosses the blood brain barrier through a saturable transport and acts on hypothalamic neurons; it inhibits expression of orexigenic AgRP/NPY/MCH and stimulates anorexigenic POMC/CART (145).

Studies demonstrate that peripheral acute administrations of leptin significantly inhibit food intake (143, 146). Plasma concentrations of leptin increase hours following a meal and, in humans, several days after overfeeding suggesting that leptin acts both at short term and long term on food intake. Leptin levels exhibit a circadian rhythm pattern where the highest concentrations of circulating leptin are at night (131). Leptin deficiency of ob/ob mice leads to an obesogenic phenotype (147).

Leptin has been found to enhance inhibitory effects of various anorexigenic gut hormones. For example, in VAN, CCK stimulates the expression of CART peptide, which induces its inhibitory effects. CCK in the presence of leptin will stimulate CART peptide levels at 1000 fold lowers concentrations than when CCK acts alone (59). It seems that the interaction of leptin with other gut hormones is necessary in order to induce short-term satiation. Specifically, leptin has been demonstrated to interact with GLP-1 and its receptor antagonist to induce satiation. Leptin receptors are found on endocrine L-cells and neurons secreting GLP-1 (148) and leptin was found to stimulate GLP-1 release in L-cells. In brain centers, LepR were found in GLP-1R-expressing neurons in the NTS and leptin was found to stimulate these neurons (149, 150). Food deprivation decreases leptin plasma levels concurrently with GLP-1 expression in the hypothalamus and it is possible that GLP-1 released from leptin-stimulated neurons modulates hypothalamic brain centers involved in appetite. Peripheral sites of mechanism of action of leptin interaction with GLP-1 seem to play an important role in modulating appetite. Peripheral

blockade of GLP-1R will attenuate leptin-induced satiation and body weight gain in rats (151). GLP-1 inhibitory effects are abolished in leptin deficient rats. One particular study found that leptin alone had no effect on food intake in normal rats; however, together with Ex-4 and GLP-1 significantly potentiates the anorexigenic effect of leptin. Furthermore, the inhibitory actions of native GLP-1 and Ex-4 are attenuated in fasted rats; however, pretreatment of leptin restored the satiating effects of GLP-1 and Ex-4 (113).

1.7.3 Summary

In summary, it is clear that GLP-1 interacts with other gut-derived hormones to control food intake. Studies have demonstrated that gut peptides act synergistically to regulate energy homeostasis and specifically these actions are mediated on the vagus nerve. Whether the satiating effect of GLP-1 is mediated by another gut peptide remains to be answered. In addition, whether these actions take place at the level of the vagus is also unclear. These questions have been elucidated in this dissertation.

The response of CCK is blunted in leptin resistant models. Furthermore, diet-induced obesity is associated with an absence of phenotypic change induced by feeding, the mechanism by which this occurs is unclear, however, may involve leptin resistance on VAN. In this second part of this chapter, we have focused on understanding the literature surrounding leptin resistance. Differently from GLP-1, leptin is a long-term regulator of food intake and evidence demonstrates that leptin receptors are present on VAN. Understanding the mechanisms underlying leptin resistance induced by obesity is necessary.

1.8 The role of leptin in obesity

Leptin is derived from the *lep* gene which transcribes to a 167-amino acid peptide. It is mainly expressed in many tissues such as the pituitary gland, skeletal muscle, and gastrointestinal tract, but its highest expression is in white adipose tissue (33). Circulating leptin concentrations are correlated with body fat. Leptin expression fluctuates according to circadian rhythm and nutritional status. Fasting decreases leptin expression, where as feeding increases leptin levels thus indicating the importance of leptin for energy homeostasis. Leptin exerts its effects through specific leptin receptors (LepR) located throughout the CNS. Six leptin receptor isoforms have been identified; the long isoform of LepR is primarily responsible for its anorexigenic effects (33, 34).

Many central circuits have been implicated in leptin signaling however; the ARC in the hypothalamus is the main site of action for leptin. Leptin acts on LepR-expressing neurons in the ARC to control food intake and differentially regulate the expression of orexgenic AgRP/NPY and POMC/CART according to nutritional status (Figure 1.11). In a fed state when leptin levels are high, leptin stimulates POMC and CART and inhibits neurons that synthesize AgRP and NPY. Conversely, in a fasted state, suppression of leptin levels decrease the expression of POMC and CART and increase AgRP and NPY expression, thus stimulating food intake and decreasing energy expenditure. The ARC is required for leptin-induced anorexia as ARC-lesioned ob/ob mice are irresponsive to central infusions of leptin (152). Deficiency in leptin signaling leads to altered expression hypothalamic neuropeptides. For example, ob/ob mice have increased levels of orexigenic AgRP expression and decreased anorexigenic POMC expression (153, 154). In mice lacking LepR in POMC neurons are mildly obese, have hyperleptinemia and surprisingly have decreased orexigenic AgRP and NPY mRNA levels (155). Leptin

signaling in the ARC induces activation of higher ordered brain regions such as the lateral hypothalamus and the ventral tegmental area to induce its effects.

Leptin resistance is the inability of obese individuals or diet-induced obese models to respond to exogenous and endogenous leptin. In most models of obesity, leptin levels are elevated indicating the importance of leptin resistance in the pathogenesis of obesity. Leptin replacement restores energy homeostasis in *ob/ob* mice but not *db/db* mice which have a mutation of the LepR (36). Similarly, in humans, obese individuals have increased fat mass and elevated leptin concentrations.

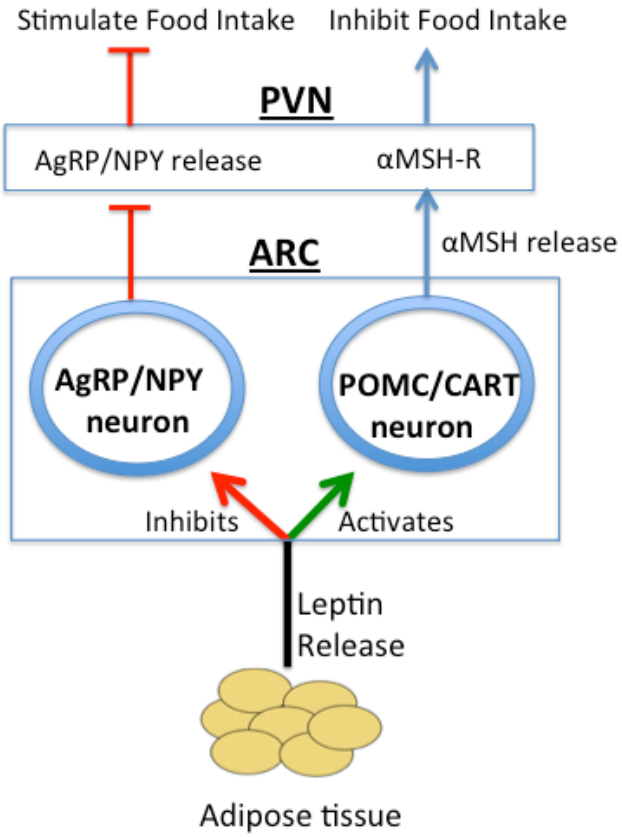

Figure 1.11 Anorexigenic effects of leptin in the hypothalamus

When leptin levels are high, leptin stimulates anorexigenic POMC neurons and inhibits orexigenic NPY and AgrP neurons in the arcuate nucleus. POMC/CART neurons stimulate αMSH release which binds to its receptors in the paraventicular nucleus and inhibits food intake.

The vast majority of obese individuals are insensitive to endogenous hyperleptinemia or leptin treatment (131). Likewise, high fat diet induced obesity in mice leads to hyperleptinemia and hyperphagia (156). Administrations of leptin induce the STAT-3 pathway, which is required to induce the satiating effects of leptin. Diet induced leptin resistance attenuates leptin-induced phosphorylation of STAT-3 in hypothalamic extracts (157). Furthermore, *in vivo*, diet induced obese mice have impaired leptin signaling in the hypothalamus (37).

LepR is expressed on the vagus nerve in the nodose ganglion. Leptin signaling on VAN has been demonstrated to play an important role in regulating energy homeostasis. Leptin increases electrophysiological activity of VAN and increases calcium release in culture (158). We have demonstrated that leptin resistance in VAN leads to an obese phenotype; there was a significant increase in body weight and food intake in parallel with a decrease in phosphorylation of STAT-3 in response to leptin, a marker of leptin signaling (159), on VAN in diet induced obese rats compared to control (2). Much attention has focused on leptin resistance in the ARC, as described above. However, in rodent models of diet-induced obesity, leptin resistance in arcuate neurons does not develop until after food intake, body weight and adiposity increase, calling into question whether leptin resistance in hypothalamic neurons drives the initial hyperphagia and obesity.

1.8.1 Summary

The discovery of leptin has highlighted that obesity is a physiological disorder. In the last few decades, much research has focused its attention on understanding the role of leptin signaling in obese individuals. Progress has been made in identifying the role and function of leptin, however, many studies are limited to the male gender. Despite the

fact that there are clear differences in energy homeostasis between male and female, there are limited studies that have explored this. In this next part of this chapter, we explore the physiological sex dimorphism in the regulation of food intake behavior and energy balance.

1.9 Sex differences influence energy homeostasis

In the United States, women are approximately twofold more susceptible to severe or morbid obesity than males (160). Moreover, burdens of the disease affect women to a greater degree than males (161, 162) and approximately 80% of bariatric surgery patients are female (163). Despite these observations, there is a strong male bias that exists in basic animal research, even in the field of obesity. Thus there is a proven need to investigate and determine the mechanisms of sex differences in the regulation of energy homeostasis.

It is well established that gonadal hormones influence feeding behavior and energy homeostasis. Fluctuations in daily energy consumption are correlated to varying concentrations of estrogen secretion across the ovarian cycle in women (164). Energy intake decreases during the estrus cycle when estradiol concentrations are high. There is evidence that during post-menopause, women tend to increase body weight and adiposity. Ovariectomy in (OVX) rats, results in rapid increases in food intake leading to increased adiposity, principally visceral fat, and body weight gain; these parameters can be normalized by treatment with exogenous estrogen (164).

The mechanisms by which estrogen may influence food intake and body weight are not clear. There is evidence for direct effects of estrogen at the level of the hypothalamus as well as the caudal hindbrain. Ovariectomy decreased hypothalamic POMC expression and α-melanocyte-stimulating hormone (αMSH) concentrations in the

ARC; levels of both are restored by estradiol treatment (165). Estradiol treatment increases excitatory synapses on POMC neurons followed by a decrease energy consumption and body weight (165). Specific knockdown of estrogen receptor (ER) in POMC neurons leads to hyperphagia (166). *Ex vivo* data have demonstrated that an administration of estradiol will induce the release αMSH (167).

Studies have also demonstrated that estrogen and leptin act similarly to regulate energy homeostasis (168). For example, central infusions of either leptin or estrogen will reduce food intake and body weight in rats (169, 170). Estrogen receptors are colocalized with leptin receptors in the hypothalamus and have similar effects on food intake following direct injection into the hypothalamus (171). Leptin and estrogen both result in increased POMC neuronal tone and excitatory inputs to the ARC (165). Moreover, the anorexigenic effect of estrogen is conserved in mice lacking leptin or its receptor (172). Recently it has been demonstrated that estradiol treatments to the hypothalamus of obese mice increases the release of αMSH despite POMC neurons being leptin resistant indicating that estrogen may act through a leptin-independent mechanism to influence food intake (167). Indeed, estrogen receptors induce the STAT-3 pathway independent of LepR-mediated STAT-3 activation (165).

Although estrogen plays an important role in maintaining energy homeostasis, testosterone has also been shown to modulate food intake and body weight. For example, orchidectomized rats results in decreased food intake and concomitant body weight, both of which are attenuated by exogenous testosterone treatment (170). Interestingly, orchiectomy decreased dark-phase meal number but increased light-phase meal size; therefore daily food intake remained unchanged compared to intact males (173). Although androgen receptor KO mice eat similarly to control littermates, androgen receptor KO mice have increased adiposity (174). Altogether, these studies

demonstrate that sex hormones can influence energy homeostasis whether it be estrogen or testosterone.

Humans display important sexual dimorphism in terms of body composition in fat, lean and bone mass. Boys are usually heavier than girls at birth which persists throughout life due to increased lean body mass (175). Males have significantly less adiposity than females and there are difference in the distribution of fat between different depots in males and females tend to have more visceral fat and females tend to have more subcutaneous fat (170). There is evidence to suggest the involvement of sex hormones in the regulation of adipose tissue in rodents and humans. In rats, chronic treatment of estrogen to intact males will redistribute fat pads to resemble those of females (170). In estrogen-reduced models, fat accumulation redistributes viscerally and estrogen treatment reverts body fat back to to subcutaneous depots (176).

There is very little research that compares male and female differences in regard to eating patterns. The total caloric intake over 24h is significantly increased in males compared to females; this increase in overall daily food intake in male mice results from more frequent meals (164). Ovariectomy will increase food intake by increasing meal size (177); in contrast, orchiectomy will decrease daily food intake by decreasing meal frequency (173).

Interestingly, males and females respond differently to gut-derived signals. For example, both peripheral and central administration of ghrelin increased food intake significantly more in intact males and OVX rats compared to intact rats or OVX-estradiol treated rats (170). Furthermore, plasma ghrelin concentrations increase after an ovariectomy (178). Asarian *et al.* demonstrated that estradiol increases the anorexigenic effect of endogenous GLP-1 and CCK in rats that had undergone Roux-en-Y gastric bypass surgery (179). Clegg *et al.* have demonstrated that the satiating effect of central

leptin differs between the sexes; exogenous central leptin has a greater inhibitory effect on females than males (178). Estrogen levels in females will alter the sensitivity to central leptin. For example, OVX rats do not respond to leptin, however, peripheral or central estradiol treatment to OVX rats restores their central leptin sensitivity and changes their body fat distribution (170). Furthermore, males that received chronic estradiol treatment responded to a lower concentration of leptin and increased their subcutaneous fat compared to their controls (170).

It is evident that there are sexual differences in mechanism regulating food intake and body weight. Mechanisms that drive differences between male and females in regulating food intake are unclear. There is a need to understand how gut-brain signaling is different between the sexes. We plan to fill this gap in our knowledge by individually investigating the intracellular signaling pathways responsible for energy homeostasis in male and female. Understanding the role of gut peptides and how their signaling alters in the sexes to regulate food intake is necessary in order to develop a non-invasive treatment.

1.10 Conclusion

The interaction between the gastrointestinal tract, vagus nerve and the CNS are necessary to process and simulate available nutrients. Most hormones and their receptors are expressed on the vagus nerve and communicate to higher order neurons in the CNS influencing food intake behavior. Impaired signaling from the periphery to the CNS leads to hyperphagia and obesity. Gut-derived hormones play an important role in regulating energy homeostasis however, the mechanism of action of anorexigenic peptides on VAN is less clear. In addition, the alteration of satiety signaling on the gut-brain axis in response to high fat feeding is less understood.

In the last decade, much progress has been made in identifying the roles, functions, and synergistic actions of gut hormones essential for nutrient sensing, as they coordinate orexigenic and anorectic signaling for food intake control via endocrine, vagal and central processes. However, the redundancy of signaling pathways of satiety signaling makes it difficult to isolate their primary functions and elucidate insight into the mechanisms.

Food intake regulation requires multiple systems to communicate and act in a coordinated manner. The gut-brain axis is necessary to regulate energy homeostasis. The identification of hormone function and the interactions between them is constantly being extended. It is clear that gut hormones work synergistically to regulate food intake. Isolating the mechanisms and interactions between gut-derived hormones is a step towards developing non-invasive anti-obesity treatments.

1.11 Objectives of Dissertation

This dissertation is aimed to determine the molecular pathways of anorexigenic peptides, particularly GLP-1 and leptin, on VAN involved in promoting satiety. In the first

part, we investigated the mechanism of action of peripheral native GLP-1 along vagal afferent pathway (Chapter 2.1). Furthermore, we identified mediators regulating GLP-1-induced satiation (Chapter 2.2). As a second study, we aimed to study the effect of leptin receptor knockdown specifically on vagal afferent neurons to understand the initiating drivers of hyperphagia and obesity (Chapter 2.3). Moreover, we compare the effect of the lack of leptin signaling on VAN in male and female mice to understand the physiological differences between genders (Chapter 2.4).

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Chapter 2: Experimental Chapter on investigating the mechanism of action of gut-derived peptides on vagal afferent neurons

Chapter 2.1: Ability of Glucagon like Peptide-1 to Decrease Food Intake is Dependent on Nutritional Status

(Manuscript published in Physiology and Behavior in August 2014)

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Running title: GLP-1 and inhibition of food intake

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2.1.1 Abstract

Gut-derived glucagon like peptide-1 (GLP-1) acts in the postprandial period to stimulate insulin secretion and inhibit gastrointestinal motor and secretory function; whether endogenous peripheral GLP-1 inhibits food intake is less clear. We hypothesized that GLP-1 inhibits food intake in the fed, but not fasted, state. There is evidence that GLP-1 acts via stimulation of vagal afferent neurons (VAN); we further hypothesized that the satiating effects of endogenous GLP-1 in the postprandial period is determined either by a change in GLP-1 receptor (GLP-1R) expression or localization to different cellular compartments in VAN. **METHODS:** Food intake was recorded following administration of GLP-1 (50 µg/kg or 100µg/kg) or saline (IP) in Wistar rats fasted for 18h or fasted then re-fed with 3g chow. GLP-1R protein expression and localization on VAN was determined by immunocytochemistry and immunoblots in animals fasted for 18h or fasted then re-fed for 40mins. GLP-1R mRNA level was detected in animals fasted for 18h or fasted and re-fed *ad libitum* for 2h. **RESULTS:** GLP-1 (100µg/kg) significantly reduced 40 min food intake by 38% in re-fed but not fasted rats ($p<0.05$). GLP-1R mRNA or protein levels in VAN were unchanged in re-fed compared to fasted rats. However, GLP-1R localization to the plasma membrane was significantly increased in VAN by feeding. **CONCLUSION:** Feeding changes the ability of peripheral GLP-1 to inhibit food intake. GLP-1Rs are trafficked to the plasma membrane in response to a meal. GLP-1 may play a role in regulating food intake in the postprandial period.

Key words: Glucagon-like-peptide-1, vagal afferent neurons, food intake, receptor trafficking

2.1.2 Highlights

- The peripheral native satiating effect of GLP-1 is dependant on feeding status
- Direct immunohistochemical evidence that GLP-1Rs are present on vagal afferent neurons
- GLP-1Rs are constitutively expressed; the cellular location of GLP-1Rs changes according to feeding status on VAN

2.1.3 Introduction

Considerable interest is focused on the mechanisms by which signals from the gastrointestinal (GI) tract influence food intake and play a role in the maintenance of body weight. The GI tract is the primary epithelial surface involved in nutrient sensing, resulting in the release of multiple peptide hormones that can influence food intake (1). The vagus nerve provides the major neuronal link between the GI tract and the central nervous system (CNS). Vagal afferent neurons (VAN) transmit information to terminals in the brainstem, resulting in activation of parasympathetic reflexes that regulate GI and pancreatic function, and activation of higher order neurons to change feeding behavior.

Glucagon-like-peptide-1 (GLP-1) is a gut hormone derived from the pre-proglucagon gene synthesized and released by intestinal L-cells found along the length of the small intestine and proximal colon (2). The insulinotropic action of GLP-1 is well established; GLP-1 directly stimulates insulin release from pancreatic β cells. GLP-1 receptor (GLP-1R) knockout mice exhibit perturbations in fasting glucose levels, glucose-stimulated insulin secretion and β-cell signal transduction (3, 4). The role of GLP-1 in regulating glucose homeostasis appears to be physiological; however, its role in feeding is less clear.

GLP-1 secretion is low in a fasting state and increases rapidly postprandially, especially with meals containing fats and carbohydrates (5). Exogenous administration of GLP-1 has been shown to inhibit food intake in humans; diabetic patients treated with GLP-1, or its stable receptor agonist, progressively lose weight (6). Intraperitoneal (IP) administration of GLP-1 decreases food intake dose-dependently in rodents (7), although this is not found consistently across studies. GLP-1 is rapidly degraded in the extracellular space; this may account for the variability in the biological activity of the native peptide to influence feeding behavior. Continuous infusion of GLP-1 either into the

hepatic portal vein or intraperitoneally in rats is effective at decreasing overall food intake through reductions in meal size and duration (8, 9). Other studies have used the stable synthetic GLP-1 analog, exendin 4 (Ex-4), to study the effects of GLP-1. However, there is good evidence that Ex-4 can access the central nervous system and activate discrete central pathways to inhibit food intake, thus these studies do not assist in understanding the role of peripheral GLP-1. There is some evidence that the ability of native GLP-1 to inhibit food intake only occurs in the fed and not the fasted state (7, 10). GLP-1 inconsistently decreases food intake when administered intraperitoneally; for example, Neary *et al*. found no difference in food intake in 20hr fasted mice following a low doses of GLP-1 compared to vehicle (11).

Under physiological conditions, because of the short plasma half-life of GLP-1 (12), it is likely that it acts locally within the intestinal wall to influence food intake. VANs are a common site of action for gut-derived satiating signals. There are reports that VAN express mRNA for GLP-1R (13) and systemic administration of GLP-1 increased electrophysiological activity in neurons of the nodose ganglion (14). Furthermore, subdiaphragmatic deafferentation attenuated the ability of peripheral GLP-1 to decrease food intake in humans (8).

Unlike other gut hormones regulating food intake, the anorexigenic effect of peripheral GLP-1 may be dependent on nutritional status with one report suggesting that exogenous GLP-1 induced satiation in freely fed rats but had no effect in fasted animals (7). Previous work has shown that VAN change their neurochemical phenotype accordingly to nutritional status (15). We hypothesized that GLP-1 inhibits food intake in the fed, but not fasted, state. Additionally, we hypothesized that the expression level of GLP-1Rs change according to feeding status, and this change in receptor expression determines the ability of GLP-1 to inhibit food intake.

2.1.4 Methods

Animals

Animals were maintained and handled in accordance with policies of the Institutional Animal Care and Use Committee at the University of California in Davis. Male Wistar rats (initial weight 200g; Harlan, San Diego) were individually housed at 22°C under a shifted 12h light-dark cycle (light 11pm-11am) with *ad libitum* access to food and water. Rats were fed a regular chow diet (Lab Diet 5001). Animals were handled for 7 days prior to experiments and habituated to the new environment and to intraperitoneal (IP) injections of 400µl physiological saline prior to food intake studies.

Feeding Studies

In all experiments, rats were fasted on wire bottom cages (6pm-11am) with *ad libitum* access to water (n=8 in each group). Experiments were performed at the start of the dark phase when the animals have the strongest drive to eat. GLP-1 7-36 amide was obtained by Bachem Bioscience Inc (King of Prussia, PA) and reconstituted in 0.9% saline. GLP-1 (50µg/kg or 100µg/kg IP) or 400µl physiological saline (IP) were administered in two different protocols. The higher dose of GLP-1 was chosen based on Williams *et al.* (7) and the lower dose was chosen as a subthreshold dose. In the first protocol, saline or GLP-1 (50µg/kg or 100µg/kg, IP) were administered at the start of dark phase (11am) after an overnight fast (6pm-11am) and food was immediately returned to the cage. Food weight and spill was recorded every 20 minutes for 2 h. In the second protocol, 40 minutes before the onset of the dark phase (11am), a small premeal (3g) was given to overnight fasted rats (6pm-10:20am). Saline or GLP-1 (50µg/kg or 100µg/kg, IP) was administered at the start of dark phase (11am) and food was immediately returned to the cage. Food weight and spill was recorded every 20

minutes for 2 h. In the third protocol, overnight fasted animals were given an *ad libitum* premeal for 40mins before the onset of the dark phase (11am) to overnight fasted rats (6pm-10:20am). Saline or GLP-1 (100µg/kg, IP) was administered at the start of dark phase (11am) and food was immediately returned to the cage. Food weight and spill was recorded every 20 minutes for 2 h (Figure 2.1.1). Food intake was determined by measuring the difference between the baseline and the weight of the food and spill every 20 minutes for 2 h. A within subject study design was used for these experiments; each animal received both saline and GLP-1 and was compared for statistical analysis. Food intake following administration of saline in one rat was statistically an outlier and was removed from the study. Two way ANOVA repeated measures was used to compare groups over 2 h and unpaired Student t-test was used for 40 min food intake.

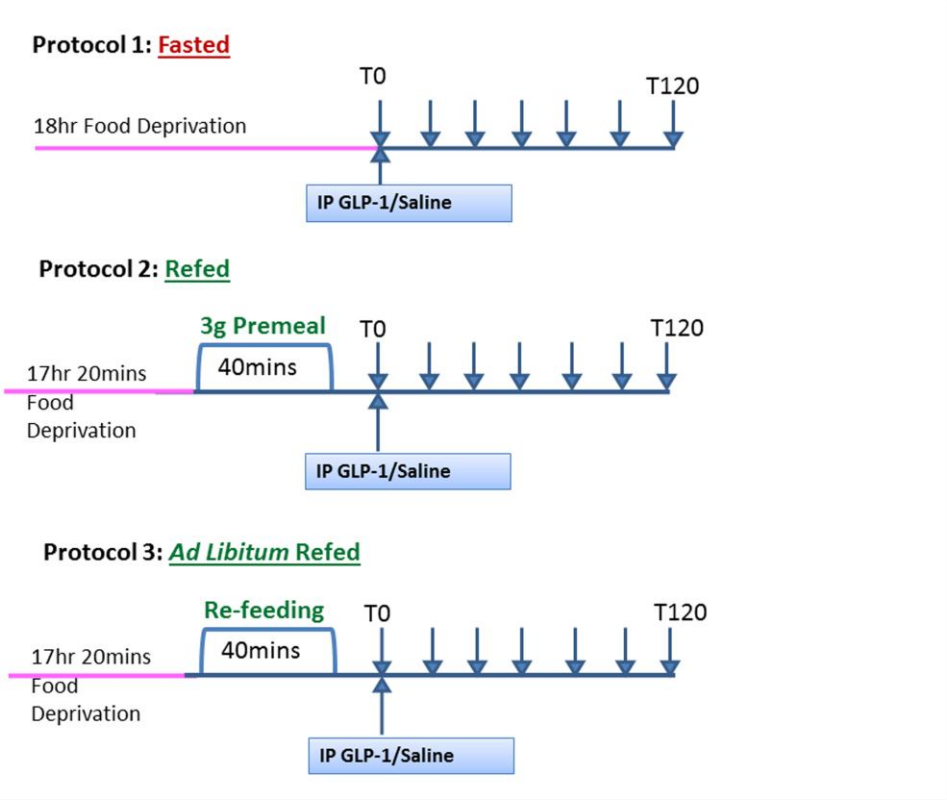

Figure 2.1.1 Protocol – Investigating whether GLP-1-induced satiation is dependant on feeding status.

Three protocols were used to investigate whether GLP-1-induced satiation is dependent on feeding status. In protocol 1, animals were fasted for 18h, and then received an administration of 100µg/kg GLP-1 or saline IP. In protocol 2, animals were fasted for 17h 20mins, and then received a 3g premeal for 40mins prior to receiving 100µg/kg GLP-1 or saline IP. In protocol 3, animals were fasted for 17h 20mins, and then received an ad libitum premeal for 40mins prior to receiving 100µg/kg GLP-1 or saline IP. Food was immediately returned to the cages after each administration and food intake was measured every 20mins for 2h.

Tissue collection

Rats were euthanized by CO_2 inhalation at least 1 h into the dark cycle and nodose ganglia was rapidly removed and post-fixed for 2 h in 4% paraformaldehyde in PBS, followed by 25% sucrose in PBS overnight at 4°C. The quantification of the total number of GLP-1R-expressing neurons on nodose ganglion sections were collected from *ad libitum* fed animals. For quantification (n=3) and localization (n=4) of GLP-1R protein, nodose ganglia were collected from rats fasted overnight or fasted overnight followed by 40 min re-fed; this time point was identified as the maximal change in food intake in response to administration of GLP-1. For mRNA analysis, nodose ganglia was collected from rats fasted overnight or fasted overnight and then re-fed for 2 h (n=5).

Immunohistochemistry

Cryostat sections of fixed nodose ganglia (9µm) were mounted onto Superfrost/Plus Slides (Fisher Scientific, Pittsburgh, PA) and processed for immunohistochemistry. Sections were blocked with 20% donkey serum (DS) (Vector Laboratories, Burlingame, CA), 0.2% Triton-X100 and 0.1% bovine serum albumin dissolved in PBS for 30mins at 37°C. Sections were incubated with an antibody raised against GLP-1R (Abcam 39072, Cambridge, MA) diluted at 1:1000 and plasma membrane marker, pan cadherin (Abcam 6528, Cambridge, MA) diluted at 1:100 in DS-PBS (2% donkey serum, 0.2% triton X100 and 0.1% bovine serum albumin dissolved in PBS) for 1 h at room temperature and then overnight at 4°C. The specificity of GLP-1R was verified in two different ways; immunostaining of kidney samples as a positive control and liver samples as a negative control and secondly by using GLP-1R immunogen blocking peptide (Abcam 39071, manufacturer's protocol was followed).

Additionally, the specificity of the GLP-1R antibody was verified by staining a pancreatic β-cell line (MIN6) which express a large quantity of GLP-1Rs (16). The primary antibody was omitted on MIN6 cells to omit the possibility of autofluorscence. Secondary antibody used was donkey anti-rabbit IgG conjugated with AlexaFluor 488 (Life technologies, Grand Island, NY) diluted at 1:1000 and donkey anti-mouse IgG conjugated with AlexaFluor 546 (Life technologies, Grand Island, NY) diluted at 1:500 in DS-PBS. Sections were incubated for 1 h at 37°C. Images were collected using an Olympus spinning disc confocal microscope (BX61 System, Olympus, Melville, NY). All images were collected using the same confocal parameters.

To determine the percentage of GLP-1R expression on VAN under fasted and refed conditions, images were taken at 40X magnification. The quantification of positive immunoreactivity for GLP-1R was analyzed by Scion Image software (Beta 4.0.2, Scion Corp., Frederick, MD, USA, 2000) and presented as percentage of positive neurons and pixels. The percentage of positive neurons and pixels were quantified by determining the total number of neurons and pixels and the total number of positive neurons and pixels within an area. Positive neurons and pixels were defined as the immunofluorescence with a gain above the threshold for background staining. The data were expressed as percentage of positive neurons, and positive pixels.

Photomicrographs were taken to determine the distribution of GLP-1R in refed and fasted rats at 60X magnification. We quantified the amount of receptor present on the membrane. This was quantified by determining the total number of pixels within an area and the total number of marked pixels. Positive pixels were defined as the immunofluorescence with a gain above the threshold for background staining. We used pan cadherin to mark the plasma membrane. We defined the plasma membrane and the cytoplasmic area using Photoshop (Adobe systems) and quantified pixels with Scion.

The plasma membrane for each neuron was outlined just outside the plasma membrane and also on the inside edge of the plasma membrane where positive pixels for pan cadherin were present. The cytoplasmic area was defined as the remaining region of the cell from the inside edge of the plasma membrane. The data is expressed as a percentage of the number of positive pixels for GLP-1R present in the plasma membrane over the total number of positive pixels on the whole cell.

Western Blots

Tissue was prepared as described by Freeman *et al.,* 2006 (17). Briefly, 5µg of protein was loaded onto the gel. Samples were loaded into precast 10% BisTris-gel and ran for 50 minutes at 200V (Invitrogen Power Case 500). The proteins were transferred on a PDVF membrane for 1 h at 30V. Membrane was blocked using 10% milk in TBST for 1 h at room temperature. GLP-1R was diluted at 1:1000, and GADPH (14C10, rabbit mAB; Cell Signaling Technology) was used as a loading control. The specificity of GLP-1R was verified in two different ways; 1) immunostaining of lung and hindbrain samples and MIN6 cells as a positive control and liver, spleen and testes samples as a negative control and 2) GLP-1R immunogen blocking peptide. Primary antibodies were applied on the membrane and developed on separate but consecutive days. Antibodies were incubated for 1 h at room temperature and then overnight at 4°C. The membrane was imaged using ECL substrate (Thermo Scientific) and with ChemiDoc XRS Imager (BioRad, Hercules, CA). The membrane was analyzed by Image Lab version 5.0 software (Hercules, CA).

MIN6 cell culture

MIN6 cells were a gift from Dr. Fawaz Haj (University of California Davis, Davis, CA). The cells between passage #22-26 were maintained in DMEM containing 25mM glucose supplemented with 15% heat-inactivated fetal bovine serum and 1% of antibiotic-antimycotic (100X) in humidified 5% CO_2, 95% air at 37°C. Media was changed every 48 hours and cells were passaged at 80% confluency. Cells that reached 80-90% confluency were used in experiments.

Real Time PCR

Vagus nerve samples were homogenized in a GenoGrinder2000 (SpexCertiprep). Total RNA was extracted from the tissue lysates using a 6100 automated nucleic acid (ANA) workstation (Applied Biosystems) according to the manufacturer's instructions. RNA concentrations were determined via absorbance at 260/280 nm using a NanoDrop (ThermoFisher Sciencific, Wilmington, D). The QuantiTect Reverse transcription kit (Qiagen) was used for cDNA synthesis following the manufactures directions. Taqman gene expression assay was used consisting of fluorescent labeled TaqMan probe (5´ end, reporter dye FAM (6-carboxyflourescein), 3´ end, quencher dye MGB (Minor Grove Binding Protein)) and primers for rGCG (Rn00562293_m1) and rGAPDH (Rn99999916_s1) from Applied Biosystems (Foster City, CA). Primers were validated using defined protocols (18). Each qPCR reaction contained 20x primer and probes for the respective qPCR system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available qPCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) and 5 μl of the diluted cDNA sample in a final volume of 12 μl. The samples were placed in 384 well plates and amplified in an automated fluorometer (ABI PRISM

7900 HTA FAST, ABI). ABI's standard amplification conditions were used: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Final quantitation was done using the comparative CT method (User Bulletin #2, Applied Biosystems). GAPDH was used to normalize expression of the target genes. Data is reported as the n-fold difference ($2^{-\Delta\Delta Ct}$) relative to a calibrator cDNA (the average of the control group). The sample expression levels show relative amounts found in the average fasted nodose ganglia expression level.

Statistical Analysis

Results are expressed as mean ± SEM. All studies were analyzed by using unpaired Student t-test or two way ANOVA repeated measures to validate statistical significance unless otherwise stated.

2.1.5 Results

Administration of GLP-1 inhibits food intake in re-fed, but not fasted, rats

In overnight fasted rats, peripheral administration of GLP-1 (50µg/kg or 100µg/kg IP) failed to reduce food intake over 2 h ($p>0.05$; Fig 2.1.2A). However, in re-fed rats, administration of GLP-1 (100µg/kg, IP) significantly decreased food intake compared to saline ($p<0.05$; Fig 2.1.2B). There was no difference in food intake following administration of GLP-1 (50µg/kg) in either fasted or re-fed rats (Fig 2.1.2A and B). GLP-1 significantly decreased food intake for 100 mins after administration in refed rats ($p<0.01$; Fig. 2.1.2B). Food intake was significantly reduced (39%) 40 min after administration of GLP-1 (100µg/kg) compared to saline treatment in re-fed rats ($p<0.01$; Fig. 2.1.3A and B).

To investigate whether GLP-1 has an effect in satiated animals, we tested the effects of GLP-1 when overnight fasted rats received an *ad libitum* premeal rather than a 3g premeal. Peripheral GLP-1 significantly decreased food intake over 2 hours (Fig 2.1.4A, $p<0.001$) or at 40mins (Fig 2.1.4B, $p<0.001$) in rats that received an *ad libitum* premeal prior to GLP-1 compared saline.

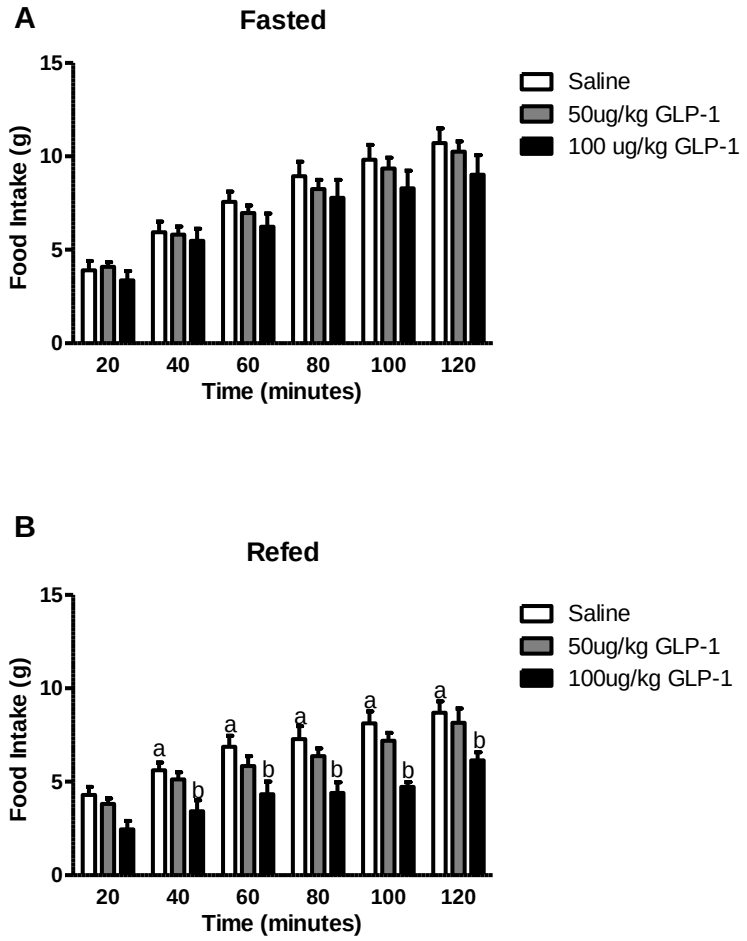

Figure 2.1.2 Administration of GLP-1 in fasted and refed animals

Cumulative food intake was measured in animals either fasted overnight or fasted then refed a 3g premeal prior to administration of GLP-1 (50µg/kg or 100µg/kg, IP) (A) GLP-1 had no effect on food intake in fasted rats; (B) 100µg/kg of GLP-1 significantly decreased food intake after 40mins and continued for 120mins in rats fasted then refed prior to administration of GLP-1 (saline vs. 100µg/kg, $P<0.05$; n=7-8 in each group). Data are represented as mean ± SEM. Statistical differences were measured using two-way ANOVA and letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b.

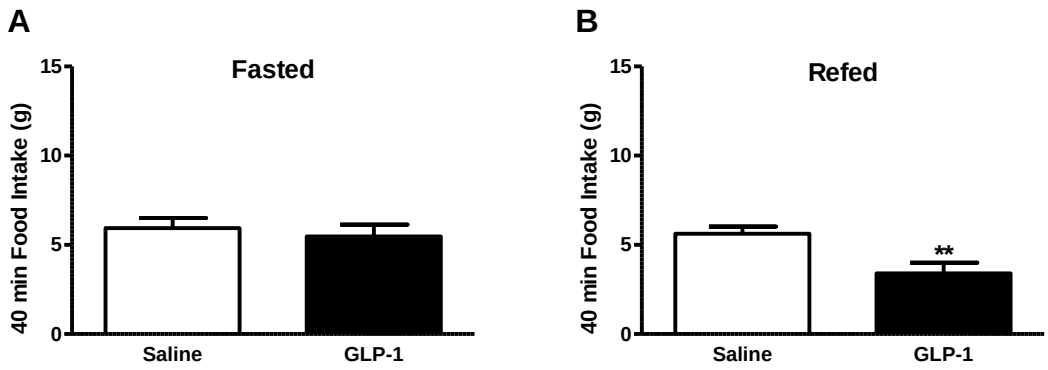

Figure 2.1.3 Effects of GLP-1 on 40min food intake in fasted and refed animals

Food intake was measured in animals either fasted overnight or fasted then refed a 3g premeal prior to administration of GLP-1 (100µg/kg, IP) (A) GLP-1 had no effect on food intake in overnight fasted rats however; (D) the anorexigenic effect of GLP-1 was significant at 40-min in refed rats (saline vs. 100µg/kg GLP-1, $P<0.05$, $n=7$ in each group). Data are represented as mean ± SEM. Statistical differences were measured unpaired Student t-test; $^{**}p<0.01$.

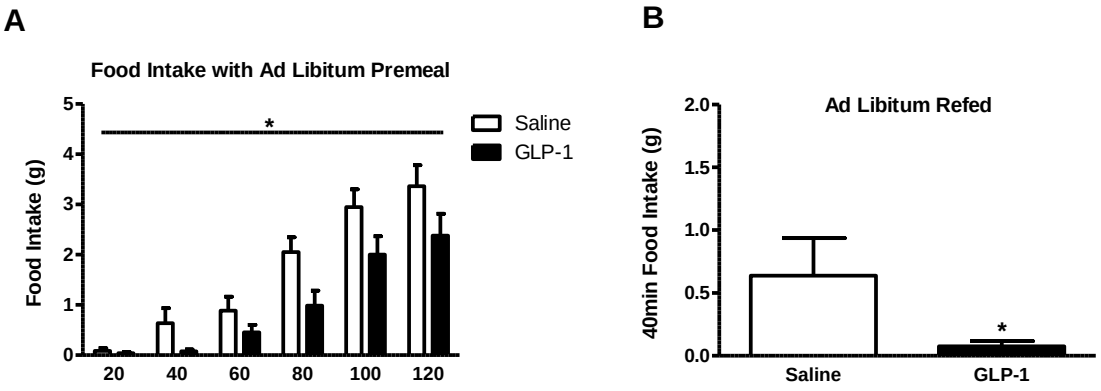

Figure 2.1.4 Administration of GLP-1 in *ad libitum* refed animals

Food intake was measured in animals either fasted overnight or fasted then refed prior to administration of GLP-1 (100µg/kg, IP) (A) GLP-1 significantly decreased food intake over 2 hours when animals were fasted then refed an *ad libitum* premeal prior to an administration of GLP-1; (B) GLP-1-induced satiation was greatest at 40mins (n=8). Data are represented as mean ± SEM. Statistical differences were measured using two-way ANOVA and unpaired Student t-test; *p <0.05 and **p<0.01.

Vagal afferent neurons express GLP-1R

Initial experiments were aimed at characterizing the GLP-1R antibody. Sections of a kidney (positive control) and liver (negative control) (19) were used to verify the specificity of a recently available GLP-1R antibody (Fig 2.1.5A). Kidney sections expressed positive immunoreactivity for GLP-1R, however, there was no immunoreactivity detected in liver. Pre-incubation of the GLP-1R with the immunogenic peptide (1:1 ratio to antibody, 1µg/ml) abolished immunoreactivity in tissue sections of kidney (Fig 2.1.5B). Additionally, immunoreactivity for GLP-1R was verified in the pancreatic cell line, MIN-6, known to express GLP-1Rs (20); cells had positive immunoreactivity for GLP-1R (Fig 2.1.6) and immunoreactivity was abolished with preincubation of GLP-1R with the immunogenic peptide and when the primary antibody was omitted (Fig 2.1.6).

The specificity of the GLP-1R antibody was also verified using immunoblots (Fig 2.1.7A-C). An immunoreactive band was present at 53kda in lung and hindbrain tissue (Fig 2.1.7A) and in MIN6 cells (Fig 2.1.7C); no bands were detected in liver, spleen, or testes tissue (Fig 2.1.7B). Further, preincubation with the immunogenic peptide abolished immunoreactivity of the 53kDa band from lung and hindbrain (Fig 2.1.7A). Taken together these data confirm the specificity of the antibody for GLP-1R in rat tissue.

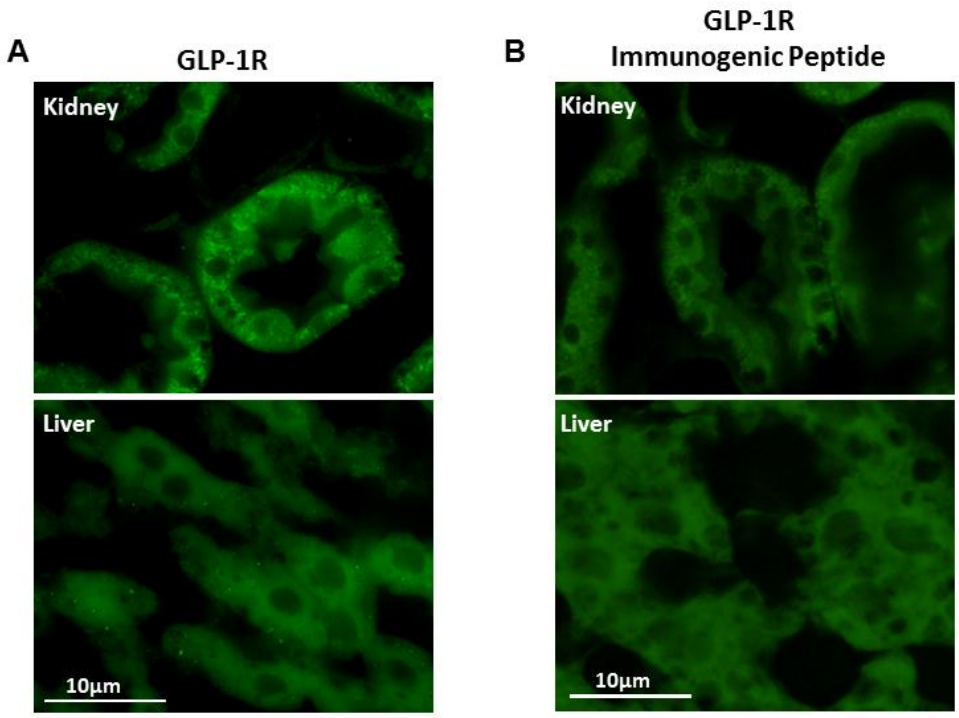

Figure 2.1.5 Specificity of GLP-1R antibody on tissue sections

The specificity of the GLP-1R antibody was verified using positive (kidney and lung tissue) and negative control (liver tissue). (A) GLP-1R immunoreactivity was present in section of kidney but not liver; (B) Pre-incubation of the immunogenic peptide abolished immunoreactivity of the kidney. All photomicrographs pictures were taken under the same conditions and at 40X magnification.

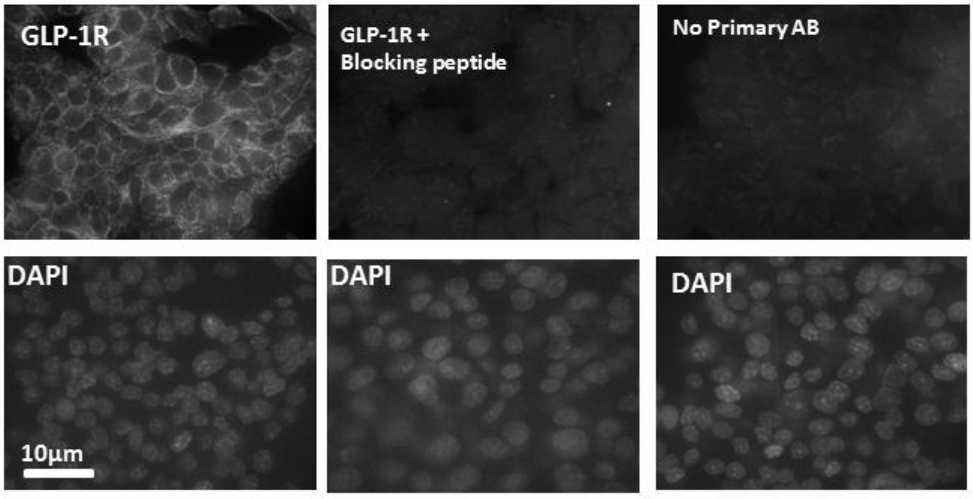

Figure 2.1.6 Specificity of GLP-1R antibody on MIN6 cells

The specificity of the GLP-1R antibody was verified using a cell line that is known to express GLP-1R. GLP-1R immunoreactivity was present in MIN6 cell line known to express GLP-1R; the immunoreactivity was abolished was pre-incubation of the immunogen peptide; a secondary antibody-only stain showed no positive staining. All photomicrographs pictures were taken under the same conditions and at 40X magnification.

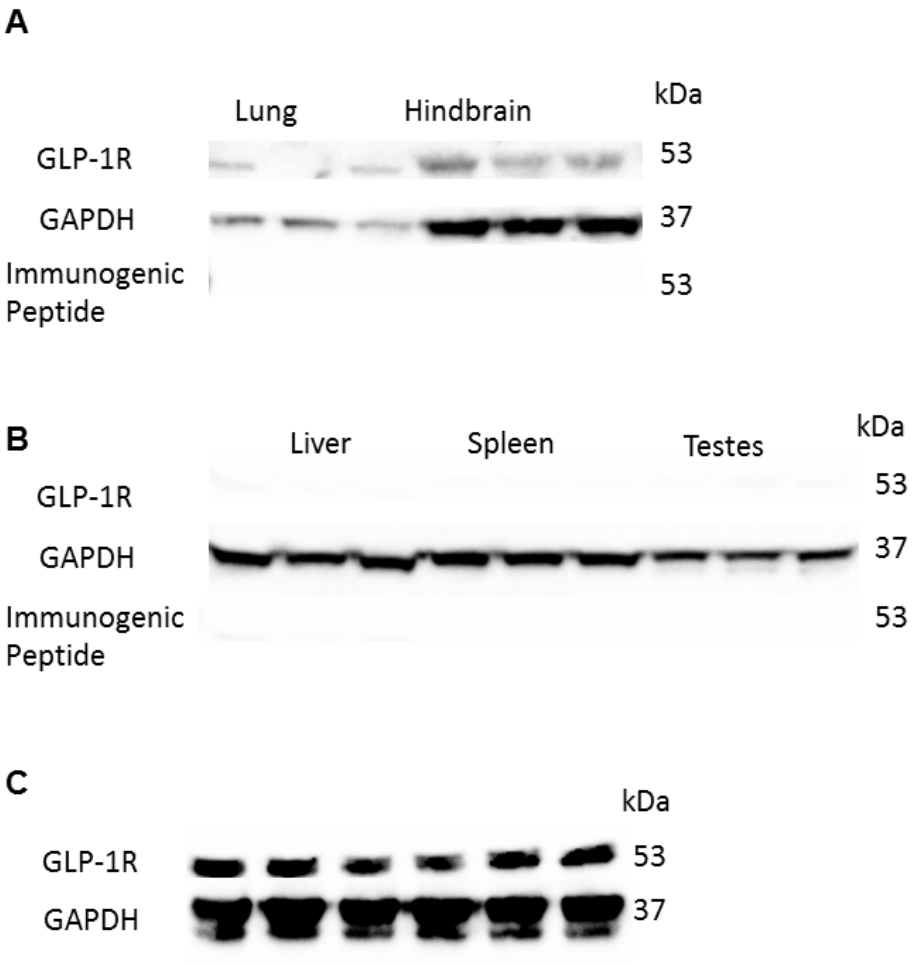

Figure 2.1.7 Specificity of GLP-1R antibody with immunoblots

Immunoblots confirmed the specificity of the antisera (A) a band was present at the correct molecular weight (53kDa) for GLP-1R in lung and hindbrain (B) but not liver, spleen and testes tissue samples. Presence of the immunogenic peptide abolished immunostaining of the lung and hindbrain. (C) A band was present at the correct molecular weight (53kDa) for GLP-1R in MIN6 cells.

Although gene expression studies have shown that VAN express GLP-1R mRNA (21), there are no published reports of protein expression. We used immunofluorescence and immunoblots to show protein expression. GLP-1R immunoreactivity was seen in tissue sections of nodose ganglia (Fig 2.1.8A). GLP-1R immunoreactivity was present in tissue sections of nodose ganglia taken from both fasted and re-fed rats. Quantification revealed that 42% of VANs were immunopositive for GLP-1; the percentage of labeled pixels and labeled neurons in nodose ganglia did not differ with feeding status ($p>0.05$; Fig 2.1.8B+C). Immunoblots confirmed these findings; GLP-1R protein levels were similar for both fasted and re-fed groups ($p>0.05$, Fig 2.1.9A+B).

We next tested GLP-1R mRNA levels in fasted and re-fed conditions. Given that it is unlikely that gene expression would change in a short amount of time, rats were fasted and re-fed for 2 h and gene expression compared to that in fasted rats. There was no difference in GLP-1R mRNA levels between fasted and 2 h re-fed nodose ganglia ($p>0.05$; Fig 2.1.10).

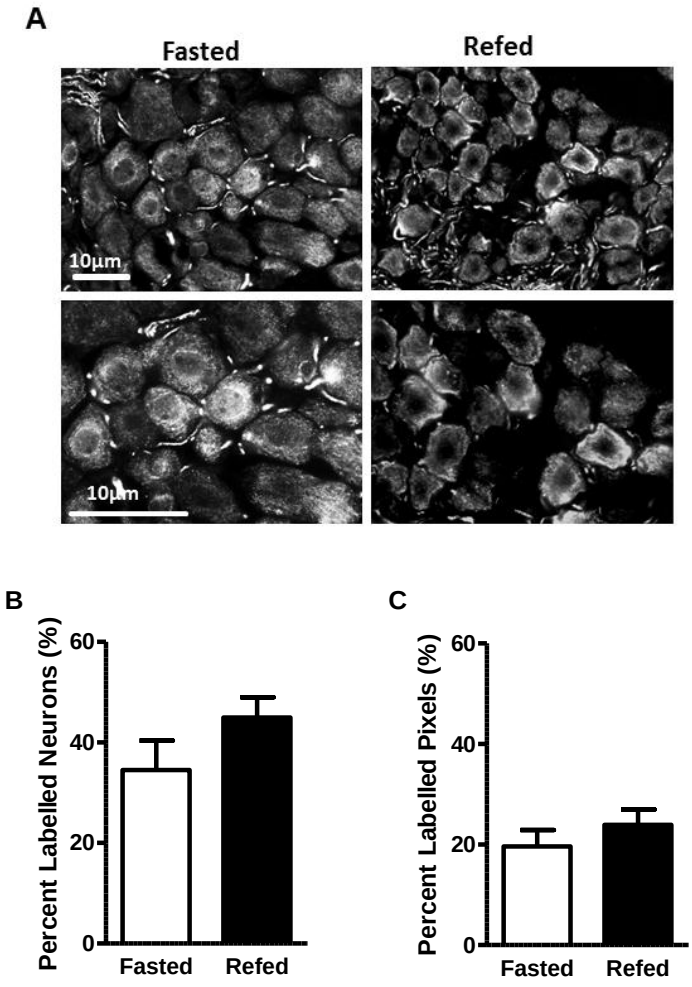

Figure 2.1.8 Measurement of protein levels of GLP-1R in VAN in fasted and refed animals by immunofluorescence

Protein levels were measured in fasted and 40-min *ad libitum* refed VAN samples. (A) photomicrographs of GLP-1R immunoreactivity in section of nodose ganglia, (B) the percent labeled neurons and (C) percent labeled pixels on NG sections did not differ between fasted and 40-min-refed animals (fasted vs. 40min refed, P>0.05, fasted and 40min refed n=4, 8 images were analyzed per rat).

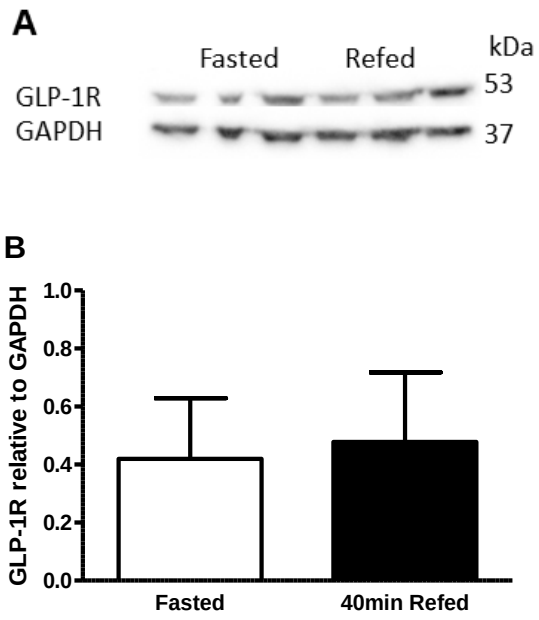

Figure 2.1.9 Measurement of protein levels of GLP-1R in VAN in fasted and refed animals by immunoblots

Protein levels were measured in fasted and 40-min *ad libitum* refed VAN samples by immunoblots; (A) and (B) protein levels relative to GAPDH did not change in a refed state compared to the fasted state (fasted vs. 40min refed, P>0.05, fasted and 40min refed n=3). Data are represented as mean ± SEM. Statistical differences were measured using unpaired Student t-test.

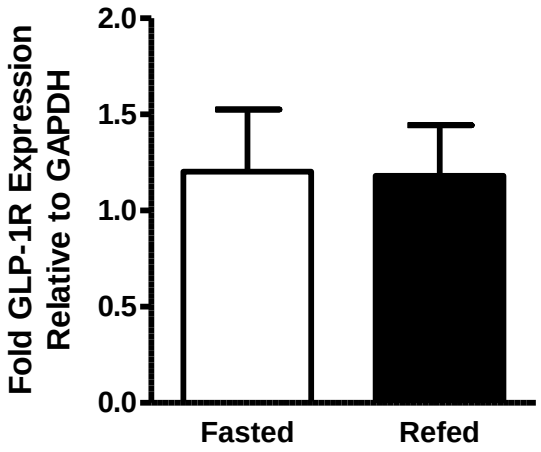

Figure 2.1.10 GLP-1R gene expression in VAN in fasted and refed animals

mRNA levels were verified in animals that were fasted overnight or fasted overnight and then refed *ad libitum* for 2 h. There was no difference between the conditions in GLP-R mRNA levels (fasted vs. 40min refed, $P>0.05$, fasted and refed $n=5$). Data are represented as mean ± SEM. Statistical differences were measured using unpaired Student t-test.

GLP-1R localization on VAN in response to feeding status

Immunohistochemistry was used to study the distribution of GLP-1R in vagal afferent neurons of re-fed and fasted rats. In tissue sections of nodose ganglia taken from fasted rats, the majority of the immunoreactivity was localized to the cytoplasm (Fig 2.1.11A). In contrast, in sections of nodose ganglia taken from re-fed rats, there was a significant increase in GLP-1R immunoreactivity at the membrane (Fig 2.1.11A). Pan cadherin was used as a plasma membrane marker (22) to quantify the immunoreactivity present on the membrane. Quantification of the immunoreactivity revealed that there was a significant increase of the number of labeled pixels at the plasma membrane of vagal afferent neurons from the re-fed compared to the fasted rats (Fig 2.1.11C). In the fasted state, 26.7 ± 1.0% of immunoreactivity for GLP-1R was localized to the plasma membrane compared to 42.3 ± 1.6% in the re-fed state; this represents a 1.8 fold increase in neurons expressing GLP-1R at the plasma membrane (Fig 2.1.11B).

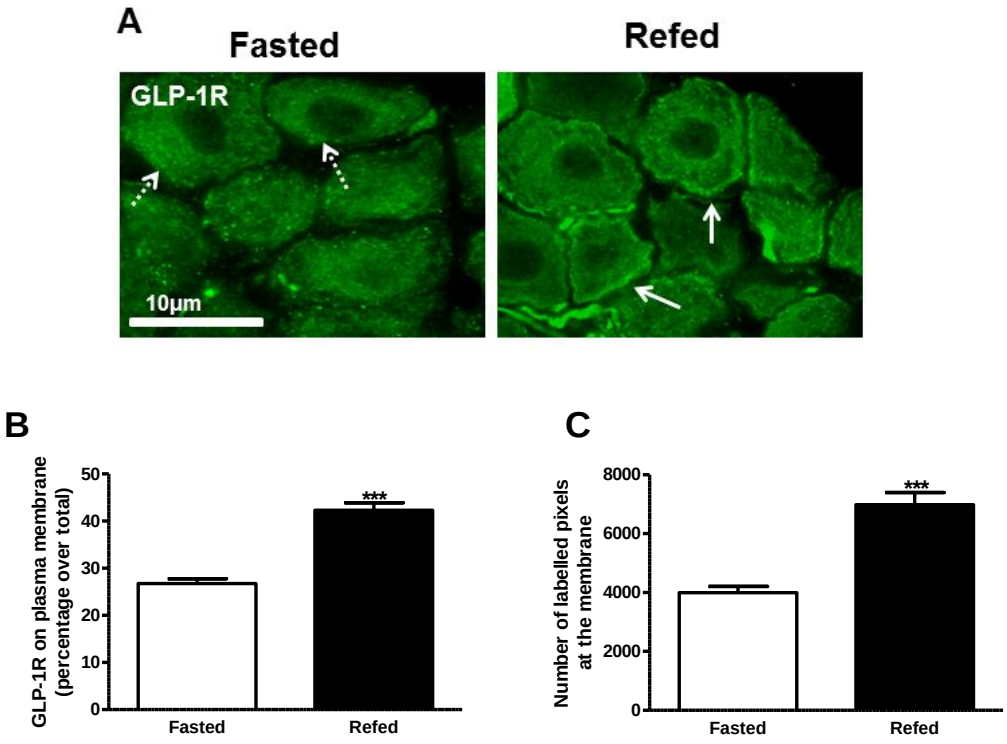

Figure 2.1.11 Localization of GLP-1R in fasted and refed VAN

The immunohistochemical colocalization of GLP-1R at the plasma membrane was analyzed on fasted or 40min *ad libitum* refed NG sections. (A) Photomicorgraphs of GLP-1R staining in green at 60X magnification. The merged images reveal that there was increased colocalization with GLP-1R at the membrane indicated with solid arrows in refed compared to fasted NG sections. The dotted arrows represent non-colocalization and the solid arrow represents colocalization. Pan cadherin was used to identify the plasma membrane and quantify the colocalization of GLP-1R at the membrane. The percent (B) and number of pixels (C) of GLP-1R present on the plasma membrane was a significantly increased in the refed condition compared to the fasted condition (fasted vs. 40-min refed, $P<0.001$, fasted vs. 40min refed n=4, 8 images per rat). Data are represented as mean ± SEM. Statistical differences were measured using unpaired Student t-test: ***$P<0.001$.

2.1.6 Discussion

In the present study, we clearly demonstrate that peripheral administration of native GLP-1 requires a postprandial state to express biological activity to inhibit food intake. In addition, we describe a novel mechanism by which GLP-1R on VAN is modulated by feeding. GLP-1R mRNA or protein expression by VAN is not altered by feeding; however, localization of the GLP-1R on the cell bodies of VAN is changed by feeding. GLP-1R co-localization at the plasma membrane of nodose ganglia neurons significantly increases in response to a short bout of food consumption compared to the fasting state. These findings provide the first line of evidence that GLP-1Rs expressed on vagal afferent neurons are trafficked to the membrane in the postprandial period. Trafficking of the receptor to the membrane provides a possible explanation to the observation that exogenously administered GLP-1 only inhibits food intake after feeding. The mechanism that is regulating trafficking of the receptor to the membrane is not known, but could depend either on the release of a signal from the gut upon intake of food that brings the receptor to the surface or the diminution of a signal that keeps the receptor localized to the cytoplasm. Our observations contribute to the understanding of the satiating mechanism of gut-derived GLP-1.

In this study, we demonstrate that peripheral GLP-1 inhibits food intake in the postprandial but not the food-deprived condition. A previous study showed that native GLP-1 failed to inhibit food intake following acute peripheral administration in fasted mice. However, in the same study, the identical dose of peripherally administered GLP-1 (30nmol/kg) decreased energy consumption in *ad libitum* fed rats (11). Taken together with the current findings, these studies suggest that GLP-1-induced satiation is dependent on feeding status. Williams *et al.* (7) provided preliminary evidence that the inhibitory effect of GLP-1 on food intake is induced postprandially; IP administrations of

GLP-1 significantly decreased food intake in rats fed *ad libitum* then fasted for 4 hours, but had no effect in overnight fasted rats. However, food intake was not recorded in the *ad libitum* fed rats; therefore the variability in quantity of food ingested or when the last meal was eaten was not controlled for. In the present study, the re-fed group was given a specific amount of food for a controlled period of time prior to a GLP-1 administration to ensure little variation in hunger and satiation. In addition, we designed a within subject study to reduce the amount of variation associated with individual differences. This study provides clear evidence that the satiating effect of GLP-1 is dependent on postprandial status.

We observed that our saline treated animals consumed the same amount of food at 40 mins irrespective of nutritional status. There are a couple of different possible reasons for this observation. The refed animals only received a small premeal prior to GLP-1, which is not sufficient to reduce the amount of food consumed during the feeding study. Alternatively, it could be that their rate of ingestion might be maximal. Since all the rats were fasted, they are very motivated to eat and it might be the maximum they can eat in 40minutes. This is supported by the fact that the animals that received a premeal ate less over the first 80 minutes as they started to become more satiated.

Previous work on anorexigenic hormones has found that their ability to suppress food intake was independent of feeding status (23, 24); GLP-1 only induces satiation postprandially and therefore differs from other gut peptides such as cholecystokinin (CCK) (7, 10). It is possible that GLP-1 requires the presence of another gut peptide to reinforce its physiological effect on regulating energy intake. There is considerable evidence that the gut hormones leptin and cholecystokinin (CCK) act synergistically to inhibit food intake (25). There is evidence that this interaction occurs at the level of VAN; sensitivity of VAN to CCK is increased in the presence of leptin (26). Leptin induces the

expression of early gene regulator 1 while CCK translocates it to the nucleus (26). There is also some evidence to suggest that GLP-1 acts synergistically with other gut hormones to potentiate its satiating effects. For example, Williams *et al.* demonstrate that sequential peripheral administrations of leptin and GLP-1 additively to reduce 24h food intake (7). In humans, infusions of PYY and GLP-1 administered together significantly decreased energy intake whereas GLP-1 alone and GLP-1 at a higher dose had no effect (11). Further work is needed to assess whether there are similar interactions in the inhibitory effects of gut-derived GLP-1 at the level of the vagus.

We used an acute peripheral administration of native GLP-1 in order to mimic endogenous GLP-1 release from the GI tract in response to a meal. Williams *et al.* (27) have previously demonstrated in rats that when central GLP-1Rs are blocked, peripheral administration of GLP-1 is still able to decrease food intake, showing a specific peripheral site of action for the inhibitory effects of GLP-1. Numerous studies demonstrate that peripheral administration of GLP-1 will decrease food intake (7, 28), though central administration will also suppress energy intake (29, 30). It remains unclear which GLP-1R population mediates the satiating effects of gut-derived GLP-1. Studies using synthetic GLP-1R agonist, exendin-4 (Ex-4), demonstrate that GLP-1 inhibits food intake in a dose-dependently manner after IP and vena cava infusions (8, 31, 32). Unlike endogenous GLP-1, Ex-4 has a prolonged half-life and crosses the blood brain barrier in its intact form resulting in a more potent energy intake suppressor than GLP-1 (33, 34). Ex-4 acts on both peripheral and central sites (34, 35); it is unclear how much endogenous GLP-1 reaches the central receptors in response to a meal. In this study, we used native GLP-1 instead of Ex-4 to produce a more physiological condition that reflects gut-derived GLP-1 release in response to a meal. Our study indicates that gut-derived GLP-1 may play a physiological role in regulating energy balance.

In the present study, we show that VANs express both mRNA and protein for GLP-1Rs. It has previously been shown that VANs express GLP-1R mRNA but there is little evidence to suggest protein expression. Vahl *et al.* provided preliminary immunocytochemical evidence that GLP-1R might be present on nodose neurons; however, the specificity of the antibody was not established (21). The present study is the first to use appropriate controls to definitively show specificity of a GLP-1R antibody. There was GLP-1 receptor immunoreactivity in tissue sections of kidney, known to express GLP-1Rs (19) but not in liver, which does not express the receptor. Moreover, immunoblots revealed a positive band at the correct molecular weight for the receptors (19) in lung, hindbrain, MIN6 cells and nodose ganglia, but not in liver, spleen and testes tissue. Finally, positive immunoreactivity was abolished by pre-incubation with the immunogenic peptide. Therefore, our data conclusively show protein expression of GLP-1Rs by VAN. Panjwani *et al*. (36) have suggested that GLP-1R antibody is not specific as an immunoreactive band was observed in tissue from GLP-1R knockout mice. It is possible that there are differences in the protocols between the current study and that of Panjawani *et al*. which explain the differences in the results (such as antibody incubation time); alternatively, it is possible that this represents a species difference. There is a possibility that the GLP-1R knockout mice have an increase in a protein that cross reacts with the GLP-1R antibody, explaining immunoreactive bands in negative control tissue. It is worth noting that we did not obtain any immunoreactive product when we used our protocols for either Western blot or immunocytochemistry in mice (Ronveaux *et al,* unpublished observations).

GLP-1 has been demonstrated to activate a vagal afferent pathway suggesting the presence of GLP-1R on VAN (37, 38). Peripheral GLP-1 increases electrophysiological activity on both *in vivo* and *in vitro* preparations of the vagus nerve

(13, 14, 38). Vagal afferent activation in response to peripheral GLP-1 injection was reduced by vagotomy (37). In clinical trials, the effect of inhibitory GLP-1 on energy intake was attenuated in vagotomized subjects indicating a possible functional role for the vagal afferent pathway in mediating the effects of GLP-1 (39). Nakagawa *et al.* (40) found GLP-1R mRNA expression in cell bodies of VAN. We provide immunohistochemical and western blot data in support of these findings.

VANs constitute an important pathway in the regulation of food intake. These neurons exhibit plasticity and switch their phenotypes between a food-deprived and a postprandial state (15). Previous data has shown that anorexigenic Y2 receptor expression by vagal afferent neurons is high following re-feeding, and conversely is decreased by 50% after a 13h fast (41). Since GLP-1-induced satiation was found to be dependent on nutrient availability, we hypothesized that, similarly to Y2 receptor, GLP-1R expression would change according to feeding status. In contrast, in the present study, we found that the GLP-1Rs are constitutively expressed on VAN, regardless of feeding status. We found no evidence of changes in GLP-1R mRNA or protein expression in response to feeding or fasting. Instead we found that GLP-1R translocates to the plasma membrane postprandially when endogenous GLP-1 is released from the gastrointestinal tract. Importantly, the rapid translocation of GLP-1R to the plasma membrane occurs within a time frame that coincides with the behavioral changes we observe in response to exogenous GLP-1. Therefore we hypothesize that this mechanism is driving the behavioral changes. Future *in vitro* studies will be needed to be able to link the behavioral changes to the change in receptor expression. Although we do not provide direct evidence of the mechanism at the vagal terminals where GLP-1 is released, we can speculate that changes in localization of the receptor in cell bodies of VAN in the nodose ganglia action is also seen at the terminals in the intestine.

In conclusion, we demonstrate that GLP-1Rs are located on the VAN providing further evidence that gut-derived GLP-1 may signal via the vagal afferent pathway to alter function. Our findings indicate that GLP-1R on VANs play a functional role in regulating food intake by a change in cellular localization which is driven by feeding status. Given that the obesity epidemic is progressively growing worldwide, it is important to understand mechanisms regulating energy balance. For example, in obese subjects there are diminished levels of GLP-1 concentrations in response to a meal (42); moreover, there is evidence to suggest that bariatric surgery will restore GLP-1 signaling in obese subjects (43). In this study, we have furthered our understanding of the potential mechanism by which peripheral GLP-1-induces satiation. Furthermore we have demonstrated a novel mechanism regulating the expression of biological activity of a gut peptide on VAN, which may play a role in the integration of gut signals and the regulation of systemic hormonal signaling to the brain.

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Chapter 2.2: Ghrelin inhibits translocation of Glucagon like Peptide-1 Receptors to the plasma membrane of vagal afferent neurons

(Manuscript in preparation)

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Running title: Ghrelin mediates the satiating effects of GLP-1 on vagal afferent neurons

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2.2.1 Abstract

Glucagon like peptide-1 (GLP-1) is released from the gut in response to a meal; GLP-1 receptors (GLP-1R) are expressed on vagal afferent neurons (VAN). We recently showed that feeding rapidly increases the ability of GLP-1 to inhibit food intake and correlates with increased GLP-1R expression at the plasma membrane (PM) in VAN. We hypothesized that either a factor is inhibiting GLP-1R translocation to the PM in a fasted state or a factor is potentiating the translocation of GLP-1R to the PM in a refed state. We investigated the role of orexigenic gut hormone ghrelin and the satiating gut hormone cholecystokinin (CCK) in GLP-1R localization on VAN and in mediating GLP-1-induced inhibition of food intake. **METHODS**: To investigate the interaction between ghrelin and GLP-1, food intake was measured in fasted animals after blockade of endogenous ghrelin with subsequent administration of GLP-1. GLP-1R localization was measured by immunohistochemistry in VAN tissue section and culture conditions. To investigate the interaction between CCK and GLP-1, food intake was measured after blockade of endogenous CCK with a subsequent administration of GLP-1 and an administration of GLP-1 in CCK1R knockout mice. **RESULTS**: Blockade of endogenous ghrelin prior to GLP-1 administration significantly decreased food intake at 20mins *in vivo* in a fasted state, (2.32±0.2 vs. 3.4±0.3,$p<0.05$ D-Ly^3-GHRP-6/GLP-1 vs. saline/saline). In nodose gangia sections, the majority of GLP-1Rs were localized at the PM in fasted animals that received D-Ly^3-GHRP-6 vs. saline. In culture, the majority of GLP-1Rs were localized in the cytoplasm in ghrelin-treated cells compared to vehicle. Pretreatment of ghrelin antagonist in ghrelin-treated cells restored GLP-1Rs to the membrane. There was no difference in food intake in either animals that received CCK1R antagonist prior to GLP-1 nor in CCK1R KO mice that received GLP-1 alone. **CONCLUSION**: In a fasted state, trafficking of GLP-1R to the membrane in VAN is

prevented by ghrelin. The data indicate that GLP-1 and ghrelin interact to modulate satiety signaling on VAN.

Key words: Glucagon like peptide-1, receptor trafficking, ghrelin, cholecystokinin, food intake, vagus nerve

2.2.2 Highlights

- Blockade of ghrelin in a fasted state restores the satiating effects of peripheral native GLP-1
- The translocation of GLP-1Rs on VAN is regulated by ghrelin *in vitro*
- Ghrelin restricts GLP-1Rs to the cytoplasm through cAMP and p38 MAPK pathway in a fasted condition on VAN

2.2.3 Introduction

Obesity is reaching epidemic portions worldwide leading to serious health complications. There is a lack of simple and effective treatments, which has stimulated research into understanding mechanisms regulating food intake and appetite. Energy homeostasis involves complex processes that coordinate the interplay between the gastrointestinal endocrine system and the central nervous system. The vagal afferent pathway is a major neural pathway by which information about ingested nutrients reaches the central nervous system and influences feeding behavior.

Glucagon like peptide 1 (GLP-1) is a hormone that is released from intestinal L-cells found in the proximal and distal gut in response to a meal (1, 2). Central and peripheral GLP-1 signaling has been shown to inhibit food intake in animals and humans (3, 4). Several lines of evidence suggest that vagal afferent neurons (VAN) are a likely site of action for GLP-1 to signal satiety 1) native GLP-1 has a very short half-life (5) and thus is more likely to act at a site close to its release 2) its receptor is present on vagal afferent neurons (6, 7) and 3) subdiaphragmatic deafferentation attenuates GLP-1-induced satiation (8).

As discussed in Chapter 2.1, GLP-1R localization changed in response to the feeding status of the animal, however, the mechanism or mechanisms regulating the translocation of GLP-1R to the membrane remains to be determined. There are two possibilities; either a hormone that is released in a fasted state (e.g. Ghrelin) that prevents GLP-1Rs from translocating to the membrane or there is an increase in another gut hormone, which is released postprandially (e.g. CCK) and allows GLP-1Rs to translocate to the membrane.

Ghrelin is the only circulating orexigenic peptide and is released from endocrine cells located in the gastric mucosa (9). Vagal afferent neurons express ghrelin receptors

(GHSR) and the actions of CCK on vagal afferent neurons are attenuated in the presence of ghrelin. For example, ghrelin has been demonstrated to inhibit CCK-induced electrophysiological activity in VAN (10). Furthermore, ghrelin attenuates CCK-induced switch in the neurochemical phenotype (11). Exogenous ghrelin dose-dependently attenuates the satiating effects of GLP-1 in rodents (12). Given that GLP-1-induced satiation is attenuated in a fasted state when ghrelin concentrations are high, we proposed that the satiating effect of GLP-1 is inhibited by ghrelin in a fasted state.

It is well established that VAN undergo a phenotypic switch between fed and fasted states that is, at least in part, dependent on cholecystokinin (CCK) (13). CCK drives changes in VAN gene expression; exogenous CCK or feeding, when endogenous CCK is high, will decrease expression of orexigenic receptors and increase expression of anorexigenic receptors on VAN. Conversely, in a fasted state, when CCK is low, gene expression of orexigenic receptors are high and anorexigenic receptors are low on VAN (14). Evidence suggests that GLP-1 and CCK may work synergistically to inhibit food intake in men (15). Therefore, we also hypothesize that the ability of GLP-1 to induce satiation in a refed but not in fasted state is mediated through CCK.

The mechanism of GLP-1R localization on VAN is unknown. Given that GLP-1 interacts with other gut peptides to regulate food intake, we hypothesized that either a hormone is potentiating the translocation of GLP-1R to the plasma membrane in a refed state or a hormone is inhibiting GLP-1R translocation to the plasma membrane in a fasted state. Therefore, we investigated the interaction between CCK and GLP-1 as well as ghrelin and GLP-1 on VAN. It is imperative to understand the mechanism of the satiating effect of GLP-1 in order to develop a noninvasive anti-obesity therapy.

2.2.4 Methods

Animals

Animals were maintained and handled in accordance with policies of the Institutional Animal Care and Use Committee at the University of California in Davis. Male Wistar rats (initial weight 180g; Harlan, San Diego) were individually housed at 22°C under a shifted 12h light-dark cycle (light 11pm-11am) with *ad libitum* access to food and water. Rats were fed a regular chow diet (Lab Diet 5001). Animals were handled for 7 days prior to experiments and habituated to the new environment and to intraperitoneal (IP) injections of 400µl physiological saline prior to food intake studies. In all feeding studies, rats were fasted on wire bottom cages (6pm-11am) with *ad libitum* access to water. Experiments were performed at the start of the dark phase when the animals have the strongest drive to eat.

Weight matched (8 weeks old) adult male CCK1R knockout mice (Mouse Biology Program, UC Davis) and their wild-type controls 129Sv (Taconic, Oxnard, CA) mice were used. Mice were individually housed at 22°C under a 12h light-dark cycle (light 6pm-6am) with *ad libitum* access to food and water. Mice were fed a regular chow diet (Lab Diet 5001). Animals were habituated to the new environment and to intraperitoneal (IP) injections of 400µl physiological saline prior to food intake studies.

Food Intake Analysis

Two feeding study protocols were performed to investigate the mediators of GLP-1 signaling. Experiment A investigated the effects of ghrelin on the satiating effects of GLP-1 in a fasted state. Experiment B investigated the effects of CCK on GLP-1 signaling in rats using the CCK1R antagonist lorglumide and CCKR1 KO mice.

Experiment A–role of ghrelin on GLP1 induced feeding

GLP-1 and D-Ly^3-GHRP-6 were obtained by Bachem Bioscience Inc (King of Prussia, PA) and reconstituted in 0.9% saline. As a controlled experiment, we tested a subthreshold of D-Ly^3-GHRP-6 on food intake. D-Ly^3-GHRP-6 (80µg/kg, IP) or saline were administered on the right side of the animal at the start of the dark phase (11am) after an overnight fast (6pm-11am). After 40mins, food was returned to the cages. In a second experiment, GLP-1 (100µg/kg IP), D-Ly^3-GHRP-6 (80µg/kg) or 400µl physiological saline (IP) were administered on the left side of the animal. The dose of GLP-1 was chosen based on Ronveaux *et al.* (6) and a 10-fold higher dose than Lee *et al.* (16) was used for D-Ly^3-GHRP-6. Saline or D-Ly^3-GHRP-6 (80µg/kg, IP) were administered at the start of dark phase (11am) after an overnight fast (6pm-11am). After 40mins, the second injection of either saline or GLP-1 (100µg/kg) was administered and food was immediately returned to the cages (Figure 2.2.1).

Food weight and spill was recorded every 20 minutes for 2 h. Food intake was determined by measuring the difference between the baseline and the weight of the food and spill every 20 minutes for 2 h. Food intake following administration of saline followed by GLP-1 in one rat was statistically an outlier and was removed from the study. One-way ANOVA was used to compare groups at 20mins.

Experiment B –role of CCK on GLP1 induced feeding

Lorglumide was obtained by Sigma Aldrich (St. Louis, MO) and CCK and GLP-1 were obtained by Bachem Bioscience Inc. (King of Prussia, PA) and reconstituted in 0.9% saline. Lorglumide (10mg/kg, IP), CCK (10µg/kg, IP), GLP-1 (100µg/kg, IP) or 400ul physiological saline (IP) were administered. The dose of lorglumide and CCK were

chosen based on de Lartigue *et al*. (11), and the dose of GLP-1 was chosen based on Ronveaux *et al*. (6).

As a control experiment, we tested effects of lorglumide on CCK induced inhibition of food intake (n=8). Saline or lorglumide (10mg/kg, IP) were administered at the start of the dark cycle (11am) after an overnight fast (6pm-11am). After 15mins, the second injection of either saline or CCK (10μg/kg, IP) was administered and food was immediately returned to cages.

Following the control experiment, we tested the effects of CCK1R blockade on GLP-1 induced inhibition of food intake (n=12). Saline or lorglumide (10mg/kg, IP) were administered at the start of the dark cycle (11am) after an overnight fast (6pm-11am). In one protocol animals received an *ad libitum* premeal 15mins following the first injection (Fig 2.2.2A). In a second protocol, animals received a 3g premeal 15mins following the first injection (Fig 2.2.2B). After 40mins of premeal, the second injection of either saline or GLP-1 (100μg/kg, IP) was administered and food was immediately returned to cages.

To confirm whether CCK mediates GLP-1 signaling (n=12), we tested the effects of GLP-1 in CCK1R KO mice. Animals were fasted either overnight (6am-6pm) or for 6 h (12pm-6pm) and then refed a 0.2g premeal for 40mins. The premeal amount was based on the smallest amount of chow consumed in 40mins. After a 40min premeal, mice received two different doses of GLP-1 (33μg/kg or 100μg/kg, IP) or saline. Food was immediately returned to the cages returned following the injection (Figure 2.2.3A-C).

In all food intake analysis experiments, food weight and spill was recorded every 20 minutes for 2 h. Food intake was determined by measuring the difference between the baseline and the weight of the food and spill every 20 minutes for 2 h. Either t-test, one way or two way ANOVA were used to compare groups.

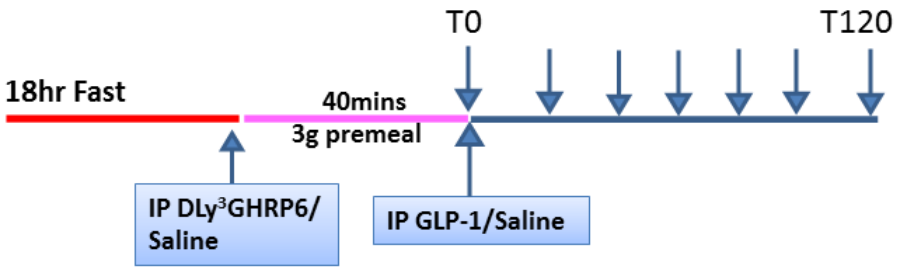

Figure 2.2.1 Protocol A - Role of ghrelin in GLP-1-induced satiation

Experiment A protocol was used to investigate the effect of ghrelin on GLP-1-induced satiation. Animals were fasted for 18 h, and then received a 3g premeal for 40mins prior to receiving 100µg/kg GLP-1 or saline IP. Food was immediately returned to cages and food intake was measured every 20mins for 2 h.

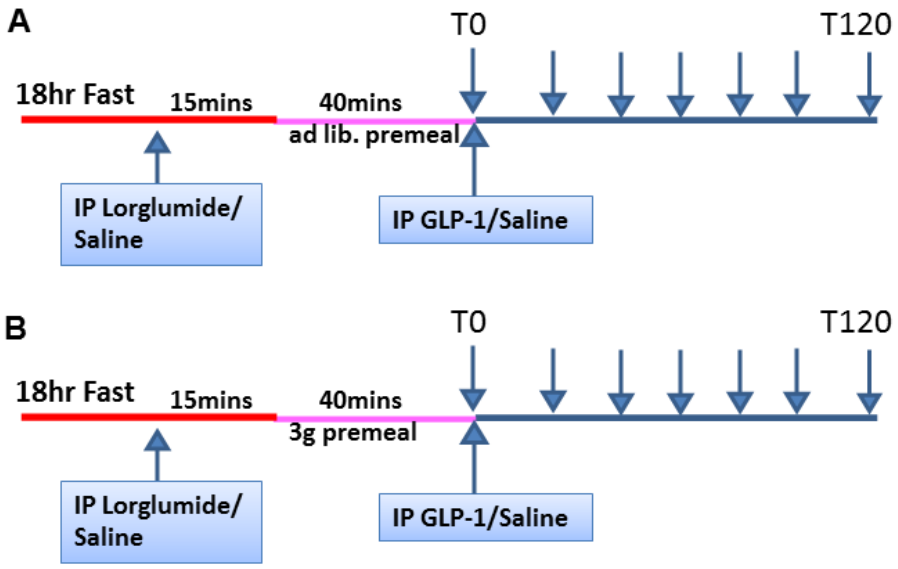

Figure 2.2.2 Protocol B - Role of CCK on GLP-1-induced satiation using CCK1R antagonist

Experiment B Two protocols were used to investigate the effect of CCK on GLP-1 signaling. Animals received IP 10mg/kg lorglumide or saline following an 18 h fast (A) In one protocol, animals received an *ad libitum* premeal 15mins following the first injection (B) In a second protocol, animals received a 3g premeal following the first injection. Following 40mins of premeal, animals received IP 100µg/kg GLP-1 or and food was immediately returned to cages.

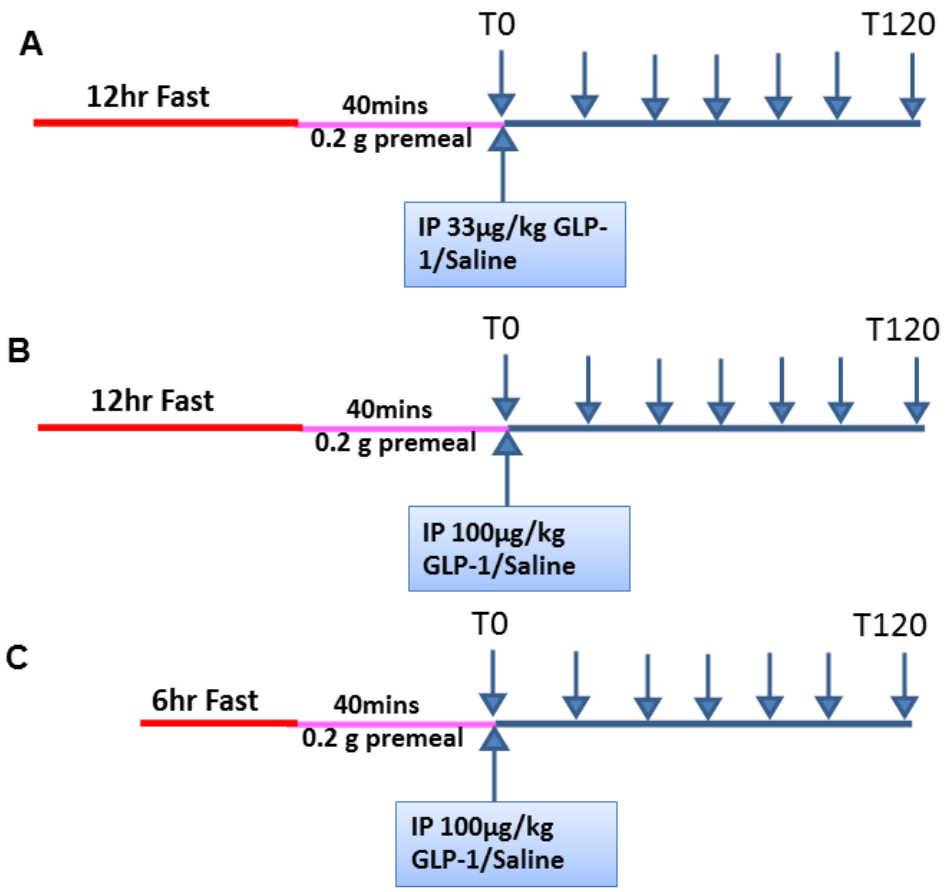

Figure 2.2.3 Protocol C - Role of CCK on GLP-1-induced satiation using CCK1R KO mice

Experiment B; Three protocols were used to investigate the effect of GLP-1-induced satiation in CCK1R KO mice. Animals were fasted for 12 h, then received a 0.2g premeal for 40mins prior to receiving either (A) 33µg/kg GLP-1 IP or saline or (B) 100µg/kg GLP-1 or saline IP. Food was immediately returned to cages and food intake was measured every 20mins for 2 h. (C) animals were fasted for 6 h prior to a 0.2g premeal. Animals received 100µg/kg GLP-1 or saline IP. Food was immediately returned to cages and food intake was measured every 20mins for 2 h.

Tissue collection

Rats were euthanized by CO_2 inhalation at least 1 h into the dark cycle and nodose ganglia was rapidly removed. For immunoblots, animals were either fasted for 18h or fasted for 18h and then refed a 3g premeal prior to receiving an administration of GLP-1 or saline. In addition, animals were fasted for 18h and received 80µg/kg D-Ly^3-GHRP-6 and then received 100µg/kg of GLP-1 40 mins later, nodose ganglia was collected 5 mins after the last injection; tissue was immediately placed in liquid nitrogen. For immunohistochemistry, nodose ganglia was post-fixed for 2 h in 4% paraformaldehyde in PBS, followed by 25% sucrose in PBS overnight at 4°C. For the localization of GLP-1R protein, nodose ganglia were collected 40mins after overnight fasted rats received either 80µg/kg D-Ly^3-GHRP-6 or saline. For primary cell cultures, rats were euthanized by CO_2 inhalation at least 1 h into the dark cycle and nodose ganglia was rapidly removed and placed immediately in ice cold Hank's Balanced Salt Solution during transport.

Western blots

Tissue was prepared as described by Freeman *et al*., 2006 (17). Briefly, 5µg of protein was loaded onto the gel. Samples were loaded into precast 10% BisTris-gel and ran for 50 minutes at 200V (Invitrogen Power Case 500). The proteins were transferred on a PDVF membrane for 1 h at 30V. Membrane was blocked using 10% milk in TBST for 1 h at room temperature. P-p44/42 MAPK was diluted at 1:1000 (9101, rabbit mAB, Cell Signaling Technology) and GADPH was diluted at 1:10,000 (14C10, rabbit mAB; Cell Signaling Technology). P-p44/42 MAPK was used as a marker for VAN activity and GAPDH was used as a loading control. Primary antibodies were applied on the membrane and developed on separate but consecutive days. Antibodies were incubated

for 1 h at room temperature and then overnight at 4°C. The membrane was imaged using ECL substrate (Thermo Scientific) on a ChemiDoc XRS Imager (BioRad, Hercules, CA). The membrane was analyzed by Image Lab version 5.0 software (Hercules, CA).

Nodose Ganglia Neuron Culture

Aseptically dissected nodose ganglia were digested with 2mg/ml of collagenase type A (Roche Diagnostics, Indianapolis, IN) in Ca^{2+} and Mg^{2+} free HBSS for 120mins at 37°C. Cells were maintained in culture for 96 h on 4-chamber well slides. To validate the protocol in which cells were not permeabilized prior to immunohistochemical staining, cells were placed in 30% glucose solution (Sigma Aldrich, St Louis, MO) and stimulated with 10nM CCK for 2 h at 37°C. To test the conditions in which GLP-1Rs translocate *in vitro*, cells were transferred to 30% glucose solution (Sigma-Aldrich, St. Louis MO) or DMEM with serum for 1 h at 37°C (n=4). Cells in 30% glucose in water were stimulated with 10nM ghrelin (Bachem Bioscience Inc., King of Prussia, PA) for 2 h at 37°C. To test the effects of ghrelin on GLP-1Rs translocation, cells were transferred to serum-free medium for 1 h at 37°C prior to treatment with 100μM D-Ly^3-GHRP-6 or saline for 30mins at 37°C followed by 10nM ghrelin (Bachem Bioscience Inc., King of Prussia, PA) for 2 h at 37°C (n=4). To investigate the downstream pathways in which ghrelin mediates GLP-1Rs translocation, cells were transferred to DMEM with serum for 1 h, stimulated with cAMPS-RP (100nM), Ly294002 (10nM), Rapamycin (10nM), SB 203580 (10nM) or U0126 (10nM) for 30mins prior to treatment with 10nM of ghrelin for 2 h (n=5). Cultured neurons were fixed with 4% paraformaldehyde in PBS for 15 mins at room temperature.

Immunohistochemistry

Cryostat sections of fixed nodose ganglia (9µm) were mounted onto Superfrost/Plus Slides (Fisher Scientific, Pittsburgh, PA) and processed for immunohistochemistry. Sections were blocked with 20% donkey serum (DS) (Vector Laboratories, Burlingame, CA), 0.2% Triton-X100 and 0.1% bovine serum albumin dissolved in PBS for 30mins at 37°C. Sections were incubated with an antibody raised against GLP-1R (Abcam 39072, Cambridge, MA) diluted at 1:1000 for 1 h at room temperature and then overnight at 4°C. The specificity of GLP-1R was previously validated by Ronveaux *et al* (6). Secondary antibody used was donkey anti-rabbit IgG conjugated with AlexaFluor 488 (Life technologies, Grand Island, NY) diluted at 1:1000 in DS-PBS. Sections were incubated for 1 h at 37°C. Images were collected using an Olympus spinning disc confocal microscope (BX61 System, Olympus, Melville, NY). All images were collected using the same confocal parameters.

Cultured neurons were blocked with 20% donkey serum (DS) (Vector Laboratories, Burlingame, CA), 0.1% bovine serum albumin dissolved in PBS for 30mins at 37°C. Cultured neurons were incubated with an antibody raised against GLP-1R (Abcam 39072, Cambridge, MA) diluted at 1:1000 or an antibody raised against CART (Phoenix Pharmaceuticals Inc., Burlingame, CA) diluted at 1:500 in DS-PBS (2% donkey serum and 0.1% bovine serum abumin dissolved in PBS) or DS-PBS with detergent (% Triton 100X) for 1 h at room temperature and then overnight at 4°C. To visualize proteins on the plasma membrane, cells were labeled without prior permeabilization. The specificity of GLP-1R was previously verified as described by Ronveaux *et al* (6). Secondary antibody used was donkey anti-rabbit IgG conjugated with AlexaFluor 488 (Life technologies, Grand Island, NY) diluted at 1:1000 in DS-PBS. Cultured neurons

were incubated for 1 h at 37°C. Images were collected using an Olympus spinning disc confocal microscope (BX61 System, Olympus, Melville, NY). 5-6 neurons were imaged for each rat. All images were collected using the same confocal parameters.

To determine the percentage of GLP-1R expression on VAN on the plasma membrane under different conditions, images were taken at either 40X or 60X magnification. The quantification of positive immunoreactivity for GLP-1R was analyzed by Scion Image software (Beta 4.0.2, Scion Corp., Frederick, MD, USA, 2000) and presented as percentage of positive neurons and pixels at the membrane. The percentage of positive neurons and pixels were quantified by determining the total number of neurons and pixels and the total number of positive neurons and pixels within an area. Positive neurons and pixels were defined as the immunofluorescence with a gain above the threshold for background staining. The data were expressed as percentage of positive neurons, and positive pixels at the membrane.

Statistical Analysis

Results are expressed as mean ± SEM. All studies were analyzed by using Student t-test, one-way or two-way ANOVA to validate statistical significance.

2.2.5 Results

Experiment A - Blockade of ghrelin receptors enhances on GLP1 induced inhibition of feeding

In experiment A, we investigated whether ghrelin plays a role in mediating the satiating effects of GLP-1 in a fasted state by pretreatment with the ghrelin receptor antagonist. Administration of D-Ly^3-GHRP-6 (80µg/kg IP) to fasted rats had no significant effect on subsequent food intake (Fig 2.2.4A). In fasted rats, administration of GLP-1 (100µg/kg, IP) alone failed to decrease food intake at 20min (Fig 2.2.4B). However, following pretreatment with D-Ly^3-GHRP-6, GLP-1 significantly decreased 20 min food intake by 33% compared to saline treated controls ($p<0.05$; Fig 2.2.4B). D-Ly^3-GHRP-6 and GLP-1 had small non-significant effects on their own. The result of combining the two together is synergistic, as the effect is bigger than the sum of both effects together.

Ghrelin receptor blockade induces GLP-1 activation of vagal afferent neurons

MAPK was used because GLP-1 is thought to act via this pathway in pancreatic acinar cells and NTS neurons (18). We found that phosphorylation of MAPK increased in animals that received a premeal compared to fasted animals (Fig 2.2.5A+B). Phosphorylation of MAPK did not change in fasted animals that received GLP-1 compared to animals that received a premeal, however, there was a slight increase in phosphorylation of MAPK in animals that received a premeal prior to GLP-1 (Fig 2.2.5A+B). GLP-1 activates the MAPK pathway in other model systems to induce its biological activities (19, 20). We investigated whether GLP-1 could induce VAN activity in a fasted state after blockade of endogenous ghrelin. We hypothesize that GLP-1 will

activate the MAPK pathway in a fasted state in the presence of a ghrelin receptor antagonist. In a fasted state, GLP-1 with prior D-Ly^3-GHRP-6 treatment induced a significant increase in MAPK activation on VAN compared to GLP-1 alone ($p<0.05$; Fig 2.2.5C+D).

Ghrelin receptor blockade induces GLP-1R localization on VAN

Immunohistochemistry was used to study the distribution of GLP-1R in vagal afferent neurons of animals that received either an administration of D-Ly^3-GHRP-6 or saline following an overnight fast. As previously show in chapter 2 the majority of GLP-1R immunoreactivity in nodose ganglia sections from fasted animals was localized in the cytoplasm (Fig 2.2.6A). Preliminary data from sections of nodose ganglia taken from animals that received D-Ly^3-GHRP-6 prior to collection, suggests that there was an increase in GLP-1R immunoreactivity at the membrane (Fig 2.2.6B).

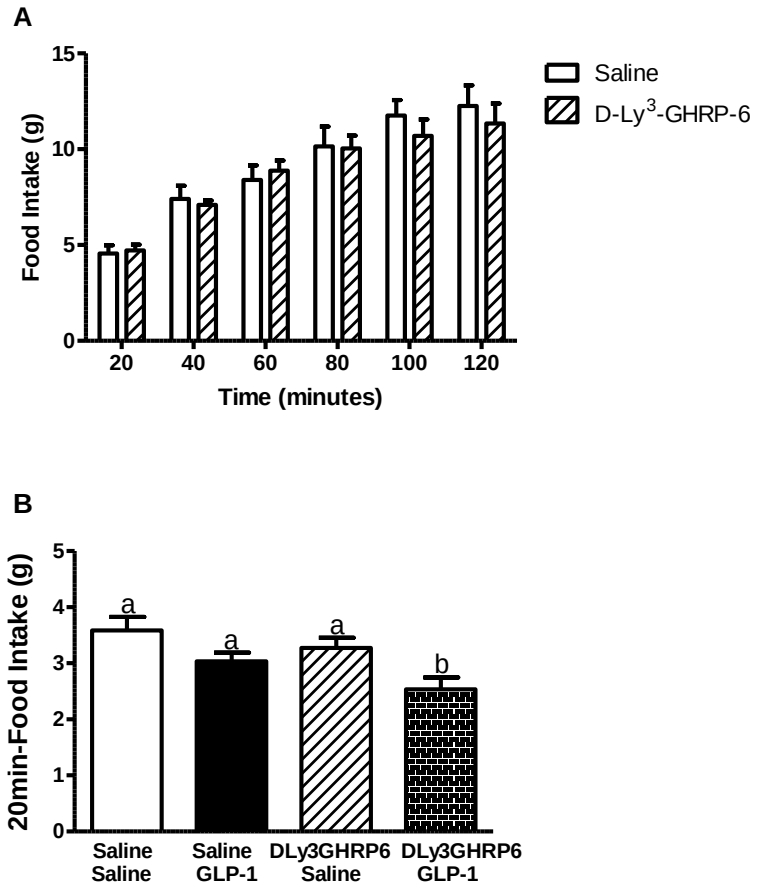

Figure 2.2.4 Blockade of ghrelin on GLP-1-induced satiation

Experiment A; Cumulative food intake is shown in grams. Rats fasted for 18 h received either 80µg/kg D-Ly^3-GHRP-6 or saline IP; (A) D-Ly^3-GHRP-6 failed to decrease food intake across time compared to saline. Animals fasted for 18 h received either 80µg/kg, followed 40 mins by 100µg/kg GLP-1 or saline IP; (B) GLP-1 and D-Ly^3-GHRP-6 alone had no effect on 20 min food intake compared to saline, however, pretreatment of D-Ly^3-GHRP-6 prior to GLP-1 significantly decreased 20 min food intake. Results are shown as means ± SEM (n=8); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (one-way ANOVA).

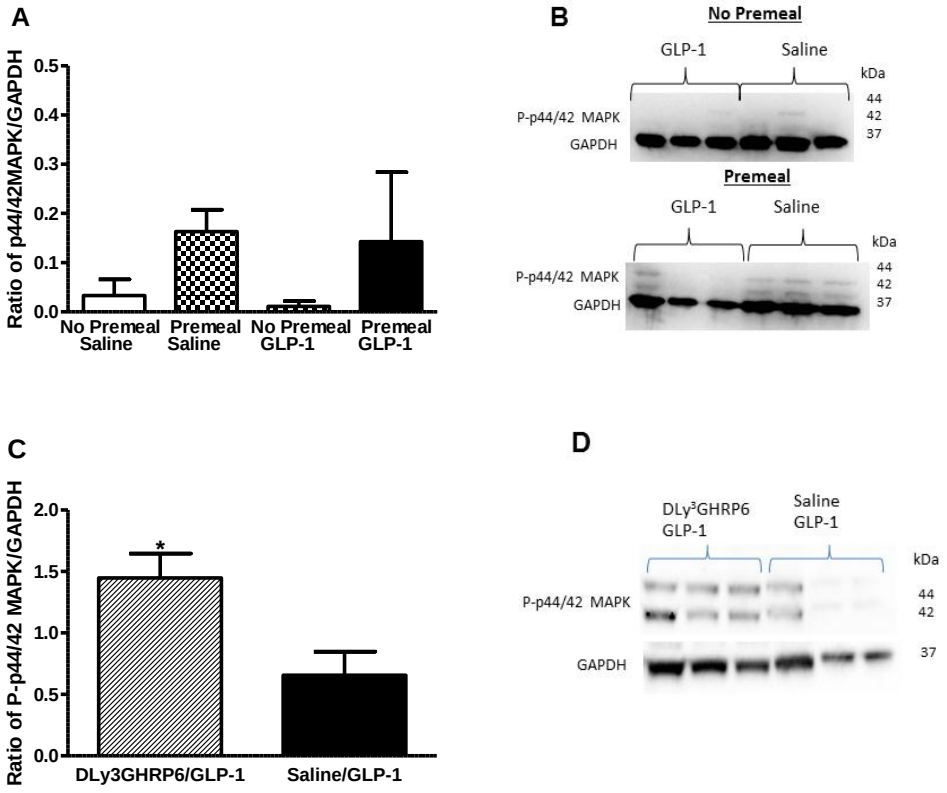

Figure 2.2.5 Blockade of ghrelin on GLP-1 signaling on VAN

In the first protocol, rats were either fasted for 18h or fasted and then refed prior to receiving an administration of GLP-1 or saline. (A) A premeal increased protein levels relative to GADPH compared to fasting condition on VAN; GLP-1 slightly increased protein levels in a refed state compared to a fasted state. (B) Immunoblots were stained with P-p44/42 MAPK and bands were at the right molecular weight (44/42kDa). In the second protocol, animals fasted for 18 h received 80µg/kg D-Ly^3-GHRP-6 or saline IP prior to an administration of 100µg/kg GLP-1 IP, followed 5 mins by nodose ganglia collection. (C) Pretreatment of D-Ly^3-GHRP-6 before GLP-1 significantly increased protein levels relative to GAPDH compared to GLP-1 alone (D) Immunoblots were stained with P-p44/42 MAPK and bands were at the right molecular weight (44/42kDa). Results are shown as means ± SEM (n=3); * P<0.05 (One-way ANOVA and Student t test).

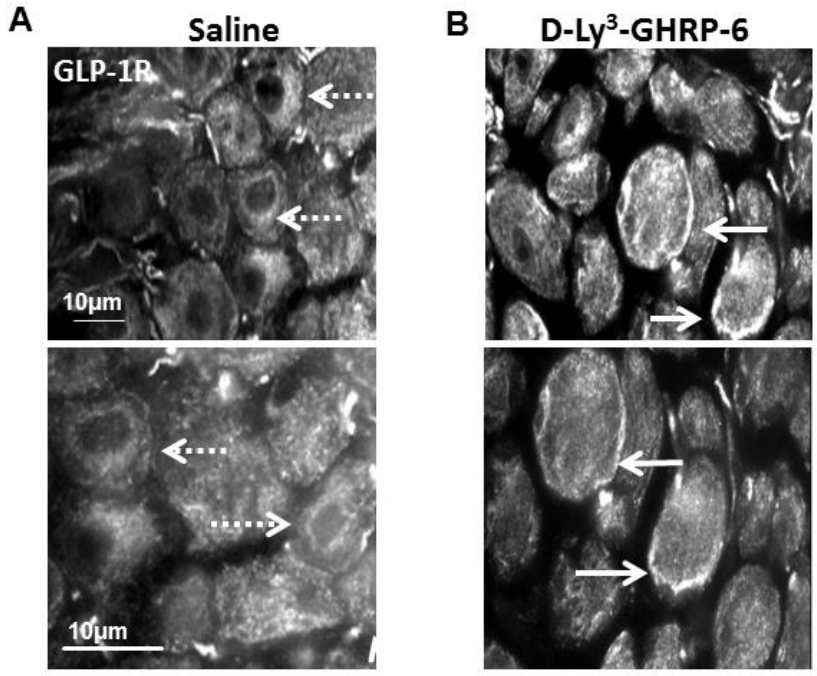

Figure 2.2.6 GLP-1R localization after blockade of ghrelin in VAN

Rats fasted for 18 h received either 80µg/kg of D-Ly^3-GHRP-6 or saline IP; representativie photomicrographs of GLP-1R immunoreactivity in sections of the nodose ganglion from animals that received (A) saline at 40X (top picture) and 60X (bottom picture) magnification (B) and D-Ly^3-GHRP-6 at 40X (top picture) and 60X (bottom picture) magnification. Dotted arrows indicate GLP-1Rs in the cytoplasm and solid arrows indicate GLP-1Rs at the membrane (n=1).

Validation of protocol to selectively localize plasma membrane bound GLP-1R

In order to exclusively visualize GLP-1R on the plasma membrane of cultured neurons we characterized an immunohistochemistry protocol lacking the permeabilization step. Antibodies are too large and hydrophilic to cross the lipid bilayer of cells, thus detergents are classically used to enable antibodies access to the interior of the cell. We hypothesized that only receptors localized on the surface of the cells would be visualized by this method, and would simplify analysis. Studies have previously demonstrated that cell surface expression can be visualized by immunohistochemistry without permeabilizing cells (21, 22).

Cultured neurons were stimulated with CCK to mimic a fed condition. Given that cocaine and amphetamine regulated transcript (CART) is a cytoplasmic peptide that is upregulated in response to CCK in VAN (14), neurons were labeled with CART antibody as a control. As we have previously shown (14), we found that CART immunoreactivity was high in cultured VAN stimulated with CCK in the presence of a permeabilizing agent (Fig 2.2.7A) but found no increase in CART expression in response to CCK in cultured VAN that were not permeabilized (Fig 2.2.7A). These data confirmed that cytoplasmic proteins are not visualized by this method.

We next labeled neurons with GLP-1R in the presence or absence of a permeabilizing agent. In CCK stimulated cultured VAN, GLP1R immunoreactivity was not significantly reduced in the unpermeabilized cells, suggesting a proportion of GLP1Rs are localized on the plasma membrane under these parameters (Fig 2.2.7B). Altogether, these data indicate that the absence of detergent in immunocytochemistry can be used to stain for GLP-1Rs on the plasma membrane.

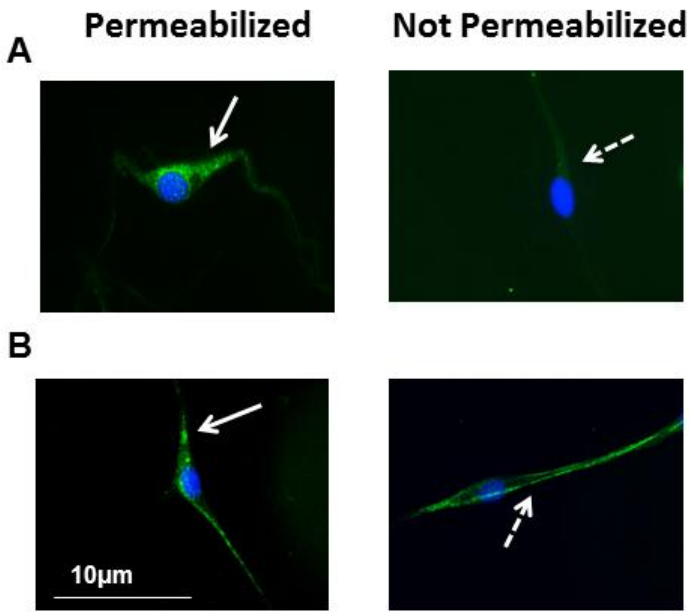

Figure 2.2.7 Validation of membrane-only surface stain protocol

To validate a membrane-only surface stain protocol we compared immunocytochemistry with and without the permeabilizing step. Cultured neurons from rats were stained with CART in green and nuclei staining with DAPI in blue (A) in cells treated with 10nM CCK immunoreactivity was increased when cells were permeabilized compared to cells that were not permeabilized. Cultured neurons from rats were stained with GLP-1R in green and nuclei staining in blue (B) in cells treated with 10nM CCK immunoreactivity was increased when cells were permeabilized compared to cells that were not permeabilized. Arrows point to immunoreactivity in the cytoplasm. Photomicrographs were taken at 60X magnification.

Determining translocation of GLP-1Rs in fasted and fed states on VAN

VAN have been shown to change phenotypes according to feeding status in culture (14). In this experiment, the effect of fasted and fed conditions in cultured VAN on localization of GLP-1Rs. Cultured neurons were transferred to baseline solution (30% glucose) to mimic fasted conditions or a stimulated solution (DMEM with serum) to mimic a fed condition. The fasted and fed conditions of VAN have previously been validated in our laboratory; VAN placed in serum-free or low glucose solution induces a fasted phenotypic switch where CART is reduced and melanin-concentrating hormone (MCH) 1R is increased. Medium containing serum on VAN mimics a fed baseline solution; the expression of CART is upregulated and the expression of MCH1R is downregulated; this phenotypic switch is also seen *in vivo* (14). Cells were labeled with GLP-1R antibody without prior permeabilization in order to visualize proteins that are exclusively on the PM. There was a 20% increase in GLP-1Rs at the membrane in cells that were in a baseline fed condition compared to a baseline fasted condition ($p<0.05$; Fig 2.2.8A+B). Cells in a fed condition were stimulated with ghrelin (10nM) for 2 h to visualize GLP-1Rs translocation. There was a significant decrease of the percentage of labeled pixels of GLP-1Rs at the membrane in cells stimulated with ghrelin compared to cells in a baseline fed condition ($p<0.001$; Fig 2.2.8C+D). This suggests that ghrelin either promotes internalization of prevents translocation to the PM.

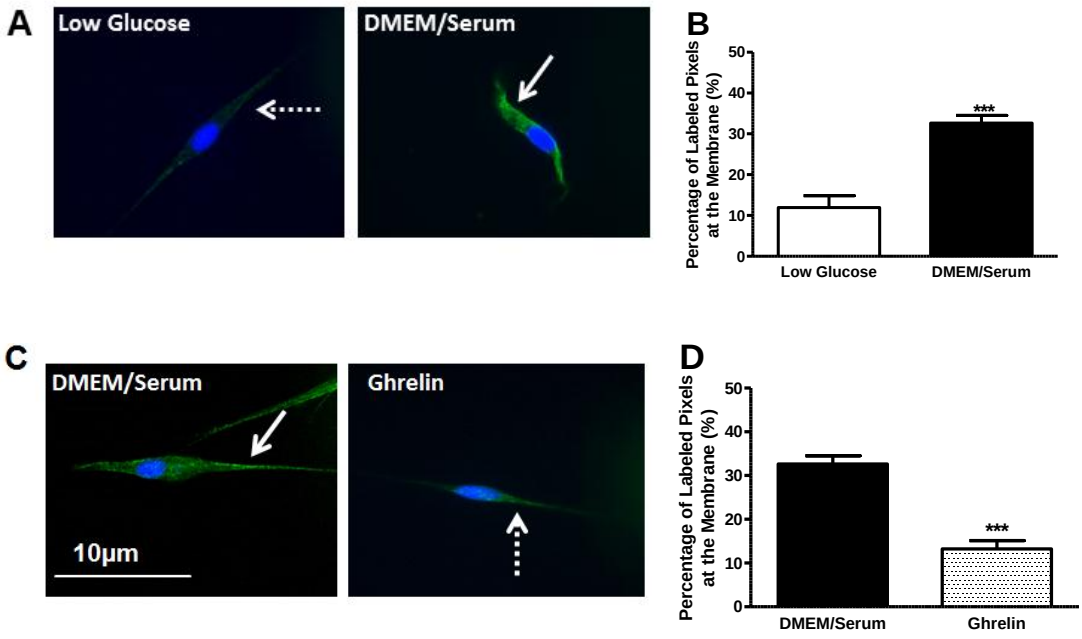

Figure 2.2.8 GLP-1Rs translocate according to feeding status *in vitro*

Membrane-only surface staining of GLP-1Rs were performed; (A) photomicrographs of GLP-1R immunoreactivity show that there is an increase in GLP-1Rs at the membrane in DMEM/serum compared to neurons in low glucose solution (B) Neurons in DMEM/serum media have a significant increase in the percentage of labeled GLP-1Rs at the membrane compared to neurones in a low glucose solution. (C) A decrease in GLP-1Rs at the membrane after cells were treated with 10nM ghrelin compared to cells in DMEM/serum media (D) this difference was significant. GLP-1R in green and nuclei stain with DAPI in blue. Results are shown as means ± SEM (n=4); *** P<0.001 (Student t-test).

Antagonizing ghrelin in VAN cultures

DMEM with serum has been shown to mimic a fed state in VAN cultures (14). To investigate the effects of blockade of ghrelin receptor on the translocation of GLP-1R, we stimulated cells either with vehicle (saline), ghrelin (10nM), D-Ly^3-GHRP-6 (100μM) or D-Ly^3-GHRP-6 and ghrelin. There was little to no GLP-1Rs present at the plasma membrane in ghrelin-stimulated neurons compared to baseline; GLP-1Rs were restored to the plasma membrane by D-Ly^3-GHRP-6 ($p<0.05$; Fig 2.2.9A). There was a 33% reduction of percentage of labeled pixels for GLP-1Rs in the ghrelin-treated neurons compared to baseline ($p<0.01$; Fig 2.2.9B). Pre-treatment of ghrelin stimulated VAN with D-Ly^3-GHRP-6 restored the percentage of labeled pixels of GLP-1Rs at the plasma membrane by 45% ($p<0.05$; Fig 2.2.9B). These data suggest that ghrelin plays a role in mediating the translocation of GLP-1Rs.

Inhibiting downstream pathways of ghrelin receptors

Actions of ghrelin are mediated by either the p38 MAPK or mTOR pathway (23, 24) therefore we examined the effects of inhibitors 100nM cAMPS-RP, 10nM Ly294002, 10nM Rapamycin, 10nM SB 203580 or 10nM U0126 on the translocation of GLP-1Rs following 10nM ghrelin treatment. There was no difference in GLP-1Rs at the membrane with treatment of PI3K inhibitor, mTOR inhibitor or MEK inhibitor compared to ghrelin alone (Fig 2.2.10A+B). Inhibiting cAMP-dependent protein kinase with cAMPS-RP and p38 MAPK with SB203580 significantly increased the percentage of labeled GLP-1Rs at the membrane compared to ghrelin alone ($p<0.05$; Fig 2.2.10A+B). The actions of ghrelin in mediating GLP-1R translocation are mediated through cAMP and p38 MAPK pathways and are not mediated by the mTOR pathway.

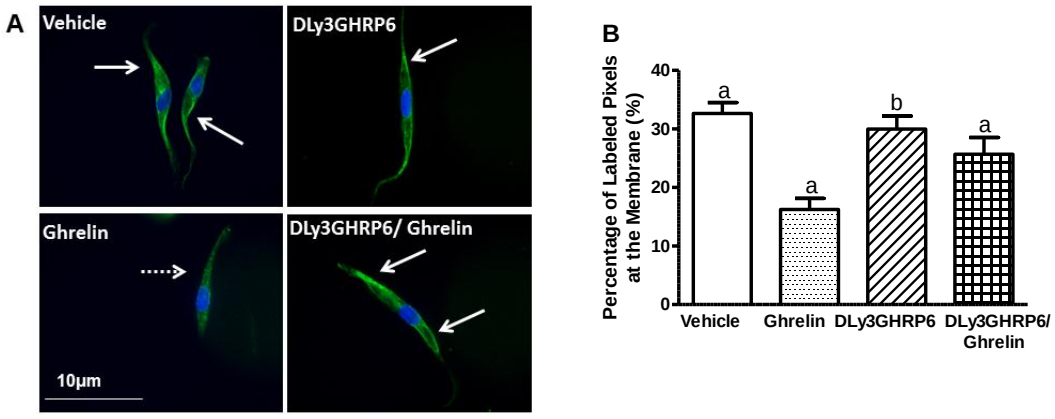

Figure 2.2.9 Ghrelin blockade on GLP-1Rs translocation *in vitro*

Cells were placed in DMEM/serum media (fed state) and stimulated with 100µM D-Ly^3-GHRP-6, 10nM ghrelin or 100µM D-Ly^3-GHRP-6 for 30mins followed by 10nM ghrelin for 2 h. Membrane-only surface staining of GLP-1Rs were performed; (A) photomicrographs of immunoreactivity of GLP-1Rs, no difference between D-Ly^3-GHRP-6-treated cells compared to vehicle, a decrease in GLP-1R at the membrane in ghrelin-treated cells and pretreatment of D-Ly^3-GHRP-6 prior to ghrelin attentuated ghrelin-induced translocation of GLP-1Rs. (B) A decrease in GLP-1Rs at the membrane in ghrelin-treated cells vs vehicle, no difference in percentage of labeled GLP-1Rs at the membrane in D-Ly^3-GHRP-6-treated cells or with pretreatment of D-Ly^3-GHRP-6 prior to ghrelin. GLP-1R in green and nuclei staining with DAPI in blue. Results are shown as means ± SEM (n=4); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (One-way ANOVA).

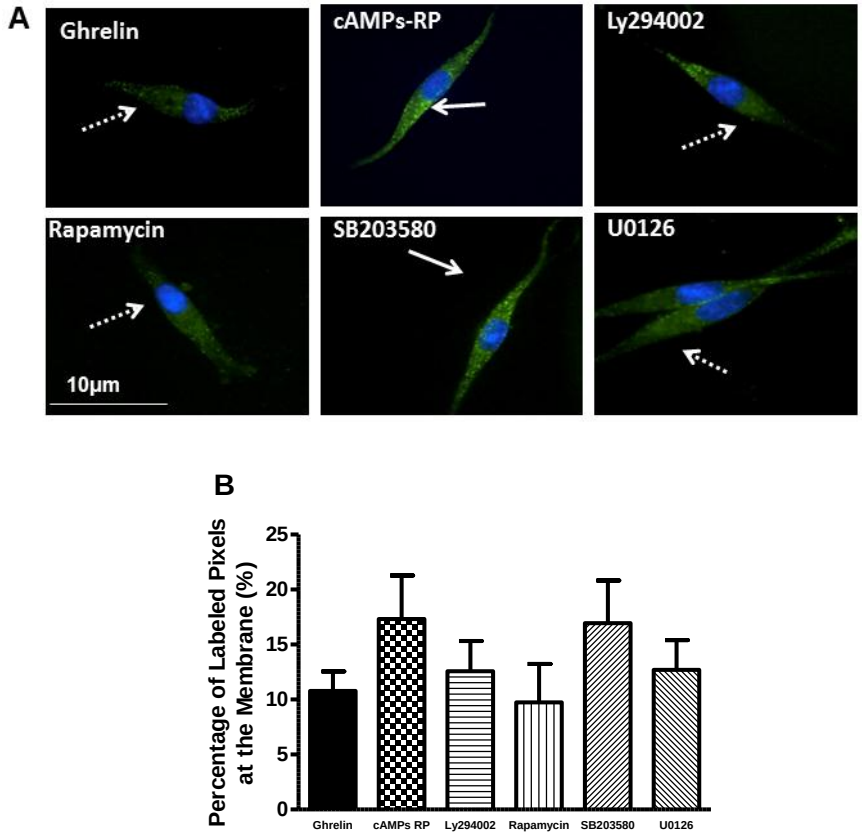

Figure 2.2.10 Pathways of ghrelin-induced GLP-1R translocation

To test downstream pathways of ghrelin-induced GLP-1R translocation in culture, cells were based in DMEM/serum media to mimick a fed state, received either 100nM cAMPS-RP, 10nM Ly294002, 10nM Rapamycin, 10nM SB 203580 or 10nM U0126 for 30mins prior to treatment with 10nM ghrelin for 2 h. (A) representative photomicrographs with GLP-1R immunoreactivity of surface-only staining (B) pretreatment of cAMPS-RP and SB203580 significantly increased the percentage of labeled GLP-1Rs at the membrane compared to ghrelin alone, all other inhibitors had no effect on the percentage of labeled GLP-1Rs at the membrane compared to ghrelin alone. GLP-1R in green and nuclei staining in blue. Results are shown as means ± SEM (n=5); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (One-way ANOVA).

Experiment B - Blockade of CCK1R has no effect on GLP1-induced inhibition of feeding

We investigated whether CCK plays a role in mediating the satiating effects of GLP-1 in a refed state. We hypothesize that, if CCK plays a role in GLP-1-induced satiation, the satiating effects of GLP-1 will be attenuated in the presence of a CCK1R antagonist or in CCK1R KO mice. After a number of experiments looking at the role of CCK in modulating GLP-1 satiation, the data still remains inconclusive.

CCK (10μg/kg, IP) significantly inhibited food intake 20, 40 and 60 mins after administration to refed rats ($p<0.001$; Fig 2.2.11A). Pretreatment with the CCK1R antagonist lorglumide alone had no effect on food intake; however, an administration of lorglumide prior to CCK attenuated the inhibitory effects of CCK at each time point ($p<0.05$; Fig 2.2.11A). To determine the role of CCK1Rs in the response to GLP-1, lorglumide was administered prior to GLP-1. Following either an ad libitum premeal (Fig 2.2.12A) or a 3g premeal (Fig 2.2.12B), GLP-1 alone trended to decrease 40min food intake, although this did not reach statistical significance. Lorglumide had no effect on GLP-1 induced inhibition of food intake (Fig 2.2.12A).

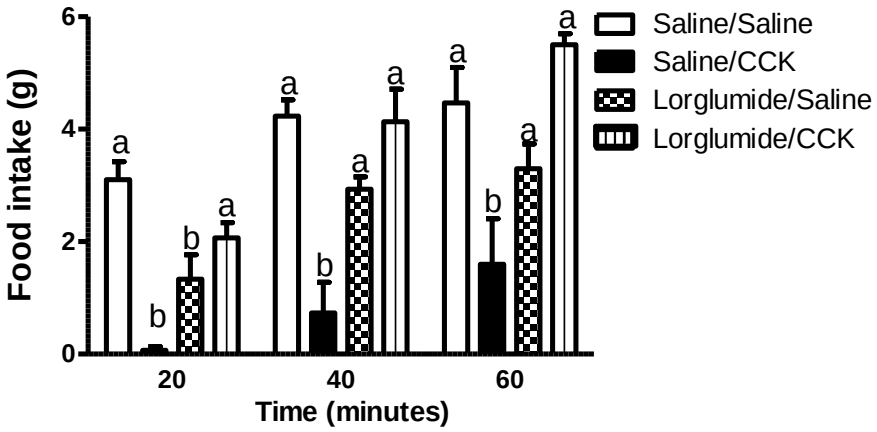

Figure 2.2.11 Blockade of CCK1R on food intake

Cumulative food intake is shown in grams. Animals fasted overnight received 10mg/kg lorglumide or saline IP, followed 15 mins by 10µg/kg CCK or saline IP; CCK alone significantly decreased food intake across time, lorglumide alone had no effect compared to saline and pretreatment of lorglumide attentuates CCK-induced satiation compared to saline. Results are shown as means ± SEM (n=8); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (Two-way ANOVA).

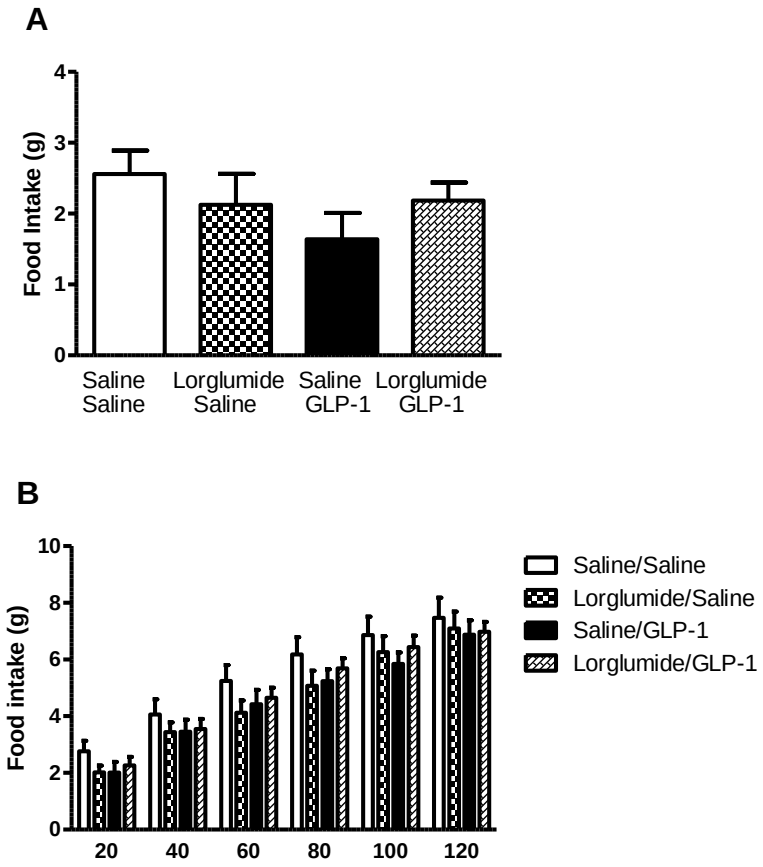

Figure 2.2.12 Blockade of CCK1R on GLP-1-induced satiation

Experiment B Cumulative food intake is shown in grams (A) Animals fasted for 18 h received 10mg/kg lorglumide or saline IP, followed 15mins by an *ad libitum* premeal, followed by 100µg/kg GLP-1 saline IP; there was no effect on food intake irrespective of treatment. (B) Animals fasted for 18 h received 10mg/kg lorglumide or saline IP, followed 15mins by a 40 min 3g premeal, followed by 100µg/kg GLP-1 or saline IP; there was no effect on food intake irrespective of treatment. Results are shown as means ± SEM (n= 8 or 12) (One-way or two-way ANOVA).

Experiment B: Effects on GLP-1 on CCK1R KO mice

To further investigate whether CCK mediates GLP-1 induced inhibition of food intake; we tested the effects of GLP-1 in CCK1R KO mice. We hypothesized that GLP-1 would inhibit food intake in WT mice fed a premeal but have no effect in KO mice. We tested two doses of GLP-1 (33µg/kg or 100µg/kg, IP). GLP-1 (33µg/kg IP) failed to decrease food intake at any time point ($p>0.05$; Fig 2.2.13A+B) compared to saline irrespective of genotype. GLP-1 (100µg/kg IP) in WT had no effect on food intake across time (Fig 2.2.13C), however, GLP-1 significantly decreased food intake 60 min after administration compared to saline ($p<0.01$; Fig 3.13D). GLP-1 (100µg/kg IP) in KO had no effect on food intake across all timepoints ($p> 0.05$; Fig 2.2.13C), but trended to decrease food intake at 60mins ($p=0.055$; Fig 2.2.13D).

Given that mice have a fast metabolism, we decided to reduce the fasting time to 6 hours and observe the effects on food intake in response to GLP-1. In WT mice, GLP-1 (100µg/kg IP) alone had no effect on food intake across time (Fig 2.2.14A) compared to saline. In KO mice, GLP-1 (100µg/kg IP) also failed to decrease food intake over time (Fig 3.14A) and at 60mins (Fig 2.2.14B) compared to saline.

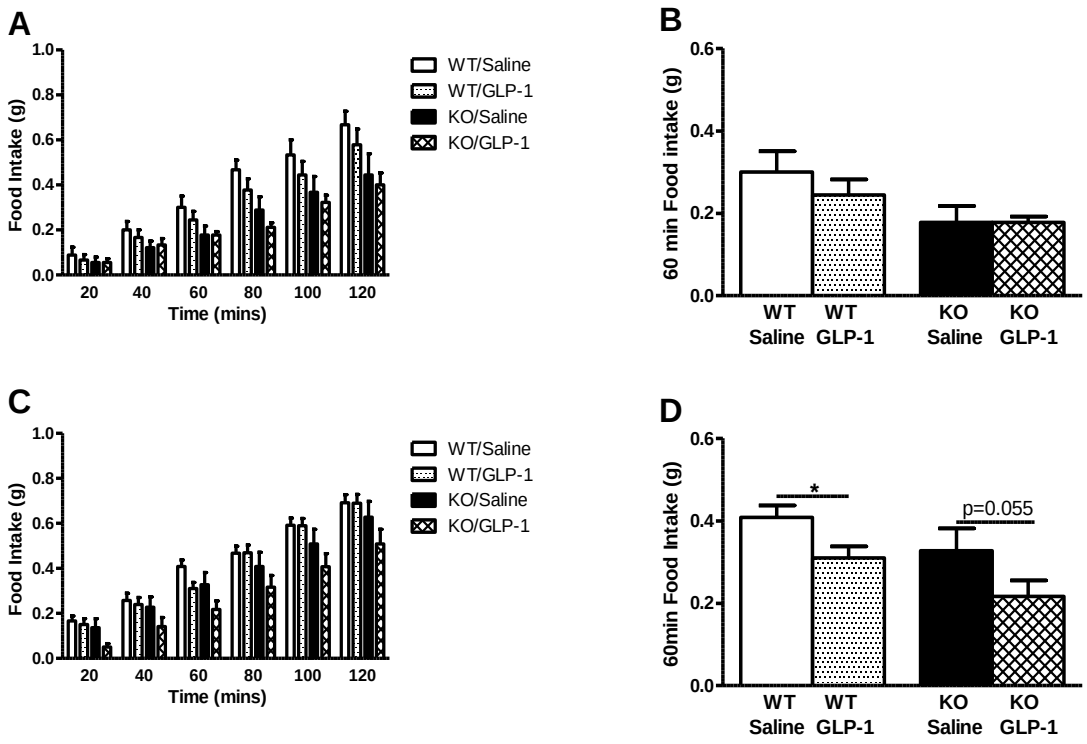

Figure 2.2.13 GLP-1 administration in 12h fasted CCK1R KO mice

Experiment B; Cumulative food intake is shown in grams. Animals fasted for 12 h received 40 min premeal prior to an administration of GLP-1or saline IP; (A) 33µg/kg of GLP-1 had no effect on food intake (B) and at 60 min irrespective of genotype (C) 100µg/kg of GLP-1 had no effect on food intake irrespective of genotype across time, however, (D) significantly decreased 60 min food intake in WT and trended to decrease 60 min food intake in CCK1R KO mice. Results are shown as means ± SEM (n=12) *P<0.05.

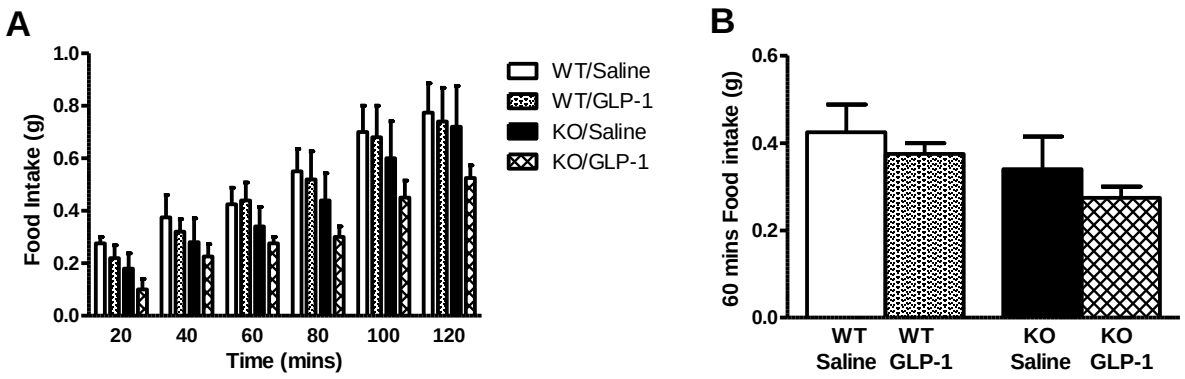

Figure 2.2.14 GLP-1 administration in 6h fasted CCK1R KO mice

Animals fasted for 6 h received a 40 min premeal prior to an administration of 100µg/kg GLP-1 or saline IP; (A) GLP-1 had no effect across time and (B) at 60 min irrespective of genotype. Results are shown as means ± SEM (n=5) (Student t-test).

2.2.6 Discussion

GLP-1Rs are constitutively expressed; the cellular location of GLP-1Rs changes according to feeding status on VAN. We sought to understand the mediators regulating the translocation of GLP-1Rs. In the present study, we demonstrate that ghrelin mediates the satiating effects of peripheral native GLP-1 *in vivo*. The role of CCK remains inconclusive. We have found that blockade of endogenous ghrelin in a fasted state restores the satiating effect of GLP-1. Furthermore, GLP-1 increases VAN activity, measured through MAPK, in the presence of a ghrelin receptor antagonist in fasted animals. We confirmed that GLP-1Rs translocate according to feeding conditions in culture. *In vitro* GLP-1Rs are not on the membrane in the presence of ghrelin, and blockade of ghrelin restores GLP-1Rs to the membrane. The data indicate that ghrelin restricts GLP-1Rs to the cytoplasm through cAMP and p38 MAPK pathway in a fasted condition.

It is well established that CCK is a master regulator of food intake and has been shown to potentiate the satiating effects of other hormones. In this study, we were unable to conclusively show that blockade of endogenous CCK or KO of CCK1R attenuated GLP-1-induced satiation. However with multiple lines of evidence failing to reach significance, it suggests that CCK is not involved in mediating the inhibitory effects of GLP-1. This is supported by the fact that very few studies show a link between CCK and GLP-1 in the literature. Leptin has been shown to potentiate the satiating effects of GLP-1 leading us to hypothesize that GLP-1 and leptin may interact at the level of the vagus to regulate energy homeostasis rather than CCK.

We used phosphorylation of p44/42 MAPK as a marker of VAN activity given that G-coupled protein receptors such as GLP-1R induce this pathway; we showed that phosphorylation of p44/42 MAPK is increased in refed animals compared to fasted

animals. In this study, phosphorylation of p44/42 MAPK was low in animals that received GLP-1 in a fasted state; however, phosphorylation of MAPK was increased in animals that received a ghrelin receptor antagonist prior to GLP-1. The data indicates that inhibiting endogenous ghrelin allows GLP-1 to activate VAN. In line with our study, GLP-1 and its analogs have been shown to activate p44/42 MAPK pathway (19, 25). Our data suggest that GLP-1 induces phosphorylation of p44/42 MAPK to activate VAN when ghrelin receptors are antagonized in a fasted state.

The satiating effect of GLP-1 is attenuated in a fasted state when ghrelin levels are high (6). This present study demonstrated that ghrelin plays a role in mediating GLP-1-induced satiation. GLP-1 induced satiation was re-instated when ghrelin receptors were antagonized in a fasted condition *in vivo*. These findings are consistent with Chelkani *et al*; ghrelin dose dependently attenuated the satiating effects of GLP-1 in rats (12). Furthermore, Exendin-4, a long lasting GLP-1 analog, dose dependently decreased plasma ghrelin levels compared to vehicle for 8 hours after an intraperitoneal injection in rats (26). Altogether, evidence supports the notion that ghrelin and GLP-1 interact to mediate food intake.

In a fasted state when ghrelin is high or in response to exogenous ghrelin, the majority of GLP-1Rs are located in the cytoplasm. *In vitro* studies confirm that GLP-1Rs are located in the cytoplasm in response to ghrelin. An intriguing question that still remains to be answered is whether ghrelin is driving GLP-1Rs to the cytoplasm or whether it is restricting GLP-1Rs in the cytoplasm. Additional work is needed to investigate the interaction of ghrelin along the GLP-1R recycling pathway.

In vivo, the translocation of GLP-1R was exclusively observed in VAN. GLP-1R translocation in culture is similar to what we observe *in vivo*. We are using a culture method validated by de Lartigue *et al* to study the neurons in which they reported a 90%

of the cells were neurons (14). Although we assume that the response we are observing in culture is in VAN, we have not tested these cells in determine the ratio of neurons to glia. A recent publication by Avau *et al.* found a significantly larger ratio of glial cells in their nodose cultures and that glial cells, rather than neurons, responded to ghrelin in culture (27). It is possible that we are looking at the communication between glial and neurons and this needs to be further elucidated.

In this study, we have shown that ghrelin is a major player in mediating the translocation of GLP-1Rs on VAN. We have furthered our knowledge on the mechanism by which GLP-1Rs are translocated according to feeding status. Ghrelin is a major player in modulating the satiating signaling of GLP-1 on VAN. Given that circulating ghrelin levels are low in obesity, these findings highlight the importance of understanding the mechanisms of gut-derived peptides on VAN.

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Chapter 2.3: Deletion of leptin signaling in vagal afferent neurons results in hyperphagia and obesity

(Manuscript Published in Molecular Metabolism in July 2014)

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Running title: leptin receptor deletion in vagal afferent neurons

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2.3.1 Abstract

The vagal afferent pathway senses hormones released from the gut in response to nutritional cues and relays these signals to the brain. We tested the hypothesis that leptin resistance in vagal afferent neurons (VAN) is responsible for the onset of hyperphagia by developing a novel conditional knockout mouse to delete leptin receptor selectively in sensory neurons (*Nav1.8/LepR*$^{fl/fl}$ mice). Chow fed *Nav1.8/LepR*$^{fl/fl}$ mice weighed significantly more and had increased adiposity compared with wildtype mice. Cumulative food intake, meal size, and meal duration in the dark phase were increased in *Nav1.8/LepR*$^{fl/fl}$ mice; energy expenditure was unaltered. Reduced satiation in *Nav1.8/LepR*$^{fl/fl}$ mice is in part due the subsequent loss of VAN plasticity. Crucially *Nav1.8/LepR*$^{l/fl}$ mice did not gain further weight in response to a high fat diet. We conclude that disruption of leptin signaling in VAN is sufficient and necessary to promote hyperphagia and obesity.

Key words: leptin resistance, vagal afferent neurons, meal patterns, hyperphagia

2.3.2 Highlights

- First reported use of the Nav1.8 cre-lox system to determine the role of proteins in gut-brain signaling
- Loss of leptin receptors in vagal afferent neurons reduces satiation
- Leptin resistance in vagal afferent neurons is sufficient and necessary to induce diet-induced obesity
- Vagal afferent neurons are important in the long-term control of energy homeostasis

2.3.3 Introduction

Obesity has become recognized as a worldwide health threat and a major public health challenge. There is currently a lack of simple and effective therapies or preventative treatments against obesity, and the mechanisms involved in the onset of diet-induced obesity remain unknown. There is growing evidence that cellular leptin resistance in the hypothalamus is important in maintenance of obesity but is unlikely to have a causative role in the onset of obesity (1). There is growing evidence that altering the strength or sensitivity to the hedonic attractiveness of food (2), availability of food (3), learned preferences (4), or signaling from the gut (5) may be involved in initiating diet-induced obesity.

Since its identification in 1994, leptin has attracted much attention as a key central and peripheral signal involved in energy homeostasis (6-8). Global deficiency in leptin or leptin receptor (LepR) results in an increase in appetite, hyperphagia, and morbid obesity in both humans and rodents (9-11). Few cases of obesity have been attributed to leptin deficiency (12, 13); rather hyperphagia and obesity are associated with cellular resistance to leptin and the consequent lack of anorexigenic action of leptin (14). Considerable attention has focused on leptin resistance in arcuate neurons of the hypothalamus as a key event in development of hyperphagia and obesity (15). However, in rodent models of diet-induced obesity, leptin resistance in arcuate neurons does not develop until after food intake, body weight and adiposity increase, calling into question whether leptin resistance in hypothalamic neurons drives the initial hyperphagia and obesity (1). Other populations of neurons important in regulation of food intake express the leptin receptor, including vagal afferent neurons (VAN) (16, 17) and neurons in the nucleus of the solitary tract (18), the site of central termination of VAN. We have shown that within 6 weeks of feeding a high-fat diet in rats, VAN become leptin-resistant; this

leptin resistance coincides with the development of hyperphagia without any measurable change in leptin signaling in the hypothalamus (19).

Leptin is a gut and adipose tissue-derived hormone that regulates a range of biological functions and processes, including energy intake and expenditure, body fat, neuroendocrine systems, autonomic function, and insulin and glucose balance (20). Multiple splice variants of the LepR (LepRa-f) have been identified with identical extracellular, transmembrane, and proximal intracellular domains (11, 21). Only LepRb, the long isoform containing a 300 amino acid intracellular tail can mediate the physiological effects of leptin (22). Binding of leptin to LepRb results in the activation of Janus tyrosine kinase 2 and leads to the phosphorylation of signal transducer and activator of transcription 3 (STAT3) (22). Mice with a neuron-specific disruption of neuronal STAT3 are hyperphagic, obese, diabetic, and infertile (23).

VAN express a plethora of receptors and carry the bulk of the information about the nutritional content of a meal from the gastrointestinal (GI) tract to the brain (24). VAN have been implicated in short term control of meal size and duration (25, 26), but whether inputs from the gut via VAN play a role in the long term regulation of food intake and body weight is not clear. In the current study, we test the hypothesis that leptin resistance in VAN is an initiating factor in the development of hyperphagia and obesity. Using a *Nav1.8*Cre-LoxP system we developed a conditional knockout mouse that lacks leptin receptor only in primary afferent neurons.

2.3.4 Methods

Animals

All experiments were approved by the UC Davis Institutional Animal Care and Use Committee (protocol #16793) and PHS animal welfare assurance to UC Davis (#A3433-01). Cre mice were generously donated by Dr. John Woods at UCL (27). LepR flox mice were purchased from Jax (28). Mice were bred to generate selective deletion of leptin receptor in primary afferent neurons. Nav1.8/LepR$^{fl/wt}$ offspring were subsequently crossed with LepR$^{fl/fl}$ mice to generate Nav1.8/LepR$^{fl/fl}$ mice. Cre-negative, Lepr$^{fl/fl}$, and Lepr$^{fl/wt}$ littermates (WT) were used as controls in all studies. Mice were individually housed after weaning under a 12h light: 12h dark schedule and allowed *ad libitum* access to food (Purina 5008) and water unless specified otherwise. All experiments were performed in 12 week old mice except for the high fat diet experiment in which 9 week old chow fed mice were either kept on chow or given *ad libitum* access to high fat diet (45% kcal/g fat; Research diets D12451) for 12 weeks.

Tissue collection

Mice were fasted overnight, or fasted and refed 2 hours. Tissue and cardiac blood was collected immediately. The quantity of food ingested during the last meal and the time of the last meal before blood collection are important confounding variables since leptin is known to be released from the gut in response to a meal and can account for as much as 20% of circulating leptin (29). Therefore we minimized the variability in circulating leptin levels between animals by collecting blood, and measuring leptin levels, from fasted animals. Leptin was measured by ELISA according to the manufacturer's protocol (Alpco Diagnostics, Salem, NH). For qPCR tissue was snap frozen. For

immunohistochemistry tissue was fixed in 4% paraformaldehyde and left in 25% sucrose overnight prior to sectioning.

PCR

Flox mice were genotyped according to prior reports (28).

To quantify *LepR* knockdown in tissue we used real-time PCR. All samples were repeated in triplicate to assure reproducibility of results. *LepR* was expression was quantified using validated TaqMan primer/probe sets (Mm01262072_m1) and conditions for the real-time RT-PCR detection of mouse leptin receptor. Specific PCR products were confirmed by demonstrating the presence on an agarose gel by electrophoresis. All samples were compared to a reference gene 18S (Mm03928990_g1). Quantification was then performed using the comparative Ct method. Nodose ganglia and superior cervical ganglia samples from 2 mice were pooled together to get sufficient cDNA, a total of 12 animals were used for these tissue.

Immunohistochemistry

As previously described (30). Primary antibodies raised against CART (H-003-63) and MCH (H-070-47) (1:200; Phoenix Peptides, Buringlame, CA), CB1 (sc20754), Y2 (sc14736), and LepR (sc8391) (1:100; Santa Cruz Biotechnology Inc., Dallas, TX), pSTAT3 (9145) (1:100; Cell Signaling Technology, Beverly, MA), and CRE recombinase (MMS-106P) (1:200, Covance Inc, Emeryville, CA) were used. Secondary antibodies were used as appropriate and included donkey anti-rabbit immunoglobulin and donkey anti-goat immunoglobulin conjugated with Alexa Fluor 488 or 555 (1:400; Molecular Probes, Eugene, OR). Percentage of positive pixels and positive neurons were quantified using Scion software as previously described.

Body compostion

Body fat and lean mass of mice were assessed by dual-energy X-ray absorptiometry (DEXA).

Metabolic analysis

Energy expenditure was evaluated in two separate cohorts of age matched (±2 days) WT and *Nav1.8/LepR*$^{fl/fl}$ mice fed powdered chow diet (LabDiet 5058) using a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Comlubus, Oh). Data was combined since no statistical difference between runs was identified (data not shown). Mice were fed powdered diet for one week and acclimated to monitoring chambers for 2 days prior to 48hr data collection; data is presented as an average of both days. Energy expenditure was calculated from the oxygen intake. Activity levels were determined by counting laser breaks along a, x, y, and z-axis. The food bout was defined as an episode of uninterrupted feeding of at least 0.02 g, and meal termination was when a bout of feeding was followed by 10 min with no measurable intake.

Feeding studies

Mice were fasted 12h prior to feeding tests. Cholecystokinin (octapeptide, sulfated) was purchased from Bachem (Torrance, CA,) and leptin (rat) from Sigma Aldrich (St. Louis, MO). CCK (0.3-30µg/kg; ip), leptin (120µg/kg; ip), or saline (100µl; ip) were administered and food was immediately returned to the cage; food intake was recorded every 20 minutes over 2 hours for CCK studies and every hour for 7 hours for leptin studies.

Statistics

Statistical analysis was performed using Prism software (Prism 5.0; GraphPad Software, La Jolla, CA). Unpaired t-test was used to make direct comparisons between WT and *Nav1.8/LepR*$^{fl/fl}$ mice. In feeding experiments paired t-test was used to compare saline with either CCK or leptin. Two-way ANOVA with Bonferroni post hoc test was used to compare the effects of leptin and saline over time in the WT and *Nav1.8/LepR*$^{fl/fl}$ mice; to compare circulating leptin concentrations in WT and *Nav1.8/LepR*$^{fl/fl}$ mice at 6 and 12 weeks; to compare the expression of CART, MCH, Y2, and CB1 expression in nodose ganglia of WT and *Nav1.8/LepR*$^{fl/fl}$ mice in response to feeding or fasting; and to compare weight gain over time in chow and high fat fed animals. One-way ANOVA with Bonferroni post hoc test was used to compare adiposity between *Nav1.8/LepR*$^{fl/fl}$ mice fed high fat or chow diets, and their starting weights before going on their respective diets. Differences were considered significant if $p<0.05$. Data are means ± SEM. * represents $p<0.05$; ** represents $p<0.01$; and *** represents $p<0.001$. For all experiments in which one-way ANOVA was performed different letters ^{a,b,c} denote significant differences between groups.

2.3.5 Results

Nav1.8 Cre selective deletion of LepR in VAN

The conditional leptin receptor allele has been used previously to generate liver- and brain-specific KO mice (28). Lox P sites flank either side of the first coding exon of *LepR* (LepRlox), which includes the signal sequence; thus cre-mediated recombination deletes all splice variants. LepRlox mice were bred with mice expressing cre driven by the Nav1.8 promoter (27) to generate selective deletion of leptin receptor in primary afferent neurons (Fig 2.3.1). Nav1.8/LepR$^{fl/wt}$ offspring were subsequently crossed with *LepR*$^{fl/fl}$ mice to generate *Nav1.8/LepR*$^{fl/fl}$ mice. Cre-negative, *LepR*$^{fl/fl}$, and *LepR*$^{fl/wt}$ littermates (WT) were used as controls in all studies.

Both WT and *Nav1.8/LepR*$^{fl/fl}$ mice were born at the expected Mendelian frequency, survived to adulthood, and were fertile. The average litter size was 6 for both genotypes and ranged from 2-13/litter in *Nav1.8/LepR*$^{fl/fl}$ mice and 1-13/litter in WT mice. Nav1.8 has previously been demonstrated to be exclusively expressed in sensory neurons, and was actively found to be absent from the cortex, cerebellum, and hippocampus in the brain (31). Here we report that *LepR* expression was unchanged in both hypothalamus and whole brain extracts of WT and *Nav1.8/LepR*$^{fl/fl}$ mice by real-time quantitative PCR analysis (Fig 2.3.2A). We confirmed by immunohistochemistry that there was no ectopic cre recombinase in discreet neurons of the arcuate nucleus or nucleus of the solitary tract (Fig 2.3.3). In addition we demonstrated that other organs that do not express Nav1.8, including liver, spleen, muscle, white adipose, heart, lung, and kidney had similar *LepR* expression in both *Nav1.8/LepR*$^{fl/fl}$ mice or WT mice (Fig 2.3.4A-C).

We did observe a significant decrease (93%) in *LepR* mRNA in neurons of the nodose ganglia in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (Fig 2.3.2B). In contrast,

there was no significant decrease in *LepR* expression in populations of other primary afferent neurons that express Nav1.8 including the trigeminal ganglia (TG), dorsal root ganglia (DRG), spinal cord, and superior cervical ganglia (SCG) in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice. We suggest that this was at least in part due to the overlap between *LepR* and *Nav1.8* expression within subsets of sensory neurons. Approximately 70% of VAN express leptin receptor (LepR) (16, 17) and a similar percentage of these neurons express Nav1.8 (27). The large reduction in LepR expression in nodose ganglia suggests that there is significant overlap between *LepR* and *Nav1.8* expression in these neurons. Around 70% of DRG neurons are positive for Nav1.8 (27), but only a small population of DRG neurons express *LepR* (32). There was a small decrease in *LepR* expression in DRG of *Nav1.8/LepR*$^{fl/fl}$ mice that did not reach statistical significance, suggesting that spinal afferent neurons expressing *Nav1.8* are a different subpopulation to those expressing *LepR*. Although TG neurons express high levels of *LepR* protein (33), very few are *Nav1.8* positive (27); we found no difference in *LepR* expression in the TG. There are currently no reports in the literature demonstrating that SCG neurons express *LepR*; although it has been proposed that cultured SCG neurons may be responsive to leptin (34), suggesting that at least a proportion of these neurons may express the *LepR* gene. Here we report that SCG neurons do express *LepR*, although in lower concentrations than in NG, DRG, and TG (Fig 2.3.2B); no change in *LepR* expression was found, presumably as a result of low *Nav1.8* expression in these neurons (27).

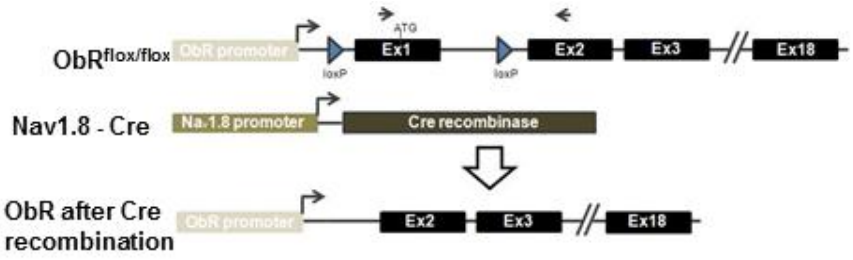


Figure 2.3.1 Generation of conditional sensory-neuron-LepR KO by Cre-loxP system

A schematic of gene targeting used to insert loxP on either side of the *LepR* coding exon. Arrows denote target site of primers.

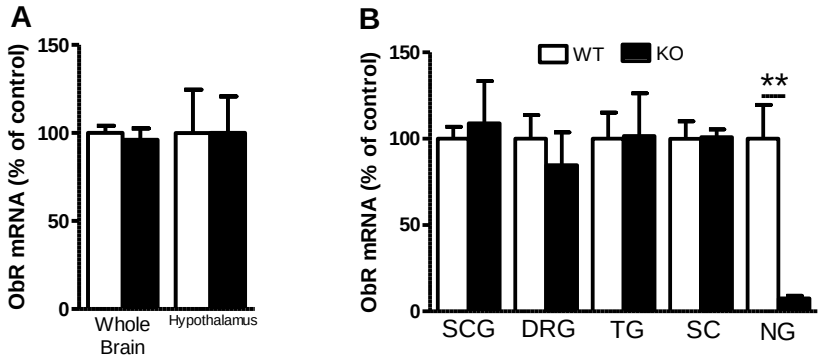


Figure 2.3.2 Verification of conditional sensory-neuron-LepR KO by Cre-loxP system

Tissues were collected to measure mRNA levels of LepR in *Nav1.8/LepR*$^{fl/fl}$ and WT mice (A) Percent change in LepR mRNA in brain and hypothalamus compared to WT, (B) in sensory neurons: superior cervical ganglia (SCG), dorsal root ganglia (DRG), trigeminal ganglia (TG), spinal cord (SC) and nodose ganglion (NG) (N=6; NG and SCG from 12 animals were used and ganglia from two animals were pooled to get sufficient cDNA) Results are shown as means ± SEM; **P<0.001 (Student t test).

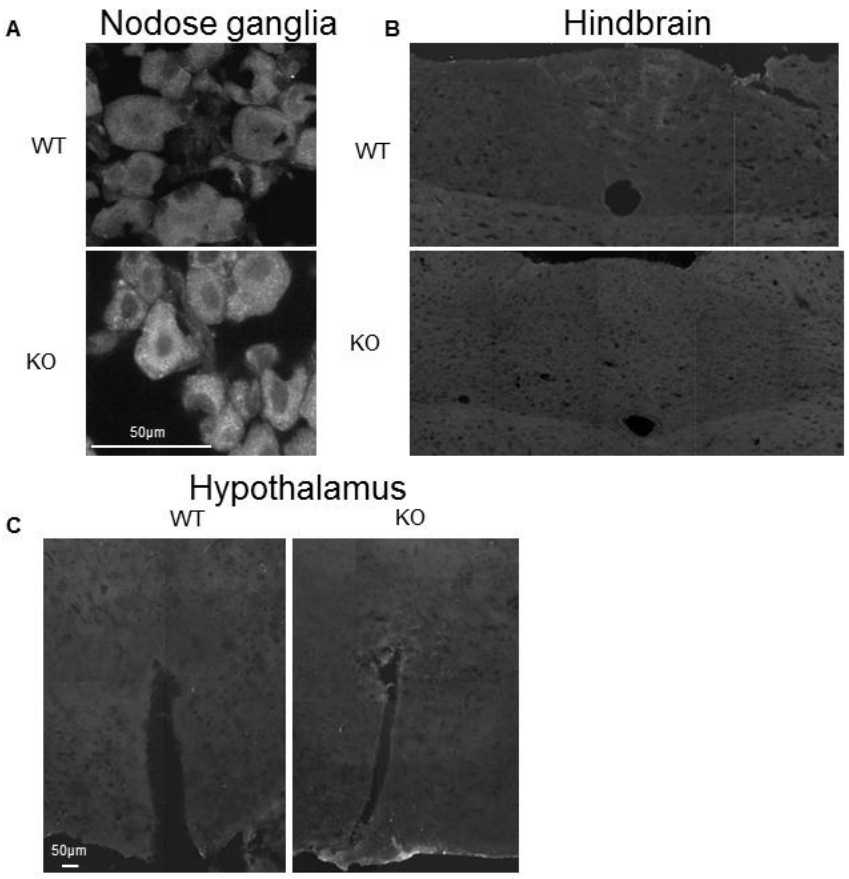


Figure 2.3.3 Analysis of CRE in nodose ganglia and brain

VAN and brain samples were stained for CRE (A) CRE immunoreactivity was positive in nodose ganglia but completely absent in the hypothalamus (B) and brainstem (C) in *Nav1.8/LepR*$^{fl/fl}$ mice (N=6).

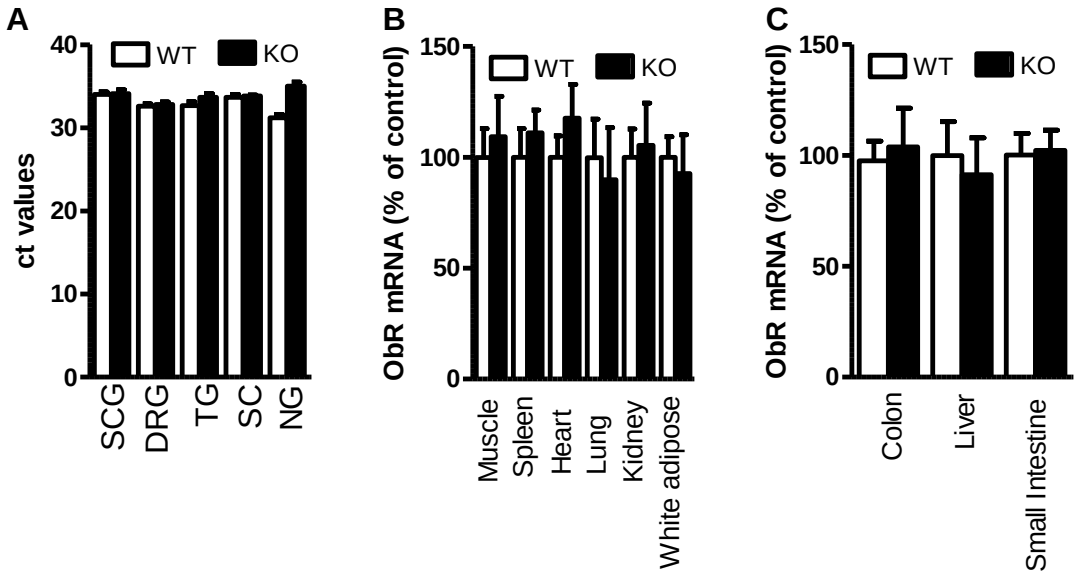


Figure 2.3.4 Verification of LepR KO in peripheral tissues

Verification of LepR in sensory neurons and peripheral tissues **(**A) ct values for LepR expression in all of the sensory neurons of both WT and KO; we found higher expression of LepR in VAN of WT mice compared to all other sensory neurons (ct value VAN 31.2 vs. DRG 32.6 vs. TG 32.7 vs. SCG 34). This reflects approximately a 2-fold increase in *LepR* expression of WT mice in VAN compared to DRG and TG, and 8-fold increase in *LepR* expression in VAN compared to SCG. (B-C) Percent change in *LepR* mRNA of *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT in non-neuronal peripheral tissue. There was no change LepR mRNA in (B) muscle, spleen, lung, heart, kidney and white adipose tissue, or in (C) gastrointestinal tissue including colon, liver and small intestine between the genotypes. Results shown as means ± SEM (n=12) (Student t test).

To demonstrate that the lack of *LepR* mRNA results in loss of LepR protein we stained nodose ganglia with a LepR antibody. WT mice express LepR on the plasma membrane, while *Nav1.8/LepR*$^{fl/fl}$ mice little to no LepR staining (Fig 2.3.5). To confirm that the absence of LepR results in the absence of functional responsiveness to leptin in VAN, we measured the ability of an intraperitoneal administration of leptin to induce nuclear translocation of phosphorylated STAT3 (pSTAT3), a known mediator of leptin signaling downstream of LepRb (35, 36) (Fig 2.3.6A). In WT mice, intraperitoneal leptin (80ug/kg) increased nuclear pSTAT3 in VAN compared to saline (27.6±3.3 vs 8.4±1.8%; $p<0.001$) (Fig 2.3.6B). Leptin failed to increase nuclear pSTAT in VAN of *Nav1.8/LepR*$^{fl/fl}$ mice compared to saline (5.6±0.8 vs 4.0±1.2%; $p>0.05$) (Fig 2.3.6B). The 93% reduction in LepR expression results in an 80% reduction in pSTAT3 nuclear expression in VAN. These data confirm that the *LepR* deletion was specific to vagal afferent neurons and we conclude that any phenotypic alteration observed in *Nav1.8/LepR*$^{fl/fl}$ mice is due to the loss of LepR in VAN.

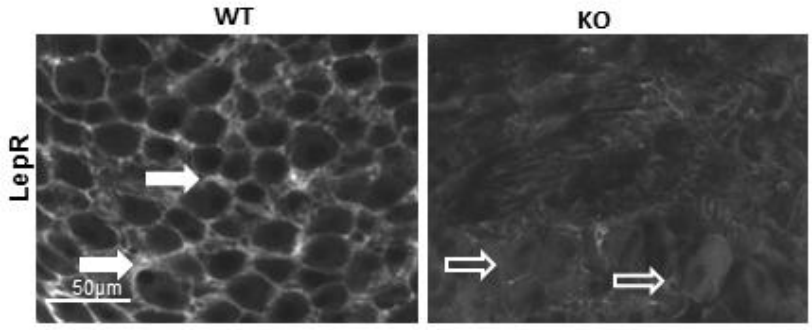


Figure 2.3.5 Leptin receptor in VAN of *Nav1.8/LepR*$^{fl/fl}$mice and WT mice

VAN of *Nav1.8/LepR*$^{fl/fl}$ and WT mice were stained with LepR antibody. Immunoreactivity was positive in WT mice and negative in *Nav1.8/LepR*$^{fl/fl}$ mice (n=6).

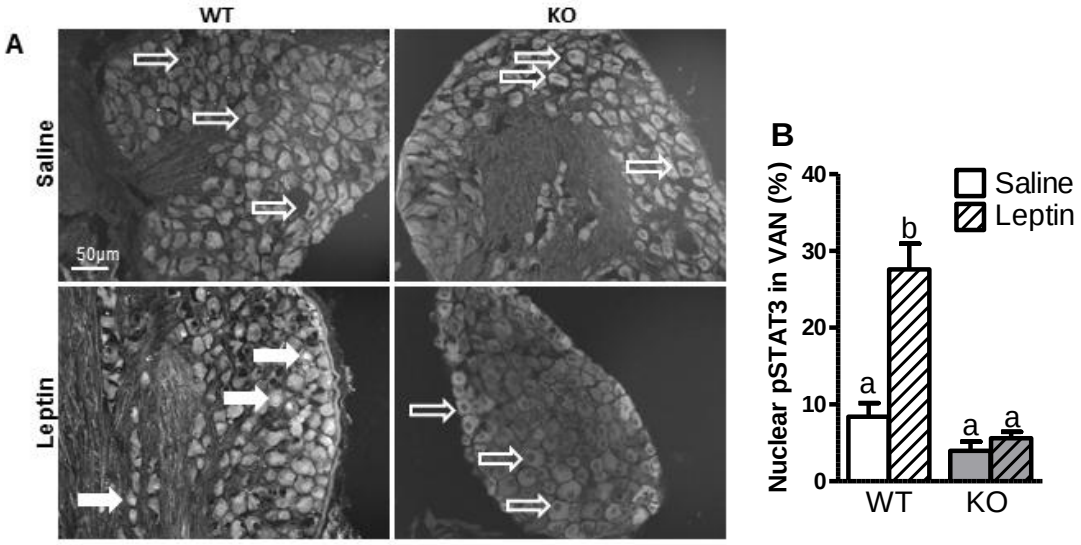


Figure 2.3.6 Leptin-induced phosphorylation of pSTAT3 in *Nav1.8/LepR$^{fl/}$* and WT mice

Animals were food deprived for 18h prior to an administration of saline or 120µg/kg leptin (A) Immunoreactivity for phosphoSTAT-3 in *Nav1.8/LepR$^{fl/fl}$* mice and WT mice in VAN (B) Quantification of percentage of positive nuclear pSTAT3 in VAN. Results are shown as means ± SEM (n=8); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (One way ANOVA).

Deletion of LepR in VAN leads to obesity

To determine the importance of endogenous leptin signaling in VAN on the regulation of energy homeostasis, we monitored the body weight of WT and *Nav1.8/LepR*$^{fl/fl}$ mice. Deletion of *LepR* in VAN of chow-fed mice led to a small but significant increase in body weight at 10 weeks ($p<0.05$) that increased further by 12 weeks ($p<0.001$; Fig 2.3.7A). The increase in body weight is less pronounced than seen in whole body (37) or neuronal *Nav1.8/LepR*$^{fl/fl}$ mice (28); however, it more closely resembles weight gain of WT mice fed a high fat diet post-weaning for 12 weeks (38). Importantly this increase in body weight was a result of increased fat mass (Fig 2.3.7B+D and 2.3.8B). In 12 week old mice, the naso-anal length was not significantly different between the groups (9.8±0.1 vs. 9.9±0.3cm; $p>0.05$); however adiposity increased 40% in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (Fig 2.3.7B+D and 2.3.9B). The weight of subcutaneous, retroperitoneal, mesenteric, and epididymal fat pads were increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (Fig 2.3.7D) as a result of increased adipocyte cell size (Fig 2.3.8A-D). The increase in adipocyte cell size of *Nav1.8/LepR*$^{fl/fl}$ mice is also seen in the shift in the frequency distribution of the subcutaneous, epididymal and mesenteric adipocyte diameter from compared to WT mice (Fig 2.3.8E-G). When the fat mass was adjusted for body weight we determined that there was a redistribution of fat pad mass to mesenteric and retroperitoneal depots in the *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (Fig 2.3.7E). This is consistent with previous studies in which disrupting vagal afferent signaling altered visceral fat depots (39-41). However, the mechanism remains unclear since there appears to be little parasympathetic supply to white adipose tissue (42). Interestingly, despite the very significant increase in adiposity, circulating plasma leptin concentrations were indistinguishable between genotypes at 6 or at 12 weeks (Fig 2.3.7C). Dissociation

between circulating leptin and adiposity has been reported in female Wistar rats fed a moderately high-fat diet and was suggested to contribute to weight gain (43). It is possible that the lack of feedback from the adipose tissue in the *Nav1.8/LepR*$^{fl/fl}$ mice contributes to the weight gain although this needs further investigation.

Deletion of LepR in VAN increases food intake in the dark phase

To determine the mechanism by which LepR knockout in VAN increases body weight, WT and *Nav1.8/LepR*$^{fl/fl}$ mice (12 weeks old, n=8) were randomly selected to be placed in metabolic cages to measure food intake, meal patterns, indirect calorimetry, and locomotor activity; based on their body weight these mice were representative of the whole population. Whole body composition analysis revealed that *Nav1.8/LepR*$^{fl/fl}$ mice weighed significantly more than WT mice as a result of increased fat mass, with no change in lean mass (Fig 2.3.9A-D). Energy expenditure and meal patterns were evaluated using a comprehensive lab animal monitoring system in which animals were fed powdered LabDiet 5058. There were no changes in energy expenditure (Fig 2.3.10A-C), activity, or dietary fuel oxidation between the groups (Fig 2.3.10D+E). There was a modest increase in energy expenditure, activity (data not shown) and respiratory quotient in all animals during the dark cycle, reflecting their nocturnal behavior.

Overall cumulative daily food intake trended to increase in *Nav1.8/LepR*$^{fl/fl}$ mice but this did not reach statistical significance ($p=0.07$); however, *Nav1.8/LepR*$^{fl/fl}$ mice ate significantly more than WT during the dark cycle (+22%; Fig 2.3.11A-C; $p<0.05$). The increase in food intake occurred predominantly in a hour window at the onset of the dark cycle (Fig 2.3.11B). Meal patterns over 24h and in the light phase were not significantly different between groups, but we found prolonged meal duration and increased meal size in the dark phase, especially in the first few hours of the dark phase. Meal pattern

analysis revealed that *Nav1.8/LepR*$^{fl/fl}$ mice ate longer meals (+26%) compared to WT mice in the early and total dark cycle ($p<0.05$; Fig 2.3.12D+C). The increased meal duration in the dark phase led to a trend towards increased 24h meal duration ($p=0.06$; Fig 2.3.12A). The quantity of food ingested in each meal during the total dark phase trended to increase (+16%) in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT ($p=0.05$; Fig 2.3.12G), and was significant in the early part of the dark phase ($p<0.05$; Fig 2.3.12H). *Nav1.8/LepR*$^{fl/fl}$ mice had a smaller satiety ratio than WT mice in the early dark phase ($p<0.05$; Fig 2.3.12L). The satiety ratio correlates meal size with the time to the subsequent meal; the smaller satiety ratio indicates that *Nav1.8/LepR*$^{fl/fl}$ mice are less satiated by a meal than WT mice. No difference in meal number, intermeal interval, rate of ingestion was observed between genotypes (Fig 2.3.13).

Thus, deletion of the leptin receptor in VAN produces a significant effect on dark phase calorie consumption and meal patterns independent of energy expenditure. These findings indicate that disruption of leptin signaling in VAN ablates a physiological satiety mechanism that controls meal termination. Notably, this mechanism primarily operates during the first hours of nocturnal feeding when rodents eat the first and largest of their daily meals.

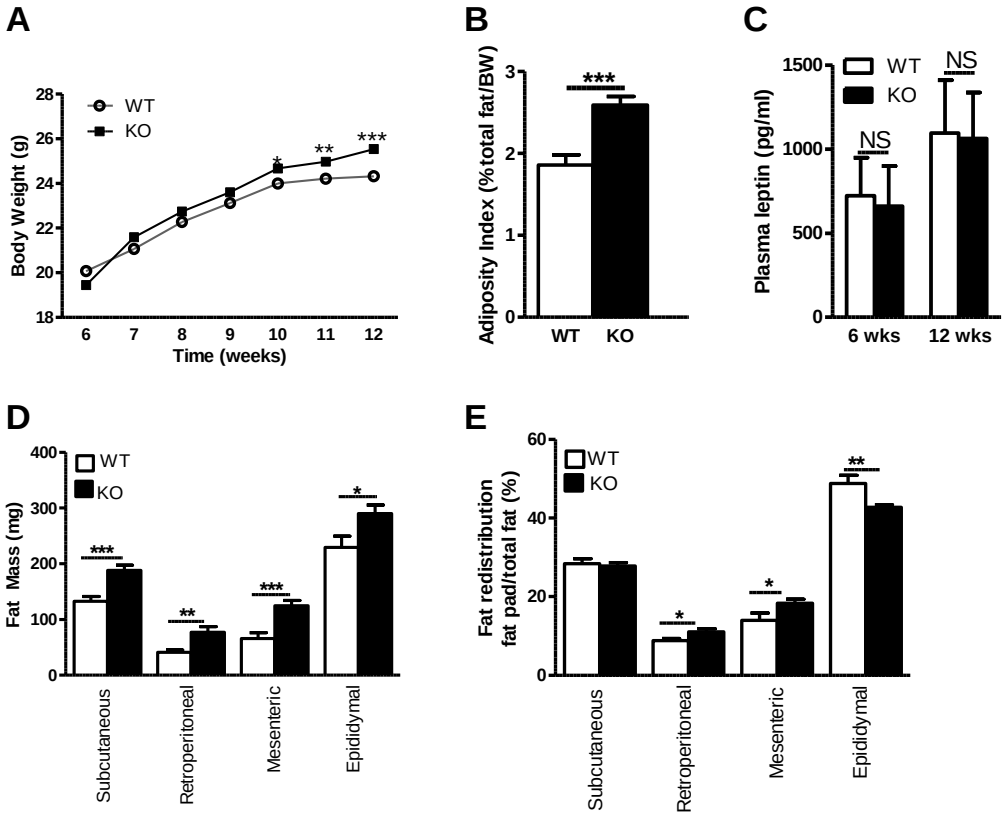


Figure 2.3.7 Analysis of energy homeostatic parameters

Weekly body weights were measured and fat pads were collected at 12 wks of age (A) Weekly body weight was significantly increased in chow-fed *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice (n=52). (B) Adiposity was significantly increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice. (C) No change in plasma leptin concentrations were found between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice at 6 or at 12 weeks of age. (D) Fat mass of subcutaneous, retroperitoneal, epididymal and mesenteric fat were all significantly increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice at 12 weeks, (E) but fat was different redistributed based on body weight. Results are shown as means ± SEM; *P< 0.05 **P<0.001 and ***P<0.001 (Two-way ANOVA and Student t test).

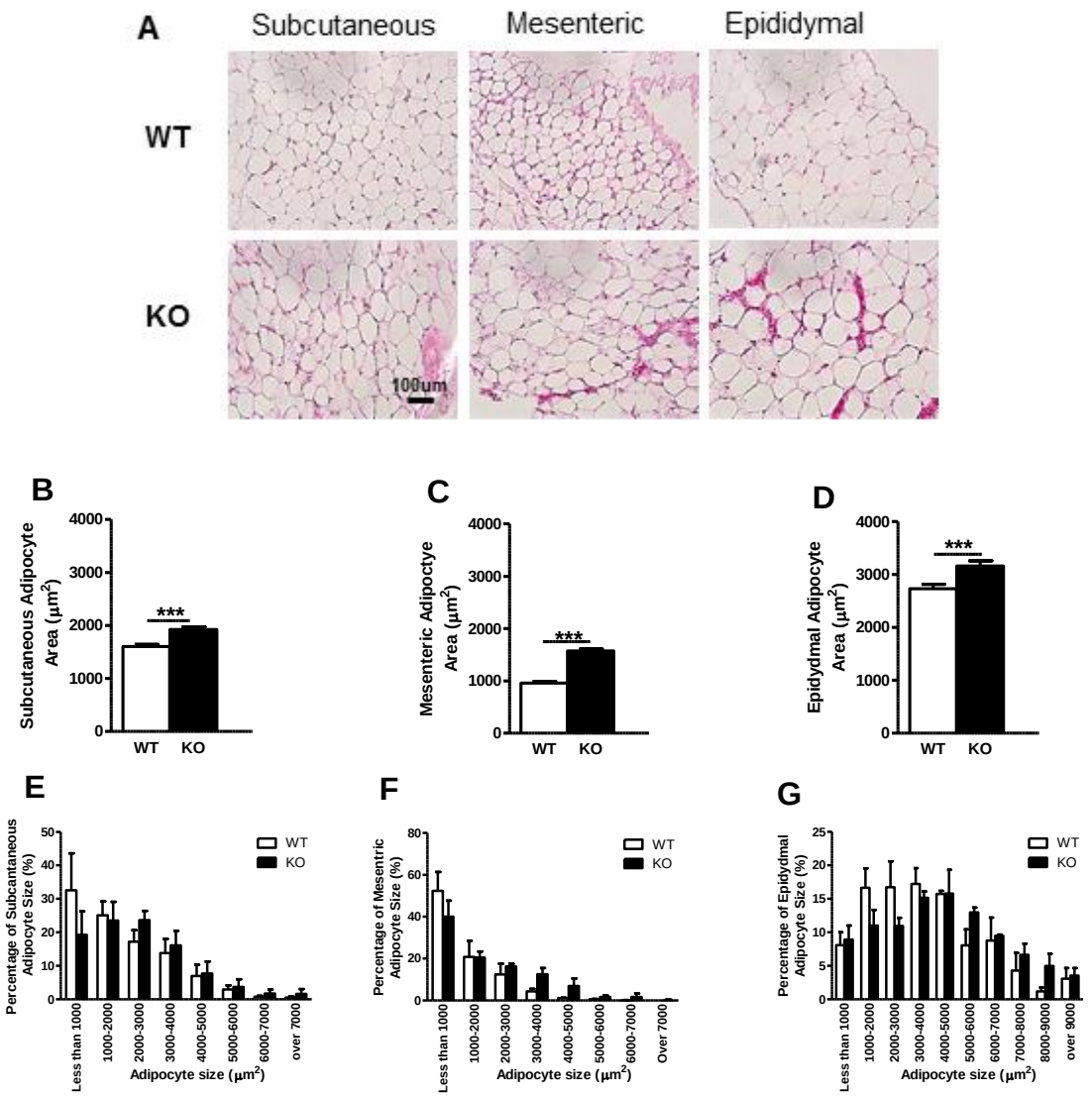


Figure 2.3.8 Analysis of fat pads in *Nav1.8/LepR$^{fl/fl}$* vs. WT mice

Fat pads were collected at 12 wks of age for analysis. (A) H&E stained section of fat pads. Quantification of subcutaneous (B), mesenteric (C) and epididymal (D) fat showing increased adipocyte area. The distribution of the percentage of adipocyte size of subcutaneous (E), mesenteric (F) and epididymal (G) fat demonstrates a right shift in the *Nav1.8/LepR$^{fl/fl}$* vs. WT mice. Results are shown as means ± SEM; ***P<0.001 (Student t test and two-way ANOVA).

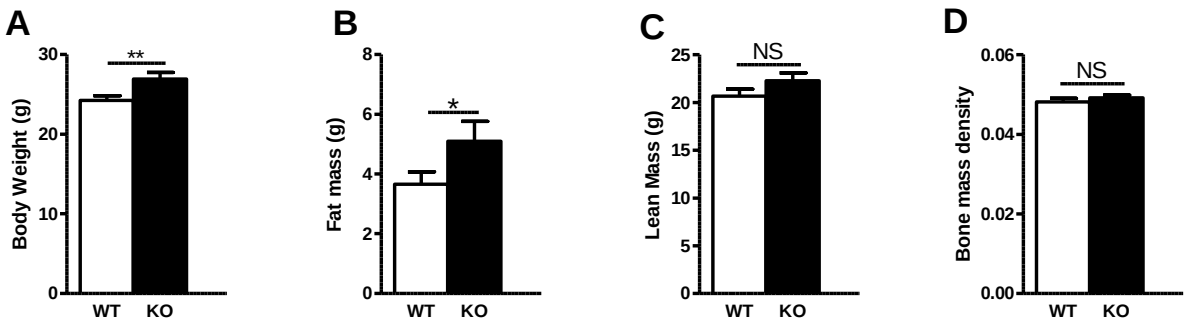
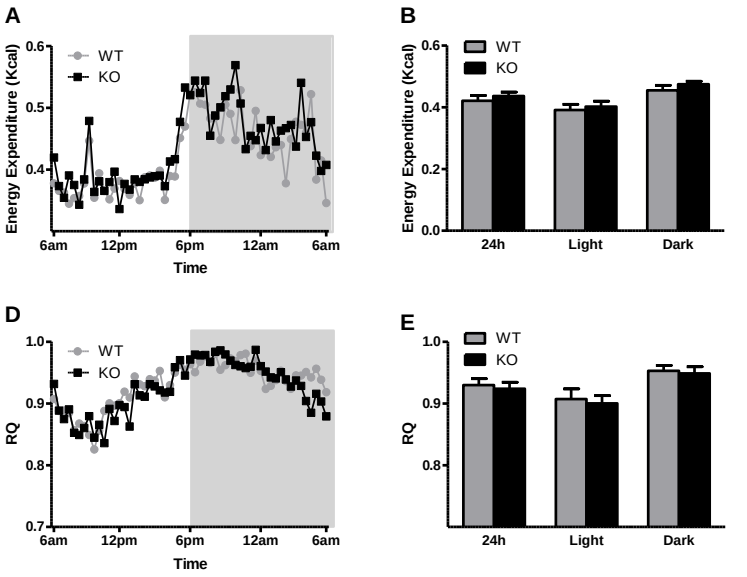


Figure 2.3.9 Analysis of body composition by DEXA

Total body composition analysis was conducted by dual energy X-ray absorptiometry (DEXA) in *Nav1.8/LepR*$^{fl/fl}$ mice and WT controls used for metabolic studies. (A) Body weight and (B) fat mass were increased in *Nav1.8/LepR*$^{fl/fl}$ mice mice compared to controls, with no change in (C) lean mass or (D) bone mass density. Results are shown as means ± SEM (n=8); *P< 0.05 and **P<0.001 (Student t test).



	Adjusted for BW	Adjusted for LBM
WT	0.422±0.011	0.427 ±0.012
KO	0.435 ±0.011	0.429 ± 0.012

Figure 2.3.10 Energy expenditure analysis by CLAMS

Nav1.8/LepR$^{fl/fl}$ mice and WT mice were placed in metabolic cages and (A-E) energy expenditure, respiratory quotient and food intake were recorded over 48 h. Average hourly energy expenditure (B) average 24h, light and dark energy expenditure, and (C) table of average energy expenditure adjusted for body weight and lean body mass was unchanged between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. (D) Average hourly respiratory quotient (RQ) over 24 hours and (E) total, light and dark RQ were unchanged between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. There were no differences in energy expenditure in *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice Results show as means ± SEM (N=8) (Student t test).

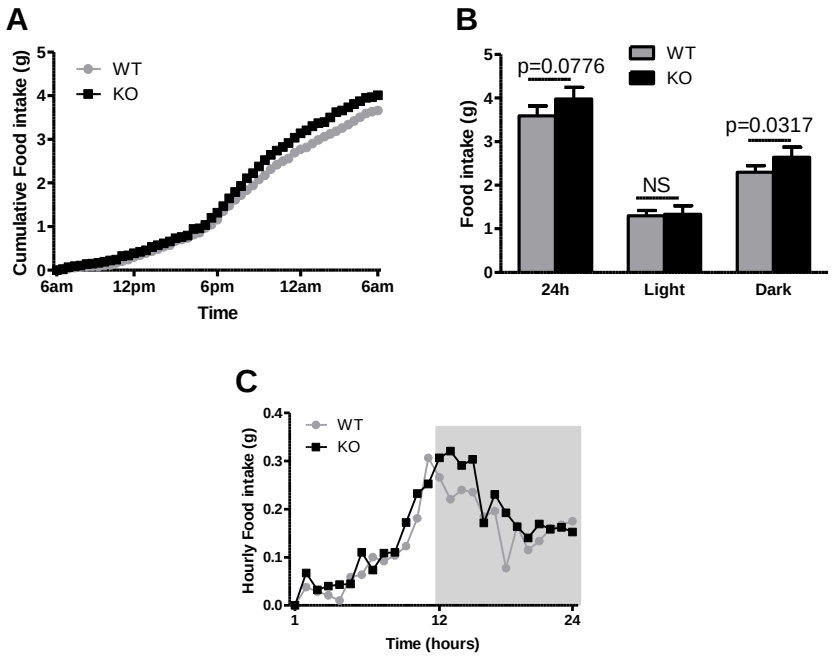


Figure 2.3.11 Food intake Analysis over 24 h measured by CLAMS

Nav1.8/LepR$^{fl/fl}$ mice and WT mice were placed in metabolic cages at 9 wks of age (A) Cumulative food intake over 24 hours was increased in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice. (B) Average food intake in *Nav1.8/LepR*$^{fl/fl}$ mice was significantly increased in the dark phase with no change in the light phase, resulting in a trend towards increased 24h food intake compared to WT mice. (C) Average hourly food intake over 24h demonstrating increased food intake in *Nav1.8/LepR*$^{fl/fl}$ mice in the first half of the dark phase. Results shown as means ± SEM (n=8) (Student t test).

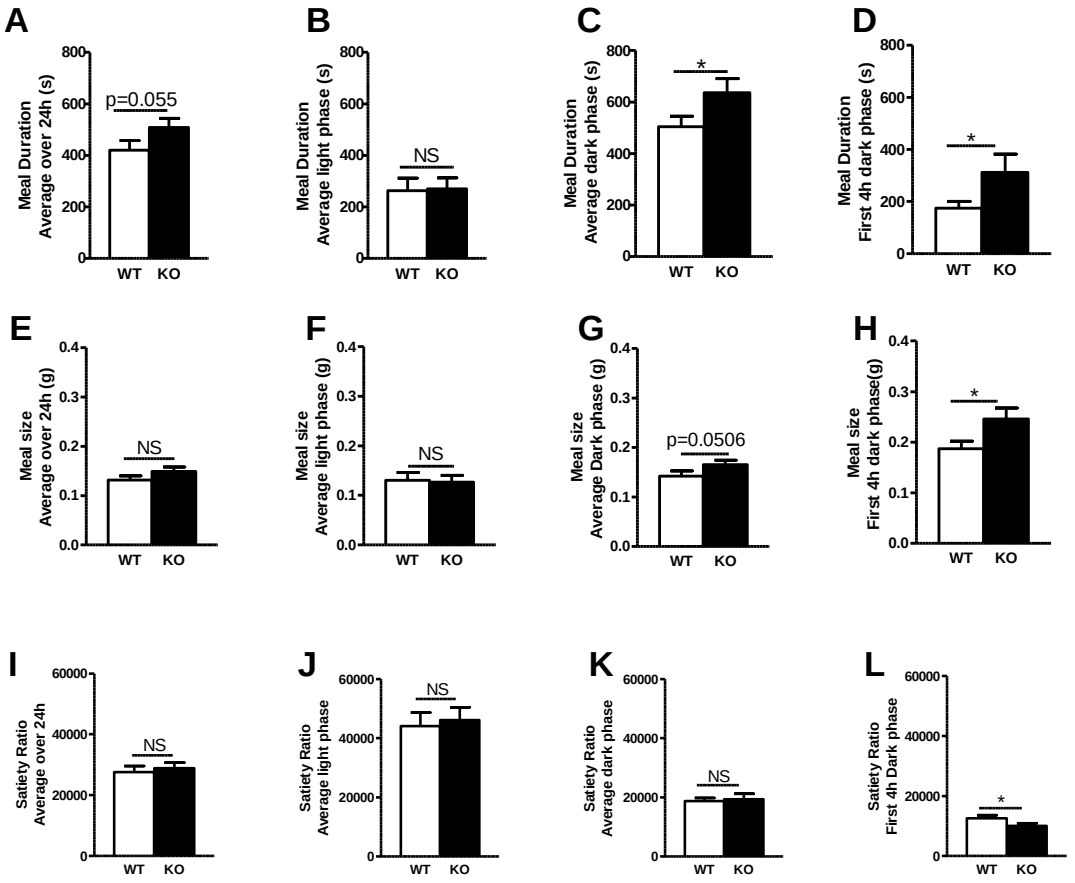


Figure 2.3.12 Meal pattern analysis by CLAMS

Meal duration (A-D) was significantly increased in the early dark phase (D), and total dark phase (C), but not the light phase (B), in *Nav1.8/LepR*$^{fl/fl}$ mice; with a trend towards 24h increase in meal duration (A). Meal size (E-H) was significantly increased in the early dark phase and trended towards increasing in total dark phase (F), but there was no change in the light phase (E) or total 24h (D) in *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice. Satiety ratio (I-L), a measure of fullness, was decreased in the early dark phase of the *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT (L), but no changes were observed in total dark phase (K), light phase (J), or total 24h (I). Results shown as means ± SEM (n=8) (Student t test).

Meal Parameter	Wildtype Control Mice	Sensory neuron-ObR Knockout Mice
Meal number	59.25 ± 5.47	57.38 ± 5.63 (NS)
Meal number in the light phase	22.50 ± 3.17	22.25 ± 3.18 (NS)
Meal number in the dark phase	36.75 ± 3.03	35.13 ± 2.99 (NS)
Intermeal interval (s)	2745 ± 306.3	2686 ± 258.4 (NS)
Intermeal interval in light phase (s)	3504 ± 368.1	3752 ± 356.6 (NS)
Intermeal interval in dark phase (s)	2082 ± 349.8	2051 ± 255.2 (NS)
Rate of ingestion (g/min)	0.064 ± 0.010	0.066 ± 0.009 (NS)
Rate of ingestion in light phase (g/min)	0.077 ± 0.009	0.078 ± 0.010 (NS)
Rate of ingestion in dark cycle (g/min)	0.052 ± 0.006	0.056 ± 0.009 (NS)

Figure 2.3.13 Table on meal parameters measured by metabolic cages

Meal number, intermeal interval and rate of ingestion did not differ between genotype irrespective of the light-dark phase. Results shown as means ± SEM (n=8) (Student t test).

Deletion of LepR in VAN reduces CCK- and leptin-induced satiation

Intestinal feedback inhibition of food intake is mediated by CCK-induced activation of the vagal afferent pathway and comprises of a decrease in meal size and duration (44). We hypothesized that a reduced sensitivity of VAN to CCK may be responsible for increasing meal size and duration in the *Nav1.8/LepR*$^{fl/fl}$ mice. Leptin and CCK synergism is well established (30, 45, 46) although the site of synergism remains unclear. CCK predominantly mediates its effects on food intake by activating CCK1 receptors on vagal afferent terminals innervating the gut. There is evidence that leptin is required for CCK to signal in VAN: in cultured VAN, leptin increases CCK signaling and leptin resistance in VAN reduces CCK-induced satiation (30). To test whether the absence of leptin signaling in VAN could inhibit CCK-induced satiation, we compared feeding responses to peripheral injections of exogenous CCK in WT and *Nav1.8/LepR*$^{fl/fl}$ mice. CCK (0.3µg/kg or 3µg/kg, IP) inhibited 2h food intake (Fig 2.3.14A), but failed to have any effect in *Nav1.8/LepR*$^{fl/fl}$ mice (Fig 2.3.14-B). A higher dose of CCK (30µg/kg, IP) significantly reduced food intake in both WT and *Nav1.8/LepR*$^{fl/fl}$ mice (Fig 2.3.14A-B). Absence of leptin signaling in VAN significantly reduces the ability of CCK to inhibit food intake. This deficit in CCK-induced signaling in the *Nav1.8/LepR*$^{fl/fl}$ mice reduces vagal afferent signaling of intestinal feedback inhibition of food intake, leading to hyperphagia, increase in body weight and a redistribution of fat to visceral depots.

In a previous study (46), leptin (120µg/kg; IP) was demonstrated to significantly reduce food intake over 7h in fasted C57BL/6J mice compared to saline. We confirmed previous reports that peripheral leptin significantly reduces food intake after 7h in WT mice (Fig 2.3.14C+D). In the *Nav1.8/LepR*$^{fl/fl}$ mice, leptin failed to significantly reduce food intake compared to saline (Fig 2.3.14C+D). At least at this dose, peripheral administration of leptin appears to mediate feeding behavior predominantly via a vagal

afferent pathway. It should be noted that the trend in reduced rate of food intake observed over the course of the 7 hours in the *Nav1.8/LepR*$^{fl/fl}$ mice may be a result of exogenous leptin crossing the BBB and acting on leptin receptors in the NTS or hypothalamus, since LepR expression remains intact in the CNS.

Deletion of LepR prevents CCK-induced plasticity in VAN

Plasticity of VAN is a well-established concept. Nerve damage or inflammation alters gene expression, changing sensitivity and excitability of VAN (47-49). More recently, nutrient availability in the gut has been associated with changes in expression of GPCRs and neuropeptide transmitters in VAN of rodents and humans (50). The neurochemical phenotype of VAN reversibly switches from an anorectic phenotype postprandially to an orexigenic phenotype under fasting conditions. Since CCK is a predominant mediator of this switch in phenotype and the absence of the leptin receptor expressed by VAN markedly compromises CCK-induced activation of VAN, we hypothesized that changes in VAN phenotype between fasted and fed conditions would be attenuated or abolished in the *Nav1.8/LepR*$^{fl/fl}$ mice.

Expression of the neuropeptide transmitter cocaine and amphetamine regulated transcript (CART; Fig 2.3.15A) and peptide YY receptor type 2 (Y2R; Fig 2.3.16A) were high, while the transmitter melanin concentrating hormone (MCH; Fig 2.3.15B) and cannabinoid receptor type 1 (CB1R; Fig 2.3.16B) expression were low in 2h refed compared to fasted WT mice, as previously described in lean rats (51-53). Conversely, food withdrawal decreased Y2R and CART expression and increased CB1R and MCH expression. However, this phenotypic switch was markedly attenuated or absent in *Nav1.8/LepR*$^{fl/fl}$ mice. Y2R and CART expression was constitutively low in VAN of KO

mice and the expression of the CB1R and MCH in VAN was high. Thus, the inability of VAN to respond to leptin results in a loss of CCK-induced neuronal plasticity.

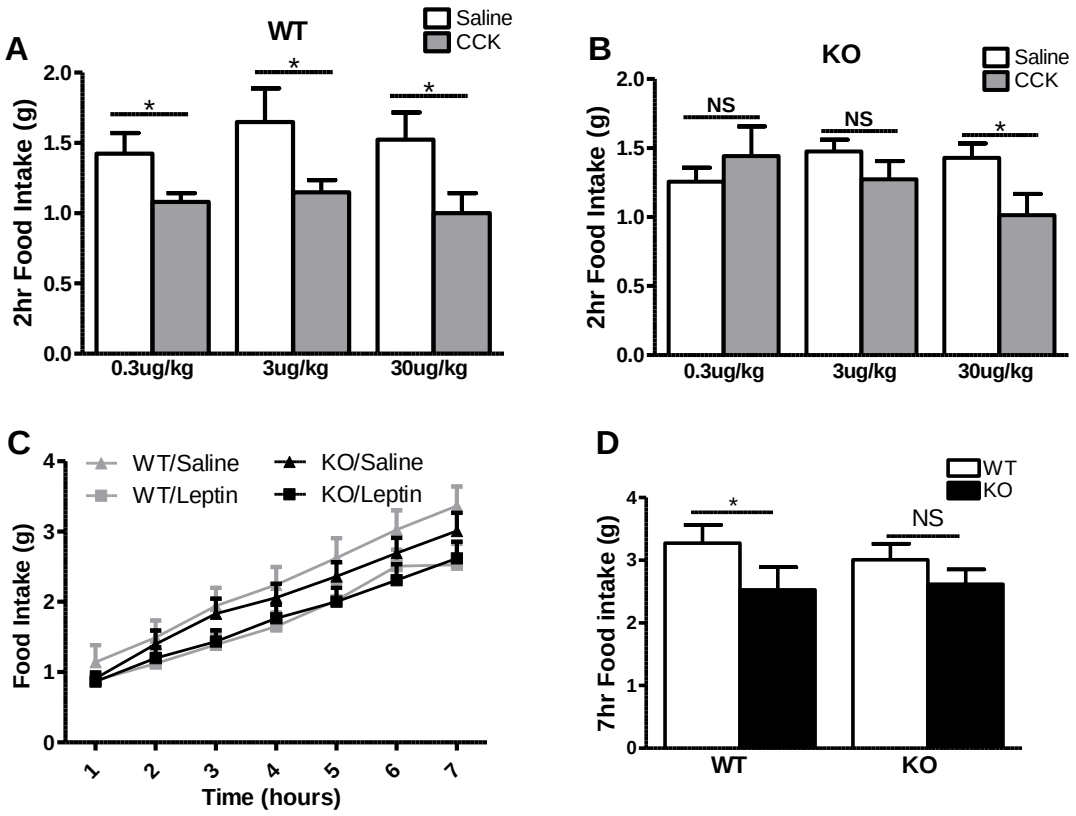


Figure 2.3.14 Satiating effects of CCK and leptin in *Nav1.8/LepR$^{fl/fl}$* and WT mice

Nav1.8/LepR$^{fl/fl}$ and WT mice were deprived of food for 18 hours and administered IP saline or CCK (A-B), and IP saline or leptin (C-D) and food intake was recorded (N=8). (A) CCK reduced 2h food intake in WT mice at doses of 0.3µg/kg, 3µg/kg of CCK and 30µg/kg; (B) satiating effects of CCK were only observed at highest dose in *Nav1.8/LepR$^{fl/fl}$* mice. (C) Food intake was measured every hour for 7 hours following IP administration of 120µg/kg of leptin (D) Leptin reduced 7hr food intake in WT mice but did not significantly inhibit food intake in *Nav1.8/LepR$^{fl/fl}$* mice. Results shown as means ± SEM (n=8) (Student t test and two-way ANOVA).

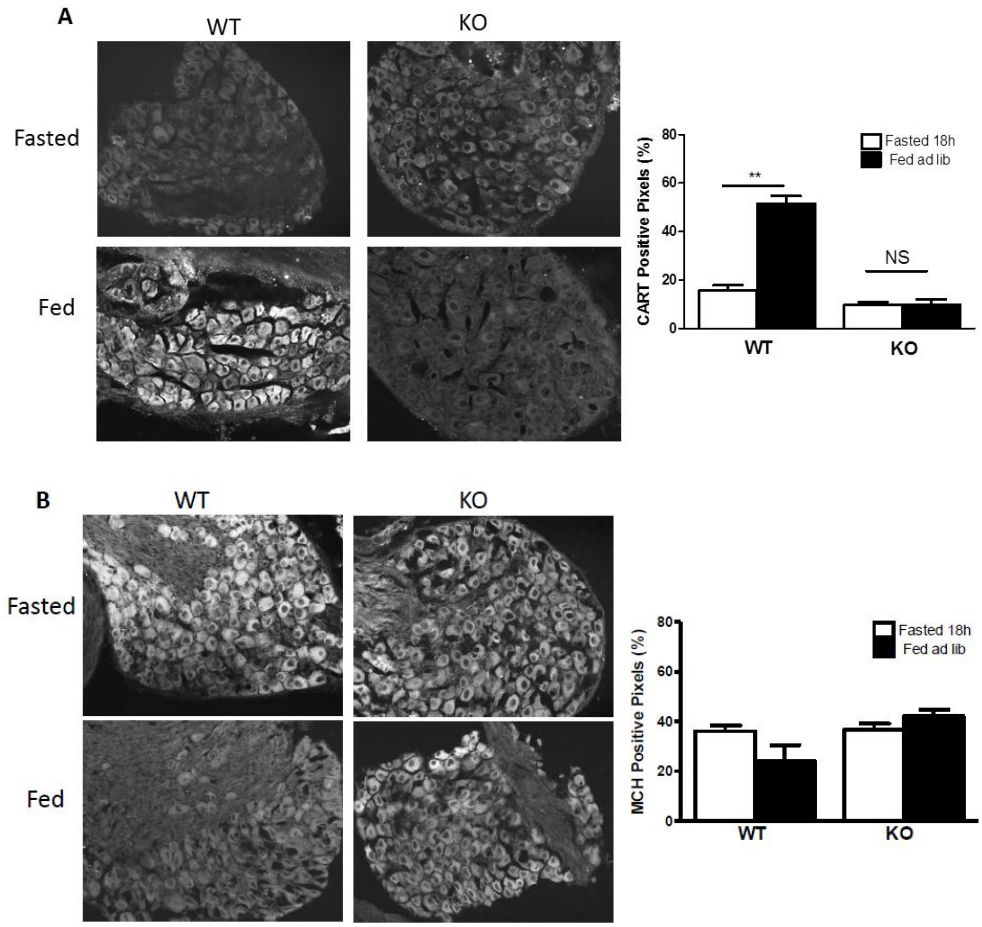


Figure 2.3.15 Analysis of VAN plasticity of CART and MCH

WT and *Nav1.8/LepR*$^{fl/fl}$ mice were either food deprived for 18hrs or fed *ad libitum*. (A) Immunoreactivity of CART protein in nodose ganglia. Percent of CART positive pixels was higher in fed compared to fasted conditions in WT mice, but constitutively low in *Nav1.8/LepR*$^{fl/fl}$ mice. (B) Immunoreactivity of MCH protein in nodose ganglia. Percent of MCH positive pixels was higher in fasted compared to fed conditions in WT mice, but constitutively high in *Nav1.8/LepR*$^{fl/fl}$ mice. Results shown as means ± SEM (n=4); *P<0.05 and **P<0.01 (Student t test).

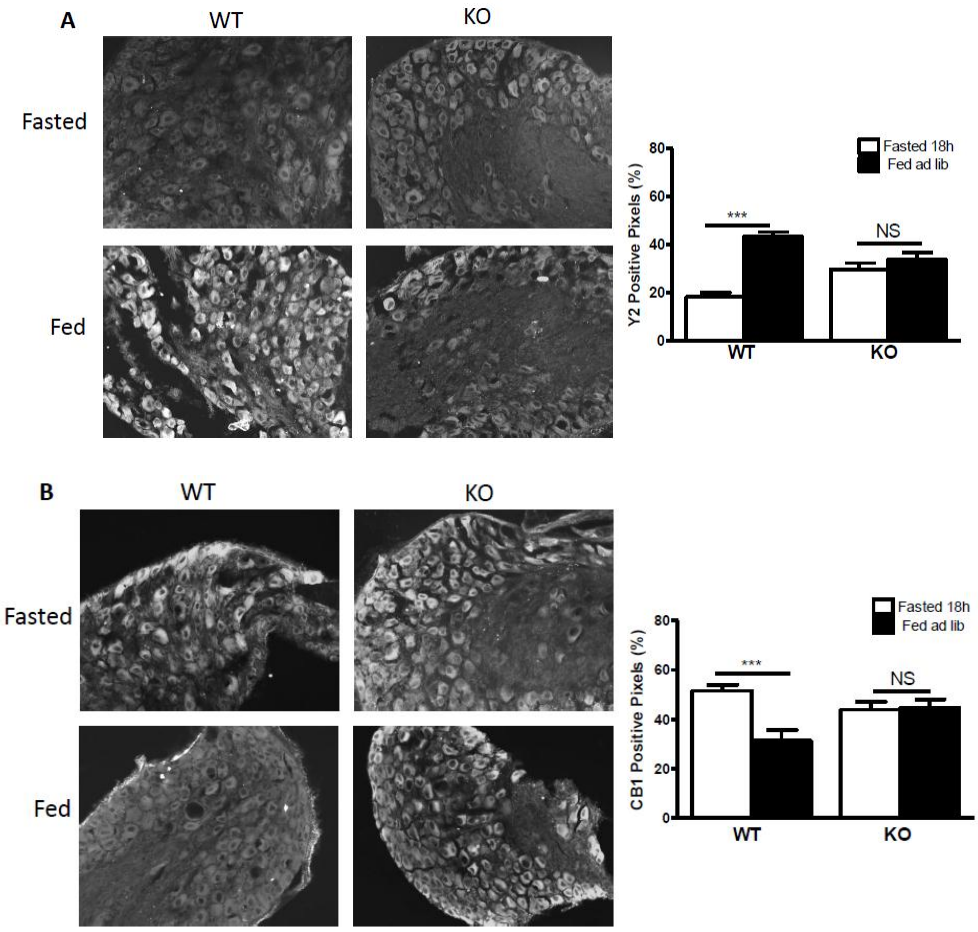


Figure 2.3.16 Analysis of VAN plasticity of Y2R and CB1

WT and *Nav1.8/LepR*$^{fl/fl}$ mice were either food deprived for 18hrs or fed *ad libitum*. (A) Immunoreactivity of Y2 receptor in nodose ganglia. Percent of Y2 positive pixels was higher in fed compared to fasted conditions in WT mice. Feeding status had no effect on Y2 positive pixels in *Nav1.8/LepR*$^{fl/fl}$ mice. (B) Immunoreactivity of CB1 receptor in nodose ganglia. Percent of CB1 positive pixels was higher in fasted compared to fed conditions in WT mice, but constitutively high in *Nav1.8/LepR*$^{fl/fl}$ mice. Results shown as means ± SEM (n=4); ***P<0.001 (Student t test).

Leptin resistance in VAN is necessary for the development of obesity

To determine whether leptin resistance in VAN is necessary for the onset of obesity we chronically fed *Nav1.8/LepR*$^{fl/fl}$ and WT mice with high fat diet. At 9 weeks, when all the animals still weighed the same (Fig 2.3.17A), mice were either kept on a chow diet or switched to a 45% high fat diet. As expected WT mice gained more weight on a high fat (HF) diet than on a chow diet (Fig 2.3.17B). After 21 weeks on chow *Nav1.8/LepR*$^{fl/fl}$ mice weigh more than WT mice (Fig 2.3.17B+C). Crucially, *Nav1.8/LepR*$^{fl/fl}$ mice failed to gain additional weight despite chronic ingestion of a HF diet for 12 weeks (Fig 2.3.17C), and weighed less than high fat fed WT mice (Fig 2.3.17B+C). Many factors are involved in weight gain following consumption of a high fat diet. We demonstrate that leptin resistance in VAN is sufficient to promote weight gain in the absence of a high fat diet, and that consumption of a high fat diet fails to increase weight gain in *Nav1.8/LepR*$^{fl/fl}$ mice. We infer from this data that other factors are involved in high fat diet-induced weight gain and that they are downstream of leptin resistance onset in VAN. We postulate that KO mice acquire compensatory mechanisms to deal with the loss of lepR in VAN during development which prevents them from gaining further weight on a high fat diet.

Adiposity comparisons in 21 week old mice fed their respective diets for 12 weeks, revealed that *Nav1.8/LepR*$^{fl/fl}$ mice gained significantly less fat than WT mice when fed a high fat diet (Fig 2.3.17D). As expected adiposity was significantly increased in WT mice fed a high fat diet compared to chow fed WT mice, however despite a large trend there was no statistical difference in adiposity between *Nav1.8/LepR*$^{fl/fl}$ mice fed chow or a high fat diet (Fig 2.3.17D). When studied at the individual fat pad level, we observed significant increases in epididymal and retroperitoneal fat pads of high fat fed *Nav1.8/LepR*$^{fl/fl}$ mice compared to chow fed *Nav1.8/LepR*$^{fl/fl}$ mice (Fig 2.3.17E).

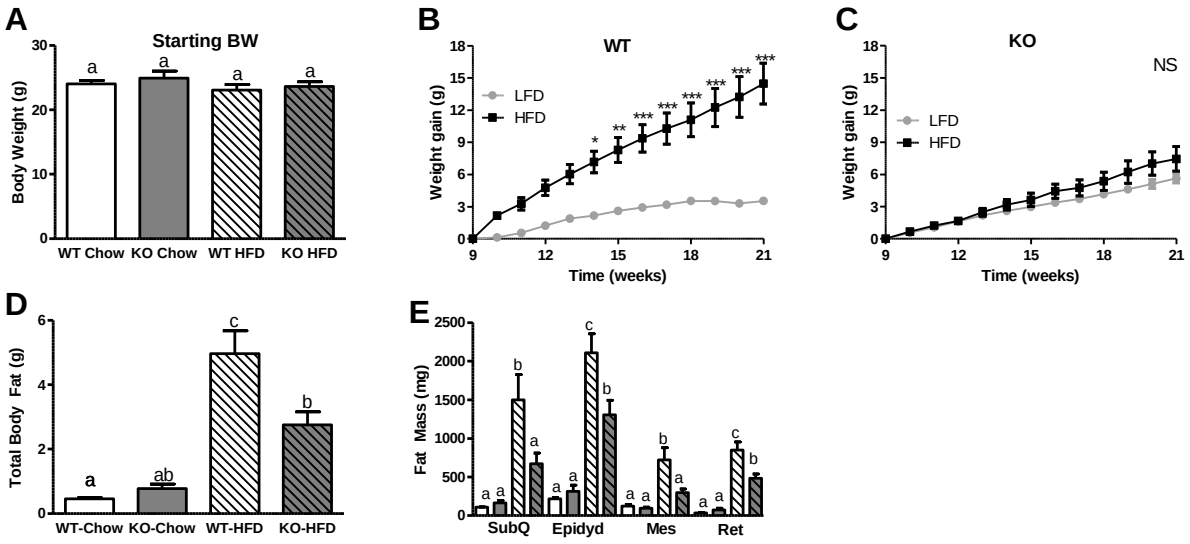


Figure 2.3.17 Analysis of weight gain and adiposity in response to a HF diet

WT and *Nav1.8/LepR*$^{fl/fl}$ mice were fed chow or high fat diet for 12 weeks (N=6-9). (A) The starting body weight of all the mice was not significantly different. (B) WT mice fed a high fat diet gained significantly more weight from 5 weeks of high fat feeding onwards. (C) *Nav1.8/LepR*$^{fl/fl}$ mice did not gain further weight in response to a high fat diet. (D) At the end of the study, high fat diet significantly increased adiposity in WT mice, but not in *Nav1.8/LepR*$^{fl/fl}$. (D) Chronic ingestion of a high fat diet for 12 weeks increased epididymal, subcutaneous, mesenteric and retroperitoneal fat pads in WT mice. (E) *Nav1.8/LepR*$^{fl/fl}$ mice fed a high fat diet had elevated epididymal and retroperitoneal fat pads, but not mesenteric and subcutaneous fat pads. Results are shown as means ± SEM (n=8); letters denotes significant differences between groups at that timepoint, i.e. a vs. b; ab not significantly different from a or b (One-way ANOVA and Two-way ANOVA).

2.3.6 Discussion

Considerable evidence has accumulated to suggest that the inability of leptin receptor-bearing neurons to respond to leptin plays a pivotal role in the development and/or persistence of an obese phenotype (7). We have developed and utilized a powerful new tool which allows the first targeted approach to determine the functional role of specific proteins in gut-brain signaling. Using the cre-lox method, we conclusively demonstrate that knocking out LepR in vagal afferent neurons is sufficient and necessary to increase food intake, weight gain and adiposity. There is evidence that LepRa, LepRb and LepRe splice variants are expressed in vagal afferent neurons (16, 17, 54). In this study we have deleted all isoforms of LepR; however, based on the fact that LepRb is currently the only isotype found to be involved in the control of food intake (22), and that LepRb signaling (ie. STAT3 activation) is severely blunted in *Nav1.8/LepR*$^{fl/fl}$ mice, our data suggest that deletion of LepRb is responsible for the phenotype of the *Nav1.8/LepR*$^{fl/fl}$ mice. The data also show that the lack of LepR expression in vagal afferent neurons leads to hyperphagia via a mechanism involving the reduction in sensitivity to gut hormones. Taken together with our previous findings that leptin resistance in vagal afferent neurons develops early in diet-induced obesity and coincides with hyperphagia, these findings demonstrate that the leptin receptor signaling in VAN mediates the hyperphagic response to chronic ingestion of a high fat diet.

The finding that VAN are important in the pathophysiology of diet-induced obesity by initiating overconsumption of food is particularly significant given that the vagal afferent pathway has largely been discounted as a putative mechanism for the onset of obesity and has only been thought to be involved in short term, meal-to-meal regulation

of food intake. VAN are well known to carry the bulk of the information about the nutritional content of a meal from the gastrointestinal tract to the brain, and lead to meal termination (24). Although we did not specifically study LepR expression in VAN innervating the gastrointestinal tract, Nav1.8-cre mice have been demonstrated to have extensive vagal innervation of the gastrointestinal tract (55) . Furthermore, retrograde tracing experiments have established that VAN innervating the gut are located in the caudal portion (56) and express CCK1 receptor (57). LepR notably colocalizes with CCK1 receptor in this population of VAN (16). We report here that LepR immunostaining is lost in the caudal region of the nodose ganglia and that CCK signaling is blunted in the *Nav1.8/LepR*$^{fl/fl}$ mice. Therefore, this is the first conclusive evidence that chronic disruption of gut-brain signaling via a vagal pathway reduces satiation over multiple meals leading to hyperphagia and obesity.

We propose that the absence of weight gain in *Nav1.8/LepR*$^{fl/fl}$ mice fed a high fat diet suggests that leptin resistance in VAN is a necessary initiating step in the development of diet-induced obesity. We demonstrate that ingestion of a high fat diet leads to weight gain in WT mice, and that deletion of LepR in VAN leads to weight gain, but that adding high fat diet to *Nav1.8/LepR*$^{fl/fl}$ mice does cause additional weight gain. Since we know that leptin resistance in VAN develops as a result of chronic ingestion of a high fat diet (19), we conclude that leptin resistance in VAN is a necessary initial step in diet-induced obesity. We suggest that the *Nav1.8/LepR*$^{fl/fl}$ mice have acquired compensatory mechanisms to deal with the loss of leptin receptor in VAN during development which prevents them from gaining further weight on a high fat diet. WT mice fed a high fat diet develop leptin resistance which initiates weight gain, and secondary mechanisms promote further weight gain. The compensatory mechanisms acquired by the *Nav1.8/LepR*$^{fl/fl}$ mice prevent the secondary mechanisms from promoting

further weight gain in response to a high fat diet. We suggest that preventing acquisition of compensatory mechanisms by knocking out LepR in VAN during adulthood would result in more pronounced weight gain on a chow diet and increased susceptible to additional weight gain in response to a high fat diet.

The data show that the hyperphagia observed in the conditional knockout mice occurs as a result of reduced meal termination rather than meal initiation. The leptin receptor knockout in VAN increases intake in the early dark phase when the animals consume the majority of food. We observed prolonged meals (reduced meal termination) with no increase in meal numbers or a reduction in the intermeal interval (meal initiation). This suggests that leptin signaling in VAN is involved in meal termination, and that knocking out leptin receptor reduces satiation. The data is consistent with previous findings that VAN are involved in meal termination (58) and that gastrointestinal hormones released post-prandially (ie. CCK, PYY, GLP-1) activate VAN to mediate satiation. There is substantial evidence in the literature that leptin potentiates CCK signaling (30, 46, 59). Here we report that leptin signaling in VAN is required for CCK-induced satiation.

We investigated the possible mechanism by which leptin receptor knockout in VAN initiates hyperphagia. We have previously demonstrated that during fasting, when there is little nutrient content in the proximal gut, the neurochemical phenotype of VAN is to express orexigenic peptides (eg MCH) and receptors (eg CB1R) (50). Post-prandial release of CCK induces a “switch” in the phenotype of VAN shown by an increase in expression of anorectic peptide (eg CART) and receptors (eg Y2R) (50). The reduced sensitivity of VAN to CCK in mice lacking leptin receptor in VAN results in loss of this plasticity; expression of the peptide transmitters, CART and MCH, and expression of the receptors, Y2R and CB1R, fail to change in response to feeding or fasting in the

Nav1.8/LepR$^{fl/fl}$ mice. There is some evidence that at least CART is released from cultured VAN and that CART can prolong satiation *in vivo* (60). Furthermore, knocking down CART expression in VAN of freely behaving rats has been shown to increase food intake in short term studies (61). Together these data suggest that CART may act as a neuropeptide transmitter involved in inhibiting food intake. Thus, the reduction in CART expression in VAN of *Nav1.8/LepR*$^{fl/fl}$ mice may account for the reduced satiation and consequently hyperphagia.

We have developed and utilized a novel tool, namely a mouse with a conditional knockout of the leptin receptor, which allows the first targeted approach to determine the functional role of vagal afferent neurons. Using this method we have been able to show for the first time that the vagal afferent pathway influences food intake, adiposity, and body weight over the long term. This result contrasts to surgical and chemical ablation studies in which long term effects on body weight or adiposity have not been reported. Prior ablation studies have lacked specificity in targeting the vagal afferent pathway. Total subdiaphragmatic vagotomy ablates both afferent and efferent pathways; the more selective subdiaphragmatic deafferentation ablates 50% of the efferent fibers, in addition to afferent fibers. Perivagal application of capsaicin, thought to cause degeneration of afferent C fibres may also damage efferent neurons (62). More recently a *Phox2b*-Cre mouse has been used to target vagal neurons (63, 64). However this cre-lox system does not discriminate between vagal afferent and vagal efferent neurons, and is also expressed in enteric neurons of the intestine (65), central noradrenergic neurons of the nucleus of the solitary tract, neurons of the area postrema, in most of the rhombencephalon (caudal to r1) at least during development, in a subset of sympathetic neurons, and extensively in neurons of the IIIrd, IVth, VIIth, IXth, Xth and XIth cranial ganglia (66). Thus, the current study is the first to selectively target vagal afferent

signaling to determine their role in food intake and body weight. It should be noted that Nav1.8 cre targets DRG and SCG neurons in addition to VAN; however, because few DRG and SCG neurons express *LepR*, the method provides knock down of leptin receptor specifically in VAN.

It is interesting to note that deleting all the splice variants of LepR using Phox2b-cre (67) or Nav1.8-cre produces similar phenotypes. Both mice lines increase weight gain on a chow diet, increase food intake at 12 weeks, had no change in respiratory quotient, and were satiated in response to a high dose of CCK. Finally, similarly to our *Nav1.8/LepR*$^{fl/fl}$ mice the Phox2b-cre LepRflox/flox mice failed to gain weight on a HFD (67). The similarity in phenotype suggests that the Phox2b-cre LepR^{floxflox} is more likely a result of LepR deletion from VAN rather than NTS neurons.

Conclusion

Vagal afferent neurons convey information about the availability of nutrients in the gut to the brain (24). In the postprandial period, the vagal afferent pathway plays a pivotal role in regulation of gastrointestinal and pancreatic function and also plays a role in the control of meal size and duration (68). Here we demonstrate that knocking out leptin receptor expression in vagal afferent neurons prevents appropriate postprandial gut-brain signaling, resulting in increased food intake, weight gain, and adiposity. Taken together with our previous data showing that leptin resistance is an early event in high-fat diet induced hyperphagia and weight gain, the current data strongly suggest that defects in leptin signaling in the vagal afferent pathway is a novel mechanism for the initiation of obesity. This novel approach may provide insight into the role of other factors (ie. hormones, cytokines, microbial products, mechanosensitivity, and nutrients) involved

in gut-brain signaling as they relate to food intake, inflammation, microbiota-brain signaling, and neurodegenerative diseases.

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Chapter 2.4: Knockdown of Leptin Receptor on vagal afferent neurons drives obesity differently in female than male mice

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Running title: Female and Male differences in Sensory neuron LepR-KO mice

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2.4.1 Abstract

Evidence demonstrates that there are sex differences in the regulation of energy homeostasis; studies using gonadectomies highlight the importance of sex hormones in regulating food intake, body weight and adiposity. Estrogen treatments helps overcome leptin resistance in global leptin receptor knockout mice. Given that estrogen receptors are present on vagal afferent neurons, we hypothesize that female mice lacking leptin receptors specifically on VAN will have different body composition and meal patterns compared to male *Nav1.8/LepR*$^{fl/fl}$ mice. **METHODS**: Body composition and meal patterns were assessed for female *Nav1.8/LepR*$^{fl/fl}$ mice. Feeding studies were used to test whether female *Nav1.8/LepR*$^{fl/fl}$ mice respond to exogenous CCK and leptin. The absolute change between *Nav1.8/LepR*$^{fl/fl}$ mice and wildtype (WT) for body composition and meal patterns were used to investigate differences between the genders. **RESULTS**: Female *Nav1.8/LepR*$^{fl/fl}$ mice exhibited similar phenotype to male *Nav1.8/LepR*$^{fl/fl}$ mice. They had increased body weight, adiposityand reduced response to gut satiety hormones. The main difference between male and female mice was their meal patterns. WT female mice ate longer and larger meals compared to males. Furthermore, female *Nav1.8/LepR*$^{fl/fl}$ mice consumed smaller and shorter meals less frequently than male *Nav1.8/LepR*$^{fl/fl}$ mice. **CONCLUSION**: Knockdown of leptin receptor on VAN leads to an obese phenotype irrespective of gender, the mechanisms leading to obesity are different.

Key words: Leptin, estrogen, vagus nerve, food intake, meal patterns, sex differences, vagal afferent neurons

2.4.2 Highlights

- Knockdown of LepR specific on VAN leads to an obesogenic phenotype in female mice.
- Male and female WT mice have inherently different body composition and meal patterns
- The pathway by which disruption of leptin signaling leads to an obese phenotype is driven by a different mechanism in male versus female mice.

2.4.3 Introduction

In the United States, females are approximately two-fold more vulnerable to severe and morbid obesity (1). In addition, the burdens of the disease have a greater impact on women (2, 3), which is reflected by the fact that over 80% of bariatric patients are women (4). Although, there has been an increase in research investigating the effects of each sex individually on the regulation of energy homeostasis, there are limited studies that have made the direct comparisons between male and females.

It is apparent that there are sex differences in body composition and control of energy homeostasis in both rodents and humans. For example, women and female rats have more total fat and higher plasma leptin levels than males (5, 6). Male rats have a greater weight gain rate compared to females although their total daily caloric intake is similar on a chow diet (7). Meal patterns between the sexes are different; female mice eat smaller meals more frequently and males eat larger meals less often (8).

Estrogen effects metabolic homeostasis by regulating appetite and food intake (9). For example the prevalence of obesity increases in post-menopausal women and estradiol treatment blunts weight gain and adiposity (10, 11). The effects of estrogen are largely mediated through α and β estrogen receptors (ER), which are expressed in the periphery and the central nervous system (12, 13). Specifically, evidence demonstrates that ER are expressed on vagal afferent neurons (VAN) (14). The vagus nerve is the primary conduit between the gastrointestinal tract and the brain and seems to play an important role in regulating ER expression. For example, in fasted female rats, ER expression is increased in the paraventricular nucleus and the nucleus of solitary tract, areas where VAN fibers are located; additionally, subdiaphramagtic vagotomies will attenuate this response (15).

Studies have implicated estrogen in the satiating effect of leptin; estrogen will mimic the actions of leptin on pro-opiomelanocortin (POMC) neurons to inhibit food intake in leptin receptor deficient female mice (16). Leptin and estrogen receptors are colocalized in neurons known to coordinate energy homeostasis, such as the arcuate nucleus (ARC) (17). Evidence suggests that leptin and estrogen crosstalk to induce their effects in the hypothalamus (18); central administraions of leptin and estrogen will both upregulate the expression of POMC and downregulate expression of neurpeptide Y (NPY) (19-21).

Leptin signaling on vagal afferent neurons (VAN) plays an important role in regulating energy homeostasis; development of leptin resistance in VAN leads to an obese phenotype (22). Leptin resistance in VAN drives hyperphagia and eventually leading to an obese phenotype which was seen in conditional knockout mice that lack leptin receptor specifically in primary afferent neurons (23). The male *Nav1.8/LepR*$^{fl/fl}$ mice mice exhibited an increase in body weight, food intake and adiposity compared to their control littermates. Furthermore, VAN of male *Nav1.8/LepR*$^{fl/fl}$ mice are locked in a fasted phenotype which may account for the reduced satiation and hyperphagia.

In the present study, we first sought to characterize the phenotype of the female *Nav1.8/LepR*$^{fl/fl}$ mice. Then we did a direct comparison of body composition and meal patterns of male and female mice. Given that estrogen and leptin acts synergically to regulate food intake, we hypothesized that female *Nav1.8/LepR*$^{fl/fl}$ mice would exhibit an obese phenotype to a lesser degree than male *Nav1.8/LepR*$^{fl/fl}$ mice.

2.4.4 Methods

Animals

All experiments were approved by the UC Davis Institutional Animal Care and Use Committee (protocol #16793) and PHS animal welfare assurance to UC Davis (#A3433-01). Cre mice were generously donated by Dr. John Woods at UCL (24). LepR flox mice were purchased from Jax. Mice were bred to generate selective deletion of leptin receptor in primary afferent neurons. Nav1.8/LepR$^{fl/wt}$ offspring were subsequently crossed with LepR$^{fl/fl}$ mice to generate Nav1.8/LepR$^{fl/fl}$ mice. Cre-negative, Lepr$^{fl/fl}$, and Lepr$^{fl/wt}$ littermates (WT) were used as controls in all studies. Mice were individually housed after weaning under a 12h light: 12h dark schedule and allowed *ad libitum* access to food (Purina 5008) and water unless specified otherwise. All experiments were performed in 12 week old mice except for the high fat diet experiment in which 9 week old chow fed mice were either kept on chow or given *ad libitum* access to high fat diet (45% kcal/g fat; Research diets D12451) for 12 weeks. All experiments were performed with male mice from Chapter 2.3.

Verification of LepR knockdown was previously performed by real-time PCR as described in Chapter 4 of thesis dissertation.

Tissue collection

Mice were euthanized by CO^2 inhalation. Epididymal, retroperitoneal and mesenteric fat pads were collected immediately after euthanization.

Body compostion

Body fat and lean mass of mice were assessed by dual-energy X-ray absorptiometry (DEXA).

Metabolic analysis

Energy expenditure at 8 wks old was evaluated in two separate cohorts of age matched (±2 days) WT and *Nav1.8/LepR*$^{fl/fl}$ mice fed powdered chow diet (LabDiet 5058) using a comprehensive lab animal monitoring system (CLAMS, Columbus Instruments, Comlubus, Oh). Data was combined since no statistical difference between runs was identified (data not shown). Mice were fed powdered diet for one week and acclimated to monitoring chambers for 2 days prior to 48hr data collection; data is presented as an average of both days. Energy expenditure was calculated from the oxygen intake. Activity levels were determined by counting laser breaks along a, x, y, and z-axis. The food bout was defined as an episode of uninterrupted feeding of at least 0.02 g, and meal termination was when a bout of feeding was followed by 10 min with no measurable intake.

Feeding studies

Mice were fasted 12h prior to feeding tests. Cholecystokinin (octapeptide, sulfated) was purchased from Bachem (Torrance, CA,) and leptin (rat) from Sigma Aldrich (St. Louis, MO). CCK (0.3-30µg/kg; IP), leptin (120µg/kg; IP), or saline (100µl; IP) were administered and food was immediately returned to the cage; food intake was recorded every 20 minutes over 2 hours for CCK studies and every hour for 7 hours for leptin studies.

Female and male mice comparison

We compared the WT female and male mice for each parameter of the meal patterns. The absolute change of *Nav1.8/LepR*$^{fl/f}$ mice compared to WT mice were calculated for each gender. The absolute change was taken relative to the average of the WT mice for each parameter. Comparisons between female and male mice were then analyzed.

Statistics

Statistical analysis was performed using Prism software (Prism 5.0; GraphPad Software, La Jolla, CA). Unpaired t-test was used to make direct comparisons between WT and *Nav1.8/LepR*$^{fl/fl}$ mice as well as the comparison between the percent changes in parameters between genders. In feeding experiments paired t-test was used to compare saline with either CCK or leptin. Two-way ANOVA with Bonferroni post hoc test was used to compare the effects of leptin and saline over time in the WT and *Nav1.8/LepR*$^{fl/fl}$ mice. Unpaired t-test was used to compare the absolute change of parameters between male and female mice. Differences were considered significant if $p<0.05$. Data are means ± SEM. * represents $p<0.05$; ** represents $p<0.01$; and *** represents $p<0.001$.

2.4.5 Results

Knockdown of LepR on VAN leads to obesity in female mice

Weekly body weight was measured over 12 wks to determine whether endogenous leptin signaling on VAN is required to maintain energy homeostasis. Female *Nav1.8/LepR*$^{fl/fl}$ mice weighed significantly more than their control littermates starting at 6 wks and further increased by 12 wks ($p<0.05$; Fig 2.4.1A). Epididymal, mesenteric and retroperitoneal fat pads were collected at 12 wks. There was a significant increase in epididymal, mesenteric and retroperitoneal fat in female *Nav1.8/LepR*$^{fl/fl}$ mice compared to WT mice ($p<0.01$; Fig 2.4.1C-E); which led to a 3-fold increase in total fat mass compared to WT mice at 12 wks ($p<0.05$; Fig 2.4.1B). Together, these findings are consistent with our data on the male *Nav1.8/LepR*$^{fl/fl}$ mice where knockdown of LepR on VAN leads to an obesogenic phenotype (23).

Lack of LepR on VAN increases food intake in female mice

To determine the mechanism by which knockdown of LepR on VAN leads to obesity, female mice were placed in metabolic cages at 9 wks. Body composition of mice was analyzed by DEXA. Whole body composition analysis revealed that female *Nav1.8/LepR*$^{fl/fl}$ mice weighed more than WT mice ($p<0.05$; Fig 2.4.2A) as a result of increased fat mass ($p<0.05$; Fig 2.4.2B) and no change in lean body mass and bone density (Fig 2.4.2C+D). Energy expenditure and meal patterns were further analyzed through CLAMS cages. There were no differences in energy expenditure and respiratory quotient between genotypes (Fig 2.4.3A-D). Cumulative food intake over 24 hours was measured (Fig 2.4.4A); female *Nav1.8/LepR*$^{fl/fl}$ mice ate significantly more in the dark phase ($p<0.05$; Fig 2.4.4B) compared to WT mice although this did not lead to an increase in daily food intake similar to that observed in the male *Nav1.8/LepR*$^{fl/fl}$ mice.

Interestingly, female *Nav1.8/LepR*$^{fl/fl}$ mice tended to eat more at the end of the light phase and beginning of the dark phase compared to their control littermates although the difference did not reach statistical significance (Fig 2.4.4C), while males ate more the first few hours after the onset of the dark phase (data in Chapter 2.3).

Meal patterns differed between genotypes. Female *Nav1.8/LepR*$^{fl/fl}$ mice ate shorter meals in the dark phase, which led to a significant difference in meal duration over 24h compared to WT mice ($p<0.05$, Fig 2.4.5A-B). Meal size was smaller compared to WT mice irrespective of time (Fig 2.4.5D-F). Female *Nav1.8/LepR*$^{fl/fl}$ mice ate 16.2% more meals compared to WT in the light and dark phase ($p<0.01$; Fig 2.4.6A-C). Furthermore, female *Nav1.8/LepR*$^{fl/fl}$ mice had shorter intermeal interval compared to WT mice ($p<0.05$, Fig 2.4.6D-F). Altogether, the data indicates that female *Nav1.8/LepR*$^{fl/fl}$ mice eat more meals more frequently than their control littermates.

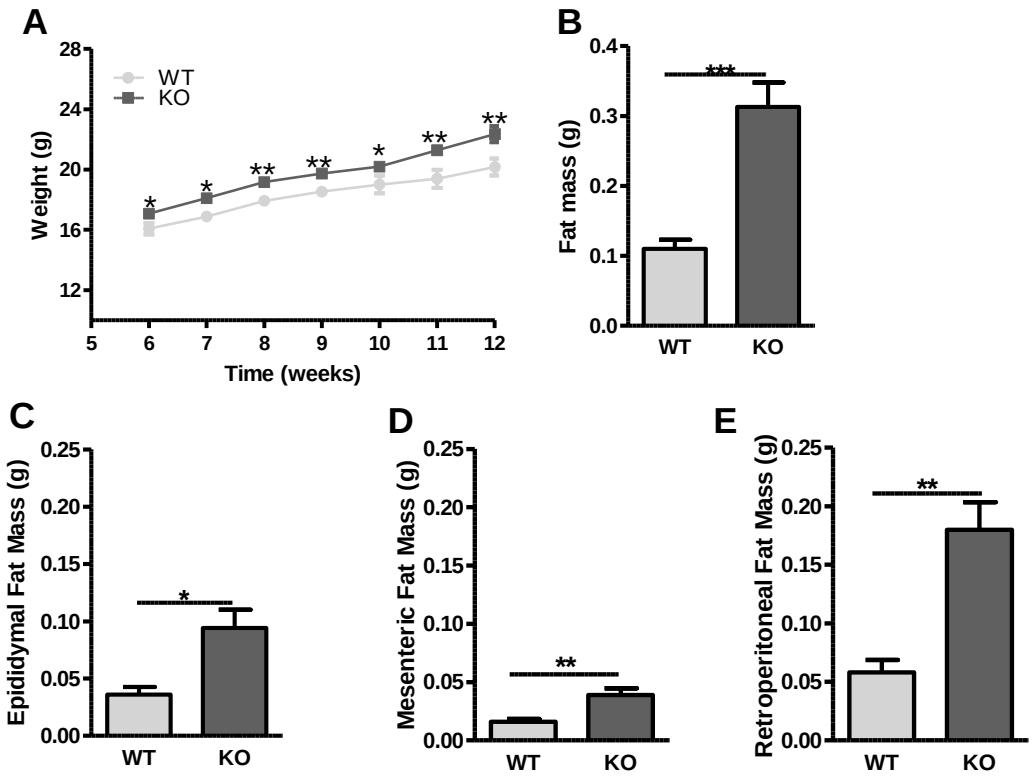

Figure 2.4.1 Analysis of energy homeostatis parameters of female *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice

(A) Weekly body weight was significantly increased in chow-fed *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice (n=20). (B) Fat mass was significantly increased in *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice. Fat mass of (C) epidydmal, (D) mesenteric and (E) retroperitoneal fat were all significantly increased in *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice at 12 weeks. Results are shown as means ± SEM (n=4-8); Two-way ANOVA was used for weekly body weight and student unpaired t-test was used for fat mass. *P <0.05 and **P<0.01.

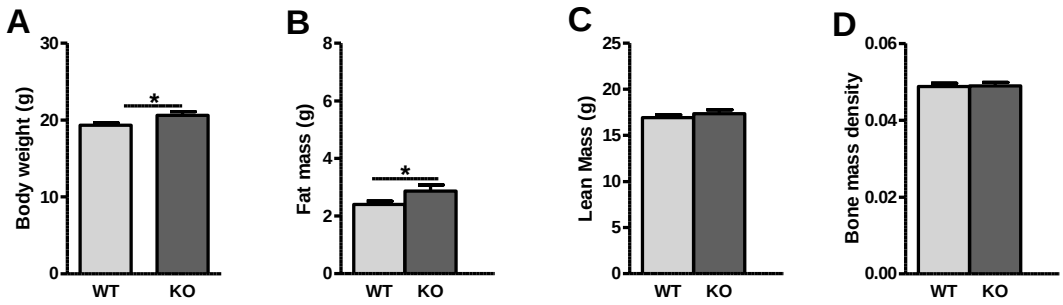

Figure 2.4.2 Analysis of body composition of female mice by DEXA

Total body composition analysis was conducted by dual energy X-ray absorptiometry (DEXA) in *Nav1.8/LepR*$^{fl/fl}$ mice and WT controls used for metabolic studies. (A) Body weight and (B) fat mass were increased in *Nav1.8/LepR*$^{fl/fl}$ mice mice compared to controls, with no change in (C) lean mass or (D) bone mass density. Results are shown as means ± SEM (n=8); (Student unpaired t-test). *P <0.05.

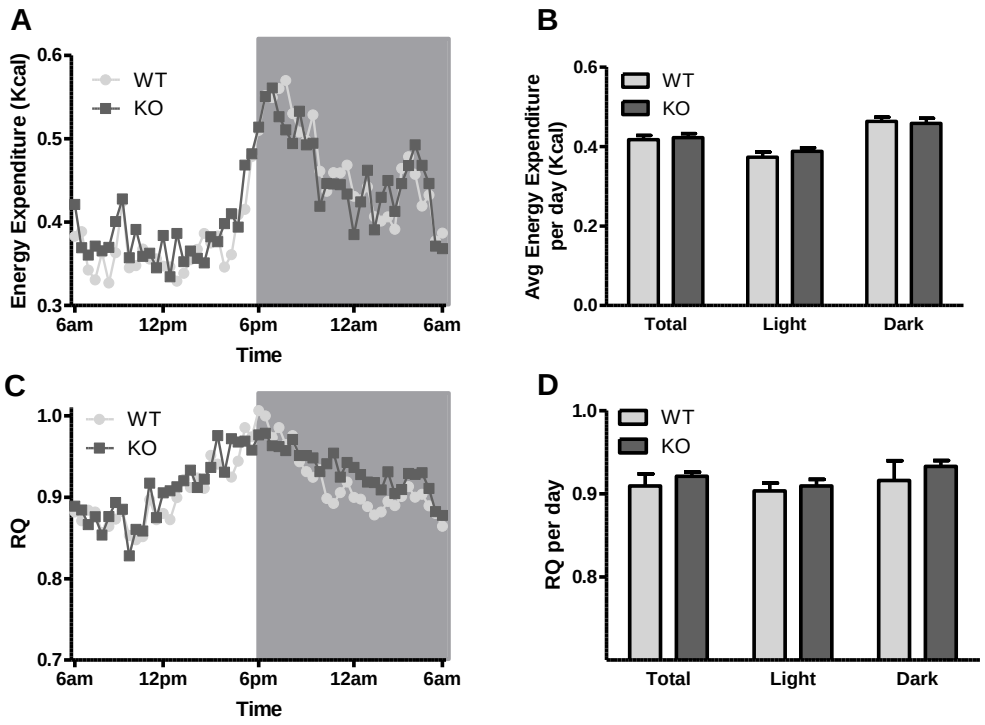

Figure 2.4.3 Analysis of energy expenditure of female mice by CLAMS

Female *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice were placed in metabolic cages and energy expenditure, respiratory quotient and food intake were recorded over 48 h. (A) Average hourly energy expenditure over 24h, (B) average 24h, light and dark energy expenditure, and (C) Average hourly respiratory quotient (RQ) over 24 hours and (D) total, light and dark RQ were unchanged between *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice. Results are shown as means ± SEM (n=8).

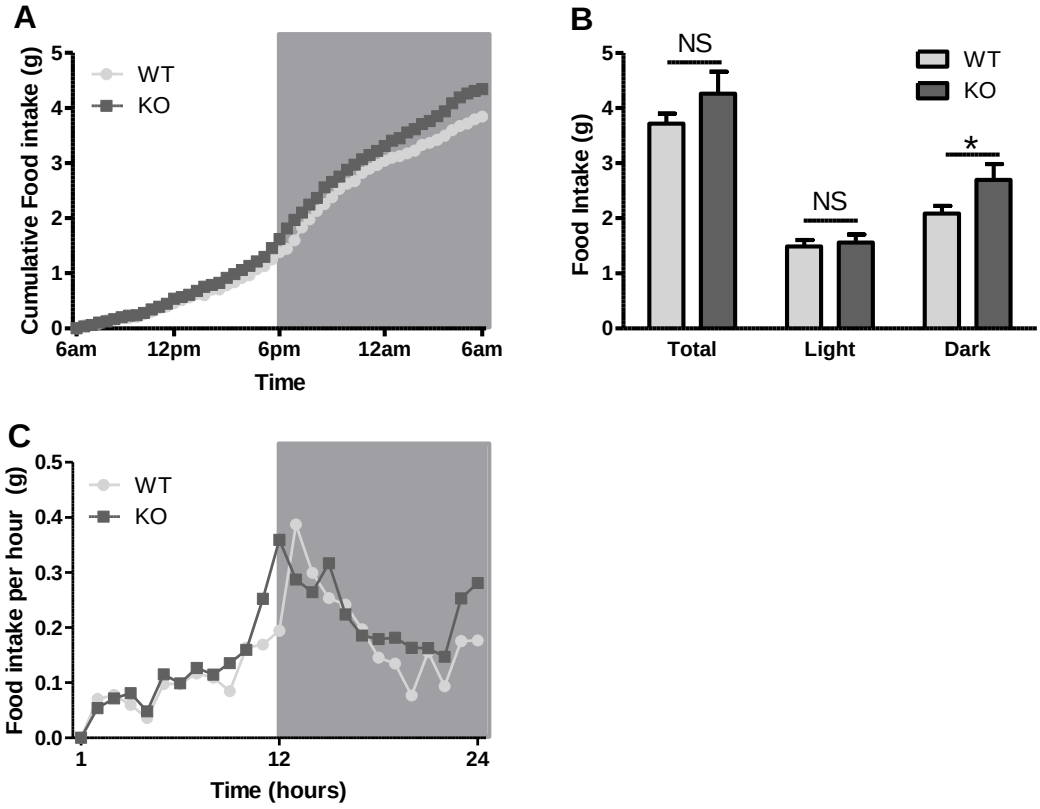

Figure 2.4.4 Food intake analysis over 24 h in female *Nav1.8/LepR$^{fl/fl}$* and WT mice

Food intake analysis was measured through CLAMS cages (A) Cumulative food intake over 24 hours was increased in *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice. (B) Average food intake in *Nav1.8/LepR$^{fl/fl}$* mice was significantly increased in the dark phase with no change in the light phase compared to WT mice and no change in total food intake. (C) Average hourly food intake over 24h demonstrating increased food intake in *Nav1.8/LepR$^{fl/fl}$* mice in the dark phase. Results are shown as means ± SEM (n=8); * P <0.05 (Student unpaired t-test).

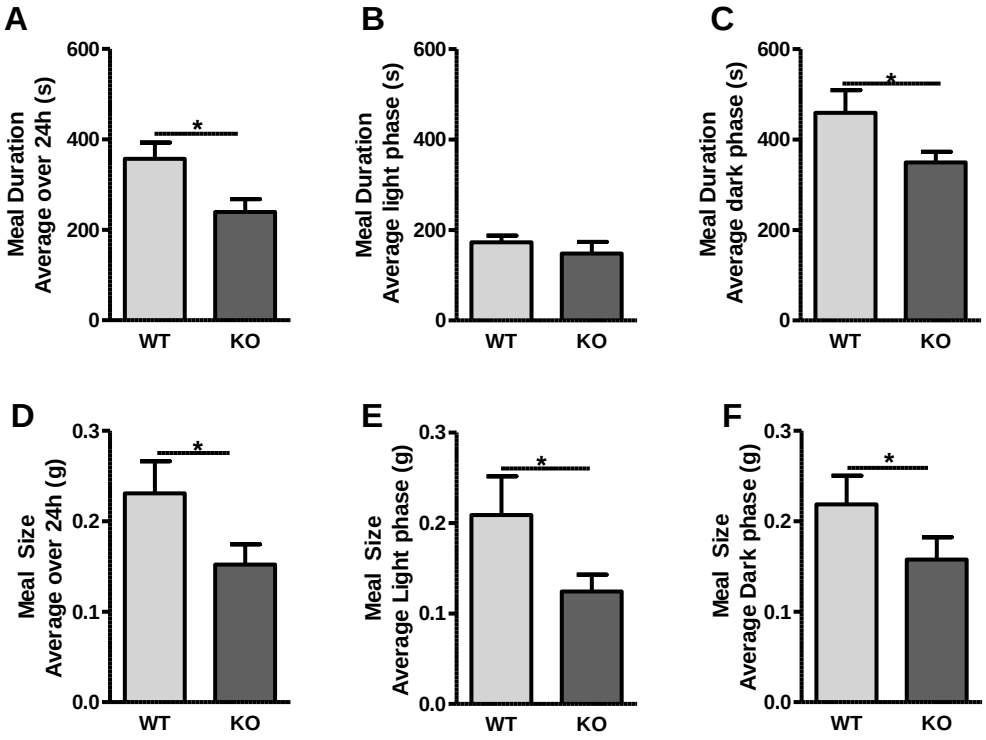

Figure 2.4.5 Meal pattern analysis in female Nav1.8/LepR$^{fl/fl}$ vs. WT mice

Meal patterns were analyzed in female Nav1.8/LepR$^{fl/fl}$ mice compared to WT mice. Meal duration (A-C) was significantly decreased in the dark phase (C), but not the light phase (B), in Nav1.8/LepR$^{fl/fl}$ mice; resulting in decreased 24h-meal duration (A). Meal size (D-F) was significantly decreased in the dark phase (F), the light phase (E) and total 24h (D) of the *Nav1.8/LepR$^{fl/fl}$* mice compared to WT (L). Results are shown as means ± SEM (n=8); * P <0.05 (Student unpaired t-test).

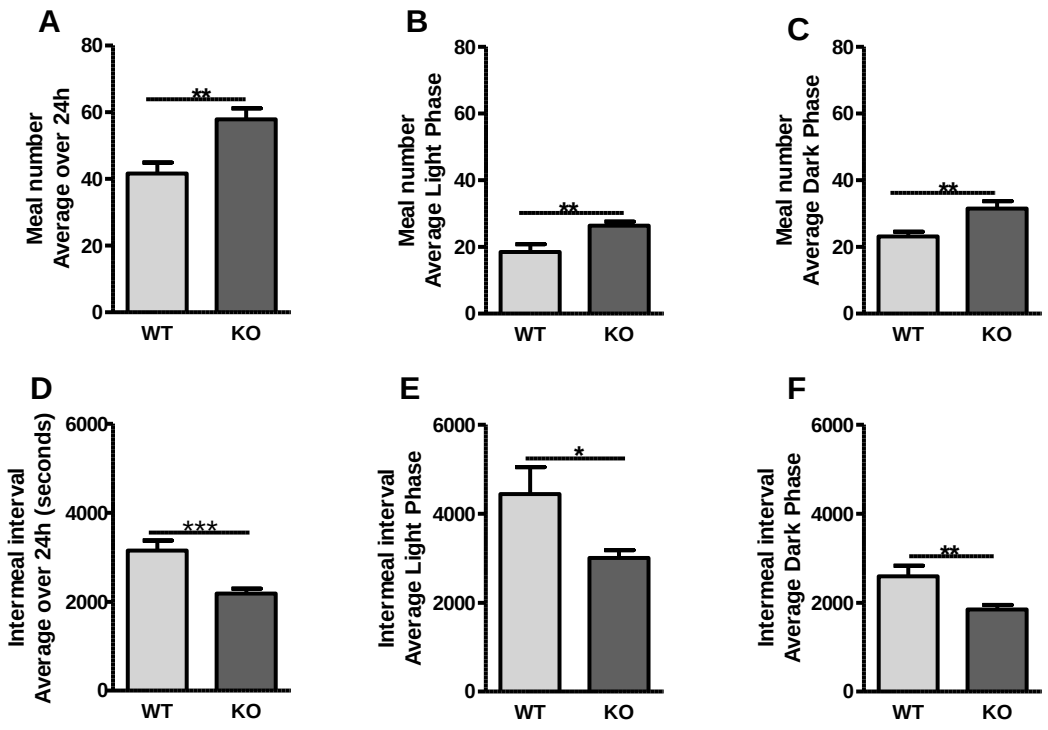

Figure 2.4.6 Meal pattern analysis in female Nav1.8/LepR$^{fl/fl}$ vs. WT mice

Meal number (A-C) was significantly increased in the dark phase (C), in the light phase (B), and total 24h (A) in *Nav1.8/LepR$^{fl/fl}$* mice compared to WT mice. Intermeal interval (D-F) was decreased in the dark phase (F), the light phase (E) and total 24h (D) of the *Nav1.8/LepR$^{fl/fl}$* mice compared to WT. Results are shown as means ± SEM (n=8) ; * P <0.05, **P<0.01 and ***P<0.001 (Student unpaired t-test).

Lack of LepR on VAN attentuates CCK and leptin-induced satiation in female mice

To determine whether satiety signaling on VAN is altered, we investigated the effects of exogenous cholecystokinin (CCK) and leptin on *Nav1.8/LepR*$^{fl/fl}$ mice. We hypothesized that the reduced sensitivity to CCK is driving the changes in meal patterns. CCK is a master regulator in controlling food intake on vagal afferent fibers innervating the gut. Leptin is required for CCK to signal; de Lartigue *et al* demonstrates that leptin increases CCK-induced signaling in culture (25). To test whether leptin deficiency leads to a blunted response to CCK, we compared feeding response to peripheral administration of exogenous CCK in female WT and *Nav1.8/LepR*$^{fl/fl}$ mice. Exogenous CCK (30μg/kg, IP) significantly decreased 2h food intake in female WT mice ($p<0.05$; Fig 2.4.7A). The same dose of CCK failed to decrease 2 h food intake in female *Nav1.8/LepR*$^{fl/fl}$ mice (Fig 2.4.7B). To investigate whether female *Nav1.8/LepR*$^{fl/fl}$ mice respond to leptin, we compared feeding response to exogenous leptin over 7h. Leptin significantly decreased food intake at 5, 6 and 7 hours in female WT mice compared to saline ($p<0.05$; Fig 2.4.7C+D). Response to leptin was attenuated in female *Nav1.8/LepR*$^{fl/fl}$ mice (Fig 2.4.7C+D).

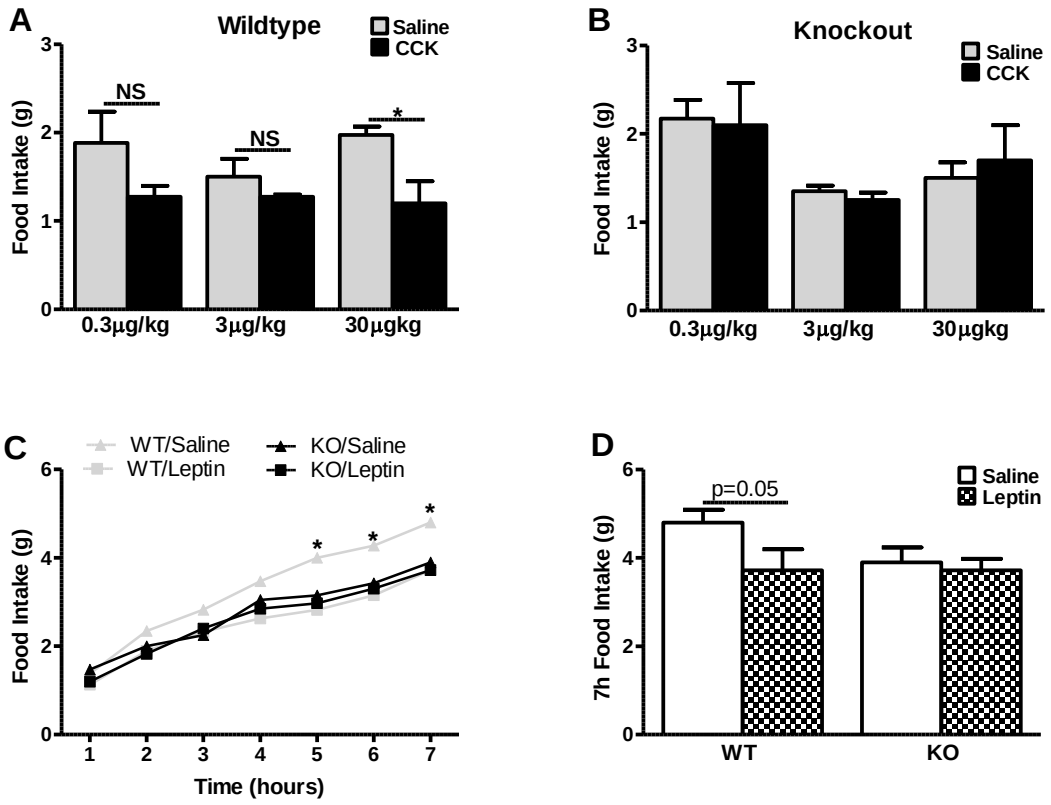

Figure 2.4.7 Satiating effects of CCK and Leptin in female *Nav1.8/LepR$^{fl/fl}$* mice

Nav1.8/LepR$^{fl/fl}$ and WT mice were deprived of food for 18 hours and administered IP saline or CCK (A-B), and IP saline or leptin (C-D) and food intake was recorded (N=4-6). (A) CCK reduced 2h food intake in WT mice at the highest dose 30µg/kg of CCK; (B) satiating effects of CCK was attenuated in *Nav1.8/LepR$^{fl/fl}$* mice. (C) Food intake was measured every hour for 7 hours following IP administration of 120µg/kg of leptin (D) Leptin reduced 7hr food intake in WT mice but did not significantly inhibit food intake in *Nav1.8/LepR$^{fl/fl}$* mice. Results are shown as means ± SEM (n=4-6); * P <0.05 (Two-way ANOVA and Student unpaired t-test).

Comparison between Male and Female mice

The general phenotype of male and female *Nav1.8/LepR*$^{fl/fl}$ mice is the same between the sexes; increased body weight, food intake and adiposity as well as a reduced sensitivity to satiating peptides CCK and leptin. In the next study, we compared the phenotypes of the male and female of WT and *Nav1.8/LepR*$^{fl/fl}$ mice. To compare the differences in the *Nav1.8/LepR*$^{fl/fl}$ mice, absolute differences between the WT and *Nav1.8/LepR*$^{fl/fl}$ mice were calculated for each gender.

There was a significant increase in the absolute change of 9 wk body weight in the male compared to the female mice (Fig 2.4.8A). There was no difference between fat mass and lean body mass between the sexes (Fig 2.4.8B+C).

First, we compared meal patterns of the WT female and male mice to investigate whether male and female mice have different innate feeding behaviors. Female WT mice had an increase in meal duration in both the light and dark phase, which resulted in a 3-fold increase over 24h compared to male WT mice ($p<0.05$; Fig 2.4.9A-C). Overall meal size was significantly increased in female WT mice versus male WT mice which was driven by the increase in the dark phase ($p<0.05$; Fig 2.4.9D+F), with no difference in meal size in the light phase (Fig 2.4.9E). Female WT mice had a decrease in meal number in the dark phase, which led to an overall 41% decrease in 24h ($P<0.05$; Fig 2.4.10A+C), with no change in the light phase (Fig 2.4.10B). The intermeal interval was increased in the dark phase in the female WT compared to male WT mice ($p<0.01$; Fig 2.4.10F), with no difference between the genders in the light phase and over 24h (Fig 2.4.10D+E).

The differences in body composition between the male and female *Nav1.8/LepR*$^{fl/fl}$ mice may be driven by changes in meal patterns. We hypothesized that the differences in meal patterns could account for the differences in body composition. We found that female *Nav1.8/LepR*$^{fl/fl}$ mice eat shorter meals over 24h than the male

Nav1.8/LepR$^{fl/fl}$ mice, which was driven by a 50% difference in meal duration in the dark phase ($p<0.05$: Fig 2.4.11A-C). There was a 30% difference in meal size over 24h between the female *Nav1.8/LepR*$^{fl/fl}$ mice compared to male *Nav1.8/LepR*$^{fl/fl}$ mice, indicating that female mice ate smaller meals in the light and dark phase ($p<0.05$; Fig 2.4.11D-F). Female *Nav1.8/LepR*$^{fl/fl}$ mice ate more meals more frequently irrespective of the time of day compared to male *Nav1.8/LepR*$^{fl/fl}$ mice ($p<0.05$; Fig 2.4.12A-F).

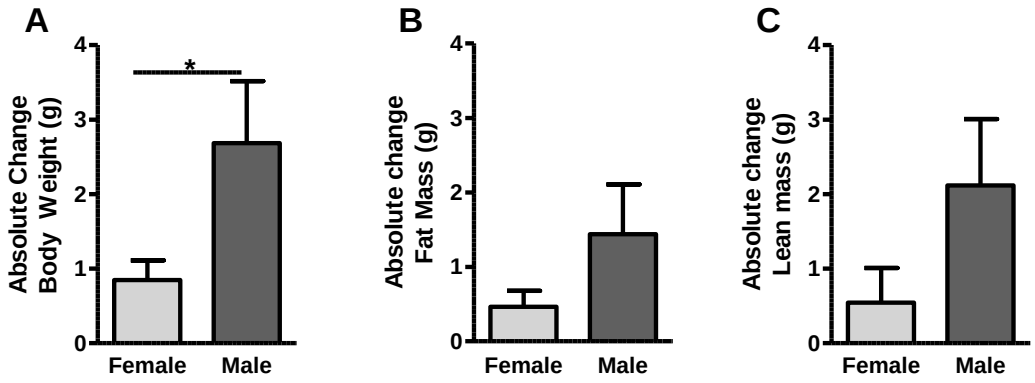

Figure 2.4.8 Absolute change of body composition between male and female *Nav1.8/LepR$^{fl/fl}$* mice

Absolute change from WT mice for female and male *Nav1.8/LepR$^{fl/fl}$* mice was calculated. Total body composition was measured using DEXA at 9 weeks; (A) absolute change of body weight was greater in male versus female *Nav1.8/LepR$^{fl/fl}$* mice, with no change in (B) fat mass or (C) lean mass. Results are shown as means ± SEM (n=8); * P <0.05 (Student unpaired t-test).

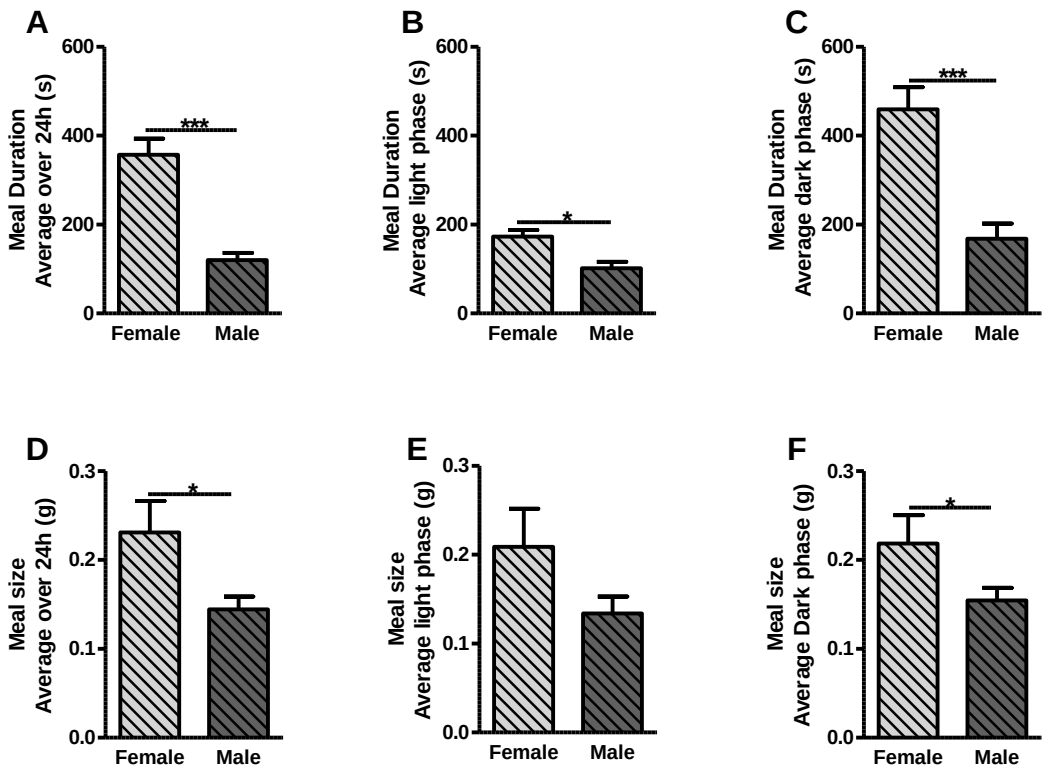

Figure 2.4.9 Comparison of meal patterns between male and female WT mice

Meal duration (A-C) was significantly more in female versus male WT mice in the light and dark phase (B+C), which led to significant increase over 24 h (A). Meal size (J-L) was significantly increased in the dark (L) and over 24h (J) in female versus male WT mice, with no change in the light phase (K). (n=8). Results are shown as means ± SEM; * P <0.05, *8*P<0.001 (Student unpaired t-test).

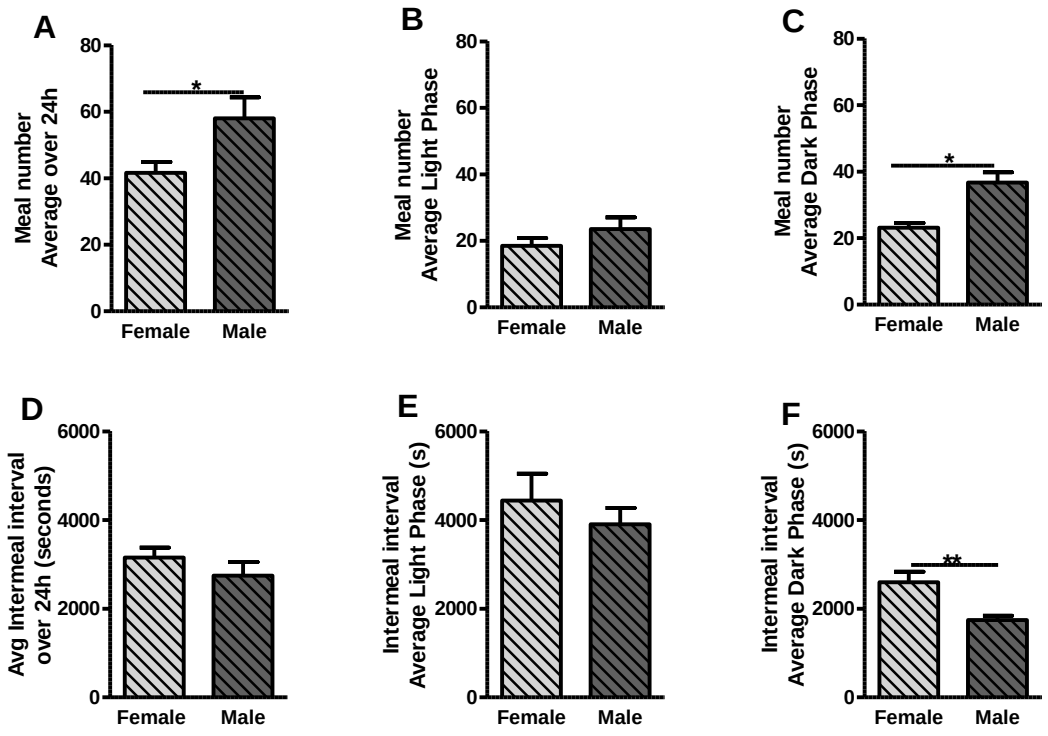

Figure 2.4.10 Comparison of meal pattern between male and female WT mice

The number of meals (A-C) over 24h (A), and in the dark phase (C) was smaller in female compared to male WT mice, with no change in the light phase (B). Intermeal interval (D-F) was increased in the dark phase (F) in female compared to male WT mice, with no change in the light phase (E) or over 24h (F). Results are shown as means ± SEM (n=8); * P <0.05, **P<0.01 (Student unpaired t-test).

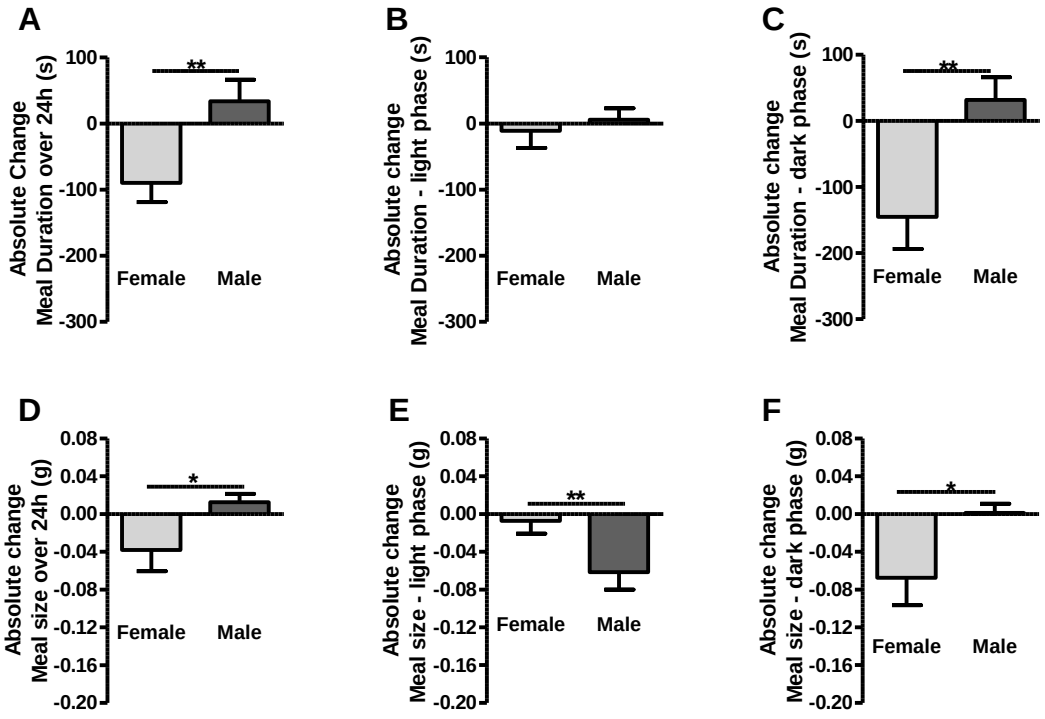

Figure 2.4.11 Absolute change of meal pattern between male and female *Nav1.8/LepR$^{fl/fl}$* mice

Absolute change of meal duration (A-C) was significantly less in female versus male *Nav1.8/LepR$^{fl/fl}$* mice in the dark phase (C), which led to significant decreased over 24 h (A) and no change in the light phase (B). Absolute change in meal size (D-F) was significantly less in the light (E) and dark phase (F) leading to a decrease over 24h (D) in female versus male mice. Results are shown as means ± SEM (n=8); * P <0.05, **P<0.01 (Student unpaired t-test).

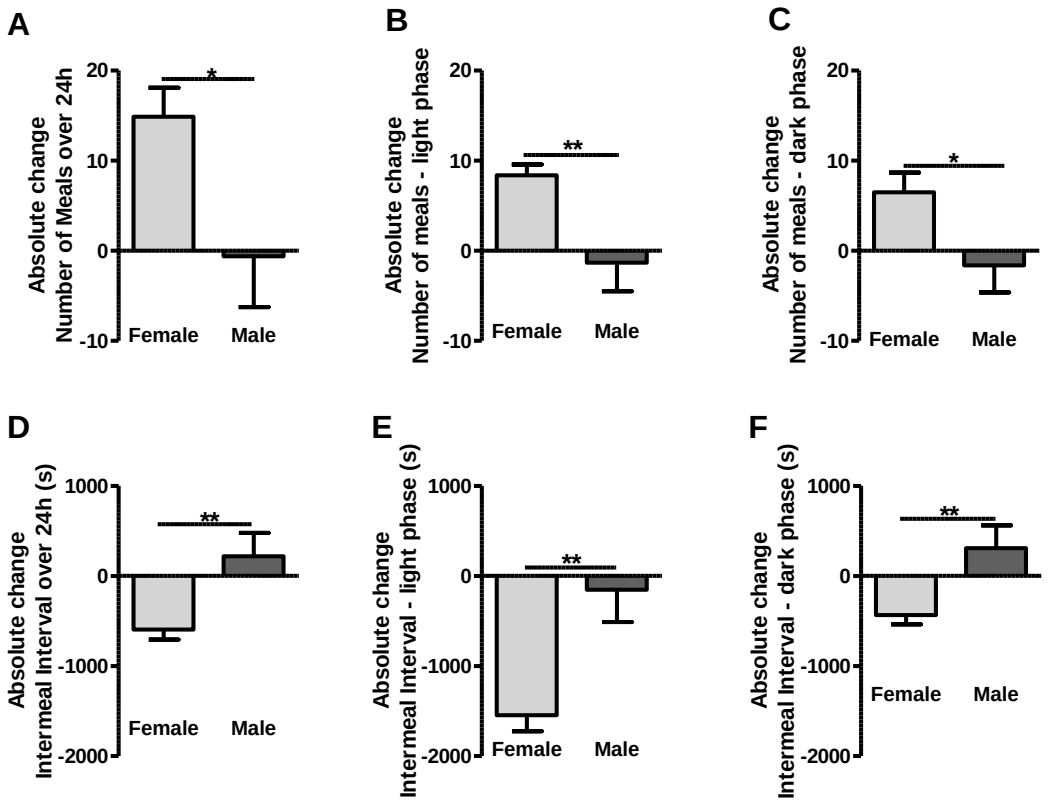

Figure 2.4.12 Absolute change of meal pattern between male and female *Nav1.8/LepR$^{fl/fl}$* mice

Absolute change of number of meals (A-C) over 24h (A), in the light phase (B) and in the dark phase (C) was greater in female compared to male mice. Absolute change in intermeal interval (D-F) was decreased over 24h (D), in the light phase (E) and in the dark phase (F) in female compared to male mice. Results are shown as means ± SEM (n=8); * P <0.05, **P<0.01 (Student unpaired t-test).

2.4.6 Discussion

In the present study, we sought to investigate differences between male and female *Nav1.8/LepR*$^{fl/fl}$ mice in order to understand the physiological differences that exist between the sexes. We found that male *Nav1.8/LepR*$^{fl/fl}$ mice had an increase in body weight compared to female *Nav1.8/LepR*$^{fl/fl}$ mice. The amount of body fat, lean mass and total daily food intake did not differ between female and male *Nav1.8/LepR*$^{fl/fl}$ mice. In addition, similar to male *Nav1.8/LepR*$^{fl/fl}$ mice, the satiating effects of CCK and leptin were attenuated in female *Nav1.8/LepR*$^{fl/fl}$ mice. Interestingly, meal patterns were different between the sexes. Female WT mice ate larger and longer meals less frequently than male WT mice. Conversely, the disruption of leptin signaling in VAN results in smaller and shorter meals than the male *Nav1.8/LepR*$^{fl/fl}$ mice.

Knockdown of LepR specific on VAN leads to an obesogenic phenotype irrespective of gender; *Nav1.8/LepR*$^{fl/fl}$ mice have increased body weight and adiposity compared to their controls, which is driven by an increase in food intake in the dark phase compared to WT mice. Furthermore, leptin deficiency on VAN in male and female mice attenuates CCK and leptin-induced satiation. This data is consistent with the notion that leptin resistance on VAN leads to an obesogenic phenotype (22, 23).

We analyzed meal patterns of male and female WT mice to investigate the difference in feeding behviors between genders. Although the total amount of food consumed didn't differ significantly between males and females, there was noticeable gender difference in meal patterns. Male WT mice were more anticipatory, while females were more reactionary, in feeding response to the start and end of the dark cycles. Female mice ate larger and longer meals less frequently compared to male WT mice. A limitation of our study is that we did not control for phases of estrous cycle. There is evidence that female rats have different meal patterns throughout their estrous cycle. Laviano *et al*

compared meal patterns between male and female rats; when female rats were in all phases of the cycle but the pro-estrus phase, females consume larger meals compared to males (7).

Fluctuations in gonadal hormone concentrations influence feeding behaviors and body composition (26). Studies using gonadectomy or hormone replacement highlight the importance of sex hormones in the regulation of energy homeostasis. For example, ovariectomized (OVX) rats become hyperphagic and gain significantly more body weight and adiposity than sham-operated controls; estradiol treatment restores energy homeostasis (27, 28). Orchidectomized rats decrease their food intake and concomitant body weight, both of which are attenuated by exogenous testosterone treatment (29).

In this study, the disruption in leptin signaling on VAN leads to obesity irrespective of gender. However, there are differences in mechanisms driving obesity between the sexes. Given that male and female WT mice are inherently different we compared the absolute change of the KO compared to the WT for each gender. We demonstrate that a lack of leptin signaling leads to differences in body composition and meal patterns between female and male; knockdown of leptin on VAN lead to a greater increase in body weight in males versus females. Furthermore, we observed a difference in the meal patterns between the male and female *Nav1.8/LepR*$^{fl/fl}$ mice. The male *Nav1.8/LepR*$^{fl/fl}$ mice ate longer and larger meals and were less satiated than their control littermates. Conversely, female *Nav1.8/LepR*$^{fl/fl}$ mice ate an increased number of shorter meals more frequently.

It is possible that estrogen may account for the differences in body composition and feeding behaviors seen in the female and male mice. For example, female *Nav1.8/LepR*$^{fl/fl}$ mice presumably still respond to estrogen, which could explain why they do not gain as much body weight as males *Nav1.8/LepR*$^{fl/fl}$ mice. Estrogen will lower

body weight and food intake in leptin and global LepR deficient mice (30). Litwak *et al* demonstrated that prior administrations of estrogen to diet-induced obese rats prevented fat accumulation and body weight gain (31). The differences seen in meal patterns between the male and female mice may also be in part due to estrogen. Exogenous estrogen decreases meal size and increases meal frequency in OVX rats (33). It is well established that there is a decrease in meal size during the estrus phase of the menstrual cycle in rodents when circulating estrogen concentrations are high (34).

There is likely a compensatory mechanism by which estrogen acts to modulate the obesogenic phenotype when there is a disruption in leptin signaling. Like leptin, estrogen targets POMC neurons; estrogen administration in mice deficient in leptin or leptin receptor will trigger upregulation of POMC neuronal activity (18). Estrogen increases sensitivity to leptin by increasing leptin-induced phosphorylation of STAT-3 (16). Interestingly, activation of estrogen receptor is capable of activating STAT-3 independently of leptin-mediated activation (16). In leptin and leptin receptor deficient mice, estrogen expression in the hypothalamus is significantly increased compared to WT mice (32). It is possible that in the female *Nav1.8/LepR*$^{fl/fl}$ mice have increased estrogen expression in the hypothalamus to compensate for the lack of leptin signaling. Further work to investigate the differences in hypothalamic neuropeptide signaling is needed.

The differences seen between male and female mice as well as *Nav1.8/LepR*$^{fl/fl}$ mice and WT may be due to differences in phases of estrous cycle in the females. However, two different cohorts of mice were placed in the metabolic cages twice and the data showed the same meal patterns for the different cohorts. Furthermore, it seems unlikely that all the *Nav1.8/LepR*$^{fl/fl}$ mice would be in one phase of the cycle and all the WT mice would be in another. It has been demonstrated that housing females in the same room

results in synchrony of the estrous cycle (35). Therefore, we can speculate that the female *Nav1.8/LepR*$^{fl/fl}$ mice and WT mice are in the same cycle. Further work is needed to control for the differences in phases of the estrous cycle.

Peripheral estrogen signaling may be transmitted via the vagus nerve to regulate estrogen receptor expression in higher order brain regions. Peripheral estrogen increases c-fos immunoreactivity in the hindbrain, the site at which VAN terminate. Estrogen receptors are expressed on VAN (14) and its receptor expression in the hindbrain decreases in vagotomized rats (15). Futhermore, there are neuroanatomical differences of VAN between male and female rats. There are more myelinated fibers on VAN of females compared males and the excitability of the fibers are dependent on estrogen; excitability of VAN from OVX rats is decreased compared to VAN from sham-operated rats. Estradiol dose-dependently increased neuronal excitability in both conditions (36). More work is needed to investigate the interaction between leptin and estrogen at the level of the vagus nerve. Much work has been done on estrogen-mediated and ovarian-cycle effect rather than on androgen-mediated effects. Thus, there is potential that androgens play a role in inducing the effects of leptin and this needs to be further elucidated.

In conclusion, we have demonstrated that male and female WT mice have inherently different body composition and meal patterns. Furthermore, a disruption in leptin signaling on VAN in females leads to increase body weight, adiposity and food intake as well as changes in meal patterns; unlike males, the increase in food intake in females *Nav1.8/LepR*$^{fl/fl}$ mice resulted from an increase in meal number and frequency and a decrease in meal size and duration. Further work is needed to understand the mechanisms driving the differences between male and females. Different mechanisms

are likely involved in males and females; therefore, different treatments may need to be developed for both sexes to treat obesity.

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Chapter 3: Discussion and Conclusion

3.1 Introduction

Consumption of a high fat (HF) diet leads to physiological changes that result in an increase in energy intake, body weight and adiposity (1). Developing novel therapeutic treatments to treat obesity clearly depends on understanding the pathways regulating energy homeostasis. Bariatric surgery produces long-term weight loss in obese subjects which coincides with increased anorexigenic circulating regulatory peptides (2). Clinical observations have highlighted the importance of the gut-brain axis in regulating food intake and body weight thus indicating that gut-derived hormones can be used as therapeutic targets. There is a gap in our knowledge of the mechanisms of action of gut-derived peptides under normal and unbalanced conditions.

The vagus nerve serves as a main conduit by which nutrient information from the periphery is integrated to the central nervous system. Destruction of vagal signaling leads to attenuated responses to satiating peptide and chronic loss of satiation leads to cumulative food intake (3). High fat (HF) diet consumption induces modulations in the neurochemical phenotypes of vagal afferent neurons (VAN); there is an increase in gene expression of orexigenic peptides and receptors in diet-induced obesity VAN, which could result in long-term alterations in gut-brain signaling that promote food intake (1). It is imperative to understand gut-derived signals at the level of the vagus as dysregulation of this pathway could be responsible for increased food intake and obesity.

3.2 Objectives and aims

Research on the effects of cholecystokinin (CCK) on regulating food intake has undoubtedly highlighted the importance of gut-derived hormones. As CCK is one of the first studied gut-derived hormones, it is a good example of how these peptides influence feeding behavior (4-6). The inhibition of food intake by CCK is mediated principally

through the vagus nerve (5). While CCK is one of the best-studied, there are other intestinal hormones that act on the vagal afferent pathway and are implicated in food intake regulation, however, our understanding of their mechanism is quite poor comparative to our knowledge of CCK. The action of gut hormones on VANs is now recognized to be an early step in controlling nutrient sensing and thereby regulating food intake and body weight. Interest in these mechanisms has grown rapidly in view of the alarming increase in obesity. Although increasing studies are demonstrating the importance of regulatory peptides, more work is needed on the receptors they act on.

In this dissertation, I have sought to understand the mechanisms of action of anorexigenic peptides along the gut-brain axis, specifically on the vagus nerve. Recent studies have indicated that GLP-1 and its analogs suppress food intake in lean humans and rodents (7, 8). Moreover, it induces its satiating effects in obese men suggesting a potential therapeutic target for promoting weight loss in obesity (9). Additionally, since its discovery, leptin has also been extensively studied as it may act as an anti-obesity treatment. However, diet-induced obesity results in the inability to respond to leptin (10). Leptin interacts with other peptides to potentiate its effects under physiological conditions and pretreatment of these peptides restores leptin responsiveness to obese models (11). Given that GLP-1 and leptin have been implicated as likely treatments to restore energy homeostasis, understanding their physiological mechanisms is imperative. Therefore, in the present studies, I investigated the pathways in which glucagon like peptide-1 (GLP-1) and leptin regulate food intake and energy homeostasis.

3.3 Summary of findings

As an initial study I sought to understand the underlying mechanism of peripheral GLP-1. Consistent with other studies, I have recently demonstrated that peripheral, native GLP-1 requires a postprandial state to induce satiation. Prolonged fasting attenuates the satiating effects of GLP-1 (see Chapter 2.1). This concept could explain the discrepancies in the literature regarding the satiating effects of peripheral GLP-1.

As peripheral and central GLP-1 signaling induces satiation, the site of action of native GLP-1 in relation to its effects on food intake was unclear. In chapter 2.1, I show direct immunohistochemical evidence that VAN express GLP-1Rs, which are functional; surprisingly, unlike other Gprotein coupled receptors (GPCR), GLP-1 gene expression on VAN did not change in VAN in response to feeding status of the animal. It is well established that gut-derived hormones induce neurochemical changes in VAN by regulating a phenotypic “switch”. VAN exists in states that either promote orexigenic or anorexigenic phenotypes (12, 13). However, I demonstrate that GLP-1Rs are constitutively expressed and that GLP-1Rs alter cellular localization according to feeding status. Under fasting conditions, GLP-1Rs are internalized; conversely, in a postprandial state GLP-1Rs are expressed on the plasma membrane (Chapter 2.1).

In Chapter 2.2, I sought to understand the mediators involved in GLP-1R translocation. It was well known that gut-derived hormones interact with each other at the level of VAN in order to regulate energy homeostasis. Specifically, several studies indicated that GLP-1 interacts with several gut peptides to regulate energy homeostasis (as discussed in Chapter 1); orexigenic ghrelin inhibits GLP-1-induced satiation in rats (14), however; anorexigenic peptides, such as leptin, potentiate its effects (15). In chapter 2.2, I demonstrate that ghrelin plays a role in mediating the satiating effects of GLP-1; GLP-1-induced satiation in fasted animals when ghrelin was blocked prior to an

administration of GLP-1. I provide evidence to suggest that ghrelin restricts GLP-1Rs on VAN to the cytoplasm and blockade of ghrelin restores GLP-1Rs to the plasma membrane. Although our data suggests that CCK does not mediate the translocation of GLP-1Rs on VAN, further work is required (Chapter 2.2).

In a second part of this dissertation, I sought to understand the role of the vagus in driving diet-induced obesity (see Chapter 2.3). Studies had established that leptin resistance in the arcuate nucleus (ARC) of the hypothalamus drives obesity (16); however, hyperphagia and increased body weight develop earlier than changes in the ARC (17). Using lepR knockout (KO) mice specifically on VAN, I found that disrupting leptin signaling on VAN is sufficient and necessary to lead to an obesogenic phenotype. On a chow diet, lepR-sensory neuron KO mice had increased food intake, body weight and adiposity compared to their wildtype (WT) littermates. The data showed that knockdown of leptin receptor on VAN leads to hyperphagia by attenuating the sensitivity to gut hormones. In addition, I found that KO mice did not gain further weight when fed on a high fat diet, which indicates that leptin resistance in VAN is required for the development of diet-induced obesity.

In Chapter 2.4, I sought to investigate the differences between male and female LepR-sensory neuron KO mice to understand the physiological differences that exist between the sexes. Investigating differences in wildtype mice, I demonstrated that male and female mice have inherently different meal patterns. Moreover, lack of leptin signaling of VAN led to an obese phenotype in male and female mice, however, the mechanism by which obesity was induced differed between the genders. LepR-sensory neuron KO male mice consumed larger and longer males more often compared to lepR-sensory neuron KO females who ate smaller and shorter meals less frequently.

3.4 Main findings

I demonstrate a novel regulatory mechanism by which intestinal hormones act on VANs. In this dissertation, I demonstrate that GLP-1Rs are constitutively expressed and that GLP-1Rs alter cellular localization according to feeding status (see Chapter 2.1). The notion that cellular localization rather than gene expression changes is important in understanding the mechanisms controlling food intake. This novel regulatory pathway implicates that the biological effects of gut-derived peptides are dependent on their receptor location. It is possible that hormones are secreted without driving any biological effects as their receptors may be internalized. Our finding insinuates that investigating only blood borne factors is not enough to understand what is physiologically happening. For example, bariatric surgeries leads to increased plasma concentrations of satiating signals, however, these concentrations may not reflect their actions.

Although vagal sensory pathways are known as a crucial neural mechanism for satiating signals, there is very little evidence to suggest that it plays a role in long-term energy homeostasis. The vagus nerve has been implicated in regulating short-term food intake; delineation to vagal afferents does not cause hyperphagia and obesity (18). Vagal destruction by surgical deafferentations or capsaicin leads to decreased meal size followed by increased meal frequency, therefore cumulative food intake is not affected (19). However, our data clearly highlights that signaling disruptions in VANs leads to meal pattern changes, which influence long term energy homeostasis (see Chapter 2.3).

The KO animal has been a very valuable tool to study the role of genes, *in vivo*, in normal physiological homeostasis. Regulatory peptides exhibit an array of biological activities, which are all comprised in a global KO animal. For example, ob/ob mice exhibit severe hyperphagia and body weight gain (20); however, the role of central versus peripheral leptin receptor is unclear. Even neuronal specific KO will target

peripheral neurons such as VAN, therefore distinguishing between the central and peripheral effects of leptin have not been systematically studied. Gut-derived peptides are pleiotropic; therefore, it is extremely hard to study the effect of one specific role for these peptides. The Cre/lox-P knockdown system allows the ablation of receptors associated with the control of food intake in a tissue-specific fashion making it easier to study the role of gut-derived peptides in one particular tissue. In Chapter 2.3, I exclusively knockdowned lepR receptors from VAN which highlights the importance of the vagus in the development of obesity. In this dissertation, I focused on the role of leptin signaling on VANs, however, this methodology can be applied to other gut-derived peptides. The ability to knockdown receptors specifically from VAN is a very powerful tool that could be used to investigate the functional importance and mechanism of action of other gut-derived hormones.

Our data highlights that disruptions in vagal signaling leads to different mechanisms to induce obesity in female versus male mice (Chapter 2.4). The prevalence of obesity is as high as in men as in women and the burdens of the disease seem to affect women particularly more so than in men; 80% of bariatric surgery patients are women (21, 22). And yet research on the regulation of energy homeostasis is biased towards the male gender. There is the assumption that research performed in males applies to females. Indeed, epidemiological and clinical studies of men often generate different results in women exemplified by sexual dimorphism in response to drugs (23). Therefore, in chapter 2.4, I sought to characterize the effects of the lack of leptin signaling on VAN in female mice and make a direct comparison between the male and female LepR KO mice. First, I demonstrated that meal patterns are inherently different between male and female mice. This is important because it suggests that there are specific physiological differences between the genders in relation to feeding behavior,

and they need to be studied in parallel for any research related to ingestive behavior. Importantly, I show that interventions produce completely different behavioral responses in male and female mice highlighting the importance of performing research in both sexes (as discussed in Chapter 2.4).

3.5 Limitations

In these present studies, I focused on investigating the mechanisms of GLP-1 and leptin on VAN, as they are associated with obesity. However, there are other peptides derived from the periphery that are involved in controlling food intake. For example, peptide YY (PYY) deficiency has been implicated in playing a role in the development of obesity (24). I also focused on the role of ghrelin and CCK in GLP-1 signaling on VAN. The pathways of gut-derived peptides are interconnected to regulate energy homeostasis, therefore, peptides other than the ones I studied may be involved in the regulatory mechanisms on VAN that I have yet to study. Gut-derived peptides are expressed throughout the peripheral and central nevous system and exhibit a variety of biological activities. It is very difficult to study one specific role of these peptides mediating appetite and energy homeostasis, especially given that they act together to exert their effects. Furthermore, studying individual hormones may be masking some of its true effects and this may explain the variability between animals and between different studies in the literature. In addition, non-hormonal factors, such as microbial products or nutrients that cross the gut barrier may modulate the effects of different hormones, and these might play a role in this integrative system. Signals controlling energy homeostasis involve extensive and interconnected hormonal systems communicating about nutrient status between different organs, and their signaling pathways are redundant. It is clear that the manipulation of one factor will not reveal the

complete mechanism underlying the control of food intake. It is important to consider these systems collectively in order to identify when, how and which tissues are driving appetite.

The vagus nerve is a functionally diverse nerve that innervates various organs such as the cardiovascular system. Lesions to the vagal afferent pathway have been used to study the importance of the vago-vagal loop in food intake. However, current methods delineate both vagal afferents and efferents leading to systemic side effects. It is unclear as to whether vagal motor deficits and sensory deficits contribute to the decrease in food intake associated in lesioned animals (25). The use of conditional gene mutations that are restricted to specific cell types *in vivo* is the most novel and best currently available method in studying the vagal afferent pathway. In the present studies (see Chapter 2.3), I used the Cre-LoxP system to investigate the role of leptin signaling at the level of the vagus. The methodologies used to manipulate vagal afferents has made significant progress in the last few years, however, they still do not allow selective ablation or stimulation of functionally specific neurons. It would be beneficial to have the ability to target specific neurons of the vagus nerve that innervate the gut as this would provide a better understanding of their direct role in communication of gut-derived signals influencing higher order neurons.

Investigating physiological pathways in female models is quite difficult as fluctuations in gonadal hormone concentrations influence feeding behaviors and body composition (26). Many studies resolve this problem by ovariectomizing rodents and administering exogenous estrogen to mimic estrus cycle concentrations. In these present studies, I did not control for the phases of the cycle of the mice at the time they were placed in the metabolic changes. Controlling for varying hormonal concentrations would help to better to limit uncertainties of cycle phase and their effect on food intake.

Furthermore, there are limitations of the techniques I used throughout these studies. Both quantitative and non-quantitative methods were used. When possible, I used quantitative techniques to eliminate research bias. In addition, I used different techniques to show the same results; thus confirming that the results are true results. For example, in chapter 2.1, I verified that the expression of GLP-1 receptors did not change according to feeding status through western blots, and immunofluorescence. My sample sizes were limited; a larger sample size would greatly increase the precision of the conclusions made from the data.

3.6 Possible future research

I have identified that disruptions in vagal signaling is sufficient and necessary to lead to obesity, however, the question remains as to what is driving leptin resistance. The mechanism by which diet leads to leptin resistance is unclear. Interestingly, the microbiota of the gastrointestinal tract exerts profound effects on host physiology (27). In recent years, there has been great interest in the contributions of the microbiota on metabolism and metabolic disease. It is well established that in obese models the microbiota is significantly altered (28). Furthermore, germ-free studies have highlighted the contributions of the microbiota to its host; germ-free mice are resistant to a high fat diet and colonization of germ free mice with conventional mice microbiota lead to an obese phenotype (28). Recently, the microbiota-gut-brain axis has become a popular topic; overall composition of the microbiota or exposures to specific bacterial strains can influence peripheral and central neural functions (29). More work is needed to investigate the mechanisms eliciting communication between the vagus and the microbiota. However, this novel avenue of research would provide a deeper understanding of the relationship between the microbiota and the vagus nerve.

I have shown that leptin resistance in the vagus nerve has profound effects, however, the question remains as to whether these effects are reversible. A study has demonstrated that reducing fat content in the diet will reverse leptin resistance in the ARC in mice; there was a downregulation of orexigenic neuropeptide Y and an increase in pro-opiomelanocortin neurons which coincided with normalized leptin sensitivity (30). Given that leptin resistance in vagal afferent neurons develops before the onset in ARC neurons, there is evidence that obesity is reversible in rats fed a high fat diet for 6-8 weeks by restricting food intake or changing the diet (30). Since leptin resistance in VAN is sufficient and necessary for diet-induced obesity, I would predict that restriction or diet change would reverse leptin resistance in VAN.

3.7 Conclusion

In conclusion, these studies provide strong evidence that the role of the vagus nerve is essential in relaying nutrient-induced signals and are necessary to maintain gut-brain communication. Moreover, a disruption in vagal signaling leads to the development of obesity. It remains unclear whether the changes occurring at the vagus are due to changes of gut derived hormone signaling or other factors such as gut luminal components (microbiota) crossing the intestinal barrier. Although there are many questions that remain to be answered, I now have clear evidence that gut-brain signaling via the vagus nerve could be an important peripheral site of action for the development of anti-obesity treatments.

3.8 References

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