Regulation of lipid metabolism by xenin

By

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Abstract

Xenin is a gastrointestinal hormone reported to supress food intake. Little is known about the role of xenin in body weight regulation. I hypothesized that enhanced xenin action will cause alterations in lipid metabolism towards reducing adiposity and body weight. To address this hypothesis, the present study investigated the effect of intracerebroventricular (i.c.v.) xenin treatment on body weight and lipid metabolism in obese mice. Effects of xenin on lipid metabolism were also investigated *ex vivo* in cultured gonadal white adipose tissue (WAT) and 3T3-L1 adipocytes. I.c.v. xenin treatment reduced body weight gain and increased phosphorylation of hormone sensitive lipase (HSL) in WAT of obese mice. Xenin treatment increased glycerol, free fatty acid (FFA) release and phosphorylation of HSL *ex vivo* in cultured WAT and 3T3-L1 adipocytes. These findings suggest that enhanced xenin action may be beneficial in reversing obesity by increasing the use of stored fats for fuel.

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"Those blessings are sweetest that are won with prayer and worn with thanks."
(Thomas Goodwin)

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Abbreviations

Ab	antibody
4-AAP	4- Amino antipyresine
ABHD5	α/β hydrolase domain containing protein 5
AC	adenylyl cyclase
ACC	acetyl CoA carboxylase
ACS	acetyl CoA synthetase
ADP	adenosine 5'-diphosphate
aCSF	artificial cerebrospinal fluid
AGRP	agouti related protein
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
α-MSH	α-melanocyte stimulating hormone
ANOVA	analysis of variance
ANGPTL4	angiopoietin like protein 4
AP	area postrema
AR	adrenergic receptors
ARC	arcuate nucleus
AT	adipose tissue
ATGL	adipose triglyceride lipase
ATP	adenosine 5'-triphosphate
ATP-CL	ATP-citrate lysate
b.w.	body weight

BAT	brown adipose tissue
BBB	blood-brain barrier
BSA	bovine serum albumin
BMI	body mass index
CART	cocaine- and amphetamine-regulated transcript
$CaCl_2$	calcium chloride
CCK	cholecystokinin
CCK-A	CCK-1 receptor
CCK-B	CCK-2 receptor
CgA	chromogranin A
CGI-58	comparative gene identification -58
ChREBP	carbohydrate-responsive element-binding protein
cDNA	complementary DNA
CNS	central nervous system
CO_2	carbon dioxide
CoA	coenzyme A
COPA	coatomer protein complex subunit alpha
CPT1b	carnitine palmitoyltransferase 1b
CRH	corticotropin-releasing hormone
CT	computer tomography
CV	coefficient of variation
DEXA	dual energy X-ray absorptiometry
DAG	diacyl glycerol

DAP	dihydroxyacetone phosphate
DGAT	acyl-coenzyme A: diacylglycerol acyltransferase
DGAT2	acyl-coenzyme A: diacylglycerol acyltransferase 2
DMN	dorsomedial nucleus
DMEM	Dulbecco's minimal essential media
DMV	dorsal motor nucleus of the vagus
DNL	*de novo* lipogenesis
DPP	dipeptidyl peptidase
DVC	dorsal vagal complex
EDTA	ethylenediaminetetraacetic acid
EP	endopeptidase
ESPA	sodium N ethyl N3 sulfopropyl-m anisidine
ERK	extracellular-signal-regulated kinases
ERK1/2	extracellular-signal-regulated kinases 1/2
Ex4	exendin-4
FDA	Food and Drug Administration
FFAs	free fatty acids
FASN	fatty acid synthase
Fgf21	fibroblast growth factor 21
fmol/ml	femtomole per milliliter
FTO	fat mass and obesity-associated
GH	growth hormone
GI	gastrointestinal tract

GIP	gastric inhibitory polypeptide
GIP	glucose dependent insulinotropic polypeptide.
GK	glucokinase
G0S2	G0/G1 switch gene 2
G3P	glycerol 3 phosphate
GK	glycerokinase
G1P	glycerol 1 phosphate
GLP-1	glucagon-like peptide 1
GLP-1R	GLP-1 receptor
GLUT	glucose transport
GPCR	G-protein coupled receptor
GSH-R	growth hormone secretagogue receptor
GSIS	glucose-stimulated insulin secretion
GWAS	genome wide association study
h	hour
HDL	high density lipoprotein
HSL	hormone sensitive lipase
H_2O_2	hydrogen peroxide
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IL-1	interleukin 1
IL-1RI	interleukin 1 type 1 receptor

IL-1β	interleukin-1β
IL-6	interleukin 6
IL-R	interleukin receptor
IR	insulin receptor
IRS1	insulin receptor substrate 1
Jak2	Janus kinase 2
Jak-Stat3	Janus kinase signal transduction- and activator of transcription 3
HFCS	high fructose corn syrup
HNTG	hepes sodium chloride TRITON X glycerol
K^+	potassium
KRB	Kreb's Ringers buffer
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
LD	lipid droplet
LHA	lateral hypothalamic area
LPA	lipophosphatide and phosphatidic acid
LPL	lipoprotein lipase
Lep	leptin
LepR	leptin receptor
M	molar
MAG	mono-acyl glyceride
MAPK	mitogen-activated protein kinase

MGL	monoglyceride lipase
MCP 1	monocyte chemotactic protein 1
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MEK1/2	MAP kinase kinase 1/2
mRNA	messenger ribonucleic acid
$MgSO_4$	magnesium sulphate
MRI	magnetic resonance imaging
MSH	melanocyte-stimulating hormone
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steato-hepatitis
NEFA	non-esterified fatty acids
NIH	National Institutes of Health
NMU	neuromedin U
NMUR2	NMU receptor 2
NP	natriuretic peptide
NPY	neuropeptide Y
NT	neurotensin
NTS	nucleus of the solitary tract
Ntsr1	neurotensin receptor 1
ob	obese

OECD	Organization for Economic Co-operation and Development
P	probability
PC1	prohormone convertase 1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	phosphodiesterase B
PDE3	phosphodiesterase 3
PDE4	phosphodiesterase 4
pERK1/2	phosphorylated ERK1/2
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PKB	protein kinase B
PLIN1	perilipin 1
PIP2	phosphotidyl inositol 4, 5 biphosphate
PIP3	phosphotidyl inositol 3, 4, 5 biphosphate
PI3K	phosphatidylinositol 3-kinase
PPAR-γ	peroxisome proliferator activated receptor – gamma
POMC	proopiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
PVN	paraventricular nucleus
PVDF	polyvinylidene diflouride
PYY	peptide YY

RNA   ribonucleic acid

RT-PCR   reverse transcriptase polymerase chain reaction

rpm   revolutions per minute

RQ   respiratory quotient

SCD-1   stearoyl CoA desaturase 1

SH2   src homology 2

SNP2   src homology 2 domain tyrosine phosphatase 2

SDS   sodium dodecyl sulphate

S.E.M   standard error of the mean

5HT2C   serotonin

SR   sustained release

Srebp-1c   sterol response element binding protein 1c

s.c.   subcutaneous

SNS   sympathetic nervous system

TBS   Tris-buffered saline

TG   triglyceride

TAG   triacylglycerol

TNF α   tumor necrosis factor α

Ucp1   uncoupling protein 1

Ucp3   uncoupling protein 3

VLDL   very low density lipoprotein

VMH   ventromedial hypothalamus

VMN   ventromedial nucleus

WAT	white adipose tissue
WHO	World Health Organization
WC	waist circumference
XENDOS	Xenical in the prevention of diabetes in the obese subjects
Y2R	neuropeptide Y receptor type 2

I. Introduction

1. Energy balance and its need

Energy balance is a basic need and requirement for every living organism. The homeostatic systems regulate this balance by determining the amount of energy intake and energy expenditure (Murphy and Bloom, 2006). Imbalance between energy intake and expenditure can either lead to obesity/overweight or underweight (Reilly, 2002). Evolutionarily our body developed a mechanism by which we can maintain normal body weight and adiposity during periods of food shortage. However, this mechanism becomes maladaptive in modern industrialized societies where high energy dense foods are almost always available with increased sedentary lifestyle. As a result, normal energy balance will be disrupted by efficiently storing fat, causing overweight and obesity.

2. Mechanism of body weight regulation

Body weight is maintained within a normal range by producing appropriate central nervous system (CNS) responses to the changes in the metabolic status of the body. The hypothalamus and the brainstem receive neural and hormonal signals from the periphery (pancreas, liver, adipose tissue, gastrointestinal tract and stomach), which encode the information about both acute and chronic nutritional and adiposity status of the body (Murphy and Bloom, 2006; Heymsfield and Wadden, 2017). There are two major theories, "glucostatic theory" and "lipostatic theory" that explain the role of metabolic signals in the regulation of energy balance.

2. 1. Glucostatic theory

According to this concept, whenever blood glucose level falls (e.g. fasting), it increases

signals that stimulate feeding, On the contrary, when blood glucose level rises it inhibits feeding (Mayer, 1952). Thus, glucose is one of the important candidate signals in regulating energy intake. There are a set of neurons within the hypothalamus that are sensitive to relatively small changes in glucose level in the periphery that are known as glucose-sensitive neurons (Wilding, 2002). These neurons were discovered in the 1960s (Anand et al., 1964; Oomura et al., 1964) and have been shown to play an important role in maintaining energy balance (Routh et al., 2014). It has been shown that the electrical activity of neurons in the hypothalamic satiety center increases when glucose was infused intravenously to both dogs and cats while it significantly reduces the activity of neurons in the hunger center. These effects were reversed when insulin was infused intravenously to these animal models (Anand et al., 1964). In addition, there are reciprocal changes in the neuronal activity of the satiety center and the hunger center after intravenous injection of glucose or insulin (Oomura et al., 1964). These findings support the glucostatic theory that hypothalamic glucose sensitive neurons control food intake by altering their activities in response to changes in glucose availability.

2. 2. Lipostatic theory

This theory describes the feedback mechanism existing between fat mass and hypothalamic signaling based on the observations that body weight and adiposity were maintained at constant level over long periods in spite of day-to-day fluctuations in energy intake. According to this theory, molecules released from the adipose tissue send signals to the brain to regulate appetite/satiety and adiposity (Kennedy, 1953). This theory was supported by the cross-circulation experiment (parabiosis) done on rats. In this experiment, a pair of rats were surgically connected each other with their blood vessels to allow exchange of humoral factors

between the animals. When an obese rat was paired with normal lean rat, the latter became hypophagic and lost weight. This finding suggests that adipose tissue of the obese rat produces a larger amount of a molecule(s) that inhibits feeding. It is likely that lean rat eats less and becomes leaner due to the increased availability of the feeding-suppressing molecule(s) from obese rat, while obese rat remains heavier because of resistance to the feeding-suppressing effect of the molecule(s) (Hervey, 1959). This concept was further strengthened after the discovery of leptin, which is released from the adipocyte and plays an important role in regulating food intake, adiposity and body weight (Zhang et al., 1994).

2. 3. Role of central nervous system (CNS) in the regulation of metabolism

The brain is a key player in the regulation of metabolism. Lesion and electrical stimulation studies have proven that the hypothalamus region of the brain has hunger and satiety centers that regulate appetite/satiety and maintain metabolic homeostasis (Berthoud et al., 2017). These studies have described the lateral hypothalamic area (LHA) and the ventromedial hypothalamus (VMH) as the "hunger centre" and the "satiety centre", respectively. In addition to these classical satiety and hunger centers, there are other hypothalamic regions that participate in the regulation of energy balance. The arcuate nucleus (ARC) has been considered as a major hypothalamic region in the regulation of energy balance after the discovery of the importance of the hypothalamic melanocortin pathway. The melanocortin system consists of neurons expressing pro-opiomelanocortin (POMC), agouti-related protein (AgRP) and melanocortin receptors (MCR) (Diano, 2011). The POMC neurons are regulated by nutrients (e.g. glucose and fatty acids) and hormonal signals such as leptin and insulin (Elias et al., 2000; Parton et al., 2007). POMC neurons increase their activity under fed conditions while fasting inhibits the

activity of these neurons (D'Agostino and Diano, 2010). On the contrary, activity of AgRP neurons are increased by fasting and reduced by feeding. Alpha melanocyte stimulating hormone (α-MSH) is a product of the *POMC* gene which potently inhibits food intake (Wilding, 2002). It has been shown that leptin triggers the production and release of α-MSH from axon terminal of POMC neurons which further activates melanocortin receptor 3 (MC3R) and 4 (MC4R) to suppress food intake and increase energy expenditure (Warne and Xu, 2013). ARC has two different subpopulations of neurons which regulate food intake. One set expresses AgRP and neuropeptide Y (NPY) which stimulates feeding, while the second set expresses POMC and cocaine-and amphetamine–related transcript (CART) which inhibits feeding. These two neuronal populations participate in the regulation of energy balance by forming neural circuits which communicate with other hypothalamic nuclei involved in appetite regulation such as paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN) and lateral hypothalamic area (LHA) (Simpson et al., 2009).

2. 4. Hormonal regulation of metabolism

Whole body metabolism is regulated by hormonal signals released from endocrine organs. There is an interplay between the neural signals released from the brain and the hormonal signals released from the endocrine organs. These hormones send signals to the specific hypothalamic region in the brain involved in the regulation of energy metabolism based on their blood concentration levels. Hormones whose secretion and circulating levels increase after meals are considered as anorexigen, while those released before meals are considered as orexigen.

2. 5. Role of adipocyte hormones in the regulation of metabolism

In the past two decades, increasing evidence suggests that adipose tissue plays an important role in the regulation of metabolism not only as a fat storing organ but also as an endocrine organ. Leptin is a 167-amino acid protein synthesized and secreted by adipose tissue and discovered in *ob/ob* mice by positional cloning (Zhang et al., 1994). These mice were deficient in leptin because of the homozygous mutation in the *leptin* gene that resulted in a phenotype expressing hyperphagia, extreme obesity, diabetes, neuroendocrine abnormalities and infertility (Zhang et al., 1994). Plasma levels of leptin are dependent on the amount of body fat. The more the body fat, the higher the leptin levels in the circulation (Considine et al., 1996). It is secreted in a pulsatile fashion with higher levels secreted in the evening and during early morning hours (Sinha et al., 1996; Licinio et al., 1997). Leptin plays an important role in regulating metabolism and neuroendocrine function by acting via binding to specific leptin receptors (LepRs) that are expressed in the CNS as well as in peripheral tissues. Five LepR isoforms are generated through alternative splicing. The LepRb isoform (also known as long leptin receptor isoform) contains a cytoplasmic domain that includes motifs for binding of intracellular signaling molecules, and therefore LepRb is crucial for the biological action of leptin (Tartaglia et al., 1995; Lee et al., 1996). Leptin regulates energy homeostasis by directly acting on the hypothalamic cells that express LepRb (Elmquist et al., 1998). It has been shown that after binding to LepRb, leptin activates the Janus kinase-signal transducer and activator of transcription-3 (JAK-STAT3) signaling pathway (Vaisse et al., 1996; Bates et al., 2003). LepRb is expressed not only in the hypothalamus but also in other brain regions such as the amygdala and the brainstem and leptin action in these brain regions is also involved in the regulation of metabolism (Robertson et al., 2008). Leptin also increases energy expenditure by increasing

sympathetic nerve activity and activating brown adipose tissue thermogenesis in mice (Collins et al., 1996; Scarpace et al., 1997). Leptin treatment improves hyperglycemia and hyperinsulinemia in *ob/ob* mice (Harris et al., 1998).

Importantly, consistent with the findings in mice, clinical evidence has shown that patients with leptin deficiency due to a mutation in the leptin (*LEP*) gene have an obese phenotype associated with hyperphagia (Farooqi et al., 2007b). In these patients leptin administration reduces food intake (Farooqi et al., 2007a) and normalizes body weight (Heymsfield et al., 1999). Similarly, lack of LepR causes impairments in metabolism such as hyperphagia, obesity and insulin resistance in rodents and humans (Strobel et al., 1998; Lee et al., 1996). Activation of LepR specifically in the hypothalamic ARC reversed these metabolic impairments in animals lacking a functional LepR (Morton et al., 2003; Coppari et al., 2005). Although leptin treatment is effective in reducing body weight in individuals without functional leptin, leptin deficiency is rare in humans. Instead leptin resistance, in particular hypothalamic leptin resistance, is a common impairment seen in obese people. Thus, leptin administration to obese individuals having already high levels of leptin and are resistant to its metabolic actions, does not cause any significant weight loss (Heymsfield et al., 1999; Kelesidis et al., 2010). The mechanism for leptin resistance is largely unknown. It has been suggested that the mechanism may involve impairments in leptin transportation into the brain and impaired functioning of downstream signaling pathways. Collectively, these findings contributed critical evidence leading to the current emphasis on the importance of hypothalamic leptin action in metabolic regulation and hypothalamic leptin resistance as a major problem in human obesity.

2. 6. Role of gut hormones in the regulation of metabolism

There are several hormones that are released from the gastrointestinal (GI) tract, which play an important role in regulating food intake. These include cholecystokinin (CCK), peptide YY (PYY), glucagon-like peptide (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and ghrelin.

2. 6. 1. Cholecystokinin (CCK)

CCK is the first gut hormone to be identified as an anorexigenic gut hormone. CCK is released from the small intestine (I-cells) after a meal and promotes its anorectic effect via the cholecystokinin 1 receptor (CCK-1R) as it has been shown that administration of specific CCK1 receptor agonist caused a dose-dependent suppression of food intake. Consistent with these findings, a CCK1 receptor antagonist (devazepide) dose-dependently inhibited the anorectic effects of CCK (Asin and Bednarz, 1992; Melville et al., 1992). Rats deficient in CCK-1R developed hyperphagia and obesity, further supporting the role of CCK/CCK-1R signaling in the regulation of food intake and metabolism (Bi and Moran, 2002). Peripheral administration of CCK increases c-fos expression in the brainstem (Zittel et al., 1999). CCK reduces food intake via the CCK1 receptor (CCK-1R) located on the pyloric/duodenal vagal afferents which terminate in the hindbrain areas that regulate food intake including area postrema (AP), dorsal motor nucleus of vagus (DMV) and the nucleus of solitary tract (NTS). Lesion of these nuclei attenuates the anorectic effect of CCK (Moran et al., 1990; Edwards et al., 1986). Continuous infusion of CCK failed to alter food intake, while intermittent injections of CCK reduced food intake (Murphy and Bloom, 2006). This suggests that the feeding-suppressing effect of CCK is short-lived. Thus, although CCK/CCK-1R signaling is a possible target for developing anti-

hyperphagia drugs, a specific treatment regimen and/or long-acting CCK analogues need to be established. Moreover, the effect of CCK analogues and CCK-1R agonists on body weight has yet to be determined.

2. 6. 2. Peptide YY (PYY)

PYY is produced in intestinal L-cells and is released into the circulation after a meal. The majority of circulating PYY is the N-terminally truncated form PYY_{3-36}. It has been shown that acute peripheral administration of PYY_{3-36} to both humans and rodents reduces food intake. Inconsistent effects of PYY_{3-36} on food intake have been reported. PYY_{3-36} failed to reduce food intake in rodents exposed to new environment (stress), suggesting the possibility that PYY_{3-36} signaling might interact with signaling pathways involved in the stress response. The anorectic effects of PYY_{3-36} are mediated via the Y2R receptor as its effects were attenuated by Y2R antagonist as well as in Y2R knockout mice (Abbott et al., 2005; Batterham et al., 2002). The Y2R receptor is an inhibitory presynaptic receptor which is highly expressed on NPY neurons present in the ARC (Broberger et al., 1997). Pyy knockout mice showed significantly higher body weight and fat mass accompanied by significantly elevated fasting or glucose stimulated serum insulin concentrations when fed a normal rodent chow compared to wild type mice (Boey et al., 2006). These findings suggest that PYY signaling plays an important role in the regulation of energy balance and glucose homeostasis. High doses of PYY_{3-36} have been shown to cause conditioned taste aversion in both animals and humans (Murphy and Bloom, 2006). It was further demonstrated that intermittent intravenous administration of PYY_{3-36} to rats caused long-term reductions in food intake, body weight and adiposity (Chelikani et al., 2006). Thus, PYY_{3-36} is involved in the regulation of food intake as a satiety factor and a possible target for anti-

obesity drugs. However, the careful consideration should be given to the dosage pattern that is essential for producing constant reductions in food intake and body weight.

2. 6. 3. Glucagon-like peptide-1 (GLP-1)

GLP-1 is an incretin that is released from the L-cells of the intestine into the circulation after a meal. It has been shown that GLP-1 when administered centrally or peripherally potently stimulates insulin secretion. Additionally, it reduces food intake in rodents and humans (Drucker, 2006). GLP-1 is rapidly inactivated by dipeptidyl peptidase (DPP) in the circulation. Exendin-4 is a potent GLP-1 receptor (GLP-1R) agonist/mimetic and resistant to degradation by DPP with a longer half-life. It has been shown that exendin-4 treatment reduces blood glucose levels in type 2 diabetic patients. Interestingly, exendin-4 treatment also caused a significant reduction of body weight in these patients (Murphy and Bloom, 2006). Thus GLP-1R agonists and GLP-1 analogues might be used as a potent target for reducing body weight. Liraglutide, a GLP-1R agonist has been approved by the FDA for the treatment of obesity and overweight (Burcelin and Gourdy, 2017).

2. 6. 4. Glucose-dependent insulinotropic polypeptide (GIP)

GIP is a 42-amino acid hormone synthesized by enteroendocrine K-cells and is released into the circulation post-prandially. It is an incretin and stimulates insulin secretion in glucose-dependent manner. In addition to the insulinotropic effect, GIP is also involved in various other functions. In the pancreas, GIP increases expression of anti-apoptotic markers and decreases pro-apoptotic markers resulting in a reduction of ß-cell death. In adipose tissue, GIP promotes lipogenesis by interacting with insulin to increase lipoprotein lipase (LPL) activity (McIntosh et

al., 2009). Evidence suggests that GIP also participates in the regulation of body weight. Paradoxically, both GIP-overexpressing mice and GIP receptor knockout mice are less susceptible to high-fat diet-induced obesity (Yamada and Seino, 2004; Kim et al., 2012). Thus, the precise role of GIP in the regulation of metabolism needs to be elucidated in future studies.

2. 6. 5. Ghrelin

Ghrelin is a 28-amino acid peptide hormone secreted from the stomach before food intake. It was initially discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) 1 which was capable of stimulating growth hormone release from the anterior pituitary gland (Kojima et al., 1999). Circulating ghrelin levels increase during fasting and decrease after a meal. Central and peripheral administration of ghrelin to rats initiates feeding behavior. In humans, subcutaneous injection and intravenous infusion of ghrelin promotes hunger and increases food intake (Kojima and Kangawa, 2005). The feeding-promoting effect of ghrelin is mediated by hypothalamic neurons that co-express orexigenic NPY and AgRP (Tschöp et al., 2000). Ghrelin receptors are expressed on almost all the NPY/AgRP neurons that are activated by ghrelin as shown by the increased firing rate of these neurons and expression of *c-fos, Npy* and *Agrp* mRNA. Both pharmacological and genetic blockade of NPY or AgRP attenuate the orexigenic actions of ghrelin (Cummings, 2006). Pharmacological blockade of ghrelin reduced food intake and body weight. Moreover, Ghsr-knockout mice and male ghrelin deficient mice also showed resistant to diet-induced obesity when fed a high fat diet. It has been shown that GHS-R antagonist potently reduces food intake in fasted mice (Cummings, 2006). Ghrelin-deficient mice showed altered metabolic fuel preferences with an increased usage of fat as a fuel source when fed a high fat diet (Murphy and Bloom, 2006). On the contrary, ghrelin

agonist administration to specific patient groups (e.g. cancer cachexia and dialysis) has been shown to be beneficial in increasing food intake and preventing body weight loss (Neary et al., 2004; Wynne et al., 2005). It has also been shown to increase gastric emptying rate in diabetic patients suffering from gastroparesis (Murray et al., 2005). These findings suggest that inhibition of ghrelin signaling is a possible target for anti-obesity drugs, while enhancement of ghrelin signaling may be beneficial in stimulating feeding in some disease conditions where appetite is reduced.

3. Obesity

3. 1. Definition of obesity and diagnosis

Obesity/overweight is defined as excessive and abnormal accumulation of fat in the body associated with various metabolic diseases. Body mass index (BMI) is widely used to classify overweight/obesity in humans. It is a ratio of weight to height and is expressed in kg/m^2. According to the World Health Organization (WHO), BMI greater than or equal to 25 is considered overweight and BMI greater than or equal to 30 is considered obese in adults (http://www.who.int/mediacentre/factsheets/fs311/en/). Although studies have shown that BMI correlates well with body fat, BMI is a measure of excess weight rather than excess body fat and may not be an accurate measure of body fat in some individuals. Factors such as age, sex, ethnicity and muscle mass can influence the relationship between BMI and fat mass. For example, in the case of athletes who carry more muscle mass and very little fat are generally categorized obese or overweight when measured using BMI. Under such conditions BMI is not a suitable tool. BMI is also not a reliable measure to define obesity in youth because it varies with age and sex in growing children. It has been suggested BMI z-score, a measure of relative weight

adjusted for age and sex, is a more accurate measure in growing children (Must and Anderson, 2006). Waist circumference (WC) is another important and independent measure in the assessment of various obesity-associated health risks such as cardiovascular disease and type 2 diabetes. In adult women, if waist circumference is greater than 35 inches and in males, if it is greater than 40 inches, they are classified under the category of obesity and they are at an increased risk of developing cardiovascular diseases in later life. More accurate assessments of body fat include measuring a person's weight underwater or in an air-displacement or using Dual Energy X-ray Absorptiometry (DEXA). Furthermore, computed tomography (CT) and magnetic resonance imaging (MRI) have emerged as advanced imaging techniques to measure adiposity. These advanced techniques allow an accurate determination of body fat distribution and quantification of fat mass in anatomically defined regions of interest (Rothney et al., 2009). Since body fat distribution pattern and the amount in specific fat depot affects the risk of cardiovascular disease, these methods provide more precise evaluation of not only obesity but also the impact of fat distribution (Mahabadi et al., 2009). However, these advanced techniques are not always available to clinical practice and are costly, so simpler methods are more routinely used to measure body fat and diagnose obesity. These simpler methods include measurement of BMI, WC (as mentioned above) and the thickness of fat layer under the skin in different areas of the body, and Bioelectrical Impedance Analysis (http://www.obesityhelp.com/content/aboutobese.html#howis).

3. 2. Prevalence

Worldwide obesity has doubled since 1980. According to the WHO report in 2016, there were >1.9 billion overweight adults by the end of 2014 and > 600 million were obese. More than

41 million children under the age of 5 were overweight or obese in 2014 (http://www.who.int/mediacentre/factsheets/fs311/en/). Not surprisingly, a similar trend can be seen in Canada. Statistics Canada data show that almost two thirds of Canadian adults are now overweight or obese. Data from the Organization for Economic Co-operation and Development (OECD) have ranked Canada among the countries with the highest obesity rates. The most recent data by Canadian Obesity Network have reported that 25.4% of adults are obese and 36% are overweight. Among children, the obesity rate for girls and boys is 11% and 15%, respectively. Childhood obesity is increasing at an alarming rate of 2 to 5% increase each year (https://sencanada.ca/content/sen/committee/421/SOCI/Reports/2016-02-25_Revised_report_Obesity_in_Canada_e.pdf). According to systematic analysis performed in 2010, overweight and obesity were estimated to contribute 3.4 million deaths and 4% of years of life lost worldwide (Lim et al., 2012). In Canada, the incidence of adult obesity has increased from 6.1% to 18.3% between 1985 and 2011. The increasing trend has also been observed in obese class 1 (BMI > 30.0-34.9) from 5.1% to 13.1%, class 2 (BMI > 35.0-39.9) from 0.8% to 3.6% and class 3 (BMI > 40.0) from 0.3% to 1.6%. It has been predicted that by 2019 this will increase by 14.8%, 4.4% and 2.0% in obese class 1, 2 and 3, respectively (Twells et al., 2014). Thus, obesity is a rapidly growing serious health issue throughout the world that needs proper treatment and attention.

3. 3. Causes

3. 3. 1. Environmental factors

Obesity is a multifactorial disease. It has been proposed that environmental factors contribute to the development of obesity. Specifically, our environment includes an unlimited

supply of high energy density foods that is coupled with a sedentary lifestyle. This type of environment promotes a positive energy balance because evolutionarily our body is designed to store energy efficiently but is not designed to increase energy expenditure when there is an abundant supply of food for an extended period of time (Hill and Peters, 1998). A study performed on Australian population has shown that lifestyle positively promotes the obesity epidemic, as the risk of developing obesity is increased in people living on highways (without sidewalk or one side walk) when compared to the people living on the street having sidewalks on both sides. Watching TV for 3 or more hours daily is also associated with an increased incidence of obesity and overweight when compared to individuals who watch TV for less than 3 hours a day (Giles-Corti et al., 2003). These data support the concept that a sedentary lifestyle contributes to the development of obesity. Consumption of high fructose corn syrup (HFCS) in beverages has also increased by > 1000% from 1970 to 1990 in U.S. HFCS alone now represents > 40% of caloric sweetener added to foods and beverages. The increased usage of HFCS is associated with rapid increase in obesity/overweight in the U.S. (Bray et al., 2004). Frequent consumption of fast food is strongly associated with increases in body weight gain in U.S. over the past 15 years. This change is also found to be strongly associated with insulin resistance in this study (Pereira et al., 2005). These findings support the concept that environmental factors play an important role in the development of obesity and obesity-associated complications.

3. 3. 2. Genetic factors

Family history is one of the major determinants of obesity. A study conducted on a Gambian population showed that subjects with family history of obesity have a higher BMI and an increased risk of developing obesity in later life (van der Sande et al., 2001). Obesity is a

complex and heritable disease which is a result of an interplay between genetic predisposition, environment and epigenetics. There are a number of genes that are associated with monogenic and polygenic forms of obesity. Monogenic obesity is rare in humans and involves mutations in genes of the leptin-melanocortin system such as leptin (*LEP*), leptin receptor (*LEPR*), proopiomelanocortin (*POMC*), prohormone convertase 1 (*PC1*) and melanocortin 4 receptor (*MC4R*) (Farooqi and O'Rahilly, 2006). MC4R deficiency represents the most common monogenic cause of human obesity. Roles of these genes in obesity have been confirmed in mouse models that are deficient in these genes (Yazdi et al., 2015). The genetic predisposition to obesity for most individuals (> 95% of cases) has a polygenic basis. An individual genetic variant itself has a small effect on the phenotype and causes a sizeable phenotypic effect only in combination with other predisposing variants. Genome-wide association studies (GWAS) have identified 227 genetic variants and their involvement in various pathways such as central nervous system, adipocyte differentiation, insulin signaling, lipid metabolism, liver and muscle biology, plus there are recently discovered associations with gut microbiota in the development of polygenic obesity (Pigeyre et al., 2016). GWAS identified associations between common genetic variants and obesity risk. The fat mass and obesity associated (*FTO*) gene was the first obesity-susceptibility gene identified through GWAS and is most widely replicated in a variety of age groups and ethnic backgrounds (Frayling et al., 2007). These findings suggest that although genetic mutations contribute to monogenic obesity, the majority of human obesity has a more complex polygenic nature.

3. 3. 3. Ethnic and cultural differences

Ethnicity and cultural disparities are linked to the development of different food-related beliefs, preferences and behaviors that may influence the risk of obesity in certain populations. These ethnic differences can contribute to the development of obesity during gestation, infancy, childhood and adolescence (Kumanyika, 2008). Ethnic differences can also contribute to the development of obesogenic behaviors in children. A study conducted in England on children from white, black and South Asian parents have shown that subjects from black and South Asian ethnicity were three times more likely to have an obesogenic lifestyle, an environment which promotes weight gaining effects than the white ethnic group (Falconer et al., 2014). These findings suggest that ethnic and cultural backgrounds affect the development of obesity.

3. 4. Treatment

There are 3 approaches to treat obesity; lifestyle intervention, pharmacological treatment and surgery.

3. 4. 1. Lifestyle Interventions

Lifestyle intervention is the most readily available therapy to most obese and overweight patients. It is recommended that patients with a BMI > 30 should be consulted intensively on lifestyle interventions such as appropriate diet and exercise. It has been shown that dietary therapy such as caloric restriction, low-fat diet and carbohydrate-restricted diet can reduce body weight (Clifton, 2008). Increasing physical activity in combination with dietary therapy has been shown to be effective in maintaining body weight within the normal range (Thomas et al., 2006). Types of fat consumed are also contributing factors in maintaining body weight within the

normal range. A high-fat diet with saturated fats causes obesity while a high-fat diet containing polyunsaturated fatty acids is protective against diet-induced obesity (Wang et al., 2002); (Dziedzic et al., 2007). However, in most cases, behavioral interventions have a major problem in that obese individuals often regain the lost weight after the completion of the weight loss program (Jakicic et al., 2008; Svetkey et al., 2008). Although lifestyle interventions are readily available to most obese/overweight patients, it is always challenging to keep the patient motivated to remain in the weight loss program.

3. 4. 2. Pharmacological treatment

Obesity is a serious health concern and also a significant risk factor for various other diseases such as type 2 diabetes, insulin resistance, cardiovascular diseases and dyslipidemia. It has been shown that approximately 5-10% of weight loss in obese individuals is associated with significant health benefits (Jensen et al., 2014). Mechanisms by which anti-obesity drugs reduce body weight can be classified into two categories: reducing energy intake by suppressing appetite or inhibiting absorption of nutrients. Over the past years, various clinical trials have been performed to develop anti-obesity drugs such as fenfluramine, sibutramine and rimonabant. Unfortunately, many clinical trials failed to produce significant health benefits mainly due to the adverse side effects of these drugs (Burcelin and Gourdy, 2017).

At present, there are 6 drugs that have been approved for the treatment of obesity by the U.S. Food and Drug Administration (FDA). Orlistat is a derivative of lipostatin isolated from soil bacteria and in the gut it inhibits the breakdown of dietary fats via blocking the active site of gastrointestinal lipase and reducing absorption of ingested fats (Hauptman et al., 1992). In a double-blind randomized placebo controlled prospective clinical trial XENDOS (XENical in the

prevention of Diabetes in Obese Subjects) obese patients who received a 4-year orlistat treatment achieved a significant weight loss, and consequently a significant reduction in the incidence of diabetes (Torgerson et al., 2004).

The other five drugs are appetite suppressants that act through the CNS. Phentermine is an atypical amphetamine analogue that mainly increases norepinephrine release in the CNS and reduces food intake (Ryan and Bray, 2013). A combination treatment of a low dose of phentermine with topiramate extended-release reduces food intake and promotes a robust weight-reducing effect. Lorcaserin is a small molecule agonist of the serotonin 2C ($5\text{-}HT_{2C}$) receptor that reduces food intake and body weight. A combination product of naltrexone sustained release (SR) and bupropion SR reduces food intake and body weight at least partly by stimulating overall activity of anorexigenic POMC neurons. Lastly, several gut hormones have also been shown to be a potential target for anti-obesity drugs. Glucagon-like peptide 1 receptor (GLP-1R) agonists are currently approved for the treatment of type 2 diabetes (Lovshin and Drucker, 2009). In addition to their use as anti-diabetic drugs, they have also been shown to reduce body weight in obese or overweight individuals without diabetes (Vilsbøll et al., 2012). Liraglutide is a long acting GLP-1R agonist that was approved for the treatment of overweight and obesity. Human studies found no effects on gastric emptying and energy expenditure (van Bloemendaal et al., 2014; van Can et al., 2014). Thus, the exact mechanism by which liraglutide reduces body weight is unknown. However, animal studies have suggested that the feeding-suppressing effect of liraglutide might be mediated via hypothalamic melanocortin signaling (Secher et al., 2014). Further investigation is required to increase the number of safe and effective anti-obesity drugs to provide additional treatment options to obese patients.

3. 4. 3. Surgery

Surgical treatment of obesity aims at restricting food intake by reducing the stomach volume or shortening the length of the small intestine. Roux-en-Y bypass (RYGB), gastric banding and laparoscopic sleeve gastrectomy (LSG) are the three surgeries that are currently available. These surgeries produce body weight loss that is much greater than what is achieved by lifestyle intervention and pharmacological treatment. Surgical therapies have been shown to reduce obesity-associated metabolic complications such as type 2 diabetes and insulin resistance. (Thaler and Cummings, 2009). It has been suggested that the beneficial effects of bariatric surgery on body weight and glycemic control are mediated by alterations in the secretion of appetite-regulating hormones such as ghrelin (Korner et al., 2005; Cummings and Shannon, 2003). The limitation is that only severely obese patients (BMI > 40) and obese patients (BMI between 35 and 40) with significant obesity related comorbidities are currently eligible for bariatric surgery. Therefore, only a small fraction of patients can benefit from surgery and the majority of obese patients have to rely on lifestyle interventions and medications.

4. Xenin

Xenin is a gut hormone that is secreted from the intestinal cells and participates in the regulation of energy balance.

4. 1. Discovery of xenin

Xenopsin is an octapeptide that was first discovered in the skin of the African clawed frog *Xenopus laevis* (amphibians) (Araki et al., 1975). Xenopsin has structural similarity to the tridecapeptide neurotensin which was first isolated from bovine hypothalamus and later found in

N cells of the ileal mucosa (Carraway and Leeman, 1973). They also have similar biological activities; both xenopsin and neurotensin increase vascular permeability, inhibit gastric acid secretion, stimulate exocrine pancreatic secretion and growth and induce contractions of isolated rat fundic strips and guinea pig ileum (Feurle, 1998). Both xenopsin and neurotensin-like peptides were present in amphibians. These findings raised the question whether or not the counterpart of amphibian xenopsin is present in mammals. Through the search for xenopsin-like peptides, xenin was discovered in the human gastric mucosa. Xenin is a 25-amino acid peptide and shows amino acid sequence homology with xenopsin at the C-terminal region (Figure 1). The C-terminal region of xenin-25 also shares homology with other peptides such as neurotensin and neuromedin N (Figure 1). Therefore, all these peptides sharing common C-terminal amino acid sequence are categorized under the neurotensin/xenopsin family of peptides (Feurle et al., 1992).
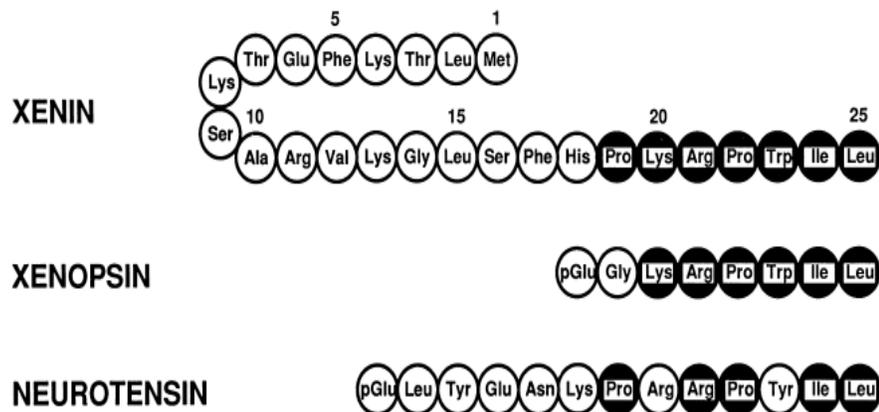
Figure 1. Xenin, xenopsin and neurotensin show amino acid sequence homology in C-terminal amino acid sequence.

4. 2. Formation and degradation of xenin

Xenin is derived from the 35-amino acid precursor peptide named proxenin (Hamscher et al., 1996). Proxenin contains the entire sequence of xenin-25 extended by 10 amino acids at the C-terminus (Figure 2). Both xenin and proxenin show 100% homology with the N-terminus of human coatomer protein complex subunit alpha (COPA) (Hamscher et al., 1996; Chow and Quek, 1997). Treatment of proxenin with pepsin cleaves off the 10 C-terminal amino acids and produces xenin-25. When acid extraction of gastric mucosa was performed in the presence of the proteinase inhibitor pepstatin, the amount of extractable xenin-25 was reduced (Hamscher et al., 1995). These findings suggest that pepsin-like proteases mediate the formation of xenin-25 from its precursor.

The aspartic proteinase, cathepsin E, which has 53% identity with pepsin, is present in the gastric mucosa at high levels and promotes the liberation of bioactive peptides from xenopsin and neurotensin precursors (Kageyama et al., 1995; Kageyama et al., 1992). The consensus sequence PXXL is present at the C-terminus of xenin-25 and generates the conformation required for the cleavage by cathepsin E (Kageyama et al., 1995; Chow and Quek, 1997) (Figure 2). Although the 4 C-terminal residues of proxenin (VIQL) differ from the conserved PXXL sequence, it has been shown that substitution of proline (P) by valine (V) in the consensus sequence of the neurotensin precursor does not change the rate of hydrolysis by cathepsin E (Kageyama et al., 1995). These data suggest that proxenin is released from the precursor COPA, followed by processing of proxenin to bioactive xenin-25 through the post-translational cleavage mediated by aspartic proteinases such as cathepsin E.

Native xenin-25 is rapidly degraded to generate C-terminally truncated fragments (Hamscher et al., 1995; Martin et al., 2012; Martin et al., 2014) (Figure 2). It has been shown

that xenin-25 and xenin 18-25 (xenin-8) have significant insulin-releasing actions in insulin-producing cell lines and perfused rat pancreas. On the contrary, xenin 9-25 (xenin-17), xenin 11-25 (xenin-15) and xenin 14-25 (xenin-12) failed to show insulinotropic effects (Martin et al., 2014; Martin et al., 2016; Silvestre et al., 2003). Although it has not been tested whether or not a 6 C-terminal amino acid fragment (xenin-6) is produced through the naturally occurring degradation of xenin-25, xenin-6 retains some biological activities of the full-length of xenin (Feurle et al., 1996; Feurle et al., 2002). The precise mechanism of xenin degradation is currently unknown. A recent study reported that the endopeptidase EC 3.4.24.15 (EP24.15, thimet oligopeptidase) is present in the stomach and small intestine and xenin is a substrate of EP24.15 (Philibert et al., 2017). EP24.15 plays a key role in the cleavage of several neuropeptides that also exist in the gastrointestinal tract such as neurotensin (Tullai et al., 2000). In summary, xenin is released from proxenin which is derived from the precursor COPA through posttranslational cleavage possibly mediated by cathepsin E. Once secreted into the circulation, xenin is quickly degraded into smaller fragments, some of which still retain biological activity, through a mechanism possibly involving the endopeptidase EP24.15.
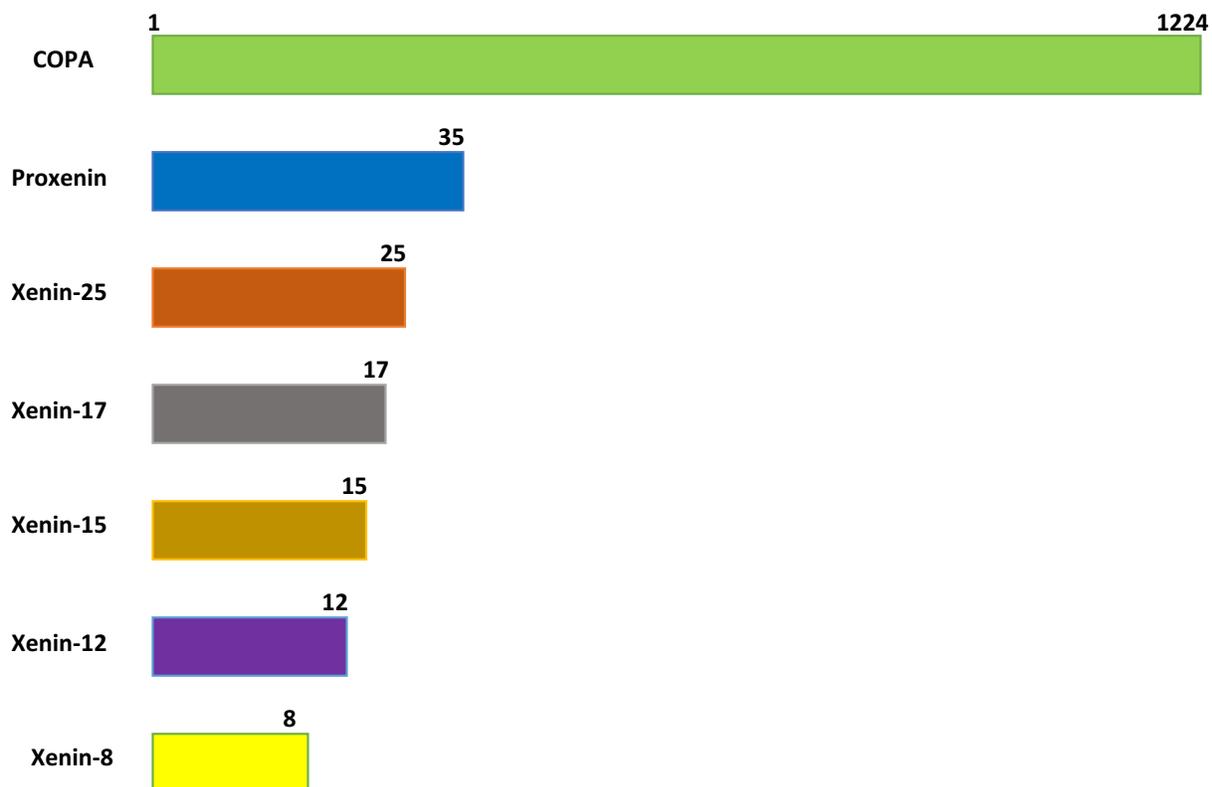
Figure 2. Formation of xenin from COPA followed by its degradation into smaller peptides.

4. 3. Species and tissue distribution of xenin

Xenin was initially isolated from the human gastric mucosa. It is also present in gastric mucosa of various other species such as dog, pig, rabbit, guinea pig and rat (Hamscher et al., 1995). Xenin is widely distributed in different organs of the body such as hypothalamus, lung, liver, heart, kidney, adrenal gland, pancreas, testis, skin and duodenal, jejunal and colonic mucosa of dog and man (Hamscher et al., 1995). Xenin is expressed in specific endocrine cells in the gastrointestinal tract. Double staining immunohistochemistry revealed that the subpopulation of chromogranin A (CgA, a marker of neuroendocrine cells)-immunoreactive cells express xenin

in the duodenum. All xenin-immunoreactive cells were positive for CgA and GIP in dog and human duodenum, indicating that xenin is present in a subset of K-cells (Anlauf et al., 2000).

Circulating xenin-25 levels are low and currently available data on xenin-25 level are not consistent. The initial study by Feurle *et al.* reported that plasma xenin-25 level rose from 6.7 fmol/ml to 14.7 fmol/ml in response to food intake in humans (Feurle et al., 1992). Another study has shown the rise in plasma concentration of xenin-25 from 10-30 fmol/ml (fasted conditions) to 90-120 fmol/ml after a meal or sham feeding in men (Feurle et al., 2003). It was also reported that the levels of plasma xenin-25 were significantly lower in obese children (198 pg/ml = 67 fmol/ml) compared to healthy children (371 pg/ml = 125 fmol/ml) (Mrózek et al., 2012). In contrast, van de Sande-Lee *et al.* reported that serum xenin-25 levels were significantly higher in adult obese patients (108 fmol/ml) compared to the control adult lean subjects (84 fmol/ml). Interestingly, circulating blood levels of xenin-25 were normalized after Roux-en-Y gastric bypass surgery in obese patients (van de Sande-Lee et al. 2013). Other studies found that circulating xenin-25 levels are below the detection limits of the assay in both mice (13.5 fmol/ml) and humans (2 fmol/ml) (Wice et al., 2010; Wice et al., 2012). Although these data suggest the possibility that circulating xenin-25 levels may be altered in response to changes in feeding condition and metabolic state, further investigation is necessary to fully understand the level and the fluctuation of circulating xenin-25.

4. 4. Role of xenin in gastrointestinal motility

Xenin belongs to neurotensin/xenopsin family and it is well established in the literature that both xenopsin and neurotensin play an important role in regulating gastrointestinal motility (Zhao and Pothoulakis, 2006). Thus, a number of studies have been focused on the role of xenin

in the regulation of gastrointestinal motility. Xenin induces canine jejunal contractions and induces a biphasic response in the jejunum of guinea pig (small relaxation followed by large contraction) (Feurle et al., 1997; Feurle et al., 1996; Kamiyama et al., 2007). Xenin induces relaxation in pre-contracted rat ileal muscle and guinea pig colonic muscle (Clemens et al., 1997; Feurle et al., 1996; Feurle et al., 2002). Xenin also stimulates gallbladder contraction in dogs (Kamiyama et al., 2007). Additionally, xenin delays gastric emptying in humans with and without type 2 diabetes. Neurotensin receptor 1 (NTSR1) was detected on enteric neurons residing within the longitudinal muscle in the human stomach (Chowdhury et al., 2014). Xenin-induced jejunal contraction and ileal and colonic relaxation are attenuated by SR48692, an antagonist of Ntsr1 (Feurle et al., 1996; Clemens et al., 1997). These findings support the role of xenin in the regulation of gastrointestinal motility at least partly via Ntsr1.

4. 5. Role of xenin in pancreatic function

Similar to neurotensin and xenopsin, xenin participates in the regulation of pancreatic function and glucose homeostasis. Xenin-25 stimulates exocrine pancreatic secretion and increases the plasma concentration of pancreatic polypeptide, vasoactive intestinal polypeptide and glucagon in dogs (Feurle et al., 1992; Feurle et al., 1997). The xenin-induced increase in exocrine pancreatic secretion is attenuated by SR48692, the Ntsr1 antagonist (Nustede et al., 1999). Xenin stimulates glucose-stimulated insulin secretion (GSIS) in perfused rat pancreas and insulin-producing BRIN-BD11 cells (Silvestre et al., 2003; Taylor et al., 2010). Degraded products of xenin-25 retain some biological activities of the full-length xenin. The longer C-terminal fragments xenin-13 (13-25) and xenin-8 (18-25) have been shown to stimulate exocrine pancreas secretion *in vivo,* while the shorter fragments xenin-5 (21-25) and xenin-4 (22-25) do

not have any effects (Nustede et al., 1999). Xenin-8 is able to reproduce xenin-25-induced augmentation of GSIS in perfused rat pancreas and BRIN-BD11 cells (Martin et al., 2014; Silvestre et al., 2003). Xenin treatment enhances GIP-induced potentiation of GSIS and reduced blood glucose levels during glucose tolerance test in mice (Wice et al., 2010; Gault et al., 2015). Xenin treatment also increases the effect of GIP on insulin release in humans with normal or impaired glucose tolerance, but not in individuals with type 2 diabetes (Wice et al., 2012). The effect of xenin on GIP-induced potentiation of GSIS is negated by an intraperitoneal (i.p.) injection of atropine methyl bromide, an antagonist of muscarinic acetylcholine receptors, which does not cross the blood-brain barrier. It is not affected by i.p. injection of hexamethonium, a nicotinic cholinergic antagonist, that blocks the activation of post-ganglionic neurons (Wice et al., 2010). Although NTSR1 is not detectable on islet endocrine cells, sympathetic neurons, blood vessels, or endothelial cells, it is expressed at high levels on axons contacting beta cells (Chowdhury et al., 2014). Collectively, these findings support the role for xenin in the regulation of glucose homeostasis. A neural relay, potentially involving NTSR1 and non-ganglionic cholinergic neurons, may mediate the stimulatory effect of xenin on GIP-induced potentiation of GSIS.

4. 6. Regulation of food intake by xenin

Xenin is released from the gastrointestinal tract after a meal, suggesting that xenin may function as a satiety signal (Feurle et al., 1992; Feurle et al., 2003). In 1998, the first paper to support this possibility was published. This study reported that a single intracerebroventricular (i.c.v.) injection of xenin at a dose of 15 μg caused a significant 43% reduction of food intake in fasted rats (Alexiou et al., 1998). Subsequently, it was also shown that i.p. injection of xenin

reduces food intake without causing a significant conditioned taste aversion in mice (Leckstrom et al., 2009). Intriguingly, i.p. injection of xenin caused a significant reduction in gastric emptying rate in mice and humans (Leckstrom et al., 2009).

It has been shown that both central and peripheral injection of xenin to chicks and mice significantly increased the expression of *c-fos* mRNA and the number of c-Fos immunoreactive cells in the hypothalamus (Cline et al., 2007). Furthermore, the i.p. xenin injection significantly increased the number of Fos-immunoreactive cells in the nucleus of solitary tract (NTS) of the brainstem (Kim and Mizuno, 2010b). All of these findings suggest that xenin inhibits feeding by activating cells in the hypothalamus and the brainstem and xenin-induced feeding suppression occurs in part through a delay in gastric emptying. Xenin belongs to the neurotensin family. Neurotensin-deficient mice do not have any obvious metabolic phenotype, whereas Ntsr1-deficient mice show a moderate increase in food intake and body weight, suggesting that Ntsr1 may play a role in regulating energy balance by mediating the effect of not only neurotensin but also other anorexigenic molecules (Remaury et al., 2002; Dobner et al., 2001). In agreement with this assumption, it was observed that xenin-induced anorexia was abolished in Ntsr1-deficient mice (Kim and Mizuno, 2010a).

Although a precise mechanism by which xenin promotes its anorectic effect is unknown, evidence suggests that xenin-induced feeding suppression is mediated by the appetite-regulating neuropeptide system in the hypothalamus. Xenin significantly stimulated the release of anorexigenic corticotropin-releasing hormone (CRH) from a hypothalamic explant cultured *ex vivo* and pre-treatment with the CHR antagonist negated the anorectic effect of xenin in rats (Cooke et al., 2009). A combination treatment with low doses of xenin and naloxone, an opioid antagonist, caused a synergistic effect on food intake (Schusdziarra et al., 2004). The i.p.

injection of xenin caused a significant increase in the hypothalamic interleukin 1 beta (*Il-1β*) mRNA levels and xenin-mediated anorectic effects were abolished in mice lacking the type 1 IL-1 receptor (IL-1R1) (Kim et al., 2014). It has been well established that CNS melanocortin signaling plays a critical role in the regulation of metabolism (Cone, 2005). Thus, it was assumed that the anorectic effect of xenin is also mediated via melanocortin signaling. Contrary to this assumption, it was shown that xenin regulates food intake independent of the melanocortin signaling pathway as its anorectic effects were not attenuated by pre-treatment with the melanocortin receptor antagonist SHU9119 and in *agouti* mice that have defects in melanocortin signaling (Leckstrom et al., 2009). Although i.p. injection of xenin significantly increased the number of pERK1/2-immunoreactive cells in the hypothalamus, i.c.v. pre-treatment with U0126 (selective inhibitor of ERK1/2 upstream kinase) failed to block the feeding-suppressing effect of xenin (Kim et al., 2016). These findings suggest that the feeding-suppressing effect of xenin is at least partly mediated via CNS signaling pathways involving neurotensin, IL-1, CRH and opioids, but is independent of signaling pathways involving melanocortins and ERK.

5. Lipid metabolism

Metabolic homeostasis is essential to sustain healthy life because its imbalance can lead to various debilitating diseases. Obesity is a metabolic complication characterized by positive energy balance which leads to the accumulation of fat in the body (Dombrowski et al., 2014). Under normal physiological conditions, excess fat is stored as triglycerides (triacylglycerol/TAG) in the adipose tissue to be used for fuel later during periods of increased energy demands. If an excessive amount of carbohydrate is consumed, it is converted to fat through lipogenesis and stored in tissues such as adipose tissue and liver. During fasting, stored

fats are released as free fatty acids (FFA) and glycerol from the adipose tissues into the circulation through lipolysis (Saponaro et al., 2015). Thus, the subtle change in the balance between lipogenesis and lipolysis is very important to maintain normal energy homeostasis and avoid metabolic impairments such as obesity.

5. 1. Lipogenesis

5. 1. 1. Sources of TAG synthesis

TAG synthesis is an important and strictly regulated process that occurs mainly in adipose tissue but also in liver, muscle, heart and pancreas. TAGs are synthesized by esterification of fatty acids with glycerol. Although, adipose tissue-derived plasma glycerol and FFA are the main source of TAG synthesis, they are also synthesized *de novo*. FFA can be derived from diet, by lipolysis or through *de novo* lipogenesis (DNL). Lipoprotein lipase (LPL) catalyzes the hydrolysis of chylomicron-triglyceride (from dietary fat) and very-low-density lipoprotein (VLDL)-triglyceride (from liver) to supply fatty acids to tissues including adipose tissue and skeletal muscle. LPL activity is increased in the fed state while reduced during fasting in adipose tissue (Lafontan and Langin, 2009). In adipose tissue, the main source of G3P is glucose via the glycolysis pathway. While in liver G3P production mainly relies on glycerol because liver expresses glycerol kinase which is responsible for conversion of glycerol to G3P. However, G3P can also be synthesized from non-carbohydrate sources such as pyruvate, lactate or amino acids through a process named as glyceroneogenesis in both adipose tissue and liver (Hanson and Reshef, 2003). Additionally, TAG can be synthesized from monoacylglycerol (MAG) through the formation of DAG in small intestine and adipocytes (Jamdar and Cao, 1992).

5. 1. 2. *De novo* lipogenesis (DNL)

DNL is the biochemical process of fatty acid formation from non-lipid precursors such as glucose. DNL occurs predominantly in liver but also in other organs such as adipose tissue (Hellerstein, 1999). Acetyl-CoA derived from glucose through glycolysis is converted to malonyl-CoA in the presence of acetyl-CoA carboxylase (ACC). Fatty acid synthase (FAS) catalyzes the conversion of malonyl-CoA to saturated fatty acids (e.g. palmitate) which are further converted to monounsaturated fatty acids (MUFA) by stearoyl-CoA desaturase 1 (SCD1) (Sanders and Griffin, 2016) (Figure 3). Genes encoding these enzymes are regulated at transcriptional levels by different transcriptional factors in response to changes in endocrine and metabolic states. Major transcription factors involved in *de novo* lipogenesis are sterol response element-binding protein 1c (SREBP1c) and carbohydrate-response element-binding protein (ChREBP) which regulate the expression of the key lipogenic genes ACC, FAS and SCD1 (Shimano et al., 1999; Dentin et al., 2004). Another important transcription factor which is involved in the lipogenesis is peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ acts not only as a master regulator of adipocyte differentiation but also plays an important role in the regulation of DNL by inducing expression of lipogenic genes such as FAS and SCD1 (Gavrilova et al., 2003; Yao-Borengasser et al., 2008). Thus, these transcription factors play an important role in regulating lipogenic processes in the body.

5. 1. 3. Hormonal regulation of lipogenesis

DNL is under complex hormonal control. Insulin is one of the major hormones that promotes triglyceride synthesis and it works by increasing the uptake of glucose by adipocytes (by stimulating translocation of glucose transporter 4, GLUT4, to the plasma membrane),

increasing adipose LPL activity, stimulating reesterification of fatty acids and stimulating the expression of lipogenic genes. Insulin promotes lipogenesis through the activation of transcription factor SREBP1c (Schwarz et al., 2003). It has been shown that SREBP1c activation by insulin is mediated via phosphoinositide-3 kinase (PI3K)-dependent activation of protein kinase B (PKB). Insulin also stimulates DNL through cAMP response element-binding protein (CREB)-mediated transcriptional activation of lipogenic genes (Klemm et al., 1998). Moreover, insulin regulates SREBP1c post translationally, specifically by promoting phosphorylation of SREBP1c and production of the transcriptionally active mature form of SREBP1c (Hegarty et al., 2005; Yellaturu et al., 2009). Glucose can stimulate lipogenesis by activating both ChREBP and SREBP1c (Musso et al., 2009) (Figure 3).

Growth hormone and catecholamines have an inhibitory influence on DNL, while thyroid hormone positively regulates DNL (Gathercole et al., 2013). Glucocorticoids can both stimulate and inhibit lipogenesis. It has been suggested that glucocorticoids produce complex effects on DNL due to the tissue-specific (i.e. adipose tissue vs. liver) effects of glucocorticoids and interactions with other hormonal and nutrient signals (Hillgartner et al., 1995). Glucagon inhibits DNL by suppressing the activity of SREBP1c and expression of its target lipogenic genes. It has been shown that glucose promotes nuclear translocation of ChREBP. Once inside the nucleus, ChREBP forms a dimer with Max-like protein X (Mlx), binds to carbohydrate response element of the promoter of target gene such as ACC to increase transcription. The activation of ChREBP is mediated via dephosphorylation of residues that are target sites of protein kinase (PKA) or AMP-activated protein kinase (AMPK) (Uyeda and Repa, 2006). Glucagon increases AMPK activity that leads to the phosphorylation of ChREBP followed by nuclear exclusion of ChREBP

and inactivation of target lipogenic genes. Thus, hormonal signals play a major role in the regulation of DNL through alterations in transcriptional regulation of lipogenic gene expression.

5. 1. 4. Abnormal lipogenesis and metabolic impairments

Impaired regulation of DNL is associated with metabolic abnormalities such as increased deposition of triglyceride in the liver of patients with non-alcoholic fatty liver disease (NAFLD) (Donnelly et al., 2005). NAFLD is characterized by hepatic steatosis which can develop into non-alcoholic steatohepatitis (NASH) followed by cirrhosis. Clinical data has shown the up-regulation of major DNL regulators such as SREBP1c, FAS and ACC1 in patients suffering from NAFLD (Higuchi et al., 2008). Moreover, phenotypes of animal models with altered SREBP1c expression support the contributory role of DNL in the development of NAFLD (Knebel et al., 2012; Moon et al., 2012). Excessive hepatic lipogenesis can result from alterations in insulin signaling. Various mouse models in which a component of the insulin signaling pathway has been ablated are hypolipidemic with reduced hepatic lipogenic gene expression (Wang et al., 2015). These findings support the contribution of the insulin pathway to lipogenesis and the development of hepatic steatosis. However, paradoxically hepatic lipid production is increased in insulin resistant states. Hepatic glucose production is increased under the same condition, suggesting that liver exhibits selective insulin resistance. Liver is resistance to the inhibitory effect of insulin on glucose production, but not lipogenesis. High glucose levels in insulin resistance may also activate ChREBP, leading to increased expression of lipogenic genes.
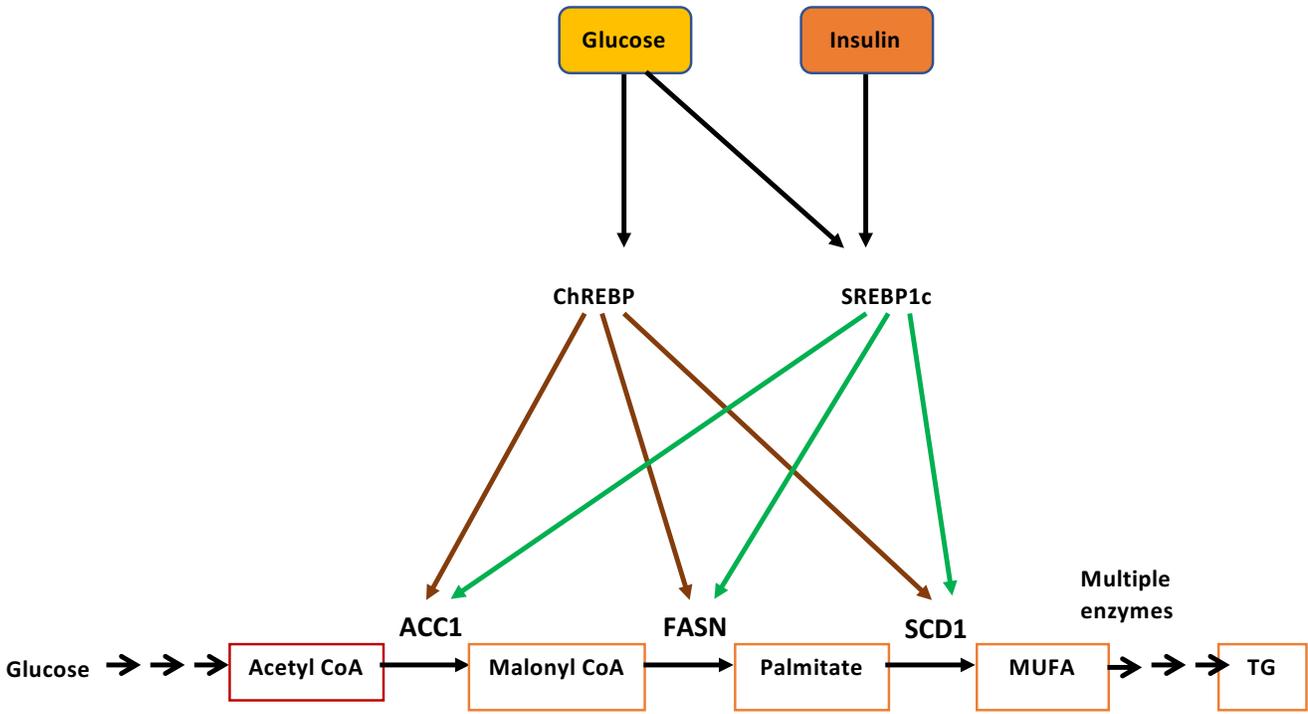
Figure 3. A schematic diagram showing the biochemical pathways involved in lipogenesis.

ChREBP: carbohydrate-responsive element-binding protein, SREBP1c: sterol regulatory element-binding protein 1, ACC1: acetyl-CoA carboxylase 1, FASN: fatty acid synthase, SCD1: stearoyl-CoA desaturase 1, MUFA: monounsaturated fatty acid, TG: triglycerides

5. 2. Lipolysis

5. 2. 1. Overview of lipolysis

Lipolysis is a strictly regulated mechanism that mobilizes endogenous energy stores (i.e. TAG) from the adipose tissue to provide other metabolically active tissues energy in the form of FFA and glycerol through the circulation during a period of increased energy demands such as fasting and exercise (Nielsen et al., 2014). Lipolysis is regulated by endocrine signals such as insulin and catecholamines. In mammals, lipolysis is catalyzed by three different lipases. The first and rate-limiting enzyme in the lipolytic pathway is adipose triglyceride lipase (ATGL) which catalyzes the conversion of TAG to DAG (Zimmermann et al., 2004). Next, hormone sensitive lipase (HSL) catalyzes the conversion of DAG to MAG and monoacylglycerol lipase (MGL) cleaves MAG into the end products; glycerol and FFA (Haemmerle et al., 2002; Fredrikson et al., 1986) (Figure 4).

5. 2. 2. Regulation of lipolysis by sympathetic nervous system

Adipose tissue is innervated by the sympathetic nervous system (SNS) which controls the lipolytic activity in response to the activation of different adrenergic receptors (ARs) present on the adipocyte membrane. There are two main classes of ARs, alpha ARs (α1AR and α2AR) and beta ARs (β1AR, β2AR and β3AR). The amount of lipolysis is controlled by the balance between α2AR tone and βAR tone (Robidoux et al., 2004). Activation of βAR promotes lipolysis in the adipose tissue, while activation of α2AR inhibits lipolysis. Activation of the SNS and βAR receptors initiates the lipolytic cascade. Beta adrenergic receptors are coupled to a Gs-protein which transmits an activation signal to adenylyl cyclase (AC), leading to increased cAMP production, followed by activation of protein kinase A (PKA) and its downstream targets

(Lafontan and Berlan, 1993) (Figure 4). Thus, SNS outflow from the CNS contributes to the regulation of lipolysis through alterations in the balance between α2AR tone and βAR tone.

5. 2. 3. Lipolytic enzymes and co-regulators

Lipolysis is regulated by complex mechanisms involving different lipases and lipid droplet associated proteins. HSL is a key regulator of lipolysis and is expressed in most tissues. Tissue specific isoforms of HSL with the size ranging between approximately 85 to 130 kDa are generated from a single gene (Langin et al., 1993; Lindvall et al., 2004). It has been shown that upon lipolytic stimulation, HSL moves from the cytosol to the surface of lipid droplets where it interacts with perilipin-1. Phosphorylation of perilipin-1 induces physical alterations of the lipid droplet surface which facilitate the action of phosphorylated HSL on triglyceride (Figure 4). Activation of HSL is dependent upon the phosphorylation status of specific serine residues. Studies have shown that Ser^{563}, Ser^{659} and Ser^{660} in rats and mice corresponding to the Ser^{552}, Ser^{649} and Ser^{650} in humans are the active sites for HSL phosphorylation (Strålfors et al., 1984; Garton et al., 1988; Contreras et al., 1998). In the inactive state, HSL resides in the cytoplasm of the adipocyte, but once it gets activated or phosphorylated it translocates to the lipid droplet surface and further activates the lipolytic cascade. It has been reported that phosphorylation of HSL at Ser^{563} site is crucial for its translocation to the lipid droplet surface, while phosphorylation at Ser^{660} and Ser^{659} are critically involved in enzymatic activation (Daval et al., 2005; Anthonsen et al., 1998). In addition to this, extracellular-signal regulated kinase (ERK) also enhances HSL activity by phosphorylating the Ser^{600} site. On the contrary, AMPK-mediated phosphorylation of the Ser^{565} site inhibits lipolysis by blocking translocation of HSL to the surface of lipid droplet.

The primary role of HSL in triglyceride hydrolysis has been challenged by findings that HSL-deficient mice have relatively normal adipose tissue mass and increased DAG (Osuga et al., 2000; Haemmerle et al., 2002). These findings suggested that HSL functions mainly a DAG hydrolase and led to a search for other lipases in the adipose tissue. Adipose triglyceride lipase (ATGL, also known as patatin-like phospholipase domain-containing protein 2) was identified as a lipase specifically against triglyceride. ATGL controls both basal and stimulated lipolysis in adipose tissue. It is expressed in almost all tissues with highest expression in WAT and BAT (Villena et al., 2004). Expression of ATGL protein is up-regulated by fasting in humans (Nielsen et al., 2011) while in mice *Atgl* mRNA is suppressed by feeding (Kershaw et al., 2006). These changes in ATGL/Atgl expression correlate with body's energy demands. There are 2 phosphorylation sites, Ser^{404} and Ser^{428} in human ATGL (corresponding to Ser^{406} and Ser^{430} in rodents) (Bartz et al., 2007). Fasting and exercise increases phosphorylation of ATGL at Ser^{406} which increase its TAG hydrolase activity (Pagnon et al., 2012; Ahmadian et al., 2011).

Comparative gene identification-58 (CGI-58), also known as alpha/beta hydrolase domain-containing protein 5 (ABHD5) interacts with patatin domain of ATGL, acts as a major regulator of the lipolytic action of ATGL. Under basal conditions (non-stimulated conditions), perilipin-1 coats the lipid droplet and binds to CGI-58. Thus, CGI-58 is unable to activate ATGL (Lass et al., 2006; Schweiger et al., 2008). Upon stimulation, elevation of intracellular cAMP levels and PKA activation promotes perilipin-1 phosphorylation followed by the dissociation of CGI-58 from perilipin-1. CGI-58 is dispersed in the cytosol and interacts with ATGL. Formation of the ATGL-CGI-58 complex activates the hydrolytic function of ATGL and promotes hydrolysis of TAG to DAG and FFA (Figure 4). Thus, CGI-58 has been shown to be a major co-regulator of ATGL under stimulated lipolytic conditions.

Lipid droplets are coated by members of the perilipin family. Perilipin-1 (PLIN1/perilipin A) is the predominant perilipin that is present in mature adipocytes and the major substrate for cAMP-dependent PKA (Nielsen et al., 2014). Phosphorylation of perilipin-1 at Ser^{517} (rodents) is crucial for the regulation of HSL- and ATGL-mediated lipolysis (Miyoshi et al., 2007). Moreover, it has been shown that perilipin promotes HSL-mediated lipolysis via both phosphorylation-dependent and −independent mechanisms (Miyoshi et al., 2006). Phosphorylation of perilipin-1 at Ser^{492} leads to lipid droplet remodeling which increases the surface area of the lipid droplet for lipase binding (Marcinkiewicz et al., 2006).

Monoacylglycerol lipase (MGL) is a hydrolase that is constitutively expressed in adipose tissue and has no affinity for TAG and DAG. It is responsible for the final step of lipolysis, that is, hydrolysis of MAG to fatty acid and glycerol (Figure 4).

5. 2. 4. Hormonal regulation of lipolysis

In adipocytes, lipolysis is tightly regulated by neuroendocrine signals during fasting or in times of elevated energy demand. Catecholamines and insulin are the two major neuroendocrine signals that control lipolysis. Lipolysis is stimulated upon the release of catecholamines to activate the βAR-cAMP-PKA pathway in adipocytes during fasting. In contrast, in the fed state, insulin activates phosphodiesterase 3B (PDE3B) that promotes degradation of cAMP in adipocytes, resulting in suppression of lipolysis.

As already described above, the effect of catecholamines on lipolysis is bi-directional depending on the receptors to be activated. Activation of ßAR stimulates lipolysis (Robidoux et al., 2004) via activation of AC which converts ATP to cAMP. Elevated levels of cAMP in the cytoplasm activate protein kinase A (PKA) (Langin, 2006) which further activates downstream

targets, such as perilipin-1 and HSL (Nielsen et al., 2014). In contrast, α2AR coupled to G-inhibitory (Gi) protein inhibits cAMP production, resulting in reduced lipolytic activity (Figure 4).

Additional endocrine signals that can promote lipolysis are growth hormone (GH) and glucocorticoids. Clinical studies have shown that GH can stimulate lipolysis which is supported by evidence that fasting-induced lipolysis was negated by blocking fasting-induced increase in plasma GH levels in humans (Hansen et al., 2002; Sakharova et al., 2008). Both GH and glucocorticoids increase responsiveness to beta-adrenergic signals by activating the cAMP-dependent pathway (Yang et al., 2004; Doris et al., 1994). GH and glucocorticoids activate adenylyl cyclase by shifting the inhibitory G protein G1α2 subunit and removing the inhibitory influence on cAMP production (Yip and Goodman, 1999). Evidence also suggests an additive effect of GH and glucocorticoids on lipolysis in humans, suggesting a shared mechanistic pathway by GH and glucocorticoids (Djurhuus et al., 2004).

Insulin functions as a major anti-lipolytic hormone that shifts lipid metabolism towards energy storage in adipose tissue. Insulin acts through the insulin receptor (IR) present on adipocyte membrane which has intrinsic tyrosine kinase activity. After feeding, insulin binds to the IR and causes its autophosphorylation followed by phosphorylation of insulin receptor substrate 1 and 2 (IRS1/2) (White, 1998). This further promotes the activation of phosphatidylinositol 3-kinase (PI3K) which catalyzes the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). Subsequently, PIP3 activates Akt or protein kinase B (PKB). Finally, PKB/Akt activates phosphodiesterase 3B (PDE3B), an enzyme which degrades cAMP to 5'-AMP and causes subsequent inactivation of PKA, thereby reducing phosphorylation of HSL and PLIN1 and thus inhibiting lipolysis (Stokoe

et al., 1997; Choi et al., 2006). A recent study demonstrated that a PI3K inhibitor blocked insulin-induced reduction of phosphorylation of perilipin, while an Akt inhibitor failed to block the effect of insulin on perilipin phosphorylation, suggesting that the anti-lipolytic action of insulin is mediated by an Akt-independent, PI3K-dependent pathway (Choi et al., 2010). These findings suggest that insulin reduces lipolysis through both Akt-dependent and –independent mechanisms (Figure 4).

5. 2. 5. Abnormal lipolysis and metabolic impairments

In obesity, there is an increased prevalence of dyslipidemia that is characterized by increased levels of FFA and TAG with reduced levels of high-density lipoprotein (HDL). The major contributing factor to dyslipidemia is uncontrolled release of fatty acid from the adipose tissue through lipolysis consequently contributing to increased delivery of fatty acid to the liver and increased synthesis and secretion of VLDL (Jung and Choi, 2014). Importantly, elevated plasma FFA levels play an important role in developing insulin resistance under obese conditions. Circulating FFA and glycerol levels are elevated in obesity. Obesity is characterized by a low-level inflammation with increased production of pro-inflammatory chemokines and cytokines such as monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-6 and IL-8 (Hotamisligil et al., 1993; Rotter et al., 2003). Moreover, obesity also shifts the polarization status of macrophages in adipose tissue from M2 (anti-inflammatory) to M1 (pro-inflammatory), which further stimulates inflammation in adipose tissue (Lumeng et al., 2007). Interestingly, inflammation induces lipolysis in adipose tissue. TNF-α stimulates basal lipolysis partly by increasing phosphorylation of perilipin. Thus, elevated TNF-α levels contribute to increased lipolysis and plasma FFA levels in obesity.

Several obesity-associated impairments in lipolysis have been documented. Obese individuals show altered responsiveness to catecholamines. Specifically, epinephrine-induced increases in plasma FFA and glycerol levels are reduced in obese individuals compared to non-obese individuals (Bougnères et al., 1997; Jocken and Blaak, 2008). This phenomenon is called catecholamine resistance in lipolysis. Obesity is associated with reduced HSL activity, which may contribute to catecholamine resistance (Large et al., 1999). Although insulin is the major hormone that inhibits lipolysis and obesity is often associated with insulin resistance, the effect of obesity on the anti-lipolytic action of insulin is less clear.

Changes in lipolytic activity in adipose tissue appear to be fat depot specific. For example, the beta adrenergic-stimulated lipolytic response of abdominal subcutaneous adipose tissue is blunted in obese individuals (Jocken et al., 2008). In contrast, catecholamine-induced lipolysis is increased in the visceral fat of obese individuals due to increased activation of β3AR and reduced activation of α2AR receptors. Thus, further studies are necessary to fully understand obesity-associated impairments in lipolysis and underlying mechanisms.
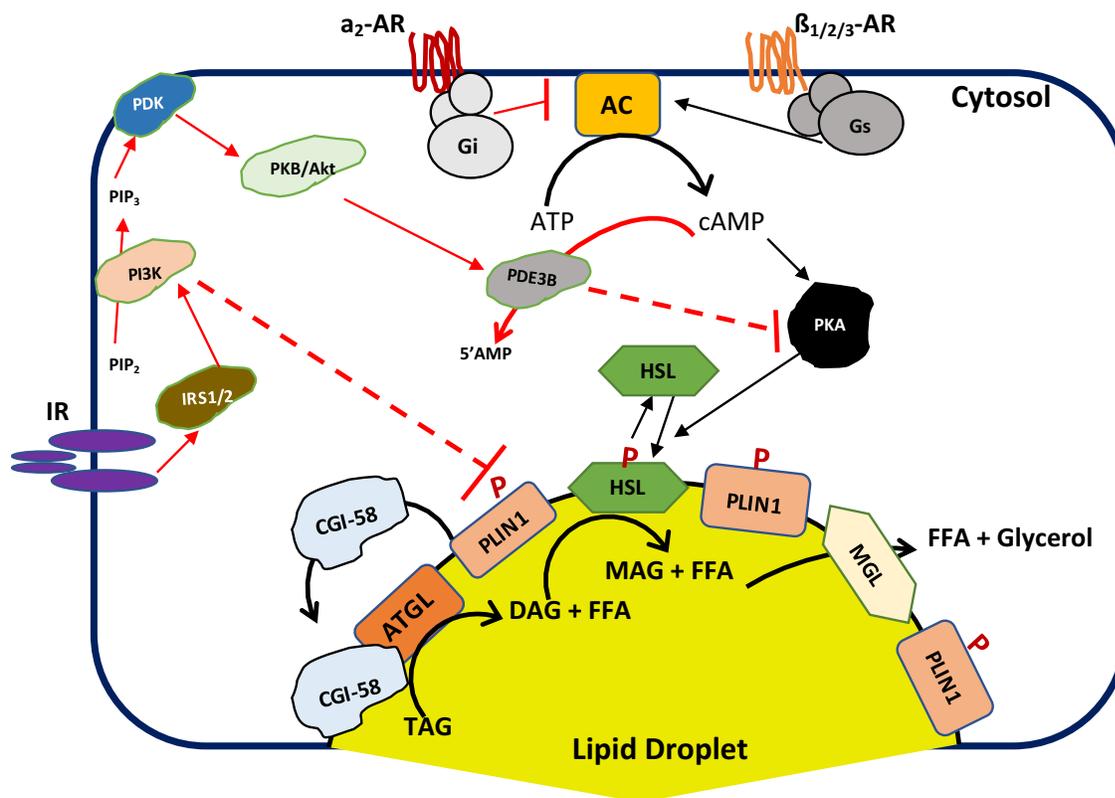
Figure 4. A Schematic diagram showing the biochemical pathways of lipolysis. AR: adrenergic receptor, AC: adenylate cyclase, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, HSL: hormone sensitive lipase, PLIN1: perilipin 1, MGL: monoglyceride lipase, TAG: triacylglycerol, DAG: diacylglycerol, MAG: monoacylglycerol, FFA: free fatty acid, ATGL: adipose triglyceride lipase, CGI-58: comparative gene identification-58, IR: insulin receptor, PIP2: phosphatidylinositol 4,5-bisphosphate, PIP3: phosphatidylinositol (3,4,5)-triphosphate, IRS1/2: insulin receptor substrate 1/2, PDK: pyruvate dehydrogenase kinase, PI3K: phosphoinositide 3-kinase, PKB: protein kinase B, PDE3B: phosphodiesterase 3B and P: phosphorylated

II. Rationale

Obesity is a major burden to the society as well as individual patients and has emerged as one of the leading health concerns over the past century. It is also associated with other chronic and metabolic complications such as insulin resistance, type 2 diabetes, cardiovascular disease, and cancer. The incidence of obesity is increasing at an alarming rate but currently there is a lack of effective pharmacological treatments for obesity. Although surgical treatment is successful in reducing obesity, it is only limited to individuals with severe obesity. Thus, a safe and effective pharmacological treatment that is readily accessible to all obese patients is urgently needed. Currently approved drugs for obesity treatment are either drugs involving the GI hormones that alter activity of metabolism-related CNS neural circuit or a drug that can alter lipid uptake by GI tract. Therefore, GI hormones are attractive candidate targets for the development of anti-obesity drugs.

GI hormones that are released after meals are known to have anorexigenic actions. Xenin is a peptide hormone produced by and secreted from a subset of intestinal K-cells after a meal (Feurle et al., 1992). Studies have shown that xenin has a feeding-supressing effect when injected intracerebroventricularly (i.c.v.) to rodents (Alexiou et al., 1998; Leckstrom et al., 2009; Cooke et al., 2009). Intraperitoneal (i.p.) injection of xenin activates cells in the hypothalamus and the brainstem (Leckstrom et al., 2009; Kim and Mizuno, 2010b; Kim et al. 2016). Moreover, xenin-induced feeding suppression has been shown to be associated with hypothalamic appetite-regulating neuropeptide systems (Schusdziarra et al., 2004; Nandar et al., 2008; Cooke et al., 2009; Kim and Mizuno, 2010a; Kim et al., 2014). These findings suggest that xenin reduces food intake at least partly through the activation of specific cells in the CNS involving the hypothalamus and the brainstem. However, the physiological role of xenin has been generally

thought to inhibit feeding, and other metabolic actions of xenin have received less attention. More specifically, it has not been tested whether or not enhanced central action of xenin affects adiposity and body weight.

Xenin shows structural similarity to neurotensin and the biological effects of xenin including its anorectic effects are mediated via the neurotensin receptor 1 (Ntsr1) (Kim and Mizuno, 2010a). Ntsr1-deficient mice are characterized by hyperphagia and mild obesity, implicating signaling through Ntsr1 in feeding and body weight regulation (Remaury et al., 2002; Opland et al., 2013). Thus, activation of Ntsr1 by xenin may influence not only food intake but also body weight. To support this possibility, previous studies in our laboratory have demonstrated that sub-chronic xenin treatment (once daily i.p. injections) causes reductions in food intake and body weight gain in normal lean mice (Kim, 2011). Interestingly, xenin treatment caused a decrease in respiratory quotient in mice (Kim, 2011). These findings suggest that the weight-reducing effect of xenin may involve both feeding-dependent and feeding-independent mechanisms. It is further suggested that xenin reduces body weight at least partly by increasing fat mobilization from adipose tissue to other tissues as a fuel. Although CNS-mediated action of xenin has been proposed, it is also possible that xenin regulates metabolism such as adipose tissue lipid metabolism by directly acting on peripheral tissues. However, this possibility has not been tested.

Overall, it is reasonable to address the hypothesis that enhanced action of xenin in the CNS and adipose tissue will lead to changes in lipid metabolism toward reducing the amount of stored fat and increasing the use of stored fat for fuel. It is expected that the outcome of the present study will provide useful information to evaluate the metabolic actions of xenin as a candidate target for the development of anti-obesity drugs.

III. Hypothesis

Enhanced action of xenin in the central nervous system (CNS) and adipose tissue will lead to changes in the levels of lipid metabolism-regulating genes and proteins in white adipose tissue and stimulate lipolysis thus leading to reduced adiposity and body weight.

IV. Objectives

1. To determine the effects of intracerebroventricular (i.c.v.) treatment of xenin on the levels of lipolytic and lipogenic marker genes and proteins in white adipose tissue (WAT), skeletal muscle and liver in obese mice.

2. To determine whether or not the neurotensin receptor 1 (Ntsr1) is expressed in mouse WAT.

3. To determine whether there is a direct effect of xenin on lipid metabolism in mouse WAT and adipocytes.

V. Materials and Methods

1. Experiment 1 (*in vivo* study)

Findings from experiment 1 have already been reported in large part in a published research article (Bhavya et al., 2017).

1.1. Animals

Male *ob/ob* mice (C57BL/6J background, 4-month-old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained under 12:12 light/dark cycle (lights on at 0600 h) with free access to standard rodent chow pellets (Prolab RMH 3000, 4.5% fat by weight, Ralston Purina) and water throughout the experiment. All studies were approved by the Institutional Animal Care and Use Committee (University of Manitoba). The animal study (i.c.v. injection study) was performed by my supervisor Dr. Mizuno before I started my graduate study.

1.2. Intracerebroventricular (I.c.v.) cannulation

Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg b.w.) and xylazine (10 mg/kg b.w.). The topical anesthetic bupivacaine (1 mg/kg b.w.) was applied to the skin before making an incision. A stainless steel guide cannula (outer diameter: 0.64 mm, inner diameter: 0.33 mm) was stereotaxically implanted into the lateral ventricle with the coordinates: 0.4 mm posterior to the bregma, 1.0 mm lateral from the midline and 1.8 mm deep to the dura in accordance with the atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). The cannula was fixed to the skull with dental cement and stainless steel screws. The

mice received a subcutaneous injection of buprenorphine (0.1 mg/kg b.w.) 3 times per day for 3 days post-surgery. Localization of the cannula was verified by assessing drinking behavior responses to the i.c.v. administration of angiotensin II (100 ng in 1 μl). It was also verified by injecting the dye through the cannula and visually inspecting the spread of the dye in the ventricular system at the end of the experiment. One mouse (out of 12) did not show the drinking response to angiotensin II and was removed from the study. Two mice (out of 11) did not have the dye spread into the ventricular system after the i.c.v. injection and were excluded from the study.

1.3. I.c.v. xenin treatment and tissue collection

Ob/ob mice fed *ad libitum* received two i.c.v. injections of xenin (5 μg/injection, American Peptide Co., Sunnyvale, CA, USA) at 1000 h and 2200 h to determine the effects of central action of xenin on metabolism. Control mice received two i.c.v. injections of artificial cerebrospinal fluid (aCSF). Xenin was reconstituted in aCSF consisting of 124 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$ and 10 mM D-glucose. Body weight and food weight were measured immediately prior to the first injection and 24 h after the first injection. Body weight change and food intake during 24 h period were calculated. Mice were euthanized by CO_2 narcosis 12-14 h after the second injection (between 1000 h and 1200 h). Immediately after euthanasia, blood was collected and blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN). Serum was separated and stored at -80 ºC for insulin, glycerol and FFA assays. White adipose tissue from gonadal fat pad, liver and, skeletal muscles were collected and stored at -80 ºC for RNA and protein analyses.

2. Experiment 2 (RT-PCR study)

To confirm the expression of neurotensin receptor 1 (*Ntsr1*) mRNA in mouse white adipose tissue, I performed reverse transcriptase polymerase chain reaction (RT-PCR) followed by agarose gel electrophoresis. Epididymal white adipose tissue from male C57BL/6 mice was homogenized in TRIzol reagent (Cat#: 15596026, Ambion by Life Technologies, Carlsbad, CA, USA) and total RNA was extracted in accordance with the manufacturer's instructions. Total RNA (1 µg) was converted to cDNA using iScript^{TM} Reverse Transcriptase SuperMix (Cat#: 1708841, Bio-Rad Laboratories, Hercules, CA, USA). Total RNA from mouse hypothalamus and liver was used as a positive control. Samples prepared without reverse transcriptase or total RNA was used as negative controls. PCR was run in a 50-µl reaction (Table 1) using specific primers for mouse Ntsr1 sequence (Accession No: NM_018766), forward: 5'-CCGCTGTATACCTGGCACTTTTG-3' and reverse: 5'-ATGGCCAGCAGCAGGATGAGCAG-3'. PCR amplification was performed for 40 cycles at 94 °C for 30 seconds (denaturing), 60 °C for 30 seconds (annealing) and 72 °C for 2 minutes (extension). PCR products were resolved by agarose gel (3%) electrophoresis in 1× TAE buffer at 100 volts for 20 minutes. The gel was stained with ethidium bromide and gel images were captured using a gel documentation system (MultiImage Light Cabinet Filter, Alpha Innotech).

Table 1. RT-PCR components

Reagent	Volume	Final concentration	Catalogue #
10X PCR buffer w/o MgCl$_2$	5 μl	1×	Y02028[a]
50 mM MgCl$_2$	1.5 μl	1.5 mM	Y02016[a]
dNTP mix (2.5 mM each)	4 μl	0.2 mM	RP001A[b]
Primer mix (5 μM)	5 μl	0.2 μM	N/A
cDNA	5 μl	N/A	N/A
Platimun Taq DNA polymerase (5 U/μl)	0.2 μl	0.04 U/μl	10966-034[a]
DEPC-water	32.3 μl	N/A	N/A
Total	50 μl	N/A	N/A

a: Invitrogen by Life Technologies, Carlsbad, CA, USA

b: Takara Bio Inc, Shiga, Japan

3. Experiment 3 (*ex vivo* study)

3.1. Animals

Male C57BL/6 mice (13-14 weeks of age) were obtained from Charles River Laboratories (Montreal, QC). Animals were maintained under 12:12 light/dark cycle (lights on at 0600 h). The mice were fed *ad libitum* with a regular rodent chow (Prolab RMH 3000, 4.5% fat by weight, Ralston Purina) and had free access to drinking water throughout the experiment. All animal studies were approved by the Institutional Animal Care and Use Committee (University of Manitoba).

3.2. *Ex vivo* adipose tissue culture

Mice were euthanized by isoflurane under *ad libitum* fed conditions between 0930 h and 1030 h. Gonadal fat pads were dissected out for culturing. Tissue explants were immediately rinsed with Krebs Ringer buffer media (KRBH) containing 135 mM NaCl, 2.2 mM $CaCl_2.2H_2O$, 1.25 mM $MgSO_4.7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4, 10 mM HEPES, 2.8 mM glucose, 100 U/ml penicillin and 0.01% streptomycin under sterile conditions. Each tissue explant was cut into small pieces (30-60 mg) and was incubated in 500 μl of KRBH containing 2% bovine serum albumin (BSA) in an incubator with 5% CO_2 and 95% air at 37 °C for 1 hour.

3.3. Treatment with xenin

After a 1-h pre-incubation, tissues were incubated with xenin at concentrations of 0.01, 1, 100, 1000 and 2000 μM for 2, 4 and 6 h. Media were collected at the three time points, and immediately frozen and stored at -80 ºC until glycerol assay. Tissues were weighed and harvested at the end of the experiment (i.e. at 6-h time point) and immediately frozen and stored at -80 ºC for RNA analysis.

In a separate study, tissues were prepared by the same protocol and treated with xenin (100, 1000 and 2000 μM) for 4 h. Culture media and tissues were collected at the end of the 4-h incubation period as above for glycerol and FFA assays and protein analysis, respectively.

Xenin concentrations for the treatment (10 nM – 2 mM) were selected based on a previous study. Our laboratory has previously demonstrated that i.p. injection of xenin at 15-50 mg/kg b.w. caused a significant reduction of food intake in normal lean mice (body weight: 25-30 g) (Leckstrom et al., 2009). The total blood volume of these mice is estimated to be 1.46-1.76 ml (58.5 [ml/kg b.w.] x 25-30 [g]). The total mass of xenin injected in this study was 0.38-1.50

mg. Consequently, it is estimated that the blood concentration of xenin after the injection was 0.26-0.85 mg/ml (88-286 μM). Thus, the 100 μM concentration used in the present *ex vivo* study is within the range of circulating xenin levels after its administration at a dose that can reduce food intake *in vivo*.

4. Experiment 4 (*in vitro* study)

3T3-L1 adipocytes were obtained from Dr. Suresh Mishra's laboratory (Passage #4). Cells were maintained and grown in regular Dulbecco's Modified Eagle Medium (DMEM, Cat#: 12100-038, Gibco by Life Technologies) supplemented with 25 mM glucose, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.01% streptomycin in an incubator with 5% CO_2 and 95% air at 37 °C. Cells (passage #9) were seeded into 12-well plate and left to grow until they reached full confluency. Preadipocytes were cultured in pre-adipocyte differentiation media (Adipocyte Nutrition Medium, Cat#: C-27438, PromoCell GmbH, Heidelberg, Germany) supplemented with 400 ng/ml dexamethasone, 44 μg/ml isobutylmethylxanthine and 0.5 μg/ml insulin (Preadipocyte Differentiation Medium SupplementMix, Cat#: C-39436, PromoCell GmbH) for 72 h to initiate differentiation. On day 3, pre-adipocyte differentiation media was replaced with culture media (Adipocyte Nutrition Medium supplemented with Adipocyte Nutrition Medium SupplementalMix, Cat#: C-39438, PromoCell GmbH) containing 3% fetal calf serum, 400 ng/ml dexamethasone and 0.5 μg/ml insulin to complete the differentiation process (12-14 days). Media were refreshed every other day. On day 12, cells were serum starved in serum-free DMEM containing 0.5% BSA and 25 mM glucose for 1 h followed by treatment with xenin at concentrations of 1 mM and 2 mM for 3 h. At the end of the experiment,

the media were collected and, immediately frozen and stored at -80 °C until assayed for glycerol and FFA.

5. Measurement of blood glucose and serum insulin levels

Blood samples were collected under ad libitum feeding condition. Blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN, USA). Serum concentrations of insulin were measured by ELISA with commercially available kits from EMD Millipore (Cat#: EZRMI-13K, St. Charles, MO, USA).

6. Glycerol assay

Culture media from *ex vivo* and *in vitro* experiments and serum samples from the *in vivo* experiment were used for the glycerol assay. Concentrations of glycerol were measured by colorimetric assay using commercially available kits (Cat#: SGA-1, Zenbio, Research, Triangle Park, NC, USA and Cat#: 10010755, Cayman Chemical Co., Ann Arbor, MI, USA). The principle of the glycerol assay is as follows. Glycerol is assayed by a coupled enzymatic reaction system. Glycerol present in the sample is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5'-diphosphate (ADP). The G-1-P gets oxidized and gives rise to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Subsequently, the peroxidase-catalyzed reaction of 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine (ESPA) with H_2O_2, produces a quinoneimine dye which absorbs light at 540 nm. The optical density at 540 nm is directly proportional to the glycerol concentration of the sample. Thus, the optical density of each sample was measured at 540 nm using a microplate reader (Model 680, Bio-Rad Laboratories,). The assay was run in duplicate

and levels were expressed as nmol glycerol per mg wet tissue for *ex vivo* studies and nmol glycerol per liter for *in vitro* studies. For the *in vivo* study, serum glycerol levels were expressed as mmol per liter.

7. FFA assay

FFA levels in culture media and serum were measured by colorimetric assay using a commercially available kit (Cat#: SFA-5, Zen-Bio). The principle of the FFA assay is as follows. In the initial step, acyl-CoA synthetase (ACS) catalyzes the formation of fatty acyl-CoA thiol esters from the non-esterified fatty acids (NEFA) in the sample. In the next step, the acyl-CoA derivatives are oxidized by acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. The resulting hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine; the reaction forms a purple product that absorbs light at 540 nm. The optical density at 540 nm is directly proportional to the NEFA concentration in the sample. The optical density of each sample was measured at 540 nm using a microplate reader (Model 680, Bio-Rad Laboratories). The assay was run in duplicates and levels were expressed as nmol FFA per mg wet tissue for the *ex vivo* studies and nmol FFA per liter for the *in vitro* studies and mmol per liter for the *in vivo* study.

8. RNA analysis

Tissues from *in vivo* and *ex vivo* studies were homogenized in TRIzol reagent (Cat#: 15596026, Ambion by Life Technologies) and total RNA was extracted in accordance with the manufacturer's instructions. Total RNA (1 μg) was converted to cDNA using iScript^{TM} Reverse

Transcriptase SuperMix (Cat#: 1708841, Bio-Rad Laboratories) and the mRNA levels were measured by real-time PCR using the ABI 7500 Fast thermal cycler (Applied Biosystem, Foster City, CA, USA) and specific primers (Table 2). The PCR reaction was run in a volume of 20 µl containing PowerUp^{TM} SYBR Green Master Mix (Cat#: A25743, Applied Biosystems), primer mix (final concentration: 300 nmol/l) and 5 µl of diluted cDNA (1:30 dilution). The reactions were incubated at 50 °C for 2 minutes for uracil-DNA glycosylase (UDG) activation and at 95 °C for 2 minutes for DNA polymerase activation, followed by 40 cycles of denaturation at 95 °C for 3 seconds and annealing/extension at 60 °C for 30 seconds. Data were analyzed by the ΔΔCt method using 7500 Fast software v2.3 (Applied Biosystems). The levels of mRNA were normalized against *cyclophilin* or *β-actin* mRNA levels. All the reactions were performed in triplicate. A range of acceptable variation was a coefficient of variation (CV) less than 5% for each triplicate. The specificity of the amplification was confirmed by performing melt curve analysis.

Table 2. List of the primers used in real-time PCR analysis

Gene	Accession No.	Direction*	Sequences (5'-3')	Exon
Lipe	U08188	F	5'-ATGAAGGACTCACCGCTGACTT-3'	2
		R	5'-CGGATGGCAGGTGTGAACT-3'	3
Atgl	BC064747	F	5'-GAGATGTGCAAACAGGGCTACA-3'	5
		R	5'-AGCAAAGGGTTGGGTTGGTT-3'	6
Adrb3	X72862	F	5'-CCGTGAAGATCCAGCAAGGA 3'	2
		R	5'-GGTTCTGGAGCGTTGGAGAGT-3'	3

Adra2	MN_007420	F	5' -CCAGACCACGACGTCACTCA -3'	1
		R	5' -GCCAGGACGATAACCGACAT -3'	1
Acc1	NM_133360	F	5'-TCTCTGGCTTACAGGATGGTTTG-3'	3
		R	5'-GAGTCTATTTTCTTTCTGTCTCGACCTT-3'	4
Fasn	NM_007988	F	5'-GCTGCGGAAACTTCAGGAAAT-3'	38
		R	5'-AGAGACGTGTCACTCCTGGACTT-3'	39
Scd1	NM_009127	F	5'-GTCAAAGAGAAGGGCGGAAAAC-3'	4
		R	5'-AAGGTGTGGTGGTAGTTGTGGAAG-3'	6
Srebp1c	NW_001030469	F	5'-GGAGCCATGGATTGCACATT-3'	1
		R	5'-GGCCCGGGAAGTCACTGT-3'	2
Pparg2	MN_011146	F	5'-TCTGGGAGATTCTCCTGTTGA-3'	B1
		R	5'-GGTGGGCCAGAATGGCATCT-3'	1
Ucp1	NM_009463	F	5'-GCTGAGTCCTTTTGTTCTTGCA-3'	1
		R	5'-GATCTGAAGGCGGACTTTGG-3'	2
Ppargc1a	NM_008904	F	5'-CTGGGTGGATTGAAGTGGTGTA-3'	2
		R	5'-GAGGGCAATCCGTCTTCATC-3'	3
Prdm16	NM_027504.3	F	5'-TCTCCGAGATCCGAAACTTCA-3'	14
		R	5'-CGGCTAAGCTGTCATCATCCT-3'	15
Lpl	NM_008509	F	5'-CCAATGGAGGCACTTTCCA-3'	5
		R	5'-CACGTCTCCGAGTCCTCTCTCT-3'	6
Ucp3	NM_009464	F	5'-CCATAGGCAGCAAAGGAACCAG-3'	2
		R	5'-GTGCCGGCCCCCAGGAACT-3'	2
Fgf21	NM_020013	F	5'-GTACCTCTACACAGATGACGACCAA-3'	1

		R	5'-GGAAGAGTCAGGACGCATAGCT-3'	3
B-actin	X03672	F	5'-CAGCTTCTTTGCAGCTCCTT-3'	1
		R	5'-TCACCCACATAGGAGTCCTT-3'	3
Cyclophilin	X52803	F	5'-AAGCATACAGGTCCTGGCATCT-3'	4
		R	5'-TGCCATCCAGCCATTCAGT-3'	4/5

*: Forward primer (F) and reverse primer (R).

9. Protein analysis

White adipose tissue from *in vivo* (100 mg) and *ex vivo* (30-60 mg) studies were homogenized in 50 µl HNTG lysis buffer containing HEPES (50 mM), NaCl (150 mM), glycerol (10%) and Triton X-100 (1%) and supplemented with EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The total protein concentration was measured by the Bradford protein assay method. Proteins (20 µg) were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat milk in 1× Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies diluted in blocking buffer at an appropriate concentration (Table 3) followed by 3 times washing (10 minutes each) in TBST. The membranes were then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilution for 1 hour at room temperature (Table 4). The protein bands were visualized using the enhanced chemiluminescence system (Advansta Inc, Menlo Park, CA, USA). The intensity of the bands on the autoradiography film was quantified using Quantity One software (Bio-Rad Laboratories). Signal intensity of phosphorylated hormone

sensitive lipase (Ser[660]-pHSL and Ser[563]-pHSL) was normalized to total HSL (tHSL) signal intensity. The signal intensity of other proteins was normalized to α-tubulin. The protein levels were expressed as a percentage of the control group.

Table 3. List of antibodies used in Western blotting

Antibody	Source	Catalogue number	Dilution
tHSL	Rabbit	4107[a]	1:1000
Ser[660]- pHSL	Rabbit	4126[a]	1:1000
Ser[563]- pHSL	Rabbit	4139[a]	1:1000
FASN	Mouse	sc-20140[b]	1:500
ATGL	Rabbit	2138[a]	1:500
Perilipin	Rabbit	3470[a]	1:200
CGI-58	Mouse	H00051099-M01[c]	1:400
SCD1	Goat	sc-14719[b]	1:200
α-tubulin	Mouse	sc-8035[b]	1:1000

a: Cell Signaling Technology, Danvers, MA, USA

b: Santa Cruz Biotechnology, Dallas, TX, USA

c: Abnova, Taipei, Taiwan

Table 4. List of secondary antibodies used in Western blotting

Antibody	Catalogue No	Company
Horse anti-mouse IgG	70765	Cell Signaling Technology, Danvers, MA, USA
Goat anti-rabbit IgG	111-035-003	Jackson ImmunoResearch Laboratories, West Grove, PA, USA
Goat anti-mouse IgG	115-035-174	Jackson ImmunoResearch Laboratories

10. Statistical analysis

Data are presented as means ± standard error of the mean (S.E.M.). Comparisons between 2 groups were performed by Student's *t*-test (for parametric data) or Wilcoxon test (for nonparametric data). For experiments with multiple groups, comparison between groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. In all cases, differences were taken to be significant if *p*-values were below 0.05.

VI. Results

1. Experiment 1

1. 1. Effects of short-term i.c.v. xenin treatment on food intake and body weight in *ob/ob* mice

To determine if the central actions of xenin reduce food intake and body weight in obese animals, the effect of i.c.v. administration of xenin on food intake and body weight was examined in obese *ob/ob* mice. A short-term i.c.v. xenin treatment significantly reduced 24-h food intake by 33.0% compared to the control aCSF treatment ($p < 0.05$ by Student's *t*-test, Fig 5A). Body weights before xenin treatment were indistinguishable between the groups (control: 61.1 ± 1.4 g, xenin: 58.5 ± 0.9 g. $p = 0.13$ by Student's *t*-test). At the end of the 24-h treatment period, xenin-treated mice had significantly lower body weight compared to controls (control: 61.4 ± 1.2 g, xenin: 57.6 ± 0.7 g, $p < 0.05$ by Student's *t*-test). Control aCSF treatment caused an increase in body weight over the 24-h period, while xenin treatment caused a reduction of body weight ($p < 0.05$ by Student's *t*-test, Fig 5B). Xenin treatment did not change the epididymal fat pad weight when compared to controls ($p = 0.99$ by Student's *t*-test, Fig. 5C).

A

B

C
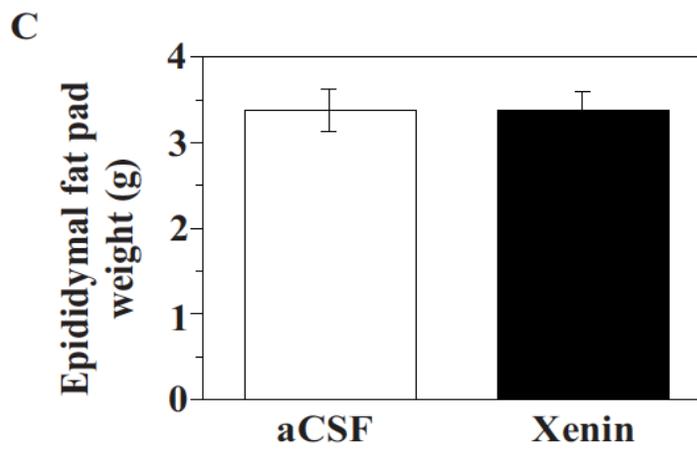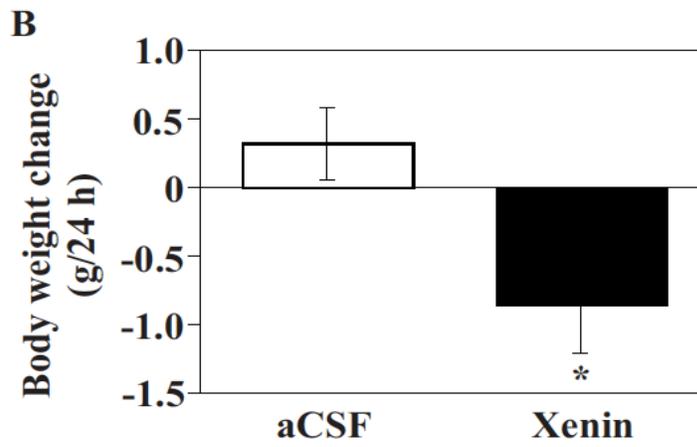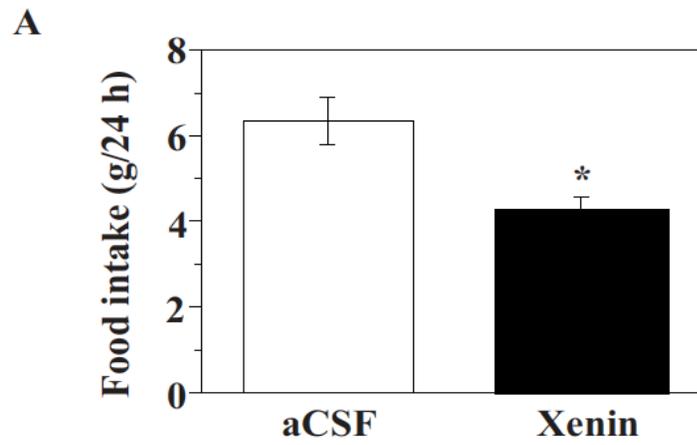
Figure 5. Effects of short-term i.c.v. xenin treatment on food intake and body weight change in obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 μg) or aCSF 12 h apart. Twenty-four-hour food intake (**A**), body weight change over 24 h (**B**) and epididymal fat pad weight **(C)** were measured. Data are means ± S.E.M (n = 4-5/group). *: $p < 0.05$ vs. aCSF by Student's *t*-test.

1. 2. Effects of short-term i.c.v. xenin treatment on blood glucose, serum insulin, FFA and glycerol levels in *ob/ob* mice

Short-term i.c.v. xenin treatment did not cause significant changes in blood glucose (p = 0.11 by Student's t-test, Fig. 6A), serum insulin (p = 0.66 by Student's t-test, Fig. 6B), serum FFA (p = 0.38 by Student's t-test, Fig. 6C) and serum glycerol (p = 0.62 by Student's t-test, Fig. 6D) levels when compared to control aCSF treatment.
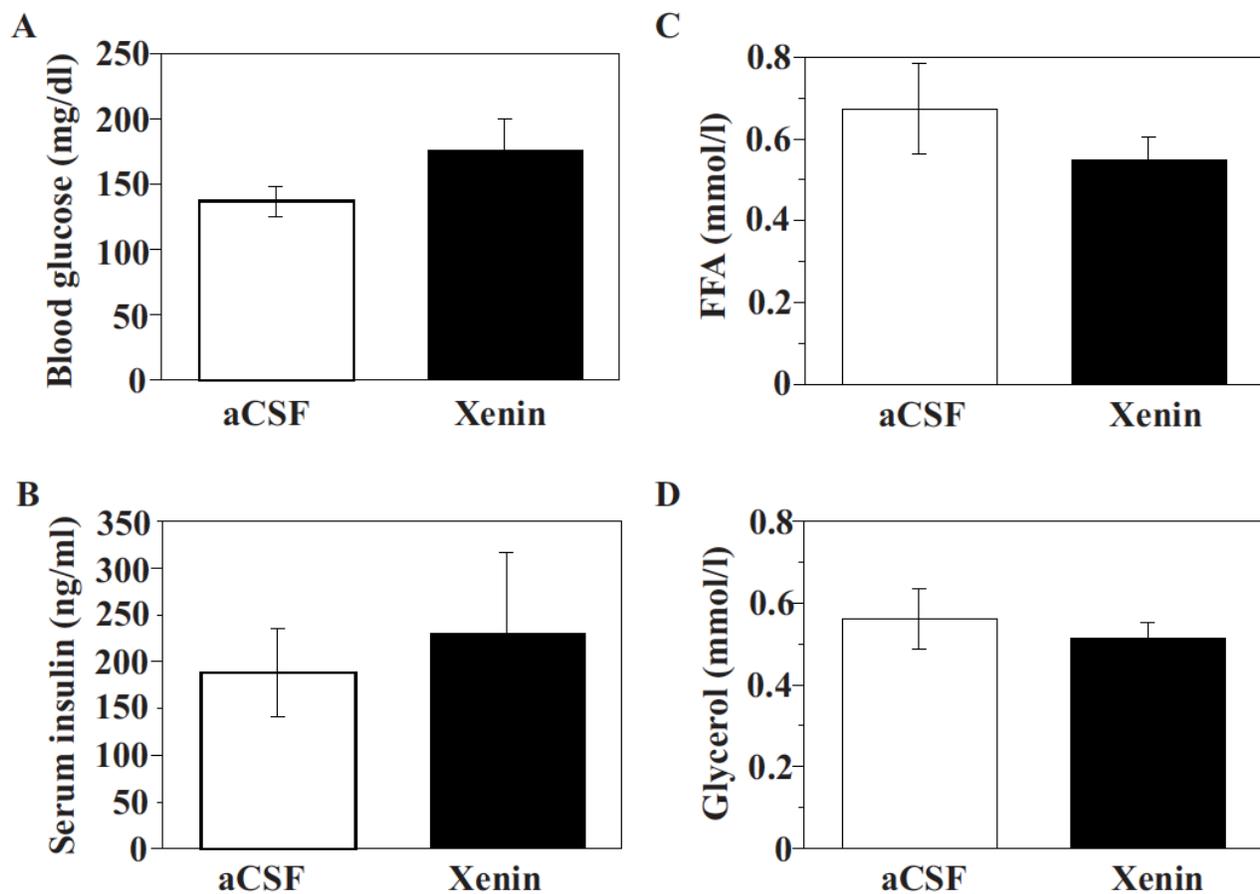
Figure 6. Effects of short-term i.c.v. xenin treatment on blood glucose, serum insulin, FFA and glycerol levels in obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 μg) or aCSF 12 h apart. Fed blood glucose (**A**), serum insulin (**B**), FFA (**C**) and glycerol (**D**) levels were measured. Data are means ± S.E.M (n = 4-5/group). *: $p < 0.05$ vs. aCSF by Student's t-test.

1. 3. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in white adipose tissue (WAT) of *ob/ob* mice

Xenin treatment significantly increased adipose triglyceride lipase (*Atgl*) mRNA levels in the WAT of obese *ob/ob* mice by 52.1% compared to control aCSF treatment ($p < 0.05$ by Student's *t*-test). There was no significant change in hormone sensitive lipase (*Lipe*) mRNA levels in response to xenin treatment ($p = 0.22$ by Student's *t*-test, Fig. 7A). Xenin treatment significantly increased beta-3 adrenergic receptor (*Adrb3*) mRNA levels by 76.8% ($p < 0.05$ by Student's *t*-test, Fig. 7A) in WAT without a significant change in alpha-2 adrenergic receptor (*Adra2*) mRNA levels compared to control aCSF treatment ($p = 0.13$ by Student's *t*-test, Fig. 7A). Levels of acetyl-CoA carboxylase 1 (*Acc1*, $p = 0.31$ by Wilcoxon test), fatty acid synthase (*Fasn,* $p = 0.73$ by Student's *t*-test) and stearoyl-CoA desaturase 1 (*Scd1*, $p = 0.47$ by Student's *t*-test) mRNA were indistinguishable between control and xenin-treated mice (Fig. 7B). Xenin treatment did not cause any significant changes in uncoupling protein 1 (*Ucp1*, $p = 0.67$ by Student's *t*-test), peroxisome proliferative-activated receptor gamma coactivator 1-alpha (*Ppargc1a*, $p = 0.66$ by Student's *t*-test) and PR domain zinc finger protein 16 (*Prdm16*, $p = 0.60$ by Student's *t*-test) mRNA levels in WAT (Fig. 7C).
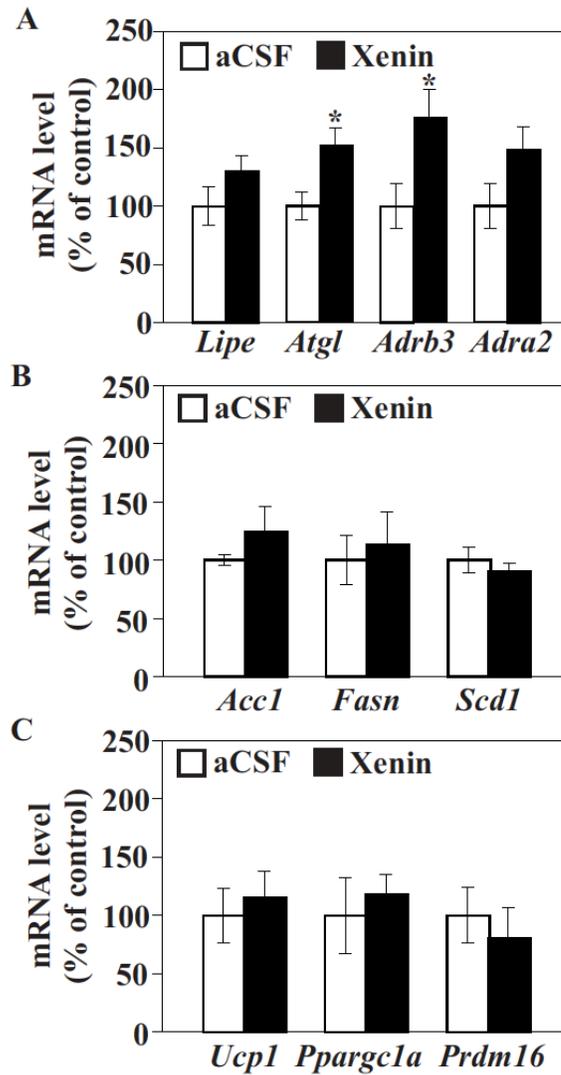
Figure 7. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in white adipose tissue of obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 µg) or aCSF 12 h apart and were euthanized 12 h after the second injection. Levels of lipolysis- (**A**), lipogenesis- (**B**) and browning/beiging-related (**C**) mRNA were measured by real-time PCR. Levels of mRNA were normalized to *cyclophilin* mRNA levels. Values in aCSF-treated mice were set to 100%. Data are means ± S.E.M (n = 4-5/group). *: $p < 0.05$ vs. aCSF by parametric Student's *t*-test or non-parametric Wilcoxon test. *Lipe*: Hormone sensitive lipase, *Atgl*: Adipose triglyceride lipase, *Adrb3*: Beta-3 adrenergic receptor, *Adra2*: Alpha-2 adrenergic receptor, *Acc1*: Acetyl-CoA carboxylase 1, *Fasn*: Fatty acid synthase, *Scd1*: Stearoyl-CoA desaturase 1, *Ucp1*: uncoupling protein 1, *Ppargc1a*: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Prdm16*: PR domain zinc finger protein 16.

1. 4. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in skeletal muscle of *ob/ob* mice

Xenin treatment significantly increased lipoprotein lipase (*Lpl*) mRNA levels by 155.6% in skeletal muscle compared to aCSF treatment ($p < 0.05$ by Wilcoxon test, Fig. 8). Levels of *Ucp3* mRNA were indistinguishable between control and xenin-treated groups ($p = 0.33$ by Student's *t*-test, Fig. 8).
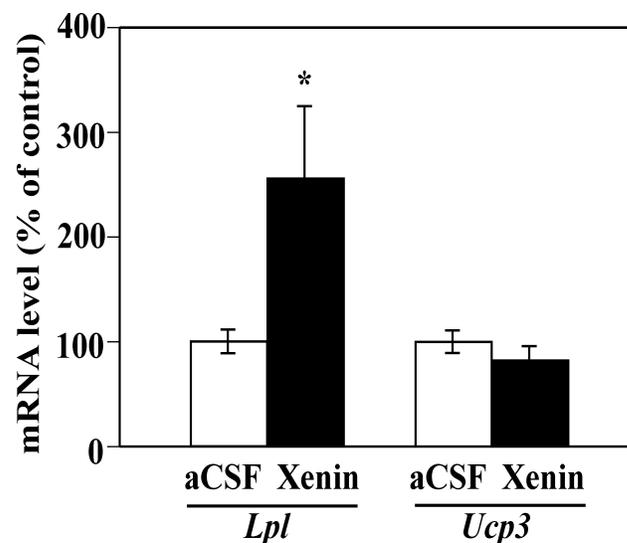

Figure 8. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in skeletal muscle of obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 μg) or aCSF 12 h apart and were euthanized 12 h after the second injection. Levels of *Lpl* and *Ucp3* mRNA were measured by real-time PCR. Levels of mRNA were normalized to *cyclophilin* mRNA levels. Values in aCSF-treated mice were set to 100%. Data are means ± S.E.M (n = 4-5/group). *: $p < 0.05$ vs. aCSF by parametric Student's *t*-test or non-parametric Wilcoxon test. *Lpl*: Lipoprotein lipase, *Ucp3*: Uncoupling protein 3.

1. 5. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in liver of *ob/ob* mice

There are no significant changes in the expression levels of *Lipe* ($p = 0.25$ by Student's t-test) and *Atgl* mRNA ($p = 0.29$ by Student's t-test, Fig. 9A). Xenin treatment significantly reduced *Fasn* and *Scd1* mRNA levels in liver compared to aCSF treatment ($p < 0.05$ by Student's t test, Fig. 9B). Xenin treatment did not cause significant changes in sterol regulatory element-binding protein 1c (*Srebp1c*, $p = 0.15$ by Student's t-test) and peroxisome proliferator-activated receptor gamma 2 (*Pparg2*, $p = 0.14$ by Student's t-test) mRNA levels (Fig. 9B). No significant change was found in the fibroblast growth factor 21 (*Fgf21*) mRNA levels in the liver after xenin treatment when compared to control aCSF treatment ($p = 0.85$ by Student's t-test, Fig. 9C).
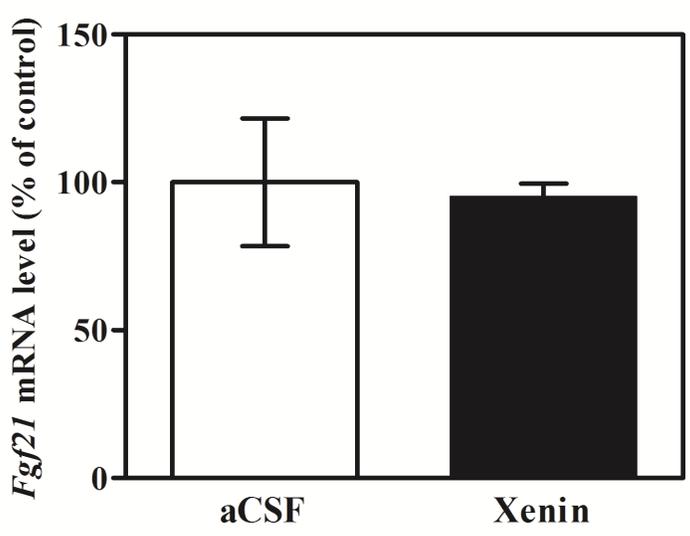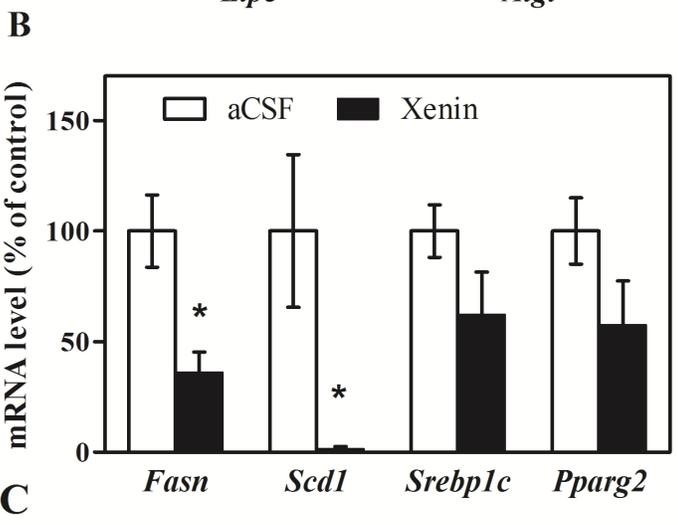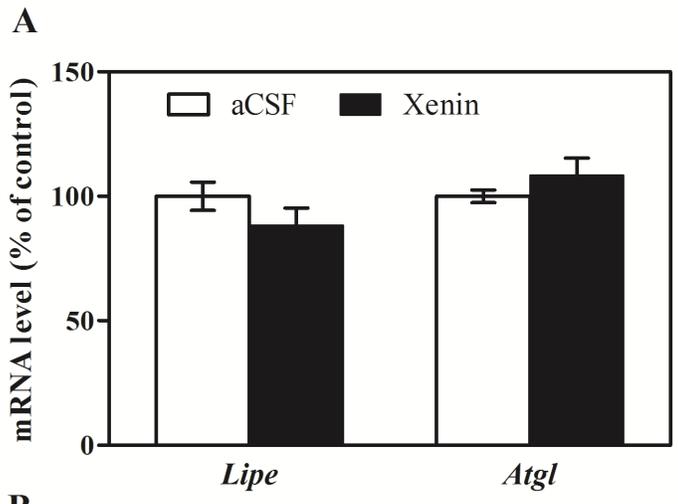
Figure 9. Effects of short-term i.c.v. xenin treatment on the expression of lipid metabolism-related genes in liver of obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 µg) or aCSF 12 h apart and were euthanized 12 h after the second injection. Levels of *Lipe* and *Atgl* **(A),** *Fasn, Scd1, Srebp1c* and *Pparg2* **(B)** and *Fgf21* **(C)** mRNA were measured by real-time PCR. Levels of mRNA were normalized to *cyclophilin* mRNA levels. Values in aCSF-treated mice were set to 100%. Data are means ± S.E.M (n = 4-5/group). *: $p < 0.05$ vs. aCSF by Student's *t*-test. *Lipe*: Hormone sensitive lipase, *Atgl*: Adipose triglyceride lipase, *Fasn*: Fatty acid synthase, *Scd1*: Stearoyl-CoA desaturase 1, *Srebp1c*: Sterol regulatory element-binding protein 1c, *Pparg2*: Peroxisome proliferator-activated receptor gamma 2, *Fgf21*: Fibroblast growth factor 21.

1. 6. Effects of short-term i.c.v. xenin treatment on the level of lipid metabolism-related proteins in white adipose tissue of *ob/ob* mice

Xenin treatment significantly increased the phosphorylation of HSL at Ser^{660} and Ser^{563} in WAT by 30.3% and 56.0% compared to control aCSF treatment, respectively ($p < 0.05$ by Student's *t*-test, Fig. 10A-C). Total HSL (tHSL) levels were not different between control (100.0 ± 15.6%) and xenin-treated *ob/ob* mice (78.9 ± 4.9%, $p = 0.28$ by Student's *t*-test). There were no significant changes in ATGL ($p = 0.97$ by Student's *t*-test, Fig. 10A, D), perilipin ($p = 0.54$ by Student's *t*-test, Fig. 10A, E) and CGI-58 ($p = 0.83$ by Student's *t*-test, Fig. 10A, F) protein levels. FASN protein levels in WAT were 41.5% lower in xenin-treated *ob/ob* mice compared to aCSF-treated control *ob/ob* mice ($p < 0.005$ by Student's *t*-test, Fig. 11).
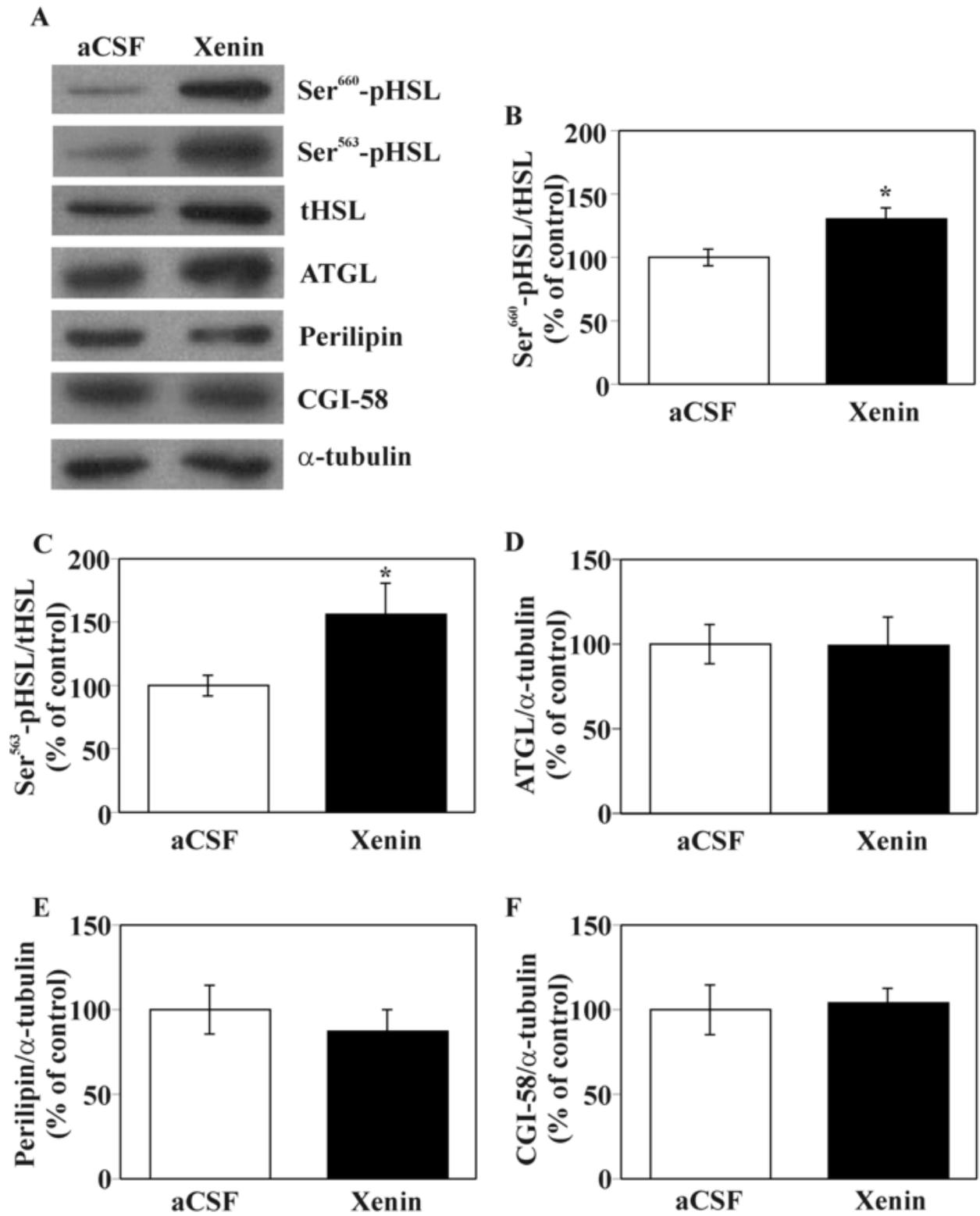
Figure 10. Effects of short-term i.c.v. xenin treatment on the level of lipolysis-related proteins in white adipose tissue of obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 μg) or aCSF 12 h apart and were euthanized 12 h after the second injection. Protein levels were measured by Western blot analysis. (**A**) Representative Western blot. Quantification of phosphorylated hormone sensitive lipase at Ser^{660} (Ser^{660}-pHSL, **B**), Ser^{563}-pHSL (**C**), adipose triglyceride lipase (ATGL, **D**), perilipin (**E**) and comparative gene identification-58 (CGI-58, **F**) protein levels. Signal intensity of Ser^{660}-pHSL and Ser^{563}–pHSL were normalized to total HSL (tHSL). ATGL, perilipin and CGI-58 levels were normalized to α-tubulin levels. Values in aCSF-treated mice were set to 100%. Data are means ± S.E.M (n = 4-5/group). *: $P < 0.05$ vs. aCSF by Student's *t*-test.
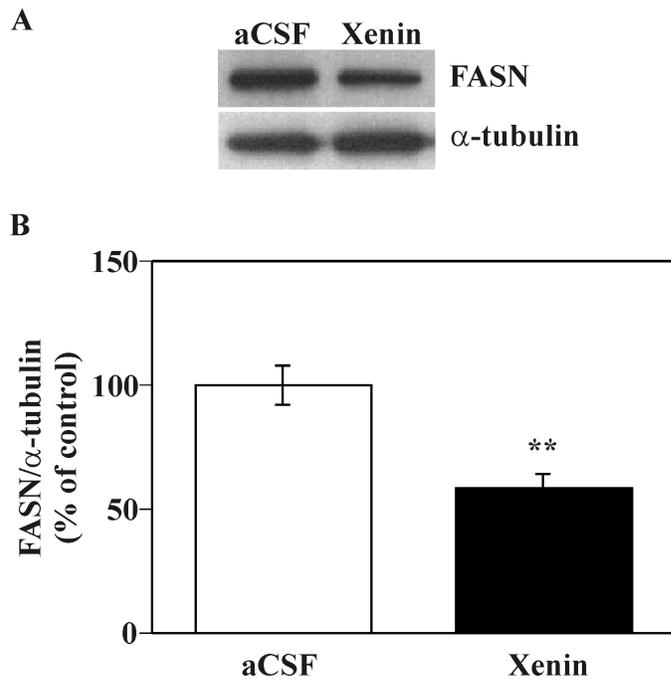
Figure 11. Effects of short-term i.c.v. xenin treatment on the level of fatty acid synthase (FASN) in white adipose tissue of obese mice. Obese *ob/ob* mice received two i.c.v. injections of xenin (5 μg) or aCSF 12 h apart and were euthanized 12 h after the second injection. FASN protein levels were measured by Western blot analysis. (**A**) Representative Western blot of FASN and α-tubulin. (**B**) Quantification of FASN protein levels. Signal intensity of FASN was normalized to α-tubulin. Values in aCSF-treated mice were set to 100%. Data are means ± S.E.M (n = 4-5/group). **: $P < 0.005$ vs. aCSF by Student's *t*-test.

2. Experiment 2

2. 1. Expression of receptors for xenin in mouse adipose tissue

Neurotensin receptor 1 (Ntsr1) has been identified as a receptor for xenin. If xenin regulates lipid metabolism by directly acting on the adipose tissue, Ntsr1 may be present in the adipose tissue. To determine whether or not Ntsr1 is expressed in white adipose tissue (WAT), RT-PCR analysis was performed using RNA prepared from mouse epididymal WAT. A 160-bp band representing *Ntsr1* mRNA was amplified from mouse WAT cDNA (WAT RT+) and positive controls (mouse hypothalamus cDNA and mouse liver cDNA) in the presence of reverse transcriptase (RT). No amplicon was found in negative controls (WAT RT-: mouse WAT without RT, Water: without RNA).
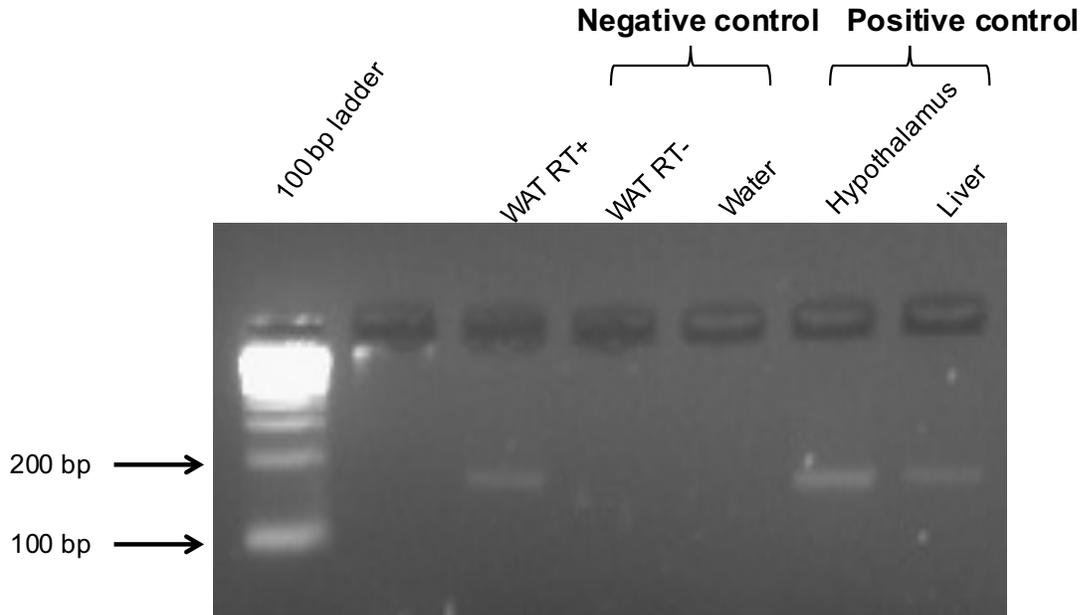
Figure 12. Expression of neurotensin receptor 1 (*Ntsr1*) mRNA in mouse white adipose tissue. RT-PCR was run using cDNA from mouse white adipose tissue (WAT RT+), positive controls (mouse hypothalamus and liver) and negative controls (WAT RT- and DEPC-water). Bands were visualized after agarose gel (3%) electrophoresis using ethidium bromide. A 100 bp DNA ladder was used as a reference. A single sharp band at 160 bp representing *Ntsr1* mRNA was observed in mouse WAT and positive controls.

3. Experiment 3

3. 1. Effects of xenin treatment on lipolysis in mouse white adipose tissue cultured *ex vivo*

Earlier findings from our laboratory have shown that peripheral (intraperitoneal) treatment with xenin reduces body weight gain in mice ((Kim, 2011). Although the present findings in experiment 1 suggest that CNS actions of xenin mediate its weight-reducing effect, these findings cannot eliminate the possibility that peripheral actions of xenin also contribute to the reduction of body weight. The finding that *Ntsr1* mRNA is expressed in adipose tissue (experiment 2) also supports the possible direct action of xenin in adipose tissue. Therefore, I decided to determine the direct effect of xenin on lipolysis in white adipose tissue. Gonadal fat pads were isolated from male C57BL/6 mice and tissue explants were cultured *ex vivo* and treated with xenin. Xenin treatment at 1 mM significantly increased glycerol release at 4 h and 6 h after the treatment compared to control vehicle treatment ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 13B and C) without a significant change at 2 h ($p = 0.42$ by one-way ANOVA followed by Dunnett's test, Fig. 13A). Xenin treatment at 2 mM significantly increased glycerol release at both 2 h and 4 h after the treatment compared to control treatment ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 13A and B) without a significant change at 6 h ($p = 0.29$ by one-way ANOVA followed by Dunnett's test, Fig. 13C). When the effect of time was analyzed within each concentration group, glycerol levels continuously increased between 2 and 6 h after the treatment with xenin at 1 mM ($F[2,30] = 16.51$, $p < 0.0001$ by one-way ANOVA, Fig. 14A) and 2 mM ($F[2,30] = 9.52$, $p < 0.001$ by one-way ANOVA, Fig. 14B). Xenin treatment at other concentrations (10 nM, 1 μM and 100 μM) did not cause any significant changes in glycerol levels at any time point examined ($p = 0.53$-0.99 by one-way ANOVA followed by Dunnett's test).

These findings were replicated in a separate experiment. Xenin treatment at 2 mM significantly increased glycerol and FFA release from *ex vivo* cultured mouse adipose tissue at 4 h compared to controls ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 15A and B). Neither glycerol nor FFA levels were significantly altered by xenin treatment at 100 μM and 1 mM (p = 0.30-0.96 by one-way ANOVA followed by Dunnett's test).

A

2 hour

B

4 hour

C

6 hour
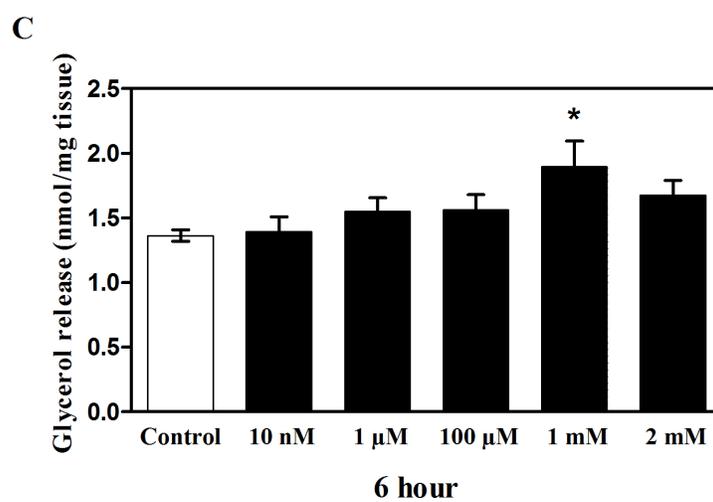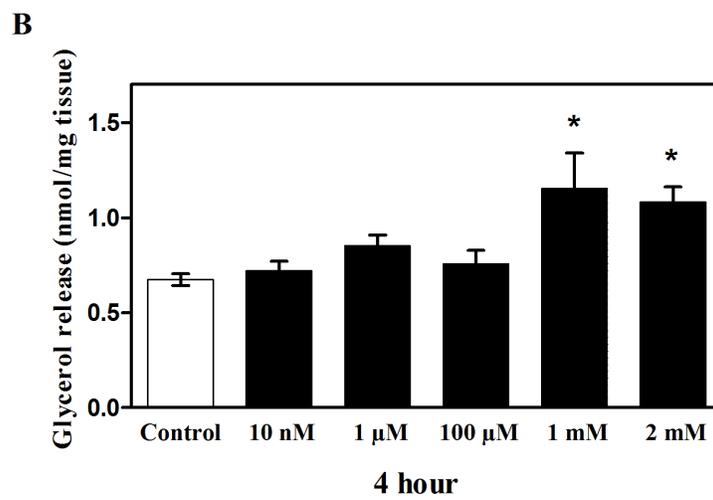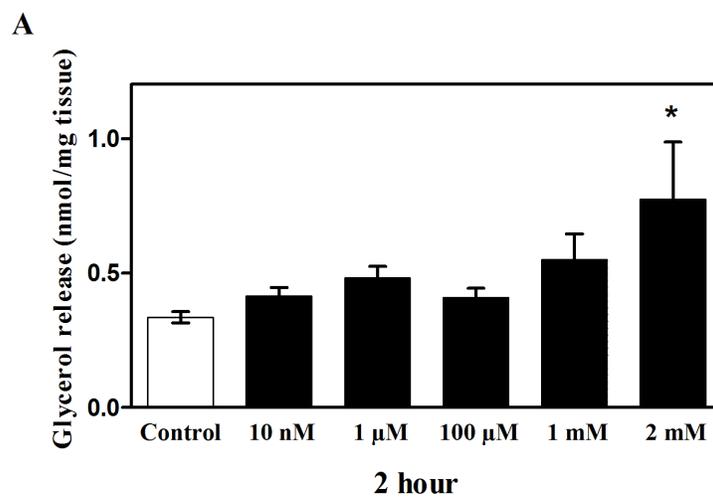
Figure 13. Effects of xenin treatment on glycerol release in mouse white adipose tissue cultured *ex vivo*. Gonadal fat pads from male C57BL/6 mice were isolated and tissue explants were cultured *ex vivo* in the presence of xenin (10 nM – 2 mM). Media was collected for glycerol release at 2 h **(A)**, 4 h **(B)** and 6 h **(C).** Glycerol levels were normalized to tissue weight. Data are means ± S.E.M (n = 10-11/group). *: $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's test.
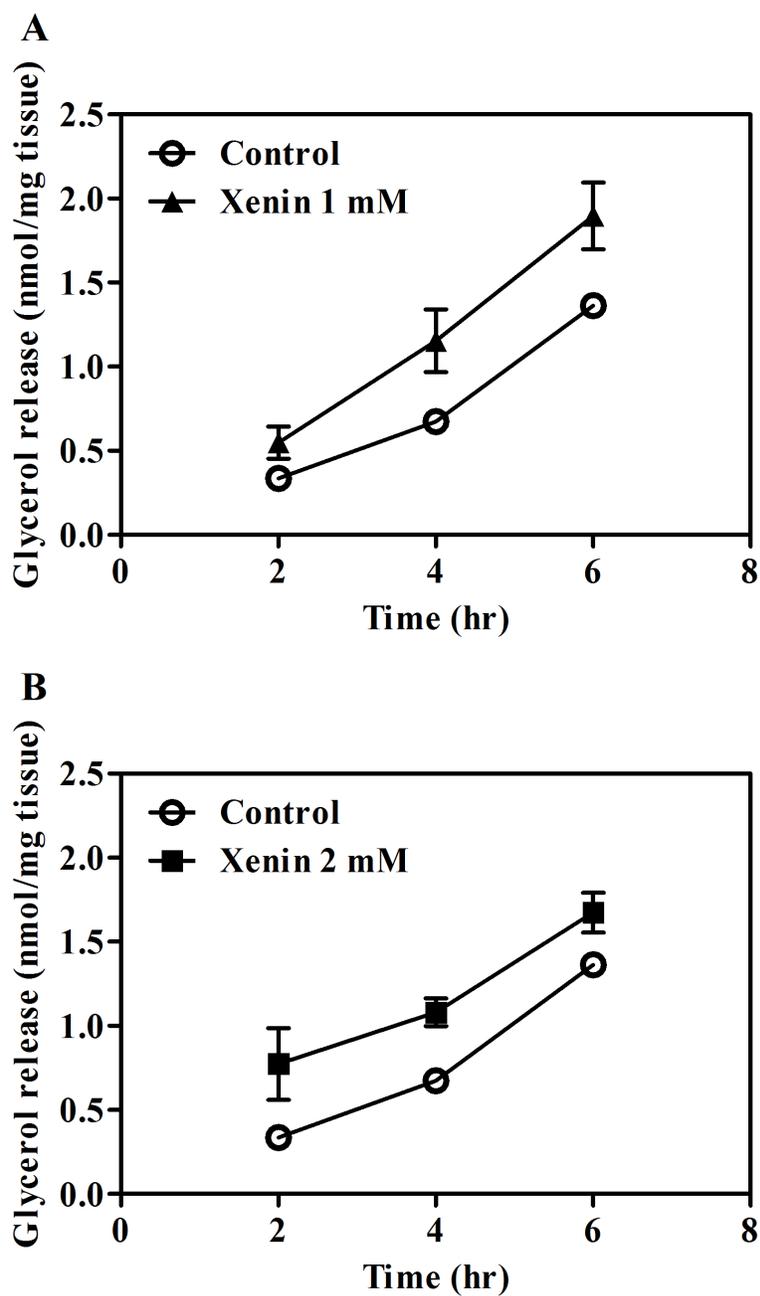
Figure 14. **Time course of xenin-induced glycerol release in mouse white adipose tissue cultured *ex vivo*.** Data from Figure 13 were plotted as a function of time in 1 mM **(A)** and 2 mM **(B)** xenin-treated groups together with control group. One-way ANOVA shows a significant effect of time on glycerol release ($p < 0.0001$ in 1 mM group and $p < 0.001$ in 2 mM group).

A

B
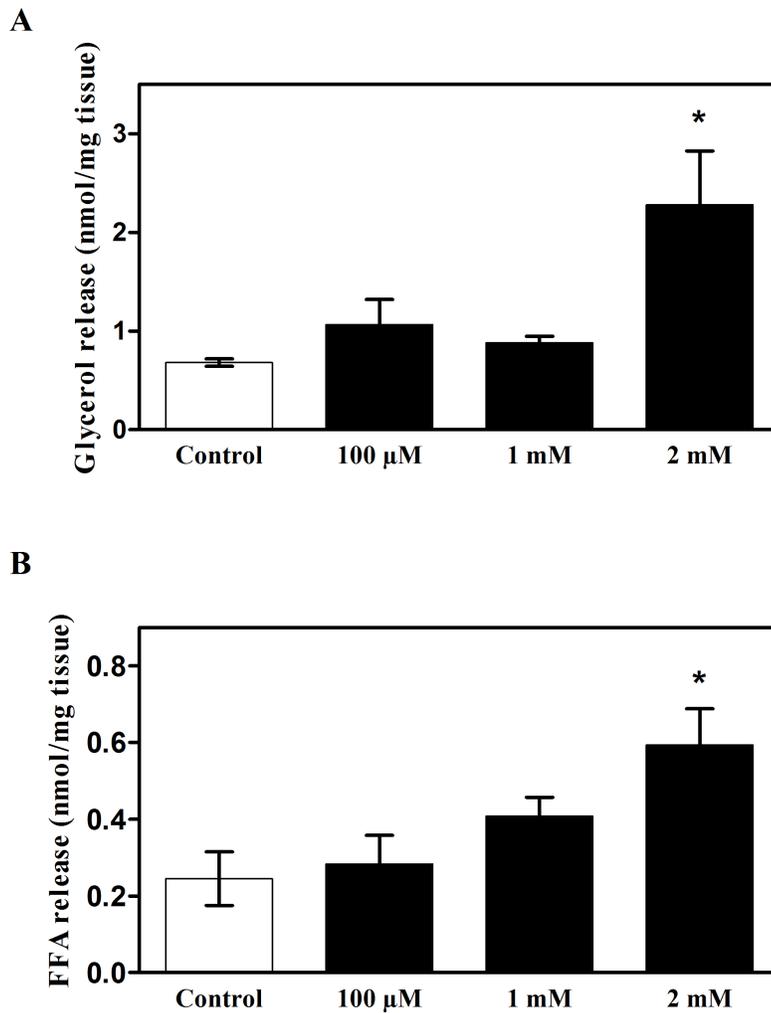
Figure 15. Effects of xenin treatment on glycerol and FFA release in mouse white adipose tissue cultured *ex vivo*. Gonadal fat pads from male C57BL/6 mice were isolated and tissue explants were cultured *ex vivo* in the presence of xenin (100 μM, 1 mM and 2 mM). Media was collected for glycerol **(A)** and FFA **(B)** release at 4 h. Levels were normalized to tissue weight. Data are means ± S.E.M (n = 8-11/group). *: $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's test.

3. 2. Effects of xenin treatment on the expression of lipid metabolism-related genes in mouse white adipose tissue cultured *ex vivo*

Direct xenin treatment significantly increased the levels of lipolytic end products in mouse white adipose tissue cultured *ex vivo*. This finding raised the possibility that xenin-induced lipolysis is associated with changes in the transcription levels of lipid metabolism-related genes. Therefore, I decided to measure the mRNA levels of lipolytic and lipogenic genes in the mouse white adipose tissue cultured *ex vivo*. Xenin treatment at 1 mM significantly reduced *Lipe* and *Atgl* mRNA levels in white adipose tissue cultured *ex vivo* by 55.0% and 51.3% compared to controls, respectively ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 16A and B). Xenin treatment at other concentrations did not cause any significant changes in *Lipe* and *Atgl* mRNA levels ($p = 0.09$-0.42 by one-way ANOVA followed by Dunnett's test, Fig. 16A and B). *Lpl* mRNA levels were not altered by xenin treatment ($p = 0.39$ by one-way ANOVA, Fig. 16C).

A
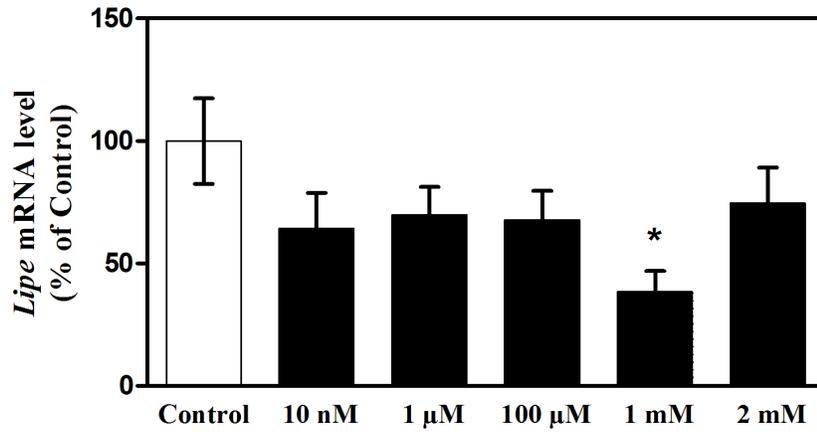
B
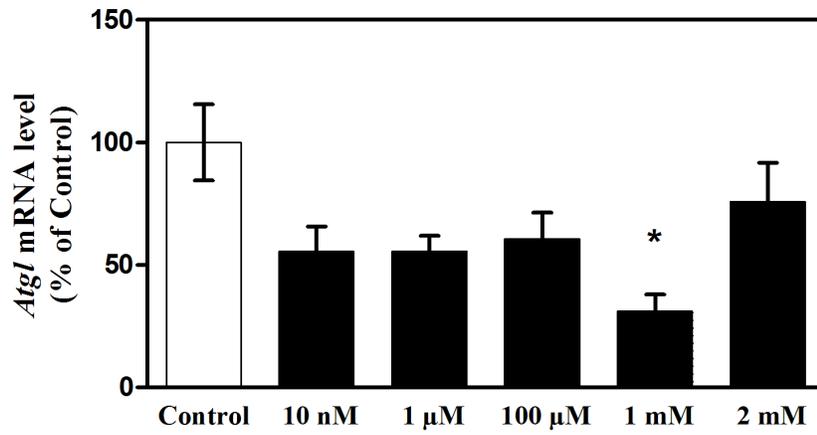
C
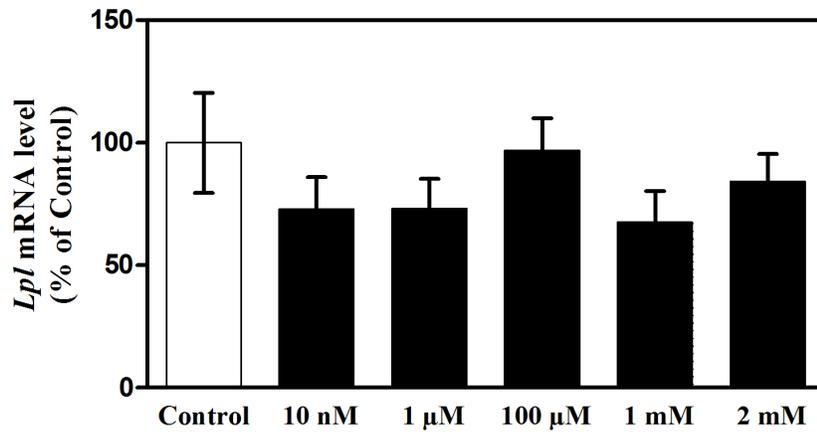
Figure 16. Effects of xenin treatment on the expression of lipid metabolism-related genes in mouse white adipose tissue cultured *ex vivo*. Gonadal fat pads from male C57BL/6 mice were isolated and tissue explants were cultured *ex vivo* in the presence of xenin (10 nM – 2 mM) and harvested at 6 h. mRNA levels of hormone sensitive lipase (*Lipe*, **A**), adipose triglyceride lipase (*Atgl*, **B**) and lipoprotein lipase (*Lpl*, **C**) were measured by real-time PCR. Levels of mRNA were normalized to *cyclophilin* mRNA levels. Values in control group were set to 100%. Data are means ± S.E.M (n = 8-11/group). *: $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's test.

## 3. 3. Effects of xenin treatment on the level of lipid metabolism-related proteins in mouse white adipose tissue cultured *ex vivo*

Xenin treatment at 100 μM and 1 mM significantly increased phosphorylation of HSL at Ser^{660} in white adipose tissue cultured *ex vivo* compared to controls ($p < 0.0001$ and $p < 0.05$, respectively by one-way ANOVA followed by Dunnett's test, Fig. 17A). Levels of total HSL (tHSL) were not statistically different between the groups ($p = 0.13$ by one-way ANOVA, Fig. 17B). Xenin treatment at 2 mM did not alter Ser^{660}-pHSL levels ($p = 0.33$ by one-way ANOVA followed by Dunnett's test, Fig. 17A). Xenin treatment did not cause any significant changes in ATGL ($p = 0.37$), CGI-58 ($p = 0.12$), FASN ($p = 0.67$) and SCD1 ($p = 0.89$) protein levels compared to controls (one-way ANOVA, Figure 17C-F).
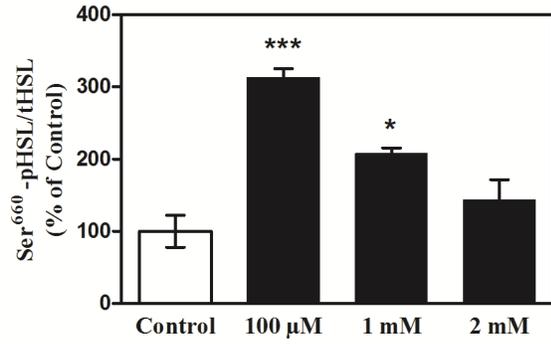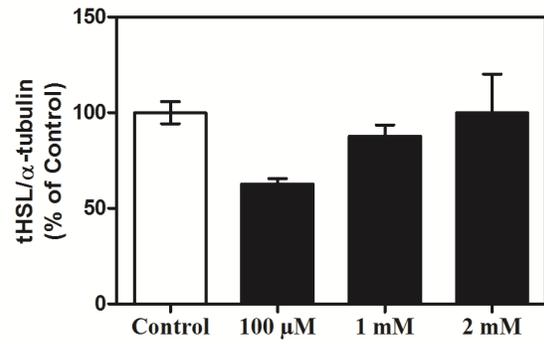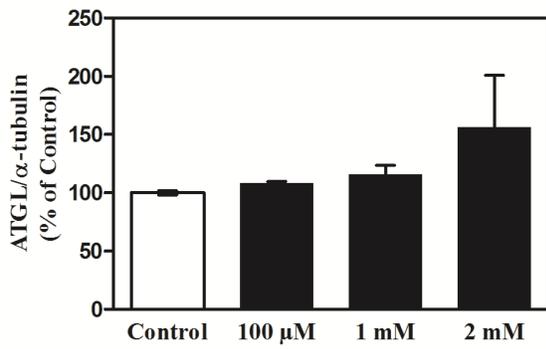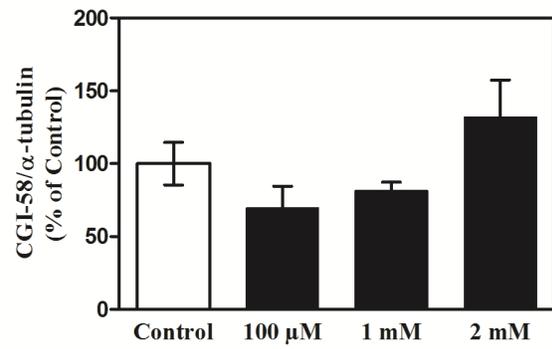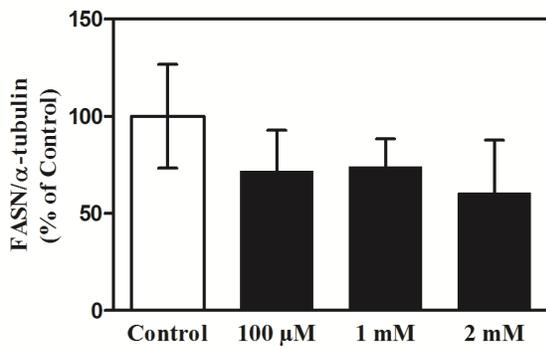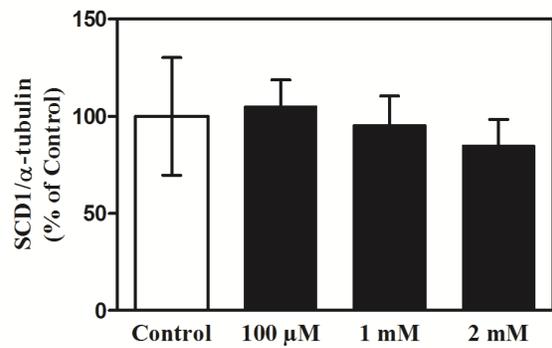
Figure 17. Effects of xenin treatment on the level of lipid metabolism-related proteins in mouse white adipose tissue cultured *ex vivo*. Gonadal fat pads from male C57BL/6 mice were isolated and tissue explants were cultured *ex vivo* in the presence of xenin (100 μM – 2 mM) and harvested at 4 h. Protein levels were measured by Western blot analysis. Quantification of phosphorylated hormone sensitive lipase (Ser^{660}-pHSL, **A**), total HSL (tHSL, **B**), adipose triglyceride lipase (ATGL, **C**), comparative gene identification-58 (CGI-58, **D**), fatty acid synthase **(**FASN, **E**) and stearoyl-CoA desaturase 1 (SCD1, **F**). Signal intensity of Ser^{660}-pHSL was normalized to signal intensity of total HSL (tHSL). Signal intensities of other targets were normalized to signal intensity of α-tubulin. Values in control group were set to 100%. Data are means ± S.E.M (n = 3/group). *: $p < 0.05$, **: $p < 0.0001$ vs. control by one-way ANOVA followed by Dunnett's test.

4. Experiment 4

4. 1. Effects of xenin treatment on glycerol and FFA release in 3T3-L1 adipocytes

Adipose tissue contains multiple cell types such as adipocytes, preadipocytes, macrophages, endothelial cells and so on. Thus, data in the *ex vivo* experiment (experiment 3) cannot determine specific contribution of these cell types to xenin-induced lipolysis. To determine the direct effect of xenin on adipocytes, 3T3-L1 cells were differentiated into mature adipocytes and treated with xenin for 3 h. Xenin treatment at 1 mM and 2 mM significantly increased glycerol levels ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 18A) and FFA levels ($p < 0.05$ by one-way ANOVA followed by Dunnett's test, Fig. 18B) compared to controls.

A

B
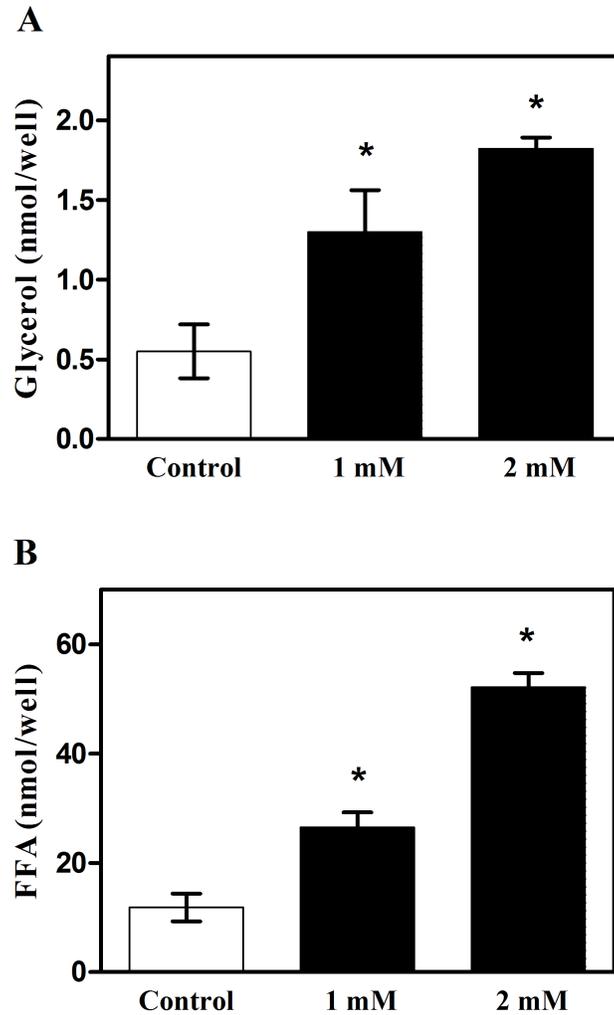
Figure 18. Effects of xenin treatment on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells were treated with xenin at 1 mM and 2 mM for 3 h. Media were collected for glycerol **(A)** and FFA **(B)** assays at the end of the experiment. Data are means ± S.E.M ($n = 3$/group). *: $p < 0.05$ vs. control by one-way ANOVA followed by Dunnett's test.

VII. Discussion

1. Role of the central action of xenin in the regulation of metabolism

1. 1. Effect of the central action of xenin on food intake and body weight

Studies have shown that xenin has feeding supressing effects when injected intracerebroventricularly (i.c.v.) and intraperitoneally (i.p.) to rats and mice (Alexiou et al., 1998; Leckstrom et al., 2009; Cooke et al., 2009). Peripheral administration of xenin reduces gastric emptying rates in mice and humans (Kim and Mizuno, 2010b; Chowdhury et al., 2014). Thus, it has been suggested that xenin acts as a satiety factor by reducing food intake at least partly by delaying gastric emptying in both rodents and humans. The feeding suppressing effect of i.c.v. xenin injection is abolished in neurotensin receptor 1 (Ntsr1)-deficient mice that develop mild obesity and hyperphagia (Kim and Mizuno, 2010a). These findings raise the possibility that xenin-mediated body weight reduction might occur through the central activation of Ntsr1. To date, no study has investigated the role of central action of xenin in the regulation of whole body metabolism and energy partitioning. Therefore, I examined the effect of increased central action of xenin on food intake, body weight and lipid metabolism in the present study using an obese mouse model. Short-term i.c.v. xenin treatment significantly reduced food intake in obese *ob/ob* mice. This finding was consistent with the previous finding that a single i.p. injection of xenin significantly reduced fasting-induced hyperphagia up to 3 h after the injection in obese *ob/ob* mice (Leckstrom et al., 2009). Interestingly, the short-term i.c.v. xenin treatment also caused a significant reduction in body weight gain in *ob/ob* mice in the present study. These findings suggest the possibility that xenin reduces body weight at least partly by reducing food intake in obese mice.

1. 2. Effect of the central action of xenin on the expression of lipolytic genes and proteins in WAT

Adipose tissue is the major organ for fat storage and has the ability to balance both storage and release of lipids in response to altered nutrient demands in the body by acting as a buffer system for lipid flux (Frayn, 2002). Thus, maintaining the balance between lipolysis and lipogenesis is an important and critical factor in regulating body weight. Homeostatic imbalance of lipid metabolism is associated with various complications such as lipotoxicity, non-alcoholic fatty liver disease (NAFLD), impaired insulin secretion, insulin resistance, and obesity (Saponaro et al., 2015). Adiposity can be reduced by increasing the lipolytic rates without affecting lipogenesis in obese mouse models (Jaworski et al., 2009). This suggests that lipolysis is a critical metabolic pathway in maintaining body weight. I compared levels of phosphorylated hormone sensitive lipase (HSL) in WAT between control and xenin-treated obese mice in the present study and found that short-term i.c.v. xenin treatment increased the phosphorylation of HSL at Ser^{563} and Ser^{660}. Phosphorylation of HSL at Ser^{659} and Ser^{660} causes activation and subsequent translocation of HSL from the cytosol to the surface of the lipid droplet, thus these sites are absolutely required for the phosphorylation-induced increase in hydrolytic activity of HSL. In contrast, although phosphorylation at Ser^{563} activates HSL, this phosphorylation site may not be necessary for HSL activation because site directed mutagenesis of serine residue 563 did not abolish HSL activity (Anthonsen et al., 1998). The present findings suggest the possibility that the central action of xenin causes an increase in enzymatic activity of HSL and its translocation from cytosol to lipid droplet surface. Thus, these findings support the hypothesis that xenin-induced weight reduction may be mediated at least partly by increased lipolysis in adipose tissue.

Adipose triglyceride lipase (ATGL) is required for the rate-limiting step of lipolysis, namely hydrolysis of TAG to DAG and FFA, and is activated by comparative gene identification-58 (CGI-58). I observed an increase in *Atgl* mRNA levels without concomitant changes in ATGL and CGI-58 protein expression in WAT following xenin treatment. It has been shown that ATGL is regulated at both transcriptional and post-translational levels which likely explains the discrepancies in its mRNA and protein levels. It is also possible that *Atgl* mRNA levels are reciprocal to its protein levels (Li et al., 2010). Additionally, the literature has reported inconclusive results on the relationship between mRNA and protein levels of ATGL. A few studies concluded that *Atgl* mRNA levels are consistent with its protein levels, while others reported that *Atgl* mRNA levels do not correlate with its protein levels (Kershaw et al., 2007; Deiuliis et al., 2008; Langin et al., 2005; Jocken et al., 2007). It is also possible that changes in HSL activity and lipolysis may occur without changes in ATGL and CGI-58 levels. For example, it was reported that the adipocyte hormone adiponectin suppresses HSL activity without causing alterations in ATGL and CGI-58 expression in adipocytes (Qiao et al., 2011). It should be noted that HSL could hydrolyze both TAG and DAG with a higher affinity for DAG. Under conditions such as beta-adrenergic stimulation, formation of the ATGL/CGI-58 complex activates hydrolytic function of ATGL and promotes hydrolysis of TAG to produce DAG (Lass et al., 2006). Thus, levels of total ATGL and CGI-58 protein may not reflect the activities of these molecules. There have been reports from both rodents and human studies that show that ATGL activity is regulated by its phosphorylation state. Phosphorylation of ATGL at Ser^{406} (mice) and Ser^{404} (humans) is observed during fasting, exercise and beta-adrenergic stimulation (Pagnon et al., 2012). However, in the present study I did not measure the levels of phosphorylated ATGL. Thus, based on the literature and the findings from the present study which showed no change in

ATGL and CGI-58 levels, I speculate that xenin-mediated lipolysis in WAT is mediated by increased HSL activity, but might not involve ATGL activation along its co-activator CGI-58. However, further studies are clearly needed to draw a definitive conclusion.

Perilipins play a key role in mediating the association of HSL with the lipid droplet surface and activating ATGL. Perilipin-1 is the predominant perilipin present in mature adipocytes. It is the most abundant protein on the lipid droplet surface and the major substrate for cAMP-dependent activated PKA. When energy demand increases, perilipin is phosphorylated by PKA, facilitating maximal lipolysis by ATGL and HSL. Phosphorylation of Ser^{492} leads to fragmentation and dispersion of the lipid droplet, which increases the surface area for lipase binding (Marcinkiewicz et al., 2006). Phosphorylation at Ser^{517} (rodents) is crucial for the regulation of HSL- and ATGL-mediated lipolysis (Miyoshi et al., 2007). In the present study, I found that levels of perilipin-1 are not altered by short-term i.c.v. xenin treatment. Although I attempted to measure levels of phosphorylated perilipin-1 using phospho-specific antibodies, unfortunately I was unable to detect phopho-perilipin-1 bands at expected sizes in Western blot analysis. However, it should be noted that HSL can translocate to the lipid droplet surface and become activated independent of phosphorylation by perilipin (Miyoshi et al., 2006). Therefore, I conclude that short-term xenin treatment may increase lipolysis without changes in total perilipin-1 levels in WAT. At present, it is unclear whether or not the central action of xenin promotes phosphorylation of perilipin-1.

1. 3. Effect of the central action of xenin on the level of lipogenic enzyme genes and protein in WAT

I.c.v. xenin treatment significantly reduced the protein levels of fatty acid synthase (FASN), an enzyme required for lipogenesis, in the present study. In contrast, I did not find any significant differences in the levels of *Fasn* mRNA as well as other lipogenic genes between the xenin-treated group and the aCSF-treated control group. There are at least 2 possible explanations for the inconsistent changes in *Fasn* mRNA (no change) and FASN protein (reduction). First, low amounts of FASN protein might increase *Fasn* gene expression (normalization) through a negative feedback loop. Second, a combined effect of translation and protein degradation determines the relative differences between mRNA and protein levels. For example, FASN protein might be quickly degraded compared to the rate of translation of *Fasn* mRNA. A recent study reported that the Src-homology 2 (SH2) domain-containing tyrosine phosphatase Shp2 negatively regulates FASN protein degradation by forming a complex with p38-phosphorylated COP1 and FASN (Yu et al., 2013). An inhibition or a lack of p38, thus an inability to form the FASN-Shp2-COP1 complex, results in reduced FASN degradation as evidenced by the observed increase in FASN protein level without a significant change in *Fasn* mRNA level (Yu et al., 2013). Taken together, it is speculated that xenin inhibits adipose tissue lipogenesis by inhibiting translation of *Fasn* mRNA, stimulating degradation of FASN or both. An examination of protein stability is warranted to address this possibility. It should be noted that human adipocyte carry out only low levels of *de novo* lipogenesis, in contrast to rodents. Thus, it is unclear whether or not the present finding (reduced *de novo* lipogenesis in mouse adipose tissue) can be translated into humans.

1. 4. Effect of the central action of xenin on the expression of browning/beiging-related genes in WAT and liver

Brown adipose tissue (BAT) plays a role in adaptive thermogenesis during cold exposure and after excess caloric intake, thereby controlling energy expenditure. BAT thermogenesis is facilitated by the energy dissipating activity of uncoupling protein 1 (UCP1) which is located in the inner mitochondrial membrane (McMillan and White, 2015). Emerging evidence has suggested that brown-like or beige adipocytes in white adipose tissue also contribute to increased energy expenditure (Wu et al., 2013). Peroxisome proliferator-activated receptor γ (PPARγ) is an essential transcription factor for differentiation and survival of all types of adipose cells. PPARγ activation promotes the formation of brown adipocyte-like cells and induces the expression of genes related to mitochondrial biogenesis and thermogenesis such as UCP1 and PPARγ coactivator-1α (PGC-1α). PR domain containing 16 (PRDM16) is a transcriptional co-regulator which controls the development and function of beige cells by regulating the expression of brown fat-specific genes (Ohno et al., 2012). PRDM16 also induces brown fat-specific genes and suppresses white fat-specific genes by interacting with PGC-1α. Evidence also suggests that fibroblast growth factor 21 (FGF21) may be an autocrine/paracrine as well as an endocrine factor controlling browning of white adipose tissue (Fisher et al., 2012; Li et al., 2014). In the present study, I found no change in the mRNA levels of brown or beige adipocyte markers (*Ucp1, Prdm16 and Ppargc1a*) in WAT and *Fgf21* in liver in xenin-treated mice compared with aCSF-treated controls. These findings suggest that the central action of xenin does not cause changes in mRNA levels that promote the phenotypic changes of WAT from white adipocytes to brown-like/beige adipocytes.

1. 5. Effect of the central action of xenin on the expression of lipid metabolism-related genes in skeletal muscle

In addition to the thermogenic tissues, there are other metabolically active organs that can also contribute to the regulation of body weight by altering lipid metabolism. Skeletal muscle takes up fatty acids either from lipoprotein-triglyceride (VLDL and chylomicrons) or from the albumin-bound plasma NEFA pools and uses them for oxidation. Changes in the levels of fatty acid uptake and fatty acid oxidation related molecules reflect alterations in energy usage in skeletal muscle, and therefore can affect body weight regulation. Lipoprotein lipase (LPL) is present at a high level in tissues such as adipose and muscle that require a large influx of fatty acids for storage or oxidation and hydrolyze VLDL- and chylomicron-triglycerides (Kersten, 2014). Muscle LPL becomes particularly active during fasting, exercise/training, or with beta adrenergic activation, conditions when the muscle has a greater need for fatty acids (Hakvoort et al., 2011; Pedersen et al., 1999; Plaisance et al., 2009). Uncoupling protein 3 (UCP3) is highly expressed in skeletal muscle and *Ucp3* mRNA levels are increased during fasting or food deprivation, acute exercise and under conditions where serum FFA levels are elevated (Cortright et al., 1999; Da-Gong et al., 1997; Millet et al., 1997). It has been suggested that activation of UCP3 promotes fatty acid oxidation (Brand and Esteves, 2005). In the present study, I found significant up-regulation in the mRNA levels of *Lpl* in skeletal muscle in the xenin-treated group compared to the aCSF-treated control group. This finding suggests that an increase in the central action of xenin stimulates fatty acids uptake by skeletal muscle. However, xenin treatment did not cause a significant change in *Ucp3* mRNA levels in skeletal muscle. I used the very short-term xenin treatment protocol in the present study and it might not be sufficient to cause a significant change in the level of the marker of fatty acid oxidation.

1. 6. Effect of the central action of xenin on the expression of lipid metabolism-related genes in liver

De novo lipogenesis (DNL) is the metabolic pathway that converts carbohydrates into fatty acids mainly in the liver and requires multiple enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN) and stearoyl CoA desaturase 1 (SCD1) (Dentin et al., 2004). Increased DNL has been linked to abnormal lipid metabolism, specifically non-alcoholic fatty liver disease (NAFLD) (Donnelly et al., 2005). It has been shown that obese *ob/ob* mice have fatty liver with increased expression of lipogenic genes, activity of lipogenic enzymes and hepatic DNL compared to control wild-type lean mice (Cohen et al., 2002; Wiegman et al., 2003; Iizuka et al., 2006). The present study demonstrated that short-term i.c.v. xenin treatment significantly reduced the levels of *Fasn* and *Scd1* mRNA in liver in obese *ob/ob* mice. These findings suggest that the central action of xenin reduces hepatic DNL, leading to reduced secretion of VLDL and reduced supply of fatty acids to adipose tissue for storage.

Although normally present at very low levels in the liver, enhanced expression of *Lipe* and *Atgl* in the liver increases fatty acid oxidation and release and ameliorates steatosis in mice (Reid et al., 2008). Thus, these lipases may play an important role in the regulation of lipid metabolism not only in adipose tissue but also in the liver. I found that levels of *Lipe* and *Atgl* mRNA in liver were not significantly altered by i.c.v. xenin treatment in the present study. Taken together, these findings suggest that xenin treatment causes alterations in hepatic lipid metabolism toward reduced hepatic triglyceride content and fatty acid supply to adipose tissue by inhibiting DNL without altering hydrolysis of triglyceride in liver.

1. 7. Effect of the central action of xenin on serum FFA and glycerol levels

In the present study, xenin treatment failed to increase serum levels of FFA and glycerol despite increased phosphorylation of HSL in WAT. The weight of the epididymal fat pad was not altered by xenin treatment. It might be possible that a one-day xenin treatment was not sufficient to cause significant changes in serum FFA and glycerol levels and adiposity. Thus, changes in gene and protein expression may represent early stage changes toward increased lipolysis and subsequent increase in serum levels of FFA and glycerol and reduction of adiposity. It is also possible that increased adipose lipolysis was offset by increased FFA uptake by skeletal muscle, resulting in no change in serum FFA level.

1. 8. Possible role of the sympathetic nervous system in mediating CNS action of xenin on lipolysis

The sympathetic nervous system (SNS) innervates WAT and alterations in sympathetic outflow from the central nervous system (CNS) to WAT affect the lipolytic capacity of adipose tissue via adrenergic receptors. Stimulation of the adipocyte's beta-3 adrenergic receptors causes an increase in cAMP levels, leading to PKA-mediated phosphorylation of HSL and perilipin which stimulates lipolysis (Bartness and Bamshad, 1998). Conversely, stimulation of alpha-2 adrenergic receptors inhibits cAMP production and lipolysis. Thus, the balance between the activity of beta-3 adrenergic receptors (lipolytic) and that of alpha-2 adrenergic receptors (anti-lipolytic) determines the net outcome of SNS-induced and catecholamine-induced lipolysis in adipose tissue. In the present study, short-term xenin treatment significantly increased the mRNA levels of the beta-3-adrenergic receptor (*Adrb3)* without a significant change in alpha-2 adrenergic receptor (*Adra2*) mRNA levels in WAT, indicating an increased beta-3 adrenergic

tone relative to alpha-2 adrenergic tone. These findings further suggest that xenin-induced lipolysis might be influenced by increased SNS outflow from the CNS to the WAT.

1. 9. Possible mechanisms by which the central action of xenin reduces body weight

The present study demonstrates for the first time that short-term i.c.v. treatment with xenin reduces food intake and body weight in obese mice. These changes are accompanied by an increase in the level of lipolytic genes and proteins in WAT, reduced expression of lipogenic genes and proteins in the liver and WAT and increased expression of lipid uptake genes in skeletal muscle, possibly contributing to xenin-induced body weight reduction. Therefore, enhancing the central actions of xenin and its downstream targets (such as SNS outflow to the WAT) may constitute a potentially effective strategy for the treatment of obesity by reducing food intake, increasing fat mobilization from the adipose tissue to other tissues (e.g. skeletal muscle) and by reducing the supply of fatty acids to adipose tissue through the reduction of hepatic *de novo* lipogenesis.

1. 10. Insulin-independent mechanism of xenin-induced changes in lipid metabolism

There might be a possibility that xenin-induced increases in lipolysis and/or reductions of lipogenesis are mediated by alterations in insulin action on adipose tissue. If xenin treatment reduces serum insulin levels that may partially explain the increased lipolysis and reduced lipogenesis. I found that i.c.v. xenin treatment did not cause a significant change in serum insulin level in the present study. Thus, it is unlikely that a reduction in insulin mediates the effect of xenin on lipid metabolism in the present study.

1. 11. Xenin-induced changes in lipid metabolism are leptin independent

The adipocyte hormone leptin plays a critical role in the regulation of whole-body metabolism. A lack of the leptin or leptin receptor leads to the development of massive obesity both in rodents and humans (Zhang et al., 1994; Lee et al., 1996; Montague et al., 1997; Clément et al., 1998). Resistant to the metabolic actions of leptin is also associated with obesity. In the present study, I used leptin-deficient *ob/ob* mice as a model of obesity and found that xenin reduced food intake, body weight gain and increased lipolytic gene and protein expression in adipose tissue of these mice. These findings are consistent with our previous finding that a single intraperitoneal injection of xenin caused an acute reduction of food intake in both lean wild-type and obese *ob/ob* mice (Leckstrom et al., 2009). Importantly, the majority of human obesity is characterized by leptin resistance whereas leptin deficiency is rare in human obesity. Therefore, the findings in *ob/ob* mice may not be directly applicable to human obesity. Although the *ob/ob* mouse is not an ideal animal model that recapitulates the phenotype of leptin resistant human obesity, it is a perfect animal model to study leptin-independent mechanisms of metabolic regulation. Findings from the present study raise the possibility that the metabolic actions of xenin are mediated via a leptin-independent mechanism(s). If the leptin-independent mechanism is intact in leptin-resistant individuals, enhancing the activity of such a mechanism (i.e. leptin-independent xenin action) should be beneficial in reversing obesity. In agreement with this assumption, our laboratory has previously demonstrated that the xenin-mediated anorectic effects were preserved in leptin-resistant obese *agouti* mice (Leckstrom et al., 2009). Taken together, these findings support the possibility that enhanced xenin action may be effective in ameliorating obesity and obesity-associated impairments through a leptin-independent mechanism. It would be important to confirm this assumption by investigating the effect of xenin treatment on

metabolism in a mouse model that mimics leptin resistant human obesity (i.e. high-fat diet-induced obese mouse model) (White et al., 2013).

1. 12. Sources of xenin

Xenin is produced in a variety of peripheral tissues with high levels in the stomach and small intestine (Hamscher et al., 1995). Intraperitoneal injection of xenin activates hypothalamic cells as represented by increased expression of the immediate-early gene *c-fos* mRNA and its product Fos in the hypothalamus (Leckstrom et al., 2009). Xenin is present in the cerebrospinal fluid (CSF) and there is a positive correlation between serum xenin levels and CSF xenin levels (van de Sande-Lee et al., 2013). Although it has not been tested whether or not xenin crosses the blood-brain barrier (BBB), these findings suggest that xenin and its degradation products cross the BBB and act on CNS cells to produce metabolic effects. However, it should not be denied that xenin and its degradation products might alter the activity of CNS cells through an indirect mechanism. It was reported that xenin stimulates secretion of vasoactive intestinal peptide (VIP) and pancreatic polypeptide (PP), both of which can reduce food intake and increase energy expenditure by activating CNS cells as a blood borne factor and/or by activating vagal afferent nerves (Feurle et al., 1997; Matsuda and Maruyama, 2007; Kojima et al., 2007). It was also demonstrated that xenin is present in the hypothalamus (Hamscher et al., 1995). Thus, it is possible that both peripherally and centrally produced xenin directly or indirectly act on specific cells in the CNS and reduce food intake and body weight and alter lipid metabolism.

1. 13. Possible role of the degradation product of xenin in the central regulation of food intake, body weight and lipid metabolism

As described in "Introduction", xenin has a short half-life (<4 h) and it is quickly degraded into smaller fragments (Hamscher et al., 1995; Martin et al., 2016; Taylor et al., 2010). The C-terminal octapeptide fragment xenin 18-25 retains biological activities similar to that of native xenin-25 (Martin et al., 2016; Silvestre et al., 2003). Therefore, changes in the expression of lipid metabolism-related genes and proteins, food intake and body weight gain in the present study are possibly contributed by the combined effect of both central action of native xenin and xenin 18-25.

2. Role of peripheral action of xenin in the regulation of lipid metabolism

2.1. Direct effect of xenin on adipose tissue lipolysis

As discussed above, the present study demonstrated that xenin affects lipid metabolism through its action in the CNS. Our laboratory has previously shown that intraperitoneal xenin treatment also causes reductions in food intake, body weight and respiratory quotient (RQ) in mice (Leckstrom et al., 2009; Kim, 2011). These findings raised the possibility that the metabolic effects of xenin may be mediated through both CNS and peripheral mechanisms. If xenin produces these metabolic effects by directly acting on peripheral tissues, receptors for xenin should be expressed in these tissues. The biological effects of xenin including its anorectic effect are mediated via the neurotensin receptor 1 (Ntsr1) as xenin-induced feeding suppression was attenuated in Ntsr1-deficient mice (Kim and Mizuno, 2010a). In the present study, I confirmed that *Ntsr1* mRNA is present in mouse white adipose tissue. This finding extends previous observations showing that Ntsr1 is expressed in preadipocytes in mouse adipose tissue and 3T3-

L1 preadipocytes (Koon et al., 2009). More importantly, these findings support the possibility that xenin acts directly on adipose tissue via Ntsr1. I also found that xenin treatment significantly increased glycerol and FFA release from mouse gonadal white adipose tissue cultured *ex vivo*. These changes occurred concomitantly with increased phosphorylation of HSL. To further determine whether or not xenin directly acts on adipocytes, I performed a similar experiment in 3T3-L1 adipocytes. Consistent with the findings in the *ex vivo* study, xenin treatment caused a significant increase in glycerol and FFA release into culture media by 3T3-L1 adipocytes. Taken together, these data are consistent with the hypothesis that xenin increases HSL activity and stimulates lipolysis by directly acting on adipocytes, possibly via Ntsr1, in adipose tissue.

2. 2. Direct effect of xenin on the expression of lipolysis-related genes and proteins in adipose tissue

Although xenin treatment increased phosphorylation of HSL, it caused a reduction of *Lipe* mRNA levels in WAT cultured *ex vivo*. As mentioned above, mRNA levels do not necessarily correlate with protein levels. The relationship between them is influenced by rates of transcription and translation as well as mRNA and protein stability. The present data suggest that xenin treatment may activate HSL while it may suppress transcription of *Lipe*. It is also possible that reduced mRNA levels in the presence of increased activity of the end product may be a consequence of negative feedback regulation.

Similar to *Lipe* mRNA, levels of *Atgl* mRNA were reduced by xenin treatment without significant changes in AGTL and its regulatory protein CGI-58, suggesting that xenin negatively regulates *Atgl* expression. Since lipase activities are regulated at the posttranscriptional level, a direct correlation between protein expression levels and activities may not exist. I also observed

an opposite pattern of *Atgl* expression in the *in vivo* i.c.v. treatment study (significant increase) and in the *ex vivo* study (significant reduction). This difference can be attributed to a different treatment protocol (direct vs. CNS-mediated indirect action, dose/concentration, time point, etc.). Additionally, consistent with the *in vivo* i.c.v. treatment study, xenin treatment did not cause a significant change in perilipin-1 protein in adipose tissue cultured *ex vivo*. As already mentioned above, at this point, there is an inconclusive picture with regard to the contribution of ATGL along with its associated proteins perilipin-1 and CGI-58 to the lipolysis-promoting effect of xenin.

2. 3. Direct effect of xenin on the expression of a gene involved in fatty acid uptake in adipose tissue

Lipoprotein lipase (LPL) plays a critical role in regulating the release of fatty acids from triglyceride-rich lipoproteins, thus affects fatty acid uptake by tissues such as adipose tissue and skeletal muscle. The present finding that xenin treatment did not alter *Lpl* mRNA levels *ex vivo* in cultured adipose tissue suggests that xenin does not directly affect fatty acid uptake by adipose tissue. The turnover of *Lpl* mRNA is slower compared to that of LPL activity (Bergö et al., 1996). LPL activity has been shown to be reduced without significant changes in the levels of mRNA and proteins in rat white adipose tissue during fasting (Doolittle et al., 1990). Thus, additional studies are necessary to determine the effect of xenin on LPL activity and fatty acid uptake.

2. 4. Direct effect of xenin on the expression of lipogenic genes in adipose tissue

In my *in vivo* study, i.c.v. treatment with xenin reduced FASN protein levels in WAT. To determine whether or not direct application of xenin onto adipose tissue can produce similar results, I measured protein levels of lipogenic enzymes in the *ex vivo* study. Xenin treatment did not cause significant changes in FASN and SCD1 protein levels in adipose tissue cultured *ex vivo*. These findings suggest that the direct action of xenin on adipose tissue does not affect levels of lipogenic enzymes and does not alter lipogenesis.

2. 5. Consideration of treatment duration

Stimulation of lipolysis leads to a rapid increase in FFA and glycerol release within 15 min and the rate of lipolysis continues to increase up to 5 hours after β-adrenergic stimulation in 3T3-L1 adipocytes *in vitro* (Schweiger et al., 2014). Significant effects of β-adrenergic agents and other lipolytic agents on lipolysis have been found between 30 min and 24 h of incubation in adipose tissue cultured *ex vivo* (Digby et al., 2006; Zu et al., 2009; Attané et al., 2011; Siegrist-Kaiser et al., 1997). I measured glycerol and FFA levels in culture media as a marker of lipolysis 2-6 h after the onset of xenin treatment in the present *ex vivo* and *in vitro* experiments. Glycerol levels show a continuous increase between 2 and 6 h after xenin treatment at 1-2 mM. These data suggest that xenin-induced lipolysis may need at least 6 h to reach a plateau. Further studies with extended range of treatment duration (> 6 h) will provide information on detailed time-course of xenin-induced lipolysis.

2. 6. Consideration of xenin concentration

Xenin treatment produced significant biological effects at concentrations with the range of 10 nM-10 μM in previous *in vitro* studies in which effects of xenin on intestinal contraction and insulin secretion were investigated (Feurle et al., 1997; Taylor et al., 2010; Martin et al., 2012; Parthsarathy et al., 2016). I used five different concentrations of xenin, 10 nM, 1 μM, 100 μM, 1 mM and 2 mM, in the present *ex vivo* as well as *in vitro* experiments and found significant increases in glycerol and FFA release only at higher concentrations (1 and 2 mM). Since the plasma level of xenin is very low in mice (< 40 pg/ml = 13.5 pM) (Wice et al., 2010), these concentrations are supra-physiological. Thus, it is likely that the effects I observed in the present *ex vivo* and *in vitro* studies are pharmacological effects of xenin and may not represent the role of endogenous xenin in normal physiology. This further suggests that endogenous xenin may not play an important role in the regulation of adipose tissue lipolysis. Nevertheless, it should be noted that one cannot deny the possibility that supra-physiological doses of xenin can be used as a pharmacological agent in obesity.

3. General discussion

Previous studies including those from our own laboratory have shown that xenin acutely reduces food intake when centrally or peripherally administered to rodents (Leckstrom et al., 2009; Cooke et al., 2009). The present study demonstrated for the first time that short-term xenin treatment (2 i.c.v. injections of xenin during a 24-h time period) reduces body weight gain in a mouse model of obesity via a leptin-independent mechanism. These changes are accompanied by increased expression of lipolytic genes and proteins in WAT, reduced expression of lipogenic genes and protein in liver and WAT and increased expression of a lipid uptake gene in skeletal

muscle. I propose that xenin produced by peripheral tissues such as intestinal K-cells or produced locally in the CNS alters activity of specific cells in the CNS via Ntsr1. This leads to global changes in lipid metabolism by increasing SNS outflow to adipose tissue, increasing adipose tissue lipolysis (at least by increasing HSL activity), increasing fatty acids uptake by skeletal muscle, reducing hepatic DNL which may cause reduced VLDL production/secretion and reduced fatty acids supply to adipose tissue for storage (Figure 19). These changes suggest the shift of lipid metabolism from fat storage to fat usage as fuel, leading to reduced adiposity and body weight. In agreement with this concept, our laboratory has previously found that xenin treatment reduced respiratory quotient in mice, suggesting xenin treatment shifts the fuel source from carbohydrate to fat (Kim, 2011). Since i.c.v. xenin treatment reduced food intake, xenin-induced reduction of body weight gain may be also assisted by reduced energy intake. Further investigations are necessary to determine relative contribution of each variable (i.e. food intake and lipid metabolism) to xenin-induced weight reduction.

As described above, both central and peripheral injections of xenin reduce food intake (Leckstrom et al., 2009; Cooke et al., 2009). These findings raised the possibility that xenin may participate in the regulation of feeding and other metabolic functions by acting through the CNS as well as by directly acting in peripheral tissues. Findings in the present *ex vivo* adipose culture and *in vitro* 3T3-L1 adipocyte studies as well as confirmation of Ntsr1 expression in adipose tissue suggest the possibility that xenin stimulates lipolysis by directly acting in adipose tissue via Ntsr1. Thus, I propose that xenin secreted by intestinal K-cells into the circulation passes through the blood brain barrier, acts on CNS cells to cause changes in lipid metabolism (including increased lipolysis) and at the same time K-cells-secreted xenin reaches the adipose tissue and increases lipolysis via Ntsr1 (Figure 19). Xenin may also affects lipid metabolism by

directly ascting in liver and skeletal muscle (Figure 19). Interestingly, although both CNS-mediated xenin action and direct xenin action increased adipose lipolysis, their effects do not completely overlap with each other. For example, i.c.v. xenin treatment reduced FASN protein levels in WAT, while xenin treatment did not alter FASN protein levels in WAT cultured *ex vivo*. Levels of *Atgl* mRNA in adipose tissue were increased by i.c.v. xenin treatment but were reduced by xenin treatment in the *ex vivo* study. These findings would indicate that xenin coordinately regulates lipid metabolism through multiple mechanisms involving both central and peripheral actions of xenin.

As already mentioned, my findings suggest that central action of xenin on lipid metabolism is independent of leptin action. Although the majority of human obesity is associated with leptin resistance, leptin-independent pathways may be intact in these patients. If that is the case, enhancing an intact leptin-independent pathway(s) should produce beneficial effects in reversing obesity in leptin resistant individuals. Consistent with this concept, anorectic effect of xenin was not impaired in leptin-resistant obese mice (Leckstrom et al., 2009). Therefore, enhancing central and/or peripheral action of xenin and its downstream targets may constitute a potentially effective strategy for the treatment of obesity by altering lipid metabolism in favour of mobilization of fat stores from adipose tissue to other tissues.
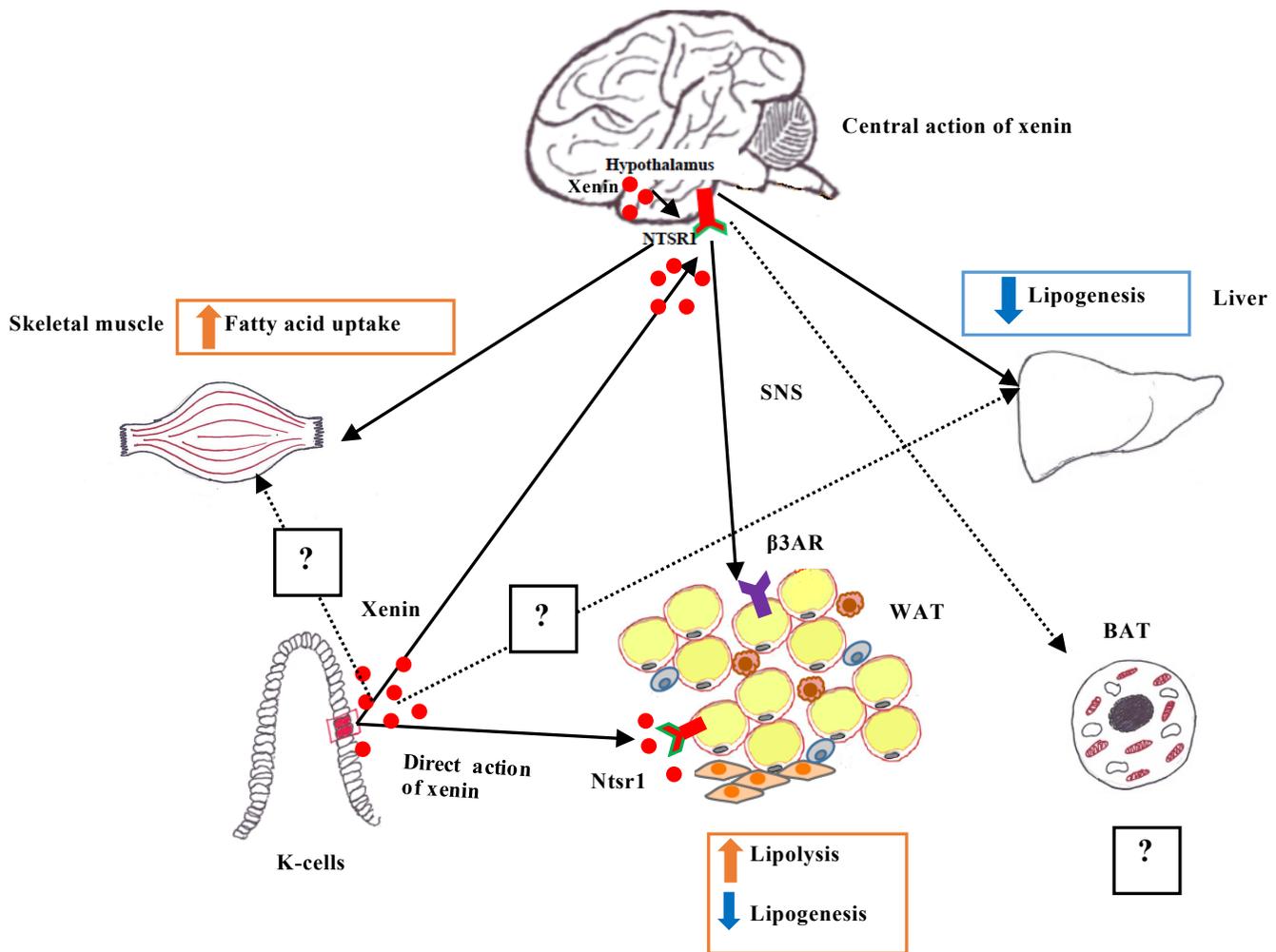
Figure 19. A schematic summary of the findings. Xenin (red closed circle) is secreted by intestinal K-cells and acts on both CNS cells and WAT adipocytes to stimulate lipolysis. Xenin may also affects lipid metabolism through its direct action in liver and skeletal muscle. Locally produced xenin in the CNS also acts on CNS cells to stimulate lipolysis. WAT: white adipose tissue, BAT: brown adipose tissue, Ntsr1: neurotensin receptor 1, SNS: sympathetic nervous system, β3AR: beta 3 adrenergic receptor.

VIII. Limitations and Future Directions

1. Animal models of obesity

Leptin resistance is commonly observed in human obesity, while leptin deficiency is very rare in human obesity. The present study was not designed for determining whether or not the enhanced central action of xenin can reverse leptin resistant obesity. Instead, I used the leptin-deficient *ob/ob* mouse model in the present study to determine the possibility that xenin operates via leptin-independent mechanism on metabolism. As already discussed, if leptin-independent mechanisms are intact in leptin-resistant individuals, the enhanced central action of xenin should be able to cause changes in lipid metabolism through a leptin-independent mechanism in these obese individuals. To further address this possibility, it will be important to determine the effect of xenin on lipid metabolism in animal models of leptin resistant obesity in future studies. A rodent model on a high-fat diet develops leptin resistance and metabolic impairments such as obesity (diet-induced obesity, DIO) and insulin resistance (White et al., 2013), and has been widely used in obesity research. Thus, DIO rodents will be used in future studies.

2. Sex differences

I used only male animals in the present study. There are sex differences in metabolic function both in rodents and humans. For example, body fat is distributed differently in a sex-dependent manner. Women have more subcutaneous fat while men have more visceral fat (Wajchenberg, 2000). The same trend has been observed in the rodents as well. There are also sex differences in sensitivity to the metabolic actions of hormones such as leptin and insulin (Clegg et al., 2003). Disparities have been observed in terms of fat loss from the different fat depots in the body in male and female rodents after weight loss interventions such as leptin

treatment and caloric restriction (Barzilai et al., 1997; Shi et al., 2007). Consequently, findings in males may not be applicable to females and vice versa. Future studies should be conducted in both sexes.

3. Differential lipid metabolism between visceral fat and subcutaneous fat

Visceral fat and subcutaneous fat are the main fat depots. Anatomically, the visceral fat depot includes the omental and mesenteric fat pads in humans. In rodents, perigonadal (epididymal in males and periovarian in females), mesenteric and retroperitoneal fat pads are considered as visceral fat depot, while inguinal fat pad is considered as subcutaneous fat depot. Perigonadal is the largest and most accessible fat pad in rodents and therefore well studied in research (Chusyd et al., 2016). Various studies have established that subcutaneous fat is associated with improved and beneficial outcomes in terms of glucose and lipid metabolism, while visceral fat is linked to metabolic complications such as insulin resistance and glucose intolerance. (Tran et al., 2008; Gabriely et al., 2002; Muzumdar et al., 2008). Moreover, it has been demonstrated that adipose tissues show different lipolytic responses to hormonal signals (e.g. catecholamines and insulin) in a fat depot-dependent manner (Frühbeck et al., 2014). Since the present study focussed on only epididymal fat pads, future studies should examine all the fat pads to determine whether or not xenin-induced lipolysis is fat depot-specific. Additionally, although markers for browning/beiging were measured in white adipose tissue, brown adipose tissue (BAT) was not studied in the present study. BAT has been shown to increase thermogenesis and energy expenditure thereby reduces adiposity in animal models of obesity (Guerra et al., 1998). Therefore, effect of xenin treatment on BAT function should also be studied in future.

4. Xenin-induced activation of the lipolytic pathway

In the present study, I measured the levels of HSL, ATGL, perilipin-1 and CGI-58 that are the key components of lipolytic pathway. The finding that xenin treatment increased levels of phosphorylated HSL at Ser^{660} and Ser^{563} let me to propose the concept that xenin stimulates lipolysis via the classical lipolysis pathway, namely cAMP/PKA/HSL pathway. However, there are gaps in our knowledge to prove this concept. First, if this concept is true, levels of cAMP should be increased following xenin treatment, and therefore cAMP levels should be measured in future studies. Second, PKA should be activated by xenin treatment. To address this point, future studies should determine the effect of PKA blockade (either by PKA inhibitor or PKA knockdown) on xenin-induced phosphorylation of HSL and lipolysis. Levels of phosphorylated perilipin-1 should be also measured as an indicator of PKA activity. Third, I measured total protein levels of ATGL as well as perilipin-1 in the present study. However, as described in "Discussion", these protein levels do not reflect enzymatic activity. To draw the definitive conclusion for the involvement of ATGL in xenin-induced lipolysis, it will be important to determine the ATGL activity using *in vitro* triglyceride hydrolase activity assay where radio-labeled fatty acids will be released from radio-labeled triolein substrate. Since *in vitro* TG hydrolase activity cannot distinguish between ATGL activity and the activity of other hydrolases, xenin-induced lipolysis will be assessed in the presence and absence of the ATGL-specific inhibitor, Atglistatin (Schweiger et al., 2017). Additionally, since CGI-58 is the co-activator of ATGL in stimulated lipolytic condition, it will be also important to determine the interaction between ATGL and CGI-58 using a co-immunoprecipitation (Co-IP) assay. Moreover, it will be interesting to determine phosphorylation status of other HSL phosphorylation sites that were not examined in the present study. Specifically, it has been

shown that phosphorylation of HSL at Ser[565] by AMPK (independent of PKA pathway) negatively regulates HSL activity and prevents the translocation of HSL to the surface of lipid droplet (Daval et al., 2005).

5. Role of Ntsr1 in the mediation of xenin-induced lipolysis

The present study is the first study that shows the direct action of xenin on adipose tissue and adipocytes. However, the exact mechanism by which xenin regulates adipose tissue lipid metabolism through its direct action is unknown. I propose that xenin may affect lipid metabolism in adipose tissue by binding to Ntsr1 expressed on adipose tissue. To prove this point, effect of xenin treatment on lipid metabolism will be examined in adipose tissue (*ex vivo*) or primary adipocyte (*in vitro*) prepared from the Ntsr1 knockout mice in future studies. Alternatively, similar experiments can be performed by blocking signalling through Ntsr1 using an Ntsr1 antagonist, siRNA or CRISPR.

6. Long-term effect of xenin treatment on metabolism

The present *in vivo* study examined the effect of short-term (1 day) xenin treatment on lipid metabolism. Our laboratory has previously demonstrated that once daily intraperitoneal injections of xenin for 6-7 days reduced body weight gain in normal lean mice (Kim, 2011). At present, it is unknown whether or not xenin treatment for an extended period of time can produce changes in lipid metabolism that were observed in the present study. Thus, future studies should determine the effectiveness of sub-chronic xenin treatment on lipid metabolism in both normal lean and obese animals. Plasma half-life of xenin is short and this may limit the effectiveness of xenin treatment in reducing body weight (Taylor et al., 2010; Wice et al., 2012; Parthsarathy et

al., 2016). To circumvent this limitation, degradation resistant xenin analogues have been generated (Martin et al., 2012; Gault et al., 2015; Parthsarathy et al., 2016). These analogues retain biological activities (such as stimulation of insulin secretion) with an extended plasma half-life. It will be interesting to examine if these analogues can alter lipid metabolism for the extended period of time compared to the native xenin.

7. Additional treatment options

Combination treatment may provide beneficial outcome by producing additive effect or synergistic effect compared to monotherapy (Halpern et al., 2010). For example, a combination of anti-hypertensive agents with different mechanisms of action has been widely used to control blood pressure in the treatment of patients with hypertension. Catecholamine is the major lipolytic factor and obesity is associated with catecholamine resistance (Bougnères et al., 1997; Jocken and Blaak, 2008). If xenin can improve sensitivity to the lipolytic action of catecholamine, combination treatment of xenin and beta-adrenergic receptor agonist may produce lipolytic response that is greater than that produced by monotherapy. Leptin also stimulates lipolysis (Zeng et al., 2015). Interestingly, I found that xenin stimulates lipolysis through a leptin-independent mechanism in the present study. This raises the possibility that leptin/xenin combination treatment may produce an additive effect on lipolysis by enhancing the activity of both leptin-dependent and leptin-independent pathways. It is also possible that xenin may enhance sensitivity to metabolic actions of leptin. These possibilities should be addressed in future studies. Additionally, recent evidence suggests that hybrid peptides integrating the complementary actions of multiple metabolism-related hormones are promising candidates for

reversing metabolic impairments (Finan et al., 2015; Finan et al., 2016). Thus, this approach should also be considered in future studies.

IX. Significance

Obesity is a global epidemic and increasing incidence of obesity and its associated complications require urgent treatment that will be accessible to all the obese individuals. At present, there are three treatment approaches that are available; behavioural modification such as dietary restriction and exercise, pharmacological treatment and surgical treatment. Pharmacological therapy generally produces modest body weight loss. However, importantly even modest weight loss can slow the progression of obesity-associated ailments. Although adverse side effects are the major obstacles for the success in developing anti-obesity drugs, pharmacological treatment has advantages over two other approaches. Once a drug is approved for treatment, it is accessible to many patients and pharmacological therapy is much easier to retain patients in treatment compared to behavioural modification. Thus, developing safe and effective anti-obesity drugs is urgently needed. Increasing the number of approved drugs will provide more options to patients and increase the chance of succeeding in weight loss.

Currently there are 6 drugs that have been approved for obesity treatment by FDA. They are either drugs that can alter the activity of appetite-controlling CNS neural circuit, the drug that can mimic metabolic actions of the GI hormone or the drug that can inhibit the absorption of dietary fats by GI tract. Thus, GI hormones that reduce food intake by acting through the CNS are attractive candidates as anti-obesity drugs. The present study focused on the role of the GI hormone xenin in the regulation of metabolism. I found that centrally administered xenin reduces food intake, body weight gain and increases lipolysis markers in obese mice. I also demonstrated that xenin stimulates lipolysis by directly acting in adipose tissue independent of its action in the CNS. Importantly, our laboratory previously reported that xenin reduces food intake without causing an adverse effect (i.e. conditioned taste aversion) and sub-chronic xenin treatment

reduced body weight gain in mice. Collectively, these findings raise the possibility that enhanced xenin action is beneficial in reversing obesity by altering lipid metabolism towards reducing fat storage and therefore xenin can be an attractive candidate target for developing anti-obesity drugs. Furthermore, the present finding that xenin produces beneficial effects on metabolism via a leptin-independent mechanism suggests that targeting xenin and its downstream signalling may be an effective approach in reversing leptin resistant obesity in which leptin-independent pathways may be functional. Since leptin resistance is very common in human obesity, establishment of a treatment protocol that ameliorates leptin resistant obesity is extremely important. Consequently, findings in the present study and future studies may put xenin and its downstream signalling pathway on the current map of metabolic regulation and opens up new avenues of research towards developing anti-obesity drugs, which may improve the quality of life for patients with obesity.

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