Roles of xenin in the regulation of energy balance: central nervous system control of food intake and body weight by xenin

by

Eun Ran Kim

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Department of Physiology

University of Manitoba

Winnipeg, Manitoba

ABSTRACT

Xenin, a gastrointestinal peptide, is structurally similar to neurotensin which functions as a satiety factor via neurotensin receptor 1 (Ntsr1). The effect of the adipocyte hormone leptin on metabolism is partially mediated through the Ntsr1 and interleukin 1 type I receptor (IL-1RI) in the central nervous system (CNS). Xenin reduces food intake when administered centrally and peripherally. However, the metabolic action of xenin and the mechanism of xenin-induced anorexia remain to be elucidated. I hypothesized that prolonged xenin treatment reduces food intake and body weight and increases energy expenditure. I also hypothesized that xenin reduces food intake by activating CNS signalling pathways including Ntsr1 and IL-1RI and by interacting with leptin. To address these hypotheses, I examined (1) the effect of xenin treatment on food intake, energy expenditure and body weight in wild-type, Ntsr1-deficient and IL-1RI-deficient mice, (2) the effect of xenin on hypothalamic Fos and interleukin 1β (IL-1β) expression in wild-type mice, and (3) the effect of co-injection of xenin and leptin on food intake and body weight in wild-type mice. Daily intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) xenin treatment (6-10 days) significantly reduced body weight gain and adiposity with a transient reduction in food intake in wild-type mice. Xenin treatment (i.p.) caused a significant reduction in respiratory quotient without changes in energy expenditure. Xenin treatment increased hormone sensitive lipase (HSL) mRNA levels and reduced acyl-coenzyme A: diacylglycerol acyltransferase 2 (DGAT2) mRNA levels in white adipose tissue. Xenin (i.p.) increased the number of Fos-immunoreactive cells in the hypothalamus and the

brainstem and increased hypothalamic IL-1β mRNA levels. The anorectic effects of xenin and leptin were abolished or attenuated in mice lacking Ntsr1 or IL-1RI. Co-administration i.p. of xenin and leptin caused greater reductions in food intake and body weight compared to leptin alone and xenin alone. These data suggest that long-term xenin treatment reduces body weight by reducing food intake and increasing fat oxidization. Xenin reduces food intake by activating CNS signalling pathways involving Ntsr1 and IL-1 possibly through the interaction with leptin. These findings implicate xenin and its downstream mediators as potential targets for anti-obesity drugs.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

5-HT	5-hydroxytryptamine
Ab	antibody
aCSF	artificial cerebrospinal fluid
AGRP	agouti related protein
AMPK	AMP-activated protein kinase
α-MSH	α-melanocyte stimulating hormone
ANOVA	analysis of variance
AP	area postrema
ARC	arcuate nucleus
b.w.	body weight
BAT	brown adipose tissue
BBB	blood-brain barrier
BMI	body mass index
Ca^{2+}	calcium
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CCK-A	CCK-1 receptor
CCK-B	CCK-2 receptor
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
DGAT	acyl-coenzyme A:diacylglycerol acyltransferase
DGAT2	acyl-coenzyme A:diacylglycerol acyltransferase 2
DIO	diet-induced obese
DMN	dorsomedial nucleus
DMV	dorsal motor nucleus of the vagus
DVC	dorsal vagal complex
ELISA	enzyme-linked immunosorbent assay
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
fmol/ml	femtomole per milliliter
FoxO1	forkhead box protein O1

FTO	fat mass and obesity-associated
GIP	gastric inhibitory polypeptide
GK	glucokinase
GLP-1	glucagon-like peptide 1
GLP-1R	GLP-1 receptor
GSH-R	growth hormone secretagogue receptor
h	hour
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
HSL	hormone sensitive lipase
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IgG	immunoglobulin G
IHC	immunohistochemistry
IL-1RI	interleukin 1 type 1 receptor
IL-1β	interleukin-1β
IL-6	interleukin 6
IL-R	interleukin receptor
IRS1	insulin receptor substrate 1

Jak2	*janus* kinase 2
K^+	potassium
LHA	lateral hypothalamic area
LiCl	lithium chloride
LPL	lipoprotein lipase
LepR	leptin receptor
M	molar
MAPK	mitogen activated protein kinase
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MEK1/2	MAP kinase kinase 1/2
min	minute
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
mTOR	mammalian target of rapamycin
NEFA	non-esterified fatty acids
NIH	National Institutes of Health
NMU	neuromedin U
NMUR2	NMU receptor 2
NPY	neuropeptide Y

NT	neurotensin
NTS	nucleus of the solitary tract
Ntsr1	neurotensin receptor 1
ob	obese
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pERK1/2	phosphorylated ERK1/2
PGC1α	peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PI3K	phosphatidylinositol 3-kinase
POMC	proopiomelanocortin
PVH	paraventricular nucleus of the hypothalamus
PVN	paraventricular nucleus
PYY	peptide YY
RNA	ribonucleic acid
rpm	revolutions per minute
RQ	respiratory quotient
s.c.	subcutaneous

S.E.M.	standard error of the mean
SCD1	stearoyl-coenzyme A desaturase 1
sec	second
SON	supraoptic nucleus
STAT3	signal transducer and activator of transcription 3
TNF-α	tumour necrosis factor-α
U/ml	unit per milliliter
UCP1	uncoupling protein 1
UCP3	uncoupling protein 3
VCO_2	production of carbon dioxide
VIP	vasoactive intestinal peptide
VMN	ventromedial nucleus
VO_2	oxygen consumption
WAT	white adipose tissue
WHO	World Health Organization
ΔΔCt	delta delta cycle threshold

I. Introduction

1. Obesity

1.1. Obesity epidemic

1.1.1. Definition of obesity

Obesity is a condition of abnormally increased body fat caused by inappropriate weight gain. The National Institutes of Health (NIH) uses body mass index (BMI) to measure overweight and obesity in its public report. A person with a BMI of 25 to 29.9 kg/m^2 is considered to be overweight and a person with a BMI greater than 30 kg/m^2 is considered obese [1]. This definition has been widely used by health professionals.

1.1.2. Diagnosis

There is a significant positive correlation between BMI and total body fat. Although BMI has been widely used to measure obesity, it may not always reflect body fat mass and it requires caution in some specific cases. For example, although athletes have a high BMI, it does not indicate that they are overweight, because their weight is accounted for by muscle mass, not by fat mass. Therefore, additional measurements have been used to diagnose obesity. Increased abdominal fat mass is a high risk factor for obesity-related diseases such as type 2 diabetes, and waist circumference provides useful estimates of

abdominal obesity. Men with a waist circumference greater than 102 cm and women with a waist circumference greater than 88 cm are considered obese. To obtain more accurate diagnosis, magnetic resonance imaging and computed tomography have been used. The advantage of the imaging technology is that it provides not only the amount of body fat, but also the distribution of body fat [1].

1.1.3. Prevalence

According to the Statistics Canada, obesity prevalence is high in Canada regardless of age, sex, ethnic background, and provinces [2]. In 2004, 23.1% of adult Canadians aged 18 or older were obese and 36.1% were overweight. These statistical data indicate that nearly 60% of adult Canadians were overweight or obese. Obesity has increased dramatically in prevalence during the past 25 years. The prevalence of obesity has also increased in the younger population. The rate of obesity has substantially increased from 8.5% to 20.5% in the age group of 25-34 between 1978/79 and 2004. Similarly, the combined rate of overweight and obesity increased from 14% to 29% in children and adolescents (12-17 year olds) during the same period. It is important to note the rate of obesity (excluding overweight) in this age group tripled from 3% to 9% during the same period. In comparison, the United States is facing a more serious situation than that in Canada. In 1999-2002, 29.7% of US adults met the criteria for obesity. According to data published in 2007 by the WHO European Region, 31.9-79.3% of adult men and 27.8-77.8% of adult women are overweight in Europe [3]. The increased prevalence of obesity is also seen in many other continents (https://apps.who.int/infobase/CountryProfiles.aspx).

As of 2000, the number of obese adults has increased to over 300 million in the world. Thus, obesity is a rapidly growing health problem throughout the world.

1.2. Causes of obesity

1.2.1. Environmental factors

There are multiple causes of obesity. Firstly, environmental factors are involved in the cause of obesity. Eating habits have changed dramatically over the past three decades [4]. In industrialized societies, unlimited quantities of foods are easily available. In the US, energy availability per person increased by 15% from 1970 to 1994. Excessive dietary fat has been considered to be one of the major causes of obesity. Availability of dietary fat was increased by 3% during a similar period. This correlates with an increased use of fats, such as butter, shortening and oils in baking and fried foods and an increased consumption of pizza and soft drinks. By contrast, consumption of vegetables and fruits was below the minimum amount recommended in the U.S. Federal dietary guidelines. Along with eating habits, lifestyle has also changed in industrialized societies. People tend to live a more sedentary lifestyle [4]. These changes in eating habit and lifestyle caused an increase in consumption of high energy dense foods and a decrease in physical activity, thus contributing to the increased prevalence of obesity and overweight people in the population.

1.2.2. Genetic factors

Genetic factors are also involved in the cause of obesity. Obesity is particularly prevalent in some ethnic groups such as African Americans and Mexican Americans. In contrast, the Asian American group has a lower obesity rate compared to the general population [1]. There are at least 18 obesity-associated genetic syndromes including Prader-Willi syndrome and Bardet-Biedl syndrome [5]. These syndromes clearly indicate that genetic factors play a role in a predisposition to obesity. Family and twin studies have demonstrated that genetic factors account for 40-70% of inter-individual differences in body weight [6, 7].

Recent advances in mouse genetics identified many genes which are associated with human obesity. Cloning of the leptin gene clearly demonstrated that leptin deficiency causes obesity in mice [8]. It was soon discovered that leptin deficiency causes severe obesity in human and leptin replacement therapy has been proven to be effective in reversing obesity in leptin-deficient obese patients [9-11]. A loss-of-function mutation in the leptin receptor gene also causes obesity in both rodents and human [12-15]. Central melanocortin system is a regulatory system of energy homeostasis, in which melanocortin 4 receptor (MC4R) and its ligands are key modulators. It is a major target of leptin action and the metabolic effect of leptin is partially mediated by increased signalling via the MC4R [16]. Mutations in MC4R have been identified in obese subjects [17-22]. These findings clearly indicate a monogenic cause of human obesity. However, congenital leptin and its receptor deficiencies are very rare in humans [9, 14]. To date, MC4R mutation is the most common monogenic cause of human obesity, but it accounts for only up to 6% of obese

subjects [23, 24]. It is likely that human obesity is not generally a monogenic disorder and that predisposition to obesity is influenced by multiple susceptibility genes.

As mentioned above, genetic variants known to influence body weight are largely restricted to mutations in several genes that cause rare monogenic obesity in human. However, it is unknown whether genetic variations are also relevant to the common form of obesity and individual variation in body weight. Recently, a genome-wide association study became a powerful tool to identify common genetic variants associated with any given phenotype through the analysis of genetic variations across the human genome. Through the search for sequence variants and their association to obesity, a strong association between adiposity and single nucleotide polymorphisms in the first intron of the FTO (fat mass and obesity-associated) gene has been identified [25, 26]. It was found that common genetic variants near MC4R were similarly associated with increased BMI [27, 28]. A subsequent study confirmed the association between common variants at FTO and the MC4R loci and body weight and identified 6 additional loci which are associated with increased BMI [29]. Although the contribution of an individual variant to increased body weight is modest, individuals with more obesity predisposing alleles have higher BMI compared to those with less of these alleles [28-30]. So far, independent variants with small but replicable effects on body weight have been identified in at least 17 gene regions [31]. These findings support the polygenic nature of inter-individual differences in body weight and predisposition to obesity.

1.2.3. Gene-environment interactions

Genetic factors modulate the impact of environmental factors on metabolism in each individual. In 1962, Neel proposed the "Thrifty genotype theory" to explain the high frequency of obesity and type 2 diabetes [32]. The core concept of this hypothesis is that our bodies are adapted to store fat and conserve energy rather than wasting energy. In ancient times, people relied on farming, hunting, and fishing for food. These people experienced alternating periods of food surplus and food shortage. To survive through these extreme changes in food availability, thrifty genes promoted the storage of body fat when foods were abundant so that they would survive (due to increase body fat stores) when food supplies were scarce. Thus, these genes were beneficial for survival in those days. However, once people adapted to the modern lifestyle, with less physical activity and a constant supply of calories, these genes promoted continuous storage of excess amounts of calories. Thus, thrifty genes are deleterious rather than beneficial in modern society, causing overweight and obesity. It has been shown that susceptibility to high-fat diet-induced obesity is altered in many genetically-engineered mouse models and different mouse strains. Genes whose absence protect against high-fat diet-induced obesity might be regarded as thrifty genes. Furthermore, a large scale human study has demonstrated that high-fat diets and low physical activity levels amplify the susceptibility to obesity in FTO risk allele carriers [33]. These findings suggest that gene-environment interactions play a role in the predisposition to obesity.

In summary, although mouse genetics has revealed numerous monogenic causes of obesity in mice, it is likely that human obesity is generally not a monogenic disorder.

Predisposition to obesity is influenced by multiple susceptibility genes and a gene-environment interaction.

1.3. Obesity-related problems

Obesity is associated with numerous serious and chronic diseases, including type 2 diabetes, coronary heart disease, cardiovascular disease, hypertension and some forms of cancer. For example, the risk of diabetes increases 93-fold in the group with a BMI of 35 or greater compared to the group with a BMI of 21 or less [34]. Adipose tissue is classically considered a passive fat storage organ. In addition to its fat-storing function, it is now generally accepted that adipose tissue is an active endocrine organ. Adipose tissue produces and secretes a number of bioactive peptides known as adipokines. An increasing number of studies suggest that inflammatory mechanisms are involved in the pathogenesis of type 2 diabetes. The excessive accumulation of body fat causes changes in adipokine secretion profile. For example, circulating levels of tumour necrosis factor-α (TNF-α), and interleukin 6 (IL-6) are elevated in obese and insulin resistant humans and animals. These adipokines cause insulin resistance by interfering with the insulin signalling pathway. Specifically, these adipokines suppress gene transcription of insulin receptor substrate 1 (IRS1) and inhibit tyrosine phosphorylation of IRS1 [35, 36]. Both TNF-α and IL-6 stimulate lipolysis and promote secretion of free fatty acids (FFAs) from adipose tissue to the circulation [37]. Increased circulating FFA levels cause insulin resistance. FFAs inhibit insulin–induced suppression of gluconeogenesis and insulin-stimulated peripheral glucose uptake [38, 39]. High blood FFA levels increase hepatic uptake and oxidization of FFA followed by increased acetyl CoA production, which in turn promotes

gluconeogenesis by increasing pyruvate carboxylase activity [40]. FFAs increase secretion of insulin which counteracts the stimulatory effect of FFA on hepatic glucose production and prevents hepatic glucose overproduction. However, chronically increased FFA levels impair insulin secretion partly by activating endoplasmic reticulum stress and promoting pancreatic β-cell apoptosis [41]. Leptin is another adipokine which plays a critical role in the regulation of energy balance. Leptin also plays a role in the regulation of glucose homeostasis and increases insulin sensitivity. Mutations in the leptin gene cause obesity and insulin resistance, and leptin replacement therapy reverses these impairments in both human and mice. However, leptin deficiency is rare in human obesity. Paradoxically, circulating leptin level is positively correlated with fat mass and high levels of leptin were found in obese subjects [42]. This suggests that leptin resistance is a common feature of human obesity. Although the precise mechanism to cause leptin resistance is not fully understood, obesity-associated insulin resistance may be mediated by leptin resistance [43]. In contrast to the levels of the afore-mentioned adipokines, adiponectin levels are inversely correlated with fat mass and adiponectin levels are reduced in obese subjects. Because adiponectin increases insulin sensitivity, an obesity-associated reduction in adiponectin secretion may contribute to the development of insulin resistance [44].

In addition to the deleterious effect of obesity on health, the obesity epidemic contributes to rising healthcare costs. The obesity-related cost was $99.2 billion in 1995 and direct medical expenses for the obesity-associated diseases such as hospital care, drug and physician care expenditures was $51.6 billion in the U.S. [1]. Therefore, it is extremely important to find a cure for obesity and to find ways to prevent obesity.

1.4. Treatment of obesity

1.4.1. Pharmacological treatment

Because obesity is a significant risk factor for many chronic diseases, even small amounts of weight loss has been shown to be beneficial in reversing the obesity-related impairments. Over the past years, many clinical trials have been performed to develop anti-obesity drugs including fenfluramine, sibutramine, and rimonabant. Unfortunately, most of these trials failed to demonstrate the effectiveness of the drugs without significant side effects. Thus, only a limited number of pharmacological treatments are currently available to obese patients.

Orlistat is a selective gastrointestinal lipase inhibitor. This drug inhibits absorption of dietary fat, and thereby prevents the deposition of dietary fat in the adipose tissue [45]. Long-term treatment of orlistat caused a significantly greater weight loss in overweight and obese individuals compared to the placebo group [46]. This compound was approved by the U.S. Food and Drug Administration (FDA) and Health Canada in 1999 and 2002, respectively, for obesity management.

There are many anti-obesity drug candidates currently undergoing clinical trials. Drugs targeting the serotonin (5-hydroxytryptamine, 5-HT) system have a long history in the treatment of obesity. Drugs such as fenfluramine compounds and sibutramine were initially approved for clinical uses, but they were eventually withdrawn from the market due to adverse side effects. Because specific subtypes of 5-HT receptors are involved in appetite suppression, lorcaserin, which selectively acts on the 5-HT 2C receptors have been developed. Clinical trials have shown promising weight-reducing effects of lorcaserin [47,

48]. Lorcaserin did not cause serious side effects in a phase 2 trial [47]. Several gut hormones have been evaluated in their potential as anti-obesity drugs. Included among these is glucagon-like peptide 1 (GLP-1). GLP-1 is an incretin peptide and long-acting GLP-1 receptor (GLP-1R) agonists are currently approved for the treatment of type 2 diabetes [49]. Clinical studies evaluated the efficacy of GLP-1R agonists to control not only glycemia but also body weight. In these studies, patients taking these agonists showed a significantly greater weight loss compared to placebo [49]. Lastly, FDA-approved tesamorelin, a growth hormone releasing factor, has been used for the treatment of lipodystrophy in human immunodeficiency virus (HIV) patients since 2010. Clinical trials demonstrated that patients taking tesamorelin experienced greater reductions in abdominal fat compared with patients administered placebo. It requires further assessments to determine whether tesamorelin can be used to reduce abdominal fat in obese patients. It is hoped that these candidate drugs continue to show minimal side effects in subsequent clinical trials and are approved for the long-term treatment of obesity.

1.4.2. Lifestyle interventions

As described above, only 1 drug (orlistat) has been approval for clinical use in the long-term treatment of obesity. However, the weight-reducing effect of orlistat is modest. Thus, it is important to develop more effective treatments. In addition to pharmacological treatment, there are lifestyle intervention strategies to control body weight. Firstly, it has been demonstrated that dietary therapy can reduce body weight. Dietary therapy includes calorie restriction, low-fat diet, and carbohydrate-restricted diet, all of which are beneficial in reducing body weight [50]. In addition to the dietary fat content, types of dietary fat

affect body weight. A high-fat diet with highly saturated fats causes obesity, while a high-fat diet with long-chain n-3 polyunsaturated fatty acids is protective against diet-induced obesity [51, 52]. Secondly, increasing physical activity level is beneficial in maintaining body weight within a normal range. Although the efficacy of exercise therapy is not robust, combination therapy with diet produces a greater weight reduction than diet alone [53]. Lastly, behavioural therapy aims to change lifelong habits that promote weight gain to healthy eating and activity patterns. One of the major problems of obesity therapy is that most obese patients regain lost weight after completion of the weight loss program. The amounts of weight regained is related to higher caloric intake, fast-food consumption, fat intake and reduced physical activity [54]. Exercise and behavioural therapies have been proven to be effective in maintaining weight loss after weight reduction [55, 56].

1.4.3. Surgical therapy

Surgical therapy for obesity involves restricting caloric intake by physically reducing the stomach volume, reducing absorption of nutrients by shortening the length of the functional small intestine, or both. The surgical treatment not only reduces body weight but also improves obesity-related metabolic abnormalities such as type 2 diabetes. This beneficial change occurs with altering endocrine secretion profiles [57]. Patients with type 2 diabetes after surgery increase insulin sensitivity with high level of GLP-1 and insulin and lower glucagon [58]. It has been also suggested that the surgery increases PYY and decreases ghrelin concentration, contributing to the weight reduction after surgery [59, 60].

To date, bariatric surgery is the most effective treatment for obesity. In the Swedish Obese Subjects trial, significantly greater weight losses (23% vs. 0.1% at 2 years and 16%

vs. 0.16% at 10 years) were observed in obese individuals who underwent bariatric surgery compared to those on conventional lifestyle therapy plus medications [61]. According to the NIH criteria, only severely obese patients whose BMI is 40 or more are potential candidates for surgery [62]. Less severely obese patients (with BMI between 35 and 40) with obesity-associated comorbidities are also considered for surgery. Thus, only a small fraction of obese patients meet the NIH criteria for bariatric surgery and the majority of patients rely on lifestyle interventions and medications which are less effective compared to surgical therapy.

In summary, currently lifestyle intervention is the only therapy readily available to most obese patients. Pharmacological treatment is recommended for the management of obese patients who are not responsive to lifestyle interventions alone. Currently, orlistat is the only approved drug for the long-term treatment of obesity in North America. Although bariatric surgery produces much greater weight loss compared to lifestyle interventions and pharmacological treatment, application of this surgery is limited to a small fraction of obese patients who meet the criteria for surgery. Thus, additional treatment options, particularly more effective and safe anti-obesity drugs, are clearly needed.

2. Regulation of energy homeostasis

Body weight is maintained within a normal range by maintaining a balance between caloric intake and energy expenditure. The intake of energy in the form of food is periodic. Similarly, levels of energy expenditure fluctuate throughout the day. Neither the amount of

calories per meal nor meal frequency exactly matches the amount of body energy requirement at a given time. Thus, our body should have a regulatory system by which storage and mobilization of energy are adjusted to meet energy demands and to sustain normal physiological functions. The central nervous system (CNS) plays a major role in the regulation of energy homeostasis by integrating peripheral signals which convey information about the energy status of the organism. These signals involve nutrients, hormones, and afferent neural inputs from visceral organs. Thus, impaired CNS integration of these peripheral signals causes abnormal energy balance, leading to obesity and obesity-associated impairments.

2.1. CNS regulation of energy balance: Role of the hypothalamus

The hypothalamus is known as a key CNS regulator of energy balance. In 1939, Hetherington and Ranson reported the observation that discrete electrolytic lesions in the ventral region of the hypothalamus result in an increase in food intake and body weight [63]. Several subsequent studies demonstrated that lesions in the ventromedial nucleus (VMN) and the ventromedial hypothalamic (VMH) area cause an increase in food intake and adiposity [64, 65]. Conversely, electrical stimulation of the VMN results in the termination of the feeding response to hunger [66]. It was demonstrated that the electrolytic lesions in the lateral hypothalamic area (LHA), a region of the ventral hypothalamus situated laterally to the VMN, reduce food intake [67]. Based on these findings, VMN and LHA were defined as the ‘satiety centre’ and the ‘hunger centre’, respectively. In addition to the VMN and LHA, other hypothalamic regions participate in the regulation of energy balance. Included among these are the paraventricular nucleus

(PVN) and dorsomedial nucleus (DMN) [68, 69]. A number of studies over the past 15 years have revealed the importance of the arcuate nucleus (ARC) in the regulation of energy balance. Similar to the VMN/VMH lesion, destruction of ARC causes hyperphagia and obesity, suggesting that ARC also functions as a satiety centre [70, 71]. The ARC lies in close proximity to the median eminence, which lacks a complete blood-brain barrier (BBB), and thus it is thought to be uniquely placed to respond to circulating nutrients and hormonal signals [72, 73]. However, other studies have demonstrated that there are numerous tight junctions between endothelial cells in the ARC, indicating that the ARC is protected by the BBB [74]. Thus, the existence of the BBB in the ARC remains a matter of continuing debate and the mechanism by which circulating substances cause changes in ARC neuronal activity remains unclear.

In addition to the anatomical characterization of the hypothalamus, neurochemical characterization of hypothalamic neurons supports the importance of ARC neurons in the regulation of food intake and energy expenditure. Two groups of neurons in the ARC integrate signals of nutritional status and influence energy homeostasis. A subpopulation of neurons in the medial ARC express the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related protein (AGRP) [75, 76]. In contrast, another subpopulation of neurons in the lateral ARC express the anorexigenic neuropeptides proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) [77, 78]. The metabolic effects of α-melanocyte stimulating hormone (α-MSH), a POMC-derived peptide, and AGRP are mediated through melanocortin 3 and 4 receptors (MC3R and MC4R) [79, 80]. Thus, hypothalamic NPY/AGRP and POMC/CART neurons and CNS cells expressing MC3R

and MC4R constitute the CNS melanocortin system. Impairments in melanocortin signalling are associated with obesity in both mice and humans [81]. These findings support the hypothesis that CNS melanocortin signalling, and in particular ARC POMC/CART and AGRP/NPY neurons play a pivotal role in the regulation of energy balance (Fig. 1).

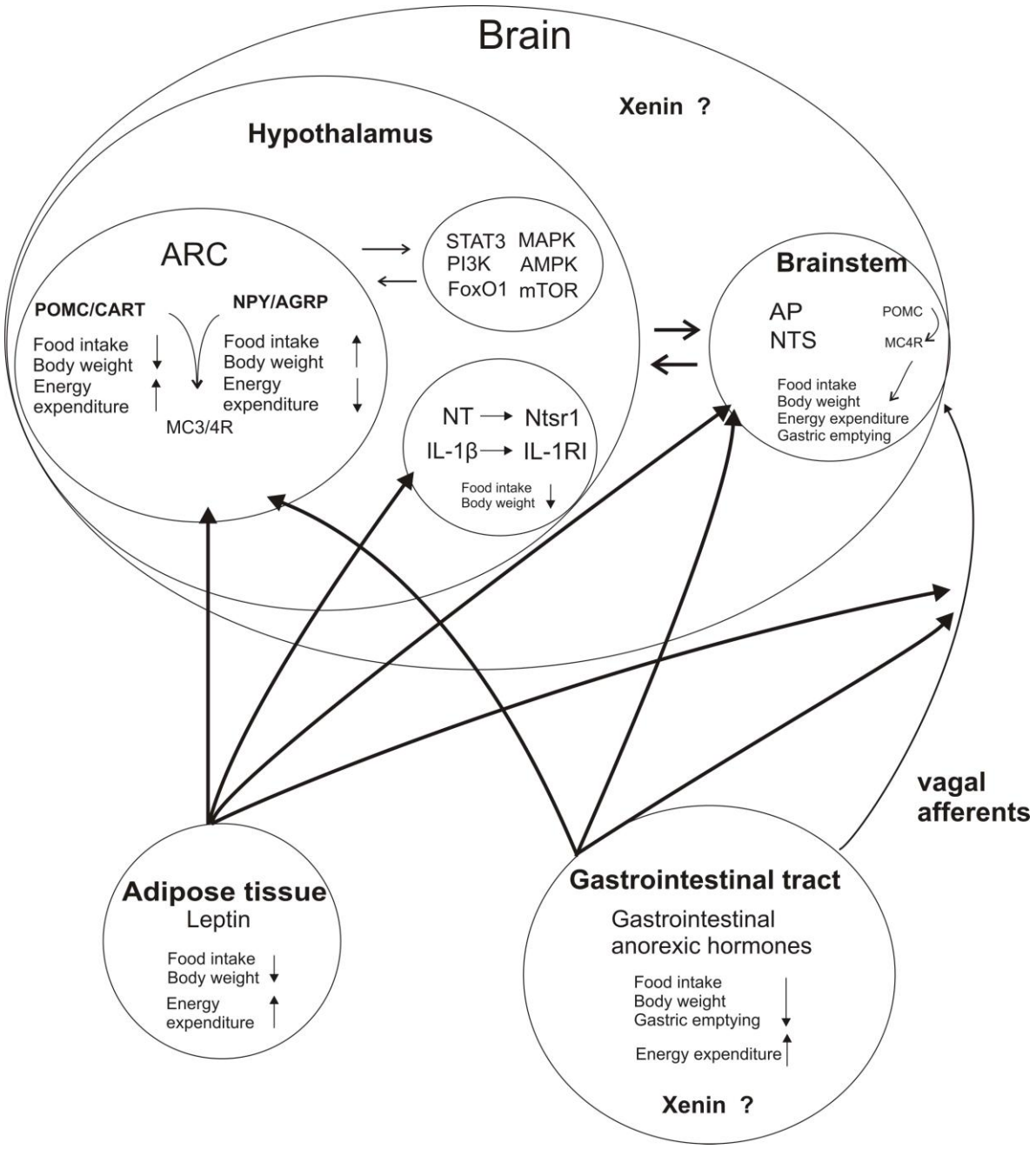

Brain
Xenin ?
Hypothalamus
ARC
POMC/CART
NPY/AGRP
Food intake
Body weight
Energy expenditure
MC3/4R
STAT3 MAPK
PI3K AMPK
FoxO1 mTOR
NT Ntsr1
IL-1β IL-1RI
Food intake
Body weight
Brainstem
AP
NTS
POMC
MC4R
Food intake
Body weight
Energy expenditure
Gastric emptying
vagal afferents
Adipose tissue
Leptin
Food intake
Body weight
Energy expenditure
Gastrointestinal tract
Gastrointestinal anorexic hormones
Food intake
Body weight
Gastric emptying
Energy expenditure
Xenin ?

Figure 1. Pathways by which anorexic gut hormones and leptin regulate energy homeostasis. Anorexic gut hormones and leptin reach the hypothalamic ARC and the NTS of the brainstem and affect neuronal activity in these CNS regions. Gut hormones and leptin also act on the vagus nerve and modify neuronal activity in the NTS. Anorexigenic POMC/CART neurons and orexigenic NPY/AGRP neurons are 2 major subpopulations of ARC neurons mediating the metabolic effects of leptin and gut hormones. Anorectic action of α-melanocyte stimulating hormone (α-MSH), POMC-derived peptide and orexigenic action of AGRP are mediated via MC3R/MC4R in the hypothalamus and other CNS regions. POMC- and MC4R-expressing neurons are also present in the brainstem and mediate anorexic effects of gut hormones. Metabolic effects of gut hormones and leptin are mediated via a number of signalling molecules including STAT3, PI3K, MAPK, AMPK, mTOR, and FoxO1. Metabolic effect of leptin is also mediated via the NT-Ntsr1 and IL-1β-IL-1RI pathways. ARC: arcuate nucleus; NTS: nucleus of the solitary tract; POMC: proopiomelanocortin; CART: cocaine- and amphetamine-regulated transcript; NPY: neuropeptide Y; AGRP: agouti-related peptide; MC3R/MC4R: melanocortin 3- and 4-receptors; STAT3: signal transducer and activator of transcription 3; PI3K: phosphatidylinositol 3-kinase; MAPK: mitogen activated protein kinase; AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; FoxO1: forkhead box protein O1; NT: neurotensin; Ntsr1: neurotensin receptor 1; IL-1β: interleukin 1β; IL-1RI: interleukin 1 type 1 receptor.

The hypothalamus regulates energy balance by integrating circulating hormonal and nutrient signals. For example, CNS regulation of energy expenditure, food intake and metabolism are under the control of the adiposity signals. In 1953 Kennedy postulated that peripheral adipocyte-related humoral factors function as a negative feedback mechanism to the hypothalamus to inhibit food intake [82]. Such factors include insulin and leptin which are secreted by pancreatic beta cells and adipose tissue, respectively, and function as an adiposity signal by conveying signals to the brain for the regulation of energy homeostasis [83, 84]. Circulating levels of insulin and leptin are proportional to body fat [42, 85]. Insulin receptors and the "long" or "signalling" form of the leptin receptors, LepRbs are expressed in several hypothalamic nuclei including the ARC [15, 86-88]. The effects of these adiposity signals on food intake and metabolism are partially mediated through hypothalamic melanocortin signalling [89, 90]. In addition to these adiposity signals, gastrointestinal hormones regulate energy balance partly through an alteration in the activity of hypothalamic neurons [91]. Furthermore, hypothalamic neurons, including melanocortinergic neurons, also respond to nutrient signals such as glucose, lipids and amino acids, and regulate energy balance [92-98]. These hormones and nutrients regulate energy balance via a variety of intracellular signalling pathways in the hypothalamus. These pathways include the signal transducer and activator of transcription 3 (STAT3), phosphatidylinositol 3-kinase (PI3K), forkhead box protein O1 (FoxO1), AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) [99-101] (Fig. 1).

Collectively, hypothalamic neurons, and in particular the ARC melanocortinergic neurons, participate in the regulation of energy balance partly by integrating hormonal and nutrient signals reflecting the body's metabolic states. Therefore, impairments in this signalling pathway lead to metabolic impairments such as obesity and insulin resistance.

2.2. CNS regulation of energy balance: Role of the brainstem

The brainstem is also involved in the regulation of energy homeostasis. The dorsal vagal complex (DVC) of the brainstem, made up of the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus (DMV) and the area postrema (AP), receives vagal afferent inputs from the gastrointestinal tract [102]. Cell bodies of these vagal afferent fibres are located in the nodose ganglia. The brainstem contains major sympathetic pathways to organs such as the adrenal gland, adipose tissue, skeletal muscle, and liver and thereby participates in the regulation of nutrient mobilization and metabolism [103]. The hypothalamus regulates food intake and body weight in response to long-term metabolic signals such as leptin and insulin, whereas the brainstem is predominantly linked to the regulation of meal initiation and termination by responding to short-term gastrointestinal signals [104]. Receptors for gastrointestinal hormones are expressed on the vagus nerve, therefore the brainstem can receive these hormonal signals via the vagal afferents [105, 106]. A unique feature of the brainstem is that the AP is a circumventricular organ with an incomplete BBB [107, 108]. The NTS is in close anatomical proximity to the AP, and thus it is in an ideal position to respond to peripheral circulating signals. Leptin receptors are expressed in the brainstem and systemic administration of leptin induces Fos expression, a marker of neuronal activation, in the brainstem. Intra-4th ventricular injection of leptin,

which primarily accesses brainstem sites, reduces food intake [109]. These findings suggest that the brainstem regulates energy balance by responding to not only the short-term gastrointestinal signals but also the long-term adiposity signals (Fig. 1).

There are extensive reciprocal neural connections between the hypothalamus and the brainstem and therefore the brainstem may regulate energy balance by interacting with the hypothalamus [110-112]. POMC and MC4R are expressed in the brainstem [113, 114]. Fourth ventricle injection of the melanocortin receptor agonist caused an increase in sympathetic outflow in rats. This response was greater in chronic decerebrate rats than in control intact rats [115]. Furthermore, transgenic overexpression of POMC in the NTS, but not in the hypothalamic ARC, attenuates obesity and insulin resistance in diet-induced obese rats [116]. These findings support the concept that the intrinsic neural circuit within the brainstem plays a role in the regulation of energy homeostasis and that the hypothalamus-brainstem connection is not necessary for the regulation of energy balance under certain conditions. NTS receives dendritic contacts from DMV neurons, making transmission between NTS and DMV possible without communicating with the hypothalamus [117-119].

In summary, the brainstem neuronal pathways regulate energy balance by integrating gastrointestinal signals as well as the adiposity signal leptin via vagal afferents and their direct action on the brainstem neurons. Despite extensive reciprocal neural connections between the brainstem and the hypothalamus, a neural circuit within the

brainstem is capable of regulating energy homeostasis without interplay between the two brain areas.

2.3. Regulation of energy balance by leptin

As mentioned above, adiposity hormones convey signals on the body's metabolic states to the brain and regulate energy balance. A number of adiposity hormones have been reported to be involved in the regulation of energy homeostasis.

Leptin, a product of the obese (*ob)* gene, is produced and secreted by the adipose tissue and its circulating level is proportional to the adiposity [42, 120, 121]. Mice with a mutation in the *ob* gene lack functional leptin and are obese with hyperphagia and hypometabolism [8]. Leptin reduces food intake, body weight, and adiposity, and increases energy expenditure, and leptin replacement reverses obesity in leptin-deficient mice [122-124]. The effects of leptin are mediated through its specific receptor, leptin receptor (LepR). The LepRs are expressed both in the CNS and peripheral tissues. The LepR is a member of the class I cytokine receptor family and at least 6 isoforms have been identified. Unlike the other LepR isoforms, the long form LepRb contains a cytoplasmic domain that includes motifs for binding of intracellular signalling molecules, and therefore LepRb is crucial for the metabolic action of leptin [12, 15]. LepRb-mediated leptin action is mediated by the activation of STAT3 and *janus* kinase 2 (Jak2) [101]. Upon leptin binding, the cytoplasmic tyrosine residue Tyr1138 of LepRb recruits and phosphorylates STAT3 proteins, which then dimerize and translocate to the nucleus and activate a specific program of gene transcription. A knockout mouse that leaves the LepR intact but specifically

disrupt the LepRb-STAT3 signal are hyperphagic and obese similar to LepRb-deficient *db/db* mice [125]. Leptin activates STAT3 in the hypothalamus, and neuron-specific STAT3 knockout mice develop obesity recapitulating phenotypes of leptin-deficient *ob/ob* mice [126, 127] These findings suggest that hypothalamic LepRb-STAT3 signalling via Tyr1138 is important for leptin regulation of energy balance. Tyr985 of LepRb recruits SH2-containing tyrosine phosphatase-2 (Shp2) followed by the activation of extracellular signal-regulated kinase (ERK) signalling [128]. Leptin induces phosphorylation of ERK1/2 in the hypothalamus and pharmacological blockade of hypothalamic ERK1/2 reverses the anorectic and weight-reducing effects of leptin [99]. These findings suggest that hypothalamic MAPK signalling is also a direct target of leptin and plays a key role in the control of food intake and body weight by leptin. Growing evidence suggests that other signalling pathways are also involved in the mediation of leptin action. These pathways include PI3K, mTOR, AMPK, and FoxO1 signalling pathways [99-101] (Fig. 1).

LepRb is widely expressed in the CNS with high expression in the hypothalamus including the VMN, ARC, DMN, PVN, and LHA [15, 87]. Systemic or central administration of leptin induces Fos expression in these hypothalamic regions, suggesting that a subset of hypothalamic neurons mediate leptin action [129, 130]. Although the CNS melanocortin system is a major signalling pathway mediating the effect of leptin on energy homeostasis [81], melanocortin signalling is not the only pathway mediating the metabolic effect of leptin. It has been suggested that the metabolic effect of leptin is at least partly mediated by the neurotensin signalling pathway. The CNS neurotensin system plays a role in the regulation of food intake and metabolism. Central administration of neurotensin

decreases food intake [131, 132]. The effect of neurotensin on food intake is mediated by neurotensin receptor 1 (Ntsr1) which is widely expressed in the CNS including the hypothalamus [133-135]. Levels of neurotensin and neurotensin mRNA are decreased in leptin-deficient *ob/ob* mice and i.c.v. administration of leptin stimulates expression of neurotensin mRNA in the hypothalamus [136, 137]. The anorectic effect of leptin is blocked by a potent Ntsr1 antagonist, SR 48692 [138]. Leptin increases phosphorylation of ERK1/2 in the hypothalamus *in vivo* and neurotensin-expressing hypothalamic cell lines *in vitro* [99, 139]. The anorexic effect of leptin is blocked by pharmacological inhibition of ERK1/2 phosphorylation in mice [99]. These findings support the role of CNS neurotensin-Ntsr1 signalling in the mediation of leptin action and that the activation of ERK1/2 is involved in this mechanism (Fig. 1).

Cytokines are known to induce anorexia and interleukin 1 (IL-1) participates in the regulation of energy homeostasis. The IL-1 system is composed of two agonist ligands IL-1α and IL-1β and an antagonist IL-1 receptor antagonist (IL-1Ra), which exert their biological activities through IL-1 receptor (IL-1R). Ablation of IL-1Ra in mice results in a lean phenotype, while IL-1 type I receptor (IL-1RI)-deficient mice exhibit an obese phenotype [140, 141]. Central administration of IL-1β reduces food intake [142, 143]. Hypothalamic IL-1β mRNA expression is reduced in leptin receptor-deficient *fa/fa* rats and leptin stimulates hypothalamic IL-1β mRNA expression [144]. The anorectic effect of leptin is abolished by over-expressing IL-1Ra and by ablation of IL-1RI, suggesting that IL-1 signalling mediates the metabolic action of leptin [145, 146]. Of particular interest is that neurotensin stimulates IL-1β expression and the production in certain cell types [147].

Taken together, these findings suggest that leptin regulation of energy homeostasis involves the hypothalamic neurotensin-IL-1 signalling pathway (Fig. 1).

Although leptin replacement therapy has been proven to be effective in reversing obesity in leptin-deficient obese mice and humans [11, 123], it fails to reverse obesity in obese rodents and patients not due to leptin deficiency. These obese animals and patients have elevated levels of plasma leptin, hence leptin resistance, which is possibly caused by several different mechanisms [101]. Impairments in the LepRb signalling have been found in animal models of leptin resistance [101], suggesting that these impairments contribute to the development of leptin resistance and that enhanced activity of signalling downstream of LepRb may be beneficial in reversing leptin-resistant obesity.

In summary, leptin regulates energy balance through LepR and activation of downstream mediators including melanocortins, neurotensin, and IL-1 in the hypothalamus. Interaction between leptin and these downstream mediators is mediated by the activation of a number of hypothalamic signalling pathways including STAT3, PI3K, MAPK, mTOR, AMPK and FoxO1. Specifically, neurotensin-MAPK-IL-1 pathway mediates some metabolic effects of leptin. Impairments in these signalling pathways result in obesity. In turn, enhanced activity of these pathways may serve as potential approach for the treatment of obesity.

2.4. Regulation of energy balance by gastrointestinal hormones

In addition to the adiposity signals, gastrointestinal hormones participate in the regulation of energy balance. Gastrointestinal peptides that are released into the circulation

in response to nutrient ingestion have been implicated in the control of food intake by limiting the size of individual meals as a satiety factor. Peptides such as cholecystokinin (CCK), glucagon like-peptide 1 (GLP-1) and peptide YY (PYY) are in this category. Plasma levels of these hormones are increased after a meal and administration of these hormones suppresses food intake. In contrast, ghrelin functions as a hunger factor. Circulating ghrelin levels rise during fasting and fall after food intake, and administration of ghrelin increases food intake. These hormones affect food intake by altering the activity of key brain areas like the hypothalamus and the brainstem, making their signalling pathways an attractive target for obesity therapy (Fig. 1).

2.4.1. CCK

CCK is a peptide hormone secreted from the intestinal I cells in response to specific nutrients in the intestine [148-150]. It is the first gastrointestinal hormone to be shown to affect food intake [151]. CCK promotes feeding suppression by reducing the size of individual meals. CCK exerts its biological effects through interaction with two subtypes of CCK receptors, CCK-1 (CCK-A) receptor and CCK-2 (CCK-B) receptor. The anorectic effect of CCK is mediated via CCK-1 receptor [152-154]. It has been suggested that delayed gastric emptying contributes to the CCK-induced feeding suppression [155]. CCK increases the activity of gastric vagal afferent fibres and vagotomy blocks CCK-induced feeding suppression and delayed gastric emptying[156-158]. Peripheral administration of CCK increases c-fos expression in the brainstem [159]. Thus the feeding-suppressing effect of CCK is primarily mediated by the activation of afferent vagus nerves innervating the NTS of the brainstem. Although CCK causes a mild taste aversion, it was suggested

that aversive effects of CCK do not mediate the effect of CCK on feeding [160-162]. Effects of CCK are short-lived and both repeated administration and continuous infusion of CCK failed to cause sustained reductions in food intake and body weight [163-165]. These characteristics make it unlikely that the manipulation of CCK signalling is a promising therapeutic strategy for the treatment of obesity.

2.4.2. PYY

PYY is produced by and secreted from the L-cells mainly on the distal intestine. Circulating levels of PYY are increased in response to food ingestion [166]. PYY_{3-36} is generated by cleaving the first two amino acids from the NH_2-terminal of the full-length PYY by dipeptidyl peptidase IV. PYY_{3-36} is able to cross the blood-brain barrier (BBB) and preferentially binds to Y2 receptors which are expressed in the hypothalamic ARC [167-169]. Although stress may interfere with the anorectic action of PYY_{3-36}, it has been generally accepted that PYY_{3-36} reduces food intake via the Y2 receptor [170-173]. High doses of PYY_{3-36} cause conditioned taste aversion, whereas low doses of PYY_{3-36} reduce food intake without aversive effects [174, 175]. Systemic infusion of the low dose of PYY delays gastric emptying rate [176]. Y2 receptors are also expressed in the NTS and nodose ganglion of the vagus nerve, and vagotomy or transection of the brainstem-hypothalamic neuronal pathways abolished the feeding-suppressing effect of PYY_{3-36} [177-179]. PYY deficiency results in obesity and overexpression of PYY reduces the susceptibility to high-fat diet-induced obesity in mice [180-182]. These data suggest that PYY_{3-36} participates in the regulation of energy balance both by directly acting on hypothalamic neurons and by activating the vagus-brainstem-hypothalamus pathway. In particular, the low-dose of

PYY_{3-36} reduces food intake possibly by slowing gastric emptying without causing aversive side effects. Phase II clinical trials of nasal PYY treatment revealed that the treatment did not meet the primary efficacy endpoint of weight loss and also the weight-reducing effect of PYY treatment was no better than that of the active control, sibutramine treatment [183]. Thus, it is not clear whether or not the PYY-Y2 receptor signalling is an attractive drug target for the treatment of obesity.

2.4.3. GLP-1

GLP-1 is produced from the precursor preproglucagon and is secreted by the L-cell of the small intestine in response to nutrient ingestion [184, 185]. GLP-1 exerts its effect through the GLP-1 receptor (GLP-1R) expressed in both central and peripheral tissues including the hypothalamus [186]. GLP-1 is a powerful incretin and clinical trials revealed that exendin-4 (Ex4), a GLP-1R agonist, treatment is effective in improving glycemic control in type 2 diabetic patients. In addition to its effect on glucose homeostasis, GLP-1 affects energy balance. Peripheral administration of GLP-1 or GLP-1R agonists reduces food intake and body weight [187-189]. Centrally administered GLP-1 also produces a similar inhibitory effect on food intake [190-192]. These two distinct effects of GLP-1 are mediated by specific brain regions. The incretin action of GLP-1 is mediated by hypothalamic ARC, whereas the anorectic effect of GLP-1 is mediated by hypothalamic PVN [190, 193] [191]. Nausea is the most frequently reported side effect of GLP-1 and GLP-1 elicits a conditioned taste aversion [194, 195]. However, it was suggested that low doses of Ex4 reduce food intake without inducing malaise [196, 197]. Systemic injection of GLP-1 increases the expression of c-Fos in both the hypothalamus and the brainstem.

The anorectic effect of GLP-1 is attenuated by vagotomy or ablation of the brainstem-hypothalamus pathways, suggesting that the brainstem plays a critical role in the mediation of the anorectic action of GLP-1 [177]. Furthermore, systemic infusion of GLP-1 delays gastric emptying rate and this effect is also attenuated in vagotomised animals [198, 199]. These data suggest that GLP-1 regulates food intake partly by delaying gastric emptying via the vagus-brainstem-hypothalamus pathway. Clinical studies demonstrated that significantly greater weight loss was achieved by GLP-1R agonists compared to placebo [49]. Thus, enhanced signalling through the GLP-1R is a promising strategy for the treatment of obesity.

2.4.4. Ghrelin

To date, ghrelin is the only known gastrointestinal hormone to increase food intake. Ghrelin was identified as an endogenous ligand for the growth hormone secretagogue receptor (GSH-R) [200]. Ghrelin is produced predominantly in the stomach and circulating ghrelin levels are increased by fasting and reduced after a meal [201-203]. Both central and peripheral administration of ghrelin increases food intake in rodents [204, 205]. Ghrelin treatment also increases food intake in healthy human volunteers [206, 207]. Both peripheral and central administration of ghrelin accelerates gastric emptying in rodents [208-211]. Similarly, the gastric emptying rate was faster in lean human volunteers who received i.v. ghrelin infusion compared to control saline infusion [212]. Furthermore, a recent study demonstrated that GSH-R antagonism lead to a reduction in food intake without causing a conditioned taste aversion [213]. These findings support the concept that ghrelin plays a role in meal initiation partly by stimulating gastric emptying. GSH-R is

expressed in the hypothalamus and injection of ghrelin increases c-fos expression in orexigenic NPY neurons and NPY mRNA expression in the hypothalamic ARC [204, 214]. Orexigenic action of ghrelin is abolished by ablation of the ARC and in mice lacking both NPY and AGRP [215, 216]. These studies provide evidence that ghrelin exerts its orexigenic action through the activation of hypothalamic orexigenic NPY/AGRP neurons. GSH-R is also expressed on the vagus nerve and ghrelin administration increases c-fos expression in the brainstem [204, 217, 218]. Ghrelin-induced feeding was abolished by vagotomy or caudal brainstem lesions [208, 217, 219, 220]. Ghrelin inhibits the activity of the vagal nerve [208]. There are two distinct populations of gastric distension-sensitive neurons in the DVC of the brainstem: gastric distension-excited neurons are activated by ghrelin, whereas gastric distension-inhibited neurons are suppressed by ghrelin [221]. Collectively, these findings suggest that ghrelin is closely involved in the regulation of gastrointestinal motility coupled with feeding behaviour and that this regulation is largely mediated via the vagal afferents. Thus, the GSH-R antagonist is a possible candidate for the treatment of obesity.

2.4.5. Gastric emptying and feeding regulation

It has been well demonstrated that ingestion of food affects gastrointestinal motility and gastric emptying is one mechanism involved in the regulation of food intake [222, 223]. An increased gastric transit time limits the rate of absorption by reducing the rate of nutrient delivery to the small intestine, leading to a prolonged satiety [224, 225]. The rate of gastric emptying is accelerated in animal models of obesity, indicating that rapid gastric emptying contributes to hyperphagia and increased body weight gain [226, 227]. As

described above, a number of anorectic gastrointestinal peptides such as CCK, PYY and GLP-1 slow gastric emptying [91, 155, 176, 198]. Conversely, orexigenic ghrelin speeds up gastric emptying [208]. The effects of these gastrointestinal peptides on gastric emptying are mediated by the vagal afferent nerves. These data suggest that changes in gastric emptying rate contribute to gastrointestinal hormone-induced changes in food intake (Fig. 1).

2.5. Interaction between leptin and gastrointestinal hormones

Gut hormone-induced feeding suppression is short-lived and repeated or frequent administration of gut hormones often leads to the development of tolerance to their effects on metabolism. In contrast, a profound effect of leptin on food intake and body weight remains significant after repetitive administration of leptin. However, the efficacy of leptin therapy in human obesity is primarily limited to extremely rare leptin deficient conditions and is not effective in reversing a more common form of obesity, leptin resistant obesity. Thus, there is a great need to establish improved therapy to overcome these limitations in obesity treatment.

A number of gut hormones regulate food intake and body weight by interacting with leptin. Co-administration of low doses of CCK and leptin that had no effect on food intake on its own caused a significant reduction in food intake [228]. Similarly, a low dose of leptin ineffective in reducing body weight when administered alone became effective when combined with a dose of CCK that was subthreshold for weight reduction [229, 230]. Pre-treatment with a low dose of leptin, which cannot reduce food intake by itself, enhanced the anorectic effect of GLP-1 agonists by increasing the duration of the anorectic effect [231].

At low doses, leptin and GLP-1R agonist injected together, but not separately, significantly reduced food intake and body weight in rats [231, 232]. Continuous infusion of PYY_{3-36} plus low dose leptin, which did not cause significant changes in food intake on its own, prolonged the anorectic effect of PYY_{3-36} [233]. Treatment with PYY_{3-36} plus leptin caused greater reductions in food intake and body weight compared with PYY_{3-36} alone or leptin alone in leptin resistant diet-induced obese (DIO) rats [234]. Similarly, co-infusion of amylin and leptin produced greater reductions in food intake, body weight and adiposity than predicted by the sum of the amylin alone and leptin alone treatments in DIO rats [234]. More importantly, combined treatment with the amylin analog pramlintide and metreleptin, a recombinant human leptin produced a significantly greater reduction in body weight compared to the treatment with pramlintide or metreleptin alone in overweight/obese humans [234]. These studies provide evidence that the efficacy of gut hormones to reduce food intake and body weight is enhanced in the presence of elevated leptin.

Receptors for both gut hormones (e.g. CCK-A receptors, GLP-1R, and Y2 receptor) and leptin are expressed in the nodose ganglion and vagal afferent neurons are activated by gut hormones and leptin [105, 106, 156, 178, 235-237]. Interestingly, almost all leptin-responsive gastric and duodenal vagal afferents are also sensitive to CCK and pretreatment with CCK enhances responsiveness of vagal afferents to leptin [237, 238]. Thus, it is likely that the interaction between gut hormones and leptin occurs at a vagal afferent level.

The CNS mediates effects of gut hormones and leptin on metabolism. Low dose of leptin did not cause changes in Fos expression in the hypothalamic PVN in mice and rats. When animals were treated with low doses of CCK and leptin together, the number of Fos-positive cells was further increased in the PVN [228, 239, 240]. Levels of phosphorylated

STAT3 (pSTAT3) in the hypothalamic ARC were higher in rats that received both CCK and leptin than in rats receiving either CCK or leptin alone [241]. Similarly, co-administration of amylin and leptin increased phosphorylation of STAT3 in the ARC without significant increases in pSTAT3 levels by amylin or leptin alone [242]. These findings suggest that the interaction between gut hormones and leptin occurs at the hypothalamic level.

In summary, gut hormones and leptin regulate energy balance through an interaction at both hypothalamus and vagal afferent levels. Gut hormones may enhance responsiveness to the anorectic and weight-reducing actions of leptin. Alternatively, leptin may extend the duration of the anorectic and weight-reducing actions of gut hormones. These characteristics of gut hormone-leptin interaction may serve as a therapeutic application to overcome drug tolerance and leptin resistance in the treatment of obesity.

3. Xenin

Xenin is another gut hormone which participates in the regulation of energy balance. The effect of xenin on food intake was first reported in 1998 and this initial finding was replicated in a subsequent independent study in 2004 [243, 244]. However, no other studies have been performed to determine its role in the regulation of metabolism at the time when I started my graduate program in 2006.

3.1. Discovery of xenin

Xenopsin is an octapeptide that was isolated from the skin of the African claw frog Xenopus [245]. A tridecapeptide, neurotensin was originally discovered and isolated from bovine hypothalamic extracts around the same time and was subsequently found in intestinal L cells [246-248]. There is a strong homology in C-terminal amino acid sequences between xenopsin and neurotensin (Fig. 2).

Proxenin	M-L-T-K-F-E-T-K-S-A-R-V-K-G-L-S-F-H-P-K-R-P-W-I-L-T-S-
	-L-H-N-G-V-I-Q-L-OH
Xenin-25	M-L-T-K-F-E-T-K-S-A-R-V-K-G-L-S-F-H-P-K-R-P-W-I-L-OH
Xenin-8	F-H-P-K-R-P-W-I-L-OH
Xenopsin	E-G-K-R-P-W-I-L-OH
Neurotensin	E-L-Y-E-N-K-P-R-R-P-Y-I-L-OH

Figure 2. Amino acid sequences of proxenin, xenin-25, xenin-8, xenopsin and neurotensin

It was shown that xenopsin and neurotensin are encoded by different genes and both xenopsin and neurotensin-like peptides are present in amphibian tissues [249-251]. These findings suggested the existence of the xenopsin-like peptide in mammals. Through the search for the mammalian counterpart of the amphibian xenopsin, a 25-amino acid peptide was identified in human gastric mucosa and was named xenin [252]. Xenin has since been identified in various mammals (dog, pig, rat, guinea pig, rabbit and monkey) [253, 254]. Sequence and mass spectrophotometry analyses revealed identical sequences and molecular weights for xenin in these species [253]. There is a high degree of homology in C-terminal amino acid sequences between xenin, xenopsin and neurotensin. In particular, six C-terminal amino acid sequences are identical between xenin and xenopsin (Fig. 2). Several smaller peptide fragments are released from the 25-amino acid xenin, and the 6 C-terminal amino acid fragment is the minimal fragment which demonstrated a biological activity similar to that of xenin. The C-terminal leucine is critical for the biological activity of xenin [253, 255, 256].

In the presence of acidified pepsin, xenin is released from a large molecule that is present in a neutral pH environment [253]. The 35-amino acid peptide extending xenin at its C-terminus was identified. Treatment of this peptide with pepsin released xenin by cleaving off 10 C-terminal amino acids [257]. Thus, this peptide was named proxenin (Fig. 2). Sequence alignment revealed that the amino acid sequences of xenin and proxenin are identical to the N-terminal amino acid residues of human coatomer protein complex subunit alpha (COPA) [257-259]. These findings suggest that xenin is likely to be released from the precursors COPA and proxenin through endogenous proteinase-mediated cleavage.

The aspartic protease, cathepsin E, is present in gastric mucosa at high concentrations and cleaves neurotensin and xenopsin precursors [260, 261]. Consensus sequences PXXL and VXXL are present at the C-terminus of xenin and proxenin, respectively, and generate the conformation for the cleavage by cathepsin E [258, 261]. Thus, it has been suggested that cathepsin E plays a major role in releasing bioactive xenin through the post-translational cleavage of COPA.

3.2. Tissue distribution of xenin

Xenin was initially identified in human stomach, but it was also found in various organs including small intestine, pancreas, liver, lung, heart, kidney, adrenal gland, testis, ovary, prostate gland, skin and brain [262]. Xenin is produced by a subset of K cells in the small intestine [254]. High levels (75-104 pmol/g wet tissue) of xenin were found in gastric mucosa both in human and dog [253]. Although xenin levels are also high in human duodenal mucosa (84 pmol/g wet tissue), xenin levels are more than 100 times lower in canine duodenal mucosa [253]. Xenin is present at lower levels in other tissues in both species [253]. Consistent with the wide distribution of xenin in the body, xenin precursor COPA mRNA is ubiquitously expressed [263]. Xenin has also been identified in secretory granules, suggesting that xenin is transported to the cell surface and secreted into the circulation [254]. The plasma concentration of xenin is 10-30 fmol/ml (30-90 pg/ml) under fasted conditions and increases to 90-120 fmol/ml (270-360 pg/ml) after a meal or sham feeding in humans [252, 264]. Precursors of xenin were not detected in plasma even after pepsin treatment [262]. The wide-spread distribution of COPA mRNA and xenin suggests that xenin plays a wide variety of roles. Furthermore, these findings suggest that the

processing of large precursors to xenin may occur in a tissue-specific manner and stomach and duodenum are the main sources of circulating xenin.

3.3. Role of xenin in the regulation of gastrointestinal motility

Because both xenopsin and neurotensin play a role in the regulation of gastrointestinal function [245, 265-269], several studies focused on the role of xenin in the regulation of gastrointestinal function. It has been demonstrated that xenin induces contraction of jejunum in guinea pig, dog, and human, while it induces relaxation of rat ileum and guinea pig colon [255, 270-272]. Pharmacological studies revealed that the effect of xenin on jejunum contraction is mediated via neural pathways involving muscarinic, tachykinin, and purinergic receptors [255]. Xenin-induced ileum and colon relaxations were due to the myokinetic action of xenin involving Ca^{2+}-dependent K^{+} channels [255, 271]. Since xenin is structurally similar to neurotensin, it was hypothesized that xenin binds and activates the neurotensin receptor, Ntsr. Both xenin-induced jejunum contraction and ileum/colon relaxation were attenuated by a Ntsr antagonist, suggesting that the effect of xenin on gastrointestinal motility is partly mediated through the Ntsr [255, 256, 271]. Xenin also induces contraction of gallbladder and jejunum in conscious dogs [273]. Xenin-induced gallbladder and jejunum contractions were abolished by pre-treatment with atropine but not by vagotomy, suggesting that the effect of xenin on the gastrointestinal motility is mediated via cholinergic nerves and not via vagal nerves. Furthermore, xenin-induced jejunum contraction was absent in cholecystectomized dogs, suggesting that jejunum contraction is induced secondary to xenin-induced contraction of gallbladder which increases bile flow into the duodenum [273]. The peak of plasma xenin level

coincides with the occurrence of phase III contraction [272]. Plasma xenin levels were increased in response to sham feeding in humans, suggesting that food-related visual, taste, olfactory and cognitive cues stimulate the secretion of xenin [264]. In summary, these findings suggest that the increased secretion of xenin during the cephalic phase plays a role in the regulation of gastrointestinal motility and that xenin-induced changes in gastrointestinal motility involve a Ntsr-mediated mechanism.

3.4. Role of xenin in the regulation of exocrine function

Because both xenopsin and neurotensin were known to stimulate exocrine pancreatic secretion [245, 265, 268], it was assumed that xenin affects exocrine pancreatic secretion. Intravenous (i.v.) infusion of the full-length of xenin and a C-terminally truncated form of xenin, xenin-8 (Fig. 2) induced an increase in exocrine pancreatic secretion in dogs [252, 270, 274]. The stimulatory effect of xenin on exocrine pancreatic secretion was greatly attenuated by the Ntsr antagonist SR48692 [274]. Although atropine blocked the ability of neurotensin to stimulate exocrine pancreatic secretion, it failed to block the effect of xenin [274-276]. Furthermore, xenin increases phosphorylation of ERK1/2 in Panc1 exocrine pancreatic cells, suggesting that xenin-induced exocrine pancreatic secretion may be mediated by the MAPK signalling pathway [277]. Xenin increases plasma levels of vasoactive intestinal peptide (VIP) which is known to have a stimulatory effect on exocrine pancreatic secretion [270, 278]. Thus, xenin-induced exocrine pancreatic secretion may be secondary to the increased VIP secretion. These findings suggest that xenin directly acts on pancreatic cells and stimulates exocrine pancreatic secretion at least partly via Ntsr, but not via muscarinic receptors.

3.5. Role of xenin in the regulation of endocrine function

It has been suggested that xenin plays a role in the regulation of endocrine function. Plasma levels of pancreatic polypeptide, VIP, insulin and glucagon were elevated by i.v. infusion of xenin in dogs [270]. Similar to these effects of the full-length xenin on pancreatic hormone secretion, xenin-8 increases secretion of insulin and glucagon in perfused rat pancreas, indicating that an intact C-terminal amino acid sequence is essential for the stimulatory effect of xenin on insulin secretion [279]. Recently, two independent groups reported the effect of xenin on insulin secretion. One group reported that subcutaneous (s.c.) injection of xenin increased plasma levels of insulin and reduced blood glucose levels during glucose tolerance tests in normal mice. Xenin increased insulin secretion both at low (5.6 mM) and high (16.7mM) glucose in an insulin-producing cell line, BRIN-BD11 cells, and there was an additive effect of xenin and glucose-dependent insulinotropic polypeptide (GIP) on insulin secretion in these cells [280]. The other group reported that although xenin alone did not affect glucose tolerance in normal mice, xenin restored the insulinotropic effect of GIP in mouse models of type 2 diabetes. Xenin did not alter insulin secretion from isolated mouse islets and in an insulin-producing cell line, MIN6, at both low (2.5 mM) and high (7.5 mM) glucose in these cells. The effect of xenin on GIP-induced insulin secretion was attenuated by atropine, suggesting that muscarinic receptors mediate the effect of xenin on insulin secretion [277]. The discrepancies between these two studies are possibly due to differences in experimental design, experimental model, and dosage of xenin. Collectively, it is likely that xenin plays a role in the regulation of glucose homeostasis as an incretin and the insulinotropic action of xenin is at

least partly mediated via muscarinic receptors and an interaction with other incretins such as GIP.

3.6. Role of xenin in the regulation of energy balance

Anorectic gastrointestinal hormones are released after food ingestion. Plasma levels of xenin were elevated above pre-feeding levels after a meal in human [252]. Postprandial elevation of plasma xenin was observed with fat-enriched, carbohydrate-enriched, protein-enriched, and mixed foods [264]. These findings led to the hypothesis that xenin participates in the regulation of food intake as an endogenous satiety factor. In 1998, the first paper reporting the effect of xenin on food intake was published. In this study, intracerebroventricular (i.c.v.) administration of xenin reduced food intake in rats in a dose-dependent manner [243]. A single i.c.v. injection of xenin at a dose of 15 μg caused a significant 43% reduction of food intake in rats compared with control vehicle injection. Consistent with the reduced food intake, the administration of xenin reduced time spent eating in rats [243]. A similar feeding-suppressing effect of i.c.v. administrated xenin was reported in a subsequent independent study [244]. These data support the role of xenin in the regulation of food intake as a satiety factor and that the feeding-suppressing effect of xenin may be mediated through the CNS. These findings also suggest the possibility that xenin plays a role in the regulation of whole body energy balance not only by regulation of food intake but also by regulating metabolism. However, this possibility had not been tested as of 2006 when I started my graduate program. Since then there have been advances in research on xenin regulation of energy balance [281]. Data reported since 2006 will be discussed in the "Discussion" section.

II. Rationale

Despite a large amount of effort over the past years, many clinical trials have failed to find safe and effective anti-obesity drugs and there is only one drug which is currently approved for the long-term treatment of obesity in North America. Currently, lifestyle intervention is the only therapy readily available to most of obese patients. Although bariatric surgery produces much greater weight loss compared to lifestyle interventions and pharmacological treatment, application of this surgery is limited to a small fraction of obese patients who meet the criteria for surgery. Thus, additional treatment options, in particular more effective and safe anti-obesity drugs, are clearly needed.

The effect of xenin on food intake was first reported in 1998 and this initial finding was replicated in a subsequent independent study in 2004. These initial findings suggest that xenin functions as an endogenous satiety factor and can be a candidate for an anti-obesity drug. However, no other studies have been performed to determine its role in the regulation of energy homeostasis. In order for xenin to become a future drug candidate for obesity treatment, it is important that the long-term treatment of xenin produces sustained weight loss without developing the tolerance and aversive response to xenin. However, effects of the long-term treatment with xenin on food intake, body weight, and energy expenditure have not been tested. No data are available on the mechanism by which xenin reduces food intake. The present study aimed at determining these points.

It has been demonstrated that several anorexigenic gut hormones, such as CCK, slow gastric emptying, suggesting that the gut hormone-induced feeding suppression is partly due to delayed gastric emptying. Therefore, it is reasonable to determine whether the dose of xenin which causes feeding suppression also reduces the rate of gastric emptying in the present study. Gut hormones exert the anorectic effect partly by activating specific signalling pathways in the CNS including the hypothalamus and the brainstem. Xenin reduces food intake when administered centrally, suggesting that xenin action in the CNS contributes to its anorectic effect. It has been shown that the effects of xenin on gastrointestinal motility and exocrine pancreatic secretion are mediated via Ntsr1 receptor which also mediates feeding-suppressing effect of neurotensin. Activation of Ntsr1 results in increased MAPK and IL-1 signallings, both of which are involved in the CNS circuit regulating energy homeostasis. Therefore, it is reasonable to address the hypothesis that xenin reduces food intake by activating specific CNS signalling pathways in the present study.

Most cases of human obesity are likely to be associated with insensitivity to leptin. Therefore, there is a huge demand for a treatment protocol which can overcome leptin resistance. Animal models of leptin resistant obesity strongly suggest a synergistic interaction between leptin and gut hormones, resulting in decreased food intake and body weight. Leptin enhances the efficacy of gut hormones in reducing food intake and body weight by prolonging the action of gut hormones. This extended effect is mediated by increasing neural activity in a signllaling pathway of gut hormones. It is unknown whether xenin regulates energy balance by interacting with leptin. As mentioned above, we propose

that the metabolic effect of xenin is mediated via the CNS signalling pathway involving Ntsr1, MAPK and IL-1. CNS Ntsr1, MAPK, and IL-1 have been shown to mediate leptin action on metabolism, suggesting that xenin and leptin converge on a common signalling pathway in the CNS. Therefore, it is reasonable to address the hypothesis that xenin and leptin reduce food intake through a synergistic interaction in the present study.

It is expected that the results of the present study will unveil a role for xenin in the regulation of energy homeostasis which has not previously been identified. The long-term effect of xenin on energy balance and an interaction between xenin and leptin are of interest as they suggest a possible strategy for the treatment of different forms of obesity including leptin-resistant obesity.

III. Hypotheses

(1) Long-term treatment with xenin will reduce food intake and body weight, and will increase energy expenditure.

(2) The anorexic effect of xenin will be associated with delayed gastric emptying, but will not be associated with aversive responses.

(3) Xenin will activate hypothalamic MAPK and IL-1 signalling pathways and the anorexic effect of xenin will be mediated through the CNS Ntsr1-MAPK-IL-1 signalling pathway.

(4) Xenin will reduce food intake and body weight through a synergistic interaction with leptin.

IV. Objectives

(1) To determine the effects of long-term xenin treatment on food intake, body weight, metabolic rate, and fat metabolism in mice.

(2) To determine the effect of xenin on gastric emptying rate in mice.

(3) To determine if xenin causes taste aversion in mice.

(4) To determine the brain areas which are activated by xenin in mice.

(5) To determine if xenin activates hypothalamic MAPK signalling and increases IL-1β gene expression in mice.

(6) To determine the effect of Ntsr1-deficiency and IL-1RI-deficiency on xenin-induced anorexia in mice.

(7) To determine the effect of co-treatment with xenin and leptin on food intake and body weight in mice.

V. Materials and Methods

1. Materials

Information on chemicals, buffers, instruments and primers is listed in the following tables (Table 1-5).

Table 1. List of chemicals and companies

Chemical	*Company*
2,2,2-tribromoethyl alcohol	Sigma–Aldrich, St. Louis, MO
Albumin, from bovine serum	Sigma–Aldrich, St. Louis, MO
Angiotensin II	Sigma-Aldrich, St. Louis, MO
Anti-c-Fos (Ab-5) (4-17) Rabbit pAb	Calbiochem, La Jolla, CA
Anti-phospho-p44/42 MAPK (Erk1/2)	Cell Signaling Technology, Danvers, MA
Bupivacaine (Sensorcaine)	AstraZeneca Canada, Mississauga, ON
Buprenorphine (Temgesic)	Schering-Plough Canada, Kirkland, QC
Calcium chloride dihydrate	Sigma–Aldrich, St. Louis, MO
Dental cement	Stoelting, Kiel, WI
D-glucose	Fisher Scientifics, Fair Lawn, NJ
Diaminobenzidine	Sigma–Aldrich, St. Louis, MO
Ethylene glycol, anhydrous	Sigma–Aldrich, St. Louis, MO
Free glycerol determination kit	Sigma–Aldrich, St. Louis, MO
Heparin	Leo Laboratories Canada LTD, Ajax, ON
HEPES	Sigma–Aldrich, St. Louis, MO
Hydrogen peroxide solution, 30% (W/W)	Sigma–Aldrich, St. Louis, MO
Insulin ELISA kit	LINCO Research, St. Charles, MO
Isoflurane	Baxter corporation, Mississauga, ON

Ketamine	Wyeth Animal Health, ON
Leptin	Sigma–Aldrich, St. Louis, MO
Leptin ELISA kit	LINCO Research, St. Charles, MO
Lithium chloride	Sigma–Aldrich, St. Louis, MO
Magnesium sulfate heptahydrate	Fluka, Buchs, Switzerland
Neuromedin U	Phoenix Pharmaceuticals, Burlingame, CA
Neurotensin	Sigma–Aldrich, St. Louis, MO
Paraformaldehyde, prills	Sigma–Aldrich, St. Louis, MO
polyvinylpyrrolidone	Sigma–Aldrich, St. Louis, MO
Potassium chloride	Sigma-Aldrich, St. Louis, Mo
Potassium phosphate monobasic	Sigma–Aldrich, St. Louis, MO
Power SYBR Green PCR Master Mix	Applied Biosystems, Foster City, CA
Saccharin sodium salt hydrate	Sigma–Aldrich, St. Louis, MO
Sodium bicarbonate	Fisher Scientifics, Ottawa, ON
Sucrose	Sigma–Aldrich, St. Louis, MO
SuperScript™ II	Invitrogen, Carlsbad, CA
Taq Man Fast universal PCR Master Mix (2X)	Applied Biosystems, Foster City, CA
tert-amyl alcohol	Sigma–Aldrich, St. Louis, MO
Triton X-100	Fluka, Sigma–Aldrich, St. Louis, MO

TRIzol	Invitrogen, Carlsbad, CA
$RT^2Profiler^{TM}PCR$ Array-Mouse obesity	SABiosciences, Frederick, MD
Urocortin	Phoenix Pharmaceuticals, Belmont, CA
Vectastain Elite ABC kit, rabbit IgG	Vector Laboratories
Xenin	American Peptide Co., Sunnyvale, CA
Xylazine	Bayer Healthcare, ON

Table 2. List of buffers and ingredients

Buffer	*Ingredient*
Artificial cerebrospinal fluid	124 mM NaCl, 26 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 10 mM D-glucose
Avertin	40% of 2,2,2-tribromoethyl alcohol, 40% of tert-amyl alcohol
Cryoprotectant	30% of Sucrose, 1% of Polyvinylpyrrolidone, 30% of Ethylene glycol in 0.1M Phosphate buffer

Table 3. List of instruments and companies

Instrument	*Company*
ABI 7500 thermal cycler	Applied Biosystems, Foster City, CA
Glucometer	ELITE XL, Bayer HealthCare, Mishawaka, IN
Metabolic cage system	AccuScan Instruments, Columbus, OH
Plate reader	Bio-Rad, Hercules, CA
Shandon Cryotome	Thermo Scientific, Waltham, MA
Stereotaxic apparatus	David Kopf Instrument, Tujunga, CA
VersaMax Animal Activity System	AccuScan Instruments, Columbus, OH

Table 4. List of primers used in real-time RCR analysis

Gene	*Accession No.*	*Primer*	*Sequence*	*Exon*
AGRP	NM_007427	Forward	5'-TGACTGCAATGTTGCTGAGTTGTG-3'	3
		Reverse	5'-TTGAAGAAGCGGCAGTAGCACGT-3'	5
β-actin	X03672	Forward	5'-CAGCTTCTTTGCAGCTCCTT-3'	1
		Reverse	5'-TCACCCACATAGGAGTCCTT-3'	3
c-fos	NM_010234	Forward	5'-GACAGCCTTTCCTACTACCATTCC-3'	1
		Reverse	5'-GGACAGATCTGCGCAAAAGTC-3'	2
Cyclophilin	X52803	Forward	5'-AAGCATACAGGTCCTGGCATCT-3'	4
		Reverse	5'-TGCCATCCAGCCATTCAGT-3'	4/5
CPT1b	NM-009948	Forward	5'-GCCGCAAACTGGACCGTGAAG-3'	8
		Reverse	5'-TTGCCTGGGATGCGTGTAGTGTT-3'	9
DGAT2	NM_026384	Forward	5'-TTCCGAGACTACTTTCCCATCCAG-3'	4
		Reverse	5'-ACCAGCCAACGTAGCCAAATAGG-3'	5

HSL	U08188	Forward	5'-ATGAAGGACTCACCGCTGACTT-3'	2
		Reverse	5'-CGGATGGCAGGTGTGAACT-3'	3
IL-1β	NM_008361	Forward	5'-TTGACGGACCCCAAAAGATG-3'	3
		Reverse	5'-TGCTGCTGCGAGATTTGAAG-3'	4
PCG1α	NM_008904	Forward	5'-GCTGAGTCCTTTTGTTCTTGCA-3'	2
		Reverse	5'-GATCTGAAGGCGGACTTTGG-3'	3
POMC	NM_008895	Forward	5'-GCCCTCCTGCTTCAGACCTC-3'	2
		Reverse	5'-CTTCCGGGGGTTTTCAGTCAG-3'	3
UCP1	NM_009463	Forward	5'-GCTGAGTCCTTTTGTTCTTGCA-3'	1
		Reverse	5'-GATCTGAAGGCGGACTTTGG-3'	1/2
UCP3	NM-009464	Forward	5'-CCATAGGCAGCAAAGGAACCAG-3'	2
		Reverse	5’-GTGCCGGCCCCCAGGAACT-3’	2

Table 5. List of primers in RT^{2}Profiler^{TM}PCR Array-Mouse obesity

UniGene	GenBank	Symbol	Description
Mm.3407	NM_009625	Adcyap1	Adenylate cyclase activating polypeptide 1
Mm.44245	NM_007407	Adcyap1r1	Adenylate cyclase activating polypeptide 1 receptor 1
Mm.3969	NM_009605	Adipoq	Adiponectin, C1Q and collagen domain containing
Mm.259976	NM_028320	Adipor1	Adiponectin receptor 1
Mm.291826	NM_197985	Adipor2	Adiponectin receptor 2
Mm.347390	NM_009633	Adra2b	Adrenergic receptor, alpha 2b
Mm.46797	NM_007419	Adrb1	Adrenergic receptor, beta 1
Mm.56995	NM_007427	Agrp	Agouti related protein
Mm.4533	NM_007468	Apoa4	Apolipoprotein A-IV
Mm.119936	NM_009730	Atrn	Attractin
Mm.1442	NM_007540	Bdnf	Brain derived neurotrophic factor
Mm.10687	NM_009766	Brs3	Bombesin-like receptor 3
Mm.19131	NM_009778	C3	Complement component 3
Mm.4361	NM_007587	Calca	Calcitonin/calcitonin-related polypeptide, alpha

Mm.4642	NM_007588	Calcr	Calcitonin receptor
Mm.75498	NM_013732	Cartpt	CART prepropeptide
Mm.2619	NM_031161	Cck	Cholecystokinin
Mm.3521	NM_009827	Cckar	Cholecystokinin A receptor
Mm.21160	NM_025469	Clps	Colipase, pancreatic
Mm.7992	NM_007726	Cnr1	Cannabinoid receptor 1 (brain)
Mm.425178	NM_016673	Cntfr	Ciliary neurotrophic factor receptor
Mm.276736	NM_007754	Cpd	Carboxypeptidase D
Mm.31395	NM_013494	Cpe	Carboxypeptidase E
Mm.1892	NM_007762	Crhr1	Corticotropin releasing hormone receptor 1
Mm.54161	NM_010076	Drd1a	Dopamine receptor D1A
Mm.41970	NM_010077	Drd2	Dopamine receptor 2
Mm.4655	NM_010253	Gal	Galanin
Mm.6219	NM_008082	Galr1	Galanin receptor 1
Mm.45494	NM_008100	Gcg	Glucagon

Mm.22329	NM_008101	Gcgr	Glucagon receptor
Mm.343934	NM_008117	Gh	Growth hormone
Mm.3986	NM_010284	Ghr	Growth hormone receptor
Mm.379095	NM_021488	Ghrl	Ghrelin
Mm.194721	NM_177330	Ghsr	Growth hormone secretagogue receptor
Mm.390969	NM_021332	Glp1r	Glucagon-like peptide 1 receptor
Mm.323523	NM_145132	Mchr1	Melanin-concentrating hormone receptor 1
Mm.20298	NM_175012	Grp	Gastrin releasing peptide
Mm.4687	NM_008177	Grpr	Gastrin releasing peptide receptor
Mm.10096	NM_010410	Hcrt	Hypocretin
Mm.246595	NM_198959	Hcrtr1	Hypocretin (orexin) receptor 1
Mm.333327	NM_008285	Hrh1	Histamine receptor H 1
Mm.391323	NM_008312	Htr2c	5-hydroxytryptamine (serotonin) receptor 2C
Mm.415	NM_010491	Iapp	Islet amyloid polypeptide
Mm.15534	NM_010554	Il1a	Interleukin 1 alpha

Mm.222830	NM_008361	Il1b	Interleukin 1 beta
Mm.896	NM_008362	Il1r1	Interleukin 1 receptor, type I
Mm.1019	NM_031168	Il6	Interleukin 6
Mm.2856	NM_010559	Il6ra	Interleukin 6 receptor, alpha
Mm.46269	NM_008386	Ins1	Insulin I
Mm.4946	NM_008387	Ins2	Insulin II
Mm.268003	NM_010568	Insr	Insulin receptor
Mm.277072	NM_008493	Lep	Leptin
Mm.259282	NM_010704	Lepr	Leptin receptor
Mm.57183	NM_008561	Mc3r	Melanocortin 3 receptor
Mm.22246	NM_026523	Nmb	Neuromedin B
Mm.425622	NM_008703	Nmbr	Neuromedin B receptor
Mm.154879	NM_019515	Nmu	Neuromedin U
Mm.389159	NM_010341	Nmur1	Neuromedin U receptor 1
Mm.154796	NM_023456	Npy	Neuropeptide Y

Mm.5112	NM_010934	Npy1r	Neuropeptide Y receptor Y1
Mm.129481	NM_008173	Nr3c1	Nuclear receptor subfamily 3, group C, member 1
Mm.130054	NM_008745	Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2
Mm.64201	NM_024435	Nts	Neurotensin
Mm.301712	NM_018766	Ntsr1	Neurotensin receptor 1
Mm.7977	NM_011011	Oprk1	Opioid receptor, kappa 1
Mm.390248	XM_001052051	Oprm1	Opioid receptor, mu 1
Mm.425181	NM_011014	Oprs1	Opioid receptor, sigma 1
Mm.277996	NM_008895	Pomc1	Pro-opiomelanocortin-alpha
Mm.212789	NM_011144	Ppara	Peroxisome proliferator activated receptor alpha
Mm.3020	NM_011146	Pparg	Peroxisome proliferator activated receptor gamma
Mm.259072	NM_008904	Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Mm.377241	NM_201615	Prlhr	Prolactin releasing hormone receptor
Mm.277916	NM_011201	Ptpn1	Protein tyrosine phosphatase, non-receptor type 1
Mm.46248	NM_145435	Pyy	Peptide YY

Mm.39884	NM_019511	Ramp3	Receptor (calcitonin) activity modifying protein 3
Mm.157119	NM_019972	Sort1	Sortilin 1
Mm.2453	NM_009215	Sst	Somatostatin
Mm.308647	NM_009217	Sstr2	Somatostatin receptor 2
Mm.376100	NM_009380	Thrb	Thyroid hormone receptor beta
Mm.1293	NM_013693	Tnf	Tumor necrosis factor
Mm.1363	NM_009426	Trh	Thyrotropin releasing hormone
Mm.377116	NM_021290	Ucn	Urocortin
Mm.4177	NM_009463	Ucp1	Uncoupling protein 1 (mitochondrial, proton carrier)
Mm.290924	XM_984218	Zfp91	Zinc finger protein 91

2. Methods

2.1. Animals

Male C57BL/6 mice were obtained from Charles River Laboratories (Montreal, QC, Canada). Male interleukin 1 type I receptor knockout mice (IL-1RI$^{-/-}$, C57BL/6J background) and wild-type C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME, US). In the neurotensin receptor 1 (Ntsr1) knockout mouse study, a pair of *Ntsr1*$^{-/-}$ mice was purchased from The Jackson Laboratories. Homozygous *Ntsr1*$^{-/-}$ mice were produced by breeding homozygous male and female *Ntsr1*$^{-/-}$ mice in our animal facility. Genotypes were confirmed by PCR using primers, 5'-CCAAGCGGCTTCGGCCAGTAACGTT-3' (forward primer to detect a targeted allele), 5'-CTCTAATGTGCCACAGCTCAGAGAG-3' (forward primer to detect a wild-type allele), and 5'-CAGCAACCTGGACGTGAACACTGAC-3' (common reverse primer). Only male mice aged at 8-24 weeks were used in the present study. Mice were individually housed under a 12 h light-dark cycle (lights on at 0600 h) throughout the experiments. The mice had free access to standard rodent chow pellets (Prolab RMH 3000, 14%, 60%, and 26% of calories from fat, carbohydrate, and protein, respectively; Ralston Purina) and water throughout the experiment except during fasting. Water was supplied with free access throughout the experiment except for the conditioned taste aversion test. All procedures were performed in accordance with approval by The University of Manitoba Protocol Management and Review Committee.

2.2. Multiple intraperitoneal (i.p.) injection study

Male C57BL/6 mice were handled and injected i.p. with saline once daily between 1730 h and 1800 h for 6 days prior to the start of the experiment to acclimatize the mice to the injection protocol. Food intake and body weight were measured every day during the acclimatization period. Although i.p. injection caused reductions in food intake and body weight in some mice, food intake and body weight returned to the pre-injection level by the third day of the acclimatization period. Mice were divided into two experimental groups. Control group received daily i.p. injections of saline for 6 days. The other group of mice received daily i.p. injections of xenin (50 µg/g b.w.) between 1730 h and 1800 h for 6 days. Food intake and body weight were measured just before the injection every day.

2.3. Multiple intracerebroventricular (i.c.v.) injection study

Male C57BL/6 mice were stereotaxically implanted with the chronic cannula in the lateral ventricle as described below. After recovery from the surgery, mice received once daily i.c.v. injections of artificial cerebrospinal fluid (aCSF) (Table 2) for 3 days prior to the experiment to acclimatize the mice to the i.c.v. injection protocol. The mice were then divided into two groups. Control group received daily i.c.v. injections of aCSF and the other group received daily i.c.v. injections of xenin (5 µg) for 10 days. The drugs were injected in a volume of 1 µl to avoid diffusion of the injected solution between 1700 h and 1800 h every day. After injection, the injection pipe remained in place for an additional 30 sec to minimize backflow of the drug. Food intake and body weight were measured just before injection every day throughout the experiment. At the end of the experiment, the

mice were sacrificed by CO_2 narcosis followed by decapitation 16 h after the eleventh and final injection. Blood and tissues (white and brown adipose tissues and skeletal muscle) were collected, immediately frozen on dry ice and kept at -80˚C until used for mRNA analysis. Sera were separated and stored at -80˚C until assayed for metabolites and hormones.

2.4. Metabolic cage study

To determine the effect of xenin on energy expenditure, metabolic rates were measured in xenin-treated mice using indirect calorimetry (PhysioScan Metabolic System, AccuScan Instruments Inc., Columbus, OH). Male C57BL/6 mice were kept in the metabolic cage and injected i.p. with saline once daily between 1730 h and 1800 h for 7 days prior to the start of the experiment to familiarize the mice with the metabolic cage system and the injection protocol. After the acclimatization period, mice were divided into two groups. Control mice received daily i.p. saline injections for 7 days. The other group of mice received daily i.p. injections of xenin (50 μg/g b.w.) for 7 days. The dose of xenin reduced food intake and body weight in multiple i.p. injection study. Food intake and body weight were measured daily throughout the experiment. Concentrations of oxygen and carbon dioxide in the metabolic cage were continuously measured throughout the 14-day experiment (7-day acclimatization period and 7-day treatment period) except between 1700 h and 1800 h. These data were used to calculate oxygen consumption (VO_2), production of carbon dioxide (VCO_2), heat production, and respiratory quotient (RQ). Metabolic cages were opened and food weight and body weight were measured and cages were cleaned between 1700 h and 1800 h every day. I.p. injections were made between 1730 h and 1800

h, and mice were immediately returned in the metabolic cage. Horizontal activity levels were simultaneously measured by counting the number of interruptions that occurred in the horizontal sensor using VersaMax Animal Activity System (AccuScan Instruments Inc.). Metabolic cage and activity data were collected in 5- or 12-min bins using the IntegraME software (Ver. 1.71, AccuScan Instruments Inc.). Hourly and daily averages of VO_2, VCO_2, heat production, RQ and horizontal activity were further calculated and used for statistical analyses. Averages of RQ during the dark cycle and light cycle were also calculated. At the end of experiment, mice were sacrificed by CO_2 narcosis followed by decapitation. Blood and tissues (white adipose and brown adipose tissues) were collected, immediately frozen on dry ice and kept at -80˚C until used for mRNA analysis. Sera were separated and stored at -80˚C until assayed for metabolites and hormones.

2.5. Conditioned taste aversion test

To determine if xenin causes aversive effects, we performed conditioned taste aversion tests. The protocol of conditioned taste aversion test was modified from previously reported methods [174, 282] (Fig. 3). Male C57BL/6 mice were adapted to a water-restriction schedule, in which they received two water bottles for 7 h (0930-1630 h) per day for 2 weeks. Total water intake for 30 min (0930-1000 h) from both bottles was measured. Under the water-restriction schedule, mice were highly motivated to drink water during the first 30 min after presentation of water. Daily food intake and body weight were stable after the 4th day of the training. On the day of conditioning, mice were given two

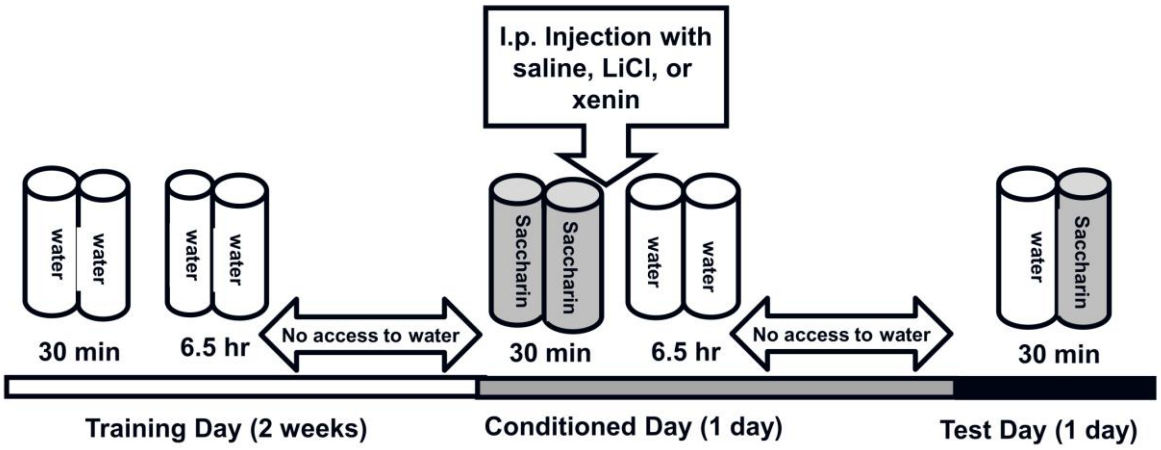


Figure 3. Treatment scheme of the conditioned taste aversion test. Mice were adapted to a restricted water schedule. They received two water bottles for 30 min (0930 h-1000 h) and total water intake from both bottles was measured. Mice were allowed free access to water for additional 6.5 h (1000 h-1630 h) and then the water bottles were removed from the cage for overnight (1630 h-0930 h). Mice were on the restricted water schedule for 2 weeks. On the day of conditioning, mice were given 2 bottles of 0.15% saccharin solution during the 30-min drinking session. Mice received i.p. injection of saline, LiCl, or xenin at the end of the drinking session. Mice were then given water for 6.5 h (1000 h-1630 h) followed by no access to water for overnight. On the test day, mice were given both saccharin solution and water for 30 min (0930 h-1000 h). Water and saccharin solution intake during the 30-min drinking session was measured.

bottles containing 0.15% saccharin solution at 0900 h instead of water. Mice were injected i.p. with xenin (50 μg/g b.w.) or saline at the end of the 30-min saccharin drinking period. LiCl (0.3 mol/l, 2% b.w., i.p.) was used as a positive control, because LiCl is known to be a strong inducer of taste aversion. Mice were then given two bottles of water for the remaining 6.5 h (1000-1630 h). On the next day, mice were given a choice of two bottles containing either 0.15% saccharin solution or water for 30 min (0930-1000 h). Consumption of saccharin solution and water was measured. Total fluid intake was the sum of the water and saccharin solution. Health condition of the mice under the restricted water regimen was monitored by observing their fur and skin condition, water and food intake, and body weight. No health problem including dehydration was observed.

2.6. Measurement of gastric emptying rate

Gastric emptying rate was determined by the method reported previously [283]. Male C57BL/6 mice were fasted overnight by removing food from the cage before lights out. Next morning pre-weighed pellets were provided to mice for 1 h from 0930 h to 1030 h and 1-h food intake was measured. Mice received i.p. injection of saline or xenin (50 μg/g b.w.) at the end of the 1-h re-feeding period. One group of mice received i.p. injection of urocortin (3 nmol/mouse) as a positive control, because this dose of urocortin is known to delay gastric emptying in mice [226]. Mice did not have access to food after the injection. Mice were sacrificed by exposing to CO_2 2 h post-injection. The stomach was removed after ligation of both pylorus and cardia. The weight of stomach and the wet content of the stomach were weighed. Gastric emptying rate was calculated by the formula: Gastric emptying (%) = {1-(wet weight of food recovered from the stomach/wet

weight of food intake)} × 100. The wet weight of food intake was calculated by the following formula: Wet weight of food intake = A × (B/C), A = dry weight of food intake, B = average wet weight of gastric content after 1-h feeding, C = average dry weight of food intake after 1-h feeding. B and C were determined in control mice. To estimate gastric distension before i.p. injection of drugs, food intake (g) during the 1-h feeding period was normalized to stomach weight (g).

2.7. Hypothalamic gene expression study

To determine whether xenin activates hypothalamic neurons, we examine the effect of xenin treatment on hypothalamic expression of the immediate early gene *c-fos*, as a marker of cell activation. Male C57BL/6 mice were fasted overnight and injected i.p. with saline or xenin (50 μg/g b.w.) at 1000 h. Mice were killed by CO_2 narcosis followed by decapitation 30 and 90 min after the injection. Mice did not have access to food after the injection. The brain was quickly removed and the hypothalamus was dissected out using the lateral edges of the optic tract and the dorsal edge of the third ventricle as landmarks, as defined by Paxinos and Franklin [284]. The dissected tissues were immediately frozen on dry ice, and stored at -80˚C until RNA analysis. Hypothalamic *c-fos* mRNA levels were determined by real-time PCR as described below. The same hypothalamic RNA samples were also used to identify possible downstream mediators of xenin action. Xenin-induced changes in hypothalamic gene expression were analyzed by PCR array as described below.

2.8. Hypothalamic and brainstem c-Fos expression study

Mice were acclimatized to handling and the i.p. injection protocol by receiving i.p. saline injection once daily for 8 days prior to the experiment. Mice were fasted for 6 h during the early light cycle and injected i.p. with saline or xenin (50 µg/g b.w.) at 1400 h. The mice were anesthetized with an i.p. injection of avertin (8 mg/g b.w.) 2 h post-injection. After opening the thoracic cavity, the heart was exposed and mice were transcardially perfused with 2-3 ml of ice-cold 0.1M phosphate buffer containing 1 U/ml heparin followed by 40 ml of 4% paraformaldehyde. Mice were decapitated, brains were removed and brain blocks were prepared. The brain blocks were post-fixed in 4% paraformaldehyde solution for 5 h at room temperature. The brain blocks were then stored in 0.1M phosphate buffer containing 10% sucrose at 4˚C until sectioning. Coronal sections (30 µm) were cut on a cryostat and collected free-floating in cryoprotectant (Table 2) and stored at -20˚C until performing immunohistochemistry. Sections were made throughout the hypothalamus (from 0.10 to 2.80 mm caudal to bregma) and the brainstem (from 6.00 to 8.00 mm caudal to bregma) [284].

2.9. Hypothalamic extracellular-signal-regulated kinases (ERK) expression study

Male C57BL/6 mice were handled and injected i.p. with saline once daily for 3 days prior to the experiment to familiarize the mice with the injection protocol. Mice were fasted for 6 h (0630-1230 h) and injected i.p. with saline or xenin (50 µg/g b.w.) at 1230 h. Fifteen minutes later the mice were deeply anesthetized with i.p. injection of avertin (8 mg/g b.w.) and perfused transcardially with 4% paraformaldehyde and coronal brain

sections (30 μm) were prepared as described above. Cells positive for phosphorylated ERK1/2 (pERK1/2) were visualized by immunohistochemistry as described below.

2.10. Xenin treatment in IL-1RI$^{-/-}$ mice

To determine the effect of IL-1RI deficiency on xenin-induced feeding suppression, the effect of i.p. xenin administration on food intake was compared between wild-type (IL-1RI$^{+/+}$) and IL-1RI-deficient (IL-1RI$^{-/-}$) mice. Male IL-1RI$^{+/+}$ and IL-1RI$^{-/-}$ mice (both C57BL/6J background) were fasted overnight and injected i.p. with saline, xenin (7.5, 15 or 50 μg/g b.w.) or leptin (0.85 or 1.2 μg/g b.w.) between 1000 h and 1100 h. Pre-weighed food pellets were provided to the mice immediately after injection and cumulative food intake was measured hourly up to 8 h after injection and 24 h after injection. Body weight was measured immediately before and 24 h after injection.

2.11. Xenin treatment in *Ntsr1*$^{-/-}$ mice

To determine the effect of Ntsr1 deficiency on xenin-induced feeding suppression, the effect of i.c.v. xenin administration on food intake was compared between wild-type (*Ntsr1*$^{+/+}$) and Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice. Male *Ntsr1*$^{+/+}$ and *Ntsr1*$^{-/-}$ mice (both C57BL/6J background) were implanted with the i.c.v. cannula in the lateral ventricle as described below. After recovery from the surgery, mice received once daily i.c.v. injection of aCSF for 3 days to acclimatize the mice to the i.c.v. injection protocol. Mice were scheduled to receive four injections at specific time points during the study. The mice were fasted for 16.5 h prior to each injection, and a resting interval of 7-10 days was provided

following the injections. For the first three injections, each mouse received an i.c.v. injection of aCSF, xenin (0.33 nmol), and neurotensin (0.33 nmol) in a random order. For the fourth and final injection, all mice were treated with neuromedin U (NMU, 3 nmol). Drugs were injected in a total volume of 1 µl over 30 s between 1000 h and 1100 h. Pre-weighed food pellets were provided to the mice immediately after injection and cumulative food intake was measured 1, 2, 3, 4, 8 and 24 h after injection. Body weight was measured immediately before and 24 h after injection.

2.12. Leptin treatment in *Ntsr1*$^{-/-}$ mice

To determine the effect of Ntsr1 deficiency on leptin-induced feeding suppression, the effect of i.c.v. leptin administration on food intake was compared between wild-type (*Ntsr1*$^{+/+}$) and Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice. Male *Ntsr1*$^{+/+}$ and *Ntsr1*$^{-/-}$ mice (both C57BL/6J background) were implanted with the i.c.v. cannula in the lateral ventricle as described below. Mice were fed *ad libitum* and injected i.c.v. with leptin (5 µg) or aCSF just before lights out. Pre-weighed food pellets were provided to mice immediately after injection. Cumulative food intake and body weight was measured 16 and 24 h after injection.

2.13. Co-injection of xenin and leptin

Male C57BL/6 mice were fasted overnight and injected i.p. with either saline, xenin (2.5 µg/g b.w.) alone, leptin (0.25 µg/g b.w.) alone, or xenin (2.5 µg/g b.w.) plus leptin (0.25 µg/g b.w.) between 1000 h and 1100 h. Pre-weighed food pellets were provided to

mice immediately after injection. Cumulative food intake was measure hourly up to 8 h after injection.

In a separate study, *ad libitum* fed male C57BL/6 mice were handled and injected i.p. with saline once daily between 0930 h and 1000 h for 3 days prior to the start of the experiment to acclimatize the mice to the injection protocol. Food intake and body weight were measured between 0900 h and 1000 h every day throughout the acclimatization and treatment periods. Mice were divided into four experimental groups: (1) saline, (2) xenin (2.5 µg/g b.w.) alone, (3) leptin (0.25 µg/g b.w.) alone, and (4) xenin (2.5 µg/g b.w.) plus leptin (0.25 µg/g b.w.). The drugs were injected i.p. twice a day for 2 days. The first and third injections were made between 0930 h and 1000 h and the second and fourth (final) injections were made between 1730 h and 1800 h. Food intake and body weight were measured 24 and 48 h after the first injection.

2.14. Intracerebroventricular (i.c.v.) cannulation

Mice were anesthetized with i.p. injection of a mixture of ketamine (100 mg/kg b.w.) and xylazine (10 mg/kg b.w.) or by exposure to isoflurane in an induction chamber. After induction, the delivery of isoflurane (1-1.25%) was maintained at a constant flow rate (0.6 ml/min) throughout the surgery. The surgical site was sterilized with chlorhexidine followed by 70% ethyl alcohol. A topical anesthetic bupivacaine (1 mg/kg b.w.) was applied to the skin before making an incision. After making a midline sagittal incision about 1.5-cm long slightly behind the eyes, the periosteal connective tissue was removed. The bregma was identified as a reference point, and location of lateral ventricle was

determined for implanting a cannula using stereotaxic coordinates. A stainless steel guide cannula (o.d.: 0.64 mm, i.d.: 0.33 mm) was stereotaxically implanted into the lateral ventricle with the following 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 [284]. The cannula was fixed to the skull with dental cement and stainless steel screws. Mice were returned to the home cage and were periodically monitored until they recovered from the anesthesia. The mice received 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 behaviour responses to the i.c.v. administration of angiotensin II (100-150 ng/μl). Drinking behaviour was observed for 10 min after the injection and the number of licking the water bottle was counted. Only mice responding with a robust drinking behaviour were used for the experiment.

2.15. RNA analysis

Total RNA was extracted from hypothalamus, brown adipose tissue, white adipose tissue and skeletal muscle using TRIzol reagent. cDNA was synthesized using SuperScript^{TM} II reverse transcriptase from 5 μg of total RNA. Target gene expression levels were measured by real-time PCR using the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA). Primers used in the present study were designed using the Primer Express Software (Ver. 3.0, Applied Biosystems). PCR reactions were run in a 25 μl reaction containing SYBR green (Table 1), primer mixture (final concentration: 0.2 mmol/L, Table 4) and 5 μl of diluted cDNA (1:30-1:100) for 40 cycles at 94 ˚C for 15 s; 60˚C for 20 s and 72 ˚C for 30 s. Data were analyzed by the ΔΔCt method using ABI7500

Fast System SDS software package (Ver. 1.3.1., Applied Biosystems) and mRNA levels were normalized to β-actin or cyclophilin mRNA levels [285]. All reactions were performed in triplicates and the coefficient of variation was less than 2% for each triplicate.

2.16. PCR array

Hypothalamic RNA samples from the hypothalamic *c-fos* mRNA expression study were used for PCR array study. For each group (saline and xenin-treated groups), 7 individual total RNA samples were pooled and used for PCR array analysis to screen xenin-induced changes in obesity-related genes. Expression levels of 84 obesity-associated genes (Table 5) in the hypothalamus were compared between saline- and xenin-treated groups by using a commercial PCR array kit ($RT^2Profiler^{TM}$PCR Array-Mouse obesity, PAMM-017C). Real-time PCR was performed according to the manufacturer's instructions. Data were analyzed using ΔΔCt method and fold changes of xenin-treated group were calculated. Because the overall average standard deviation across the entire PCR array is 0.25 cycles, the PCR array analysis reliably detects greater than 2-fold changes in gene expression. Therefore, a greater than 2-fold increase or a greater than 50% decrease in expression was used as criteria for a meaningful change in gene expression in the present PCR array study. Because the PCR array analysis was performed by using pooled cDNA samples without replication, intra-group variations might be large and might have outweighed inter-group variations. Therefore, some genes were selected, which are up regulated in the result of the PCR array and expressed in a hypothalamus. We performed conventional real-time PCR using larger numbers of individual unpooled cDNA samples (n = 7/group) to verify the results of the PCR array analysis.

2.17. Immunohistochemistry (IHC)

Cryosections were washed in 1X PBS for 3 h at room temperature. Sections were incubated with a polyclonal rabbit anti- Fos (Ab-5, 1:20,000 dilution) or anti-pERK1/2 specific antibody (polyclonal rabbit anti-pERK1/2, 1:200 dilution) in 1X PBS containing 0.3% Triton X-100 overnight at room temperature. Sections were washed three times for 1 h in 1X PBS followed by a 1-h incubation with a biotinylated goat anti-rabbit IgG (Vector Laboratories, 1:200 dilution) in 1X PBS containing 0.3% Triton X-100 at room temperature for 1 h. The sections were then incubated with avidin-peroxidase complex (Elite ABC kit, rabbit IgG) at room temperature for 1 h. Immunoreactive cells were visualized with 0.1% 3, 3'-diaminobenzidine in 0.1 M Tris (pH 7.4) with 0.0025% H_2O_2 at room temperature. Sections were washed three times for 15 min in 1X PBS, mounted on slides followed by drying overnight and dehydration and coverslipping with VectaMount Permanent Mounting Medium (Vector Laboratories).

For the analysis of hypothalamic c-Fos expression, IHC was performed every fifth tissue section throughout the anterior-posterior length of the hypothalamus covering the paraventricular nucleus (PVN), the ventromedial nucleus (VMN), the arcuate nucleus (ARC), the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN) and the supraoptic nucleus (SON). For the analysis of brainstem c-Fos expression, 2 sections were processed for IHC to cover the nucleus of the solitary tract (NTS), the area postrema (AP), and dorsal motor nucleus of the vagus (DMV). One section was at 7.3 mm and the other section was at 7.5 mm caudal to the bregma, respectively. For the analysis of hypothalamic

pERK1/2-immunoreactive cells, 8 sections per animal covering the ARC were processed for immunohistochemistry.

2.18. Histological quantification

To perform histological analysis and quantification, sections were viewed at 4X magnification under a microscope and images were captured using a Neurolucida image analysis system (MicroBrightField, Colchester, VT). The same scanning parameters were used for all images taken from sections involving comparisons between treatment groups. Numbers of Fos-immunoreactive cells were counted in the PVN, VMN, ARC, LHA, DMN and SON in 2-6 sections per animal. Numbers of Fos-immunoreactive cells were counted in the NTS, AP and DMV in 2 sections per animal. Cell count was performed, at least, 3 times per section in a blinded fashion. The sum of Fos-immunoreactive cells on both sides of the brain was calculated. The total number of Fos-positive cells per animal was calculated by adding the sum of all sections for each brain region, respectively, and used for statistical analysis. Numbers of pERK1/2-immunoreactive cells in the ARC were counted in 8 sections per animal and total number of pERK1/2-positive cells was calculated by the same method as described above.

2.19. Blood chemistry

Blood glucose levels were measured immediately after decapitation using a glucometer (Table 3). For other assays, sera were separated from clotted blood by centrifugation at 13,000 rpm for 10 min. The serum samples were frozen and stored at -

80˚C until assay. Serum levels of insulin and leptin were determined by direct sandwich method of enzyme-linked immunosorbent assay (ELISA) using commercial kits, according to the manufacturer's instruction. The lowest detection limit of the assay was 0.2 ng/ml for both insulin and leptin ELISA. Serum NEFA concentrations were determined by colorimetric methods using commercial kits (Wako Chemicals, Richmond, VA). All assays were run in duplicate.

2.20. Statistical analysis

Data were shown in mean ± S.E.M. For analysis of significant differences between groups, data were initially analyzed by one-way or two-way analysis of variance (ANOVA). Comparisons between specific groups were performed either by Dunnett's test or Tukey-Kramer test. Comparisons between two groups were done by Student's *t*-test. When the data were not normally distributed, a non-parametric Kruskal-Wallis test or a Wilcoxon test was used. Significant differences were considered if P values were < 0.05.

VI. Results

1. Effects of daily i.p. or i.c.v. injections of xenin on food intake and body weight in mice

To determine the long-term effects of xenin on food intake and body weight, mice were treated by daily i.p. or i.c.v. injections of xenin for 6 days or 10 days, respectively. Daily i.p. xenin treatment significantly reduced food intake compared to control saline treatment on day 1 and day 2 of the treatment. The effect of xenin on food intake was no longer significant after day 3 (Fig. 4A). There was no significant difference in 6-day cumulative food intake between the groups (saline: 23.4 ± 0.8 g, xenin: 21.9 ± 0.5 g, P = 0.15, Student's *t*-test). Cumulative body weight change was significantly lower in xenin-treated group compared to the control group throughout the experimental period except for day 2 and 5 (Fig. 4B). Daily i.c.v. injections of xenin caused similar effects on food intake and body weight in mice. Although daily i.c.v. injections of xenin caused a trend of reduction in food intake, the effect of xenin treatment on food intake did not reach statistical significance (main effect of treatment on food intake: F (1, 100) = 3.10, P = 0.08, two-way ANOVA, Fig. 5A). There was no difference in 10-day cumulative food intake between the groups (aCSF: 45.9 ± 2.7 g, xenin: 40.8 ± 3.3 g, P = 0.22, Student's *t*-test). In contrast, the i.c.v. xenin treatment significantly reduced cumulative body weight change compared to the control group throughout the experimental period except for day 9 (Fig.

5B). There was no difference in serum levels of insulin, leptin and NEFA between the groups (Table 6).

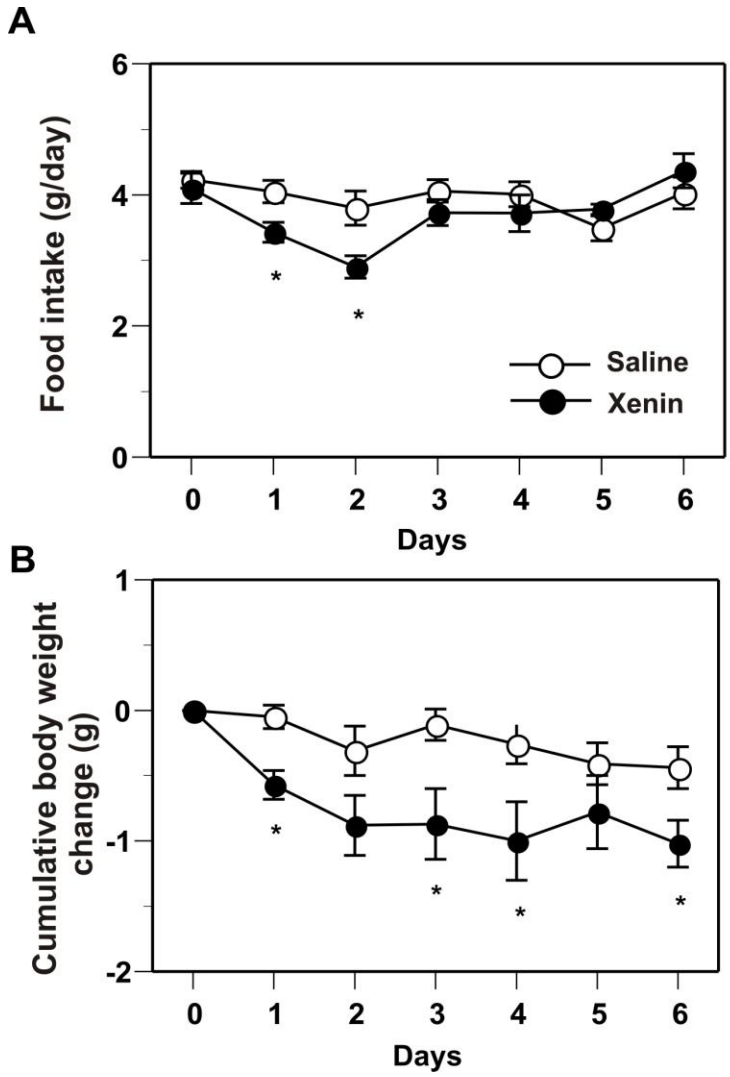


Figure 4. Reductions of food intake and body weight in mice injected i.p. with xenin. Mice received daily i.p. injections of saline or xenin (50 μg/g b.w.) for 6 days. Daily food intake (A) and cumulative body weight change (B) were measured. Data are means ± SEM (n = 6-8/group). *: $P < 0.05$ (vs. control at same time point, Student's *t*-test).

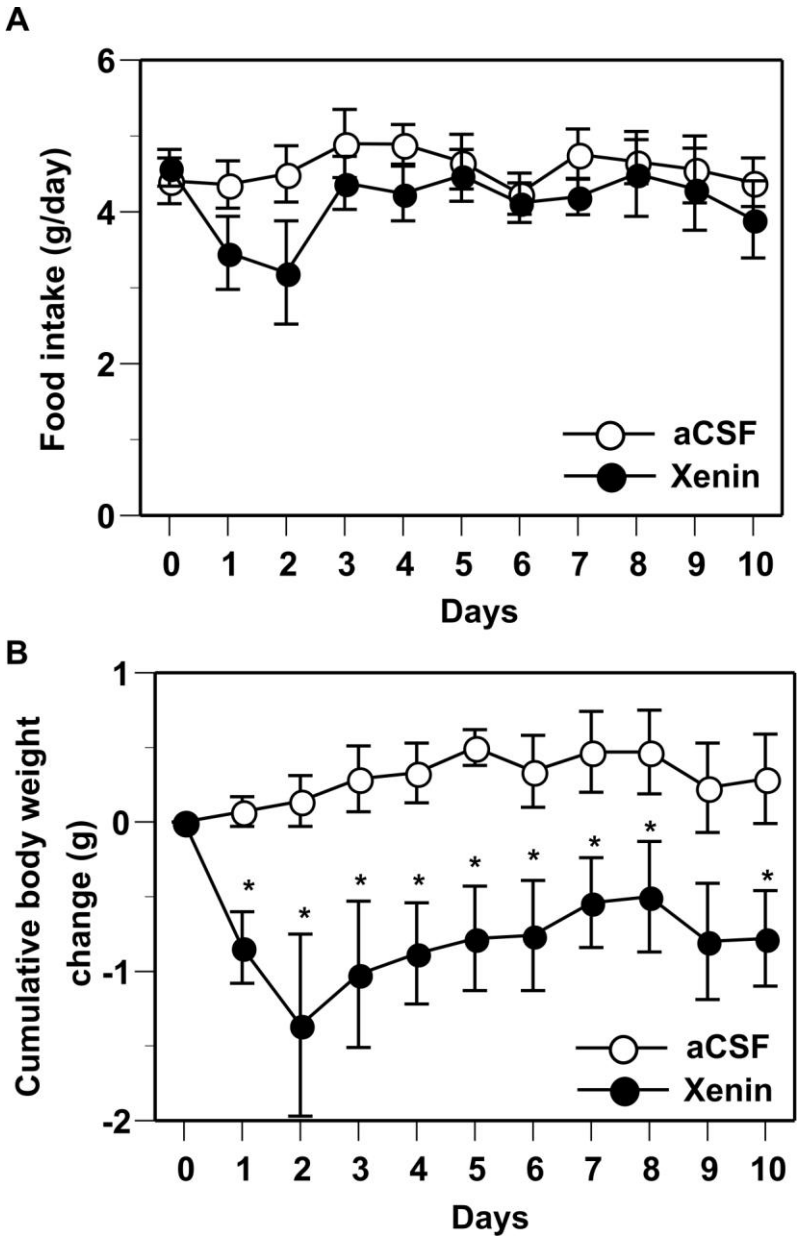


Figure 5. Reduction of body weight in mice injected i.c.v. with xenin. Mice received daily i.c.v. injections of aCSF or xenin (5 μg) for 10 days. Daily food intake (A) and cumulative body weight change (B) were measured. Data are means ± SEM (n = 5-7/group). *: $P < 0.05$ (vs. control at same time point, Student's *t*-test).

Table 6. Effect of i.c.v. xenin treatment on blood levels of hormones and metabolite

	aCSF (n = 4-5)	Xenin (n = 6-7)	P^*
Serum insulin (ng/ml)	1.82 ± 0.34	1.41 ± 0.43	0.42
Serum leptin (ng/ml)	3.73 ± 0.88	3.21 ± 0.35	0.84
Serum NEFA (mEq/L)	0.57 ± 0.11	0.78 ± 0.18	0.27

Values are means ± SEM.

*Differences between groups were analyzed by Student's *t*-test.

2. Effects of daily i.p. injection of xenin on energy expenditure in mice

To determine whether xenin-induced reduction in body weight is associated with increased energy expenditure, metabolic rates were measured in mice receiving daily i.p. injection of xenin for 7 days. Consistent with the previous daily i.p. injection study, xenin-treated mice maintained significantly lower body weight gain compared to control saline-treated mice (Fig. 6A). Cumulative food intake over a 7-day treatment period was not statistically different between the groups (saline: 30.0 ± 1.1 g, xenin: 27.6 ± 0.8 g, $P = 0.09$, Student's *t*-test). Daily i.p. injection of xenin significantly reduced epididymal fat pad weight by 29% compared to control saline treatment (Fig. 6B). Xenin treatment did not cause any significant changes in levels of circulating glucose, insulin, leptin and NEFA (Table 7).

Two-way ANOVAs showed no significant main effect of xenin treatment on VO_2 [$F(1, 78) = 0.02$, $P = 0.90$], VCO_2 [$F(1, 78) = 0.18$, $P = 0.68$], and heat production [$F(1, 78) = 0.004$, $P = 0.95$] (Fig. 7A-C). There was no difference in activity levels between control and xenin-treated mice [$F(1, 78) = 0.05$, $P = 0.82$, two-way ANOVA]. A large reduction of the activity between day 0 and 1 in both groups would not affect the result of comparison of the levels between saline and xenin treated mice. Mice from both groups presented similar activity levels during 7 days acclimation period (Fig. 7D).

To determine whether xenin-induced reduction in body weight is associated with alterations in substrate utilization, respiratory quotient (RQ) was calculated in the same

experiment. Xenin treatment significantly reduced daily average RQ during the first 2 days of the treatment compared to the control group (Fig. 8A). Xenin-induced reductions in RQ were significant during the dark phase, while RQ was not significantly different between the groups during the light phase (data from day 2, Fig. 8B). RQ during a 24-h period exhibited a diurnal fluctuation with higher levels during the dark cycle than during the light cycle in control mice (Fig. 8B). RQ was increased by 4.1-7.0% during the dark cycle compared to the light cycle in control mice (Fig. 8C). In contrast, the nocturnal increase in RQ was not observed throughout the treatment period in xenin-treated mice (Fig. 8C).

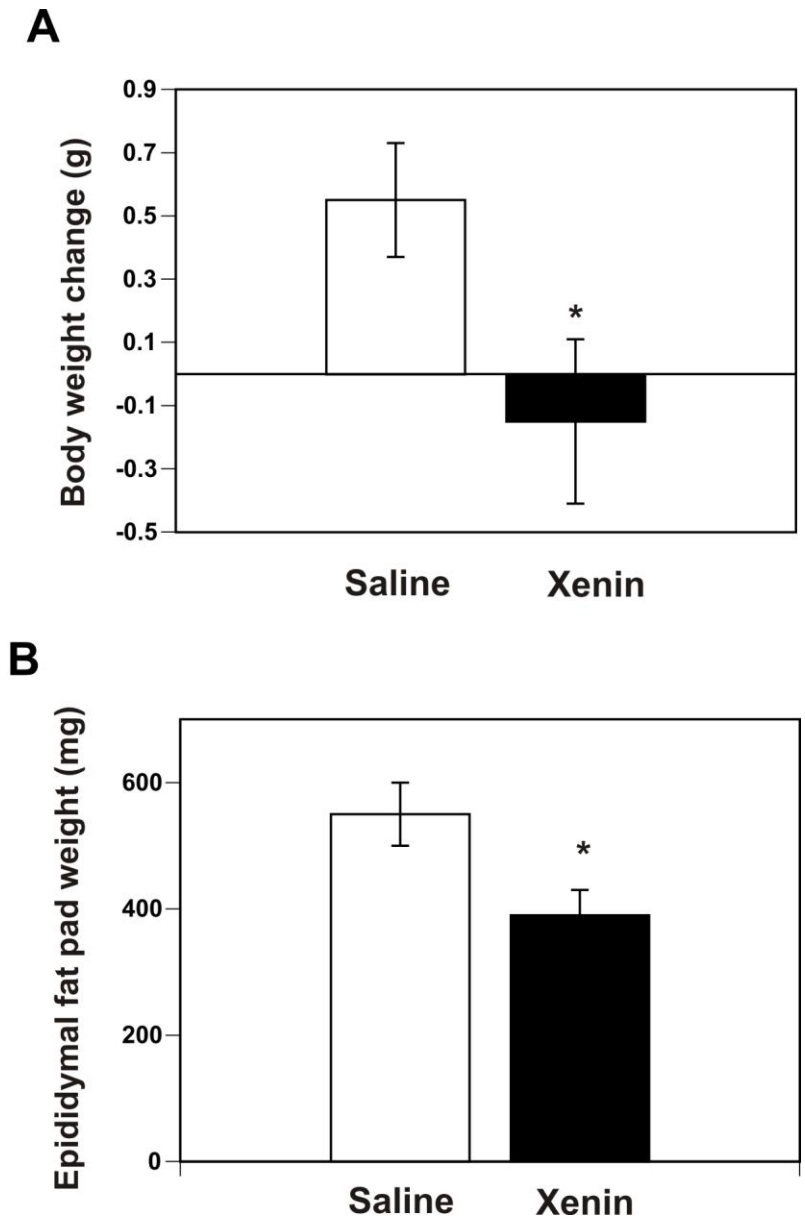


Figure 6. Reductions of body weight and fat pad weight in mice injected i.p. with xenin. Mice received daily i.p. injections of saline or xenin (50 μg/g b.w.) for 7 days. Cumulative 7-day body weight change was measured (A). Mice were sacrificed at the end of the 7-day treatment period and epididymal fat pad was removed and weighed (B). Data are means ± SEM (n = 11/group). *: Statistically significant differences vs. saline-injected control group ($P < 0.05$, Student's t-test).

Table 7. Effect of daily i.p. xenin treatment for 7 days on blood levels of hormones and metabolites

	Saline (n = 10-11)	Xenin (n =8-11)	P^*
Blood glucose (mg/dl)	170.8 ± 14.9	143.2 ± 5.7	0.10
Serum insulin (ng/ml)	1.32 ± 0.26	0.95 ± 0.08	0.21
Serum leptin (ng/ml)	6.43 ± 0.73	5.34 ± 1.55	0.18
Serum NEFA (mEq/L)	0.57 ± 0.11	0.69 ± 0.12	0.43

Values are means ± SEM.

*Differences between groups were analyzed by Student's *t*-test.

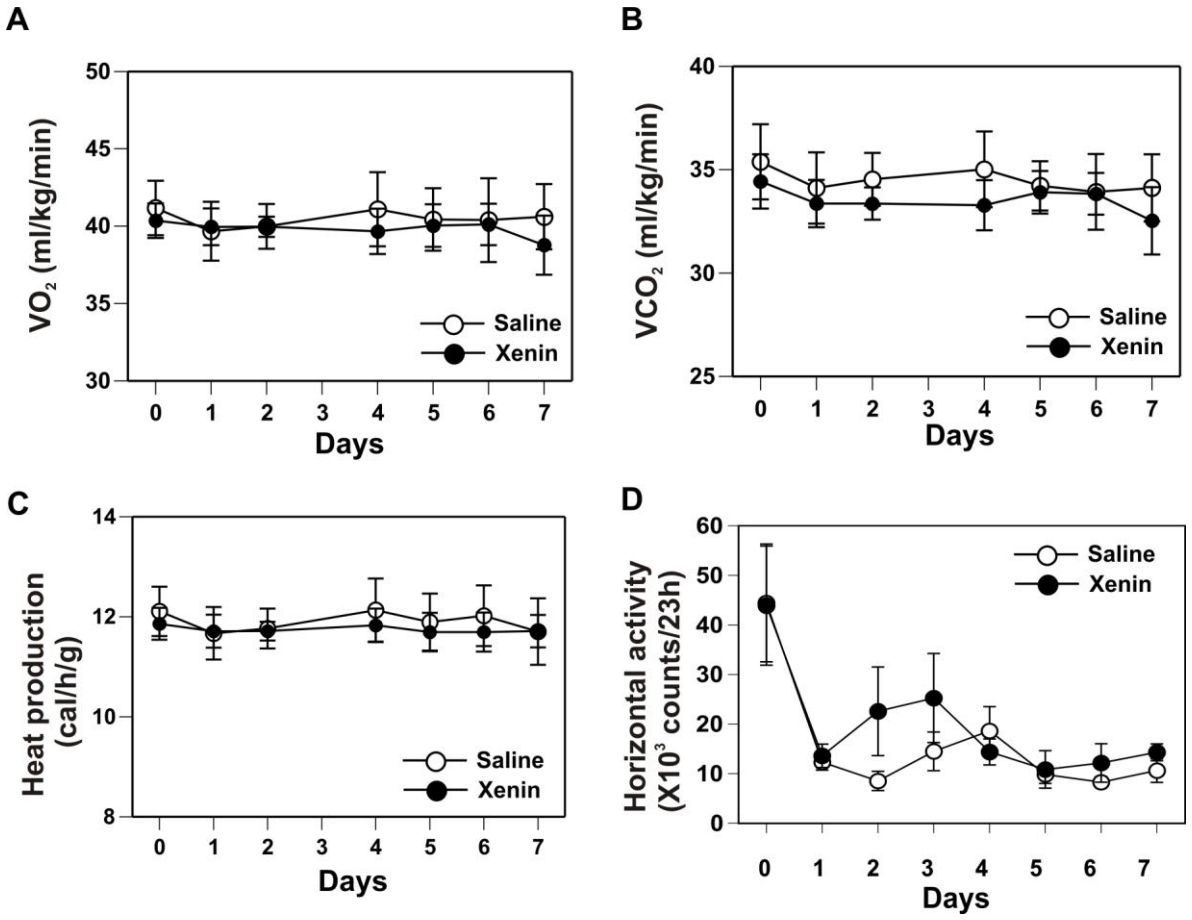


Figure 7. Effect of daily i.p. injection of xenin on energy expenditure in mice. Mice received daily i.p. injections of saline or xenin (50 μg/g b.w./day) for 7 days. Oxygen consumption (VO_2, A), carbon dioxide production (VCO_2, B), heat production (C), and horizontal activity (D) were continuously measured throughout the experiment and were expressed as daily average (VO_2, VCO_2 and heat production) or daily total (horizontal activity). Data are means ± SEM (n = 7-11/group). VO_2, VCO_2, and heat production data on day 3 are not available due to mechanical problems associated with the metabolic cage system.

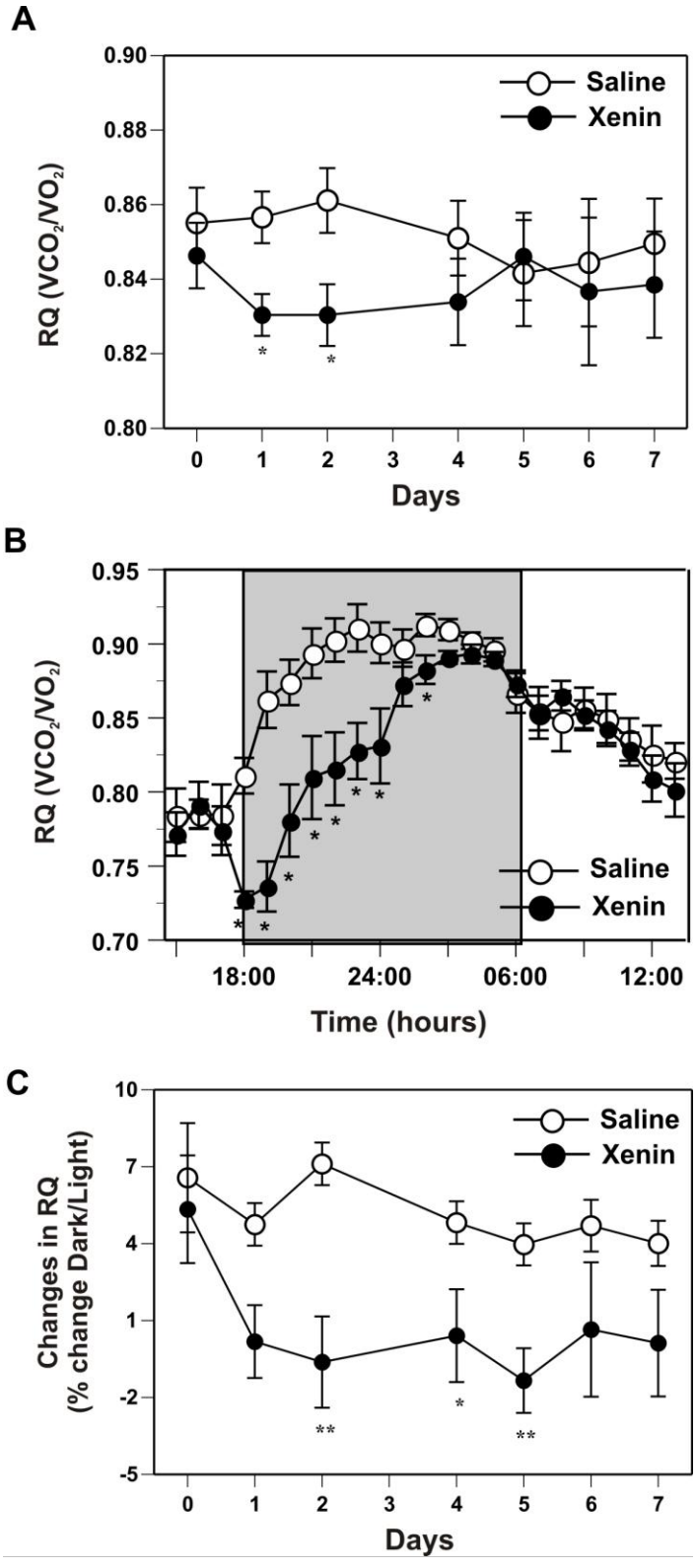

A
0.90
0.88
0.86
0.84
0.82
0.80
RQ (VCO2/VO2)
Saline
Xenin
*
*
0
1
2
3
4
5
6
7
Days
B
0.95
0.90
0.85
0.80
0.75
0.70
RQ (VCO2/VO2)
Saline
Xenin
18:00
24:00
06:00
12:00
Time (hours)
C
10
7
4
1
-2
-5
Changes in RQ
(% change Dark/Light)
Saline
Xenin
**
*
**
0
1
2
3
4
5
6
7
Days

Figure 8. Reduction in respiratory quotient (RQ) in mice injected i.p. with xenin. Mice received daily i.p. injections of saline or xenin (50 μg/g b.w./day) for 7 days. RQ was continuously measured throughout the experiment and was expressed as daily average (A). Hourly averages of RQ on day 2 were calculated (B). Shaded area in (B) represents the dark phase (1800 h-0600 h). Percentage changes in RQ during the dark cycle relative to those during the light cycle were calculated (C). Data are means ± SEM (n = 7/group). *: $P < 0.05$; **: $P < 0.005$ vs. saline-injected control group at same time point (Student's t-test).

3. Effects of daily i.p. injection of xenin on the expression of fat metabolism-related genes in mice

There was no significant difference in UCP1 mRNA levels in brown adipose tissue (BAT) between the control group and xenin-treated group (Fig. 9A). Xenin treatment significantly reduced DGAT2 mRNA levels in white adipose tissue (WAT) by 39% compared to the control saline treatment (Fig. 9B).

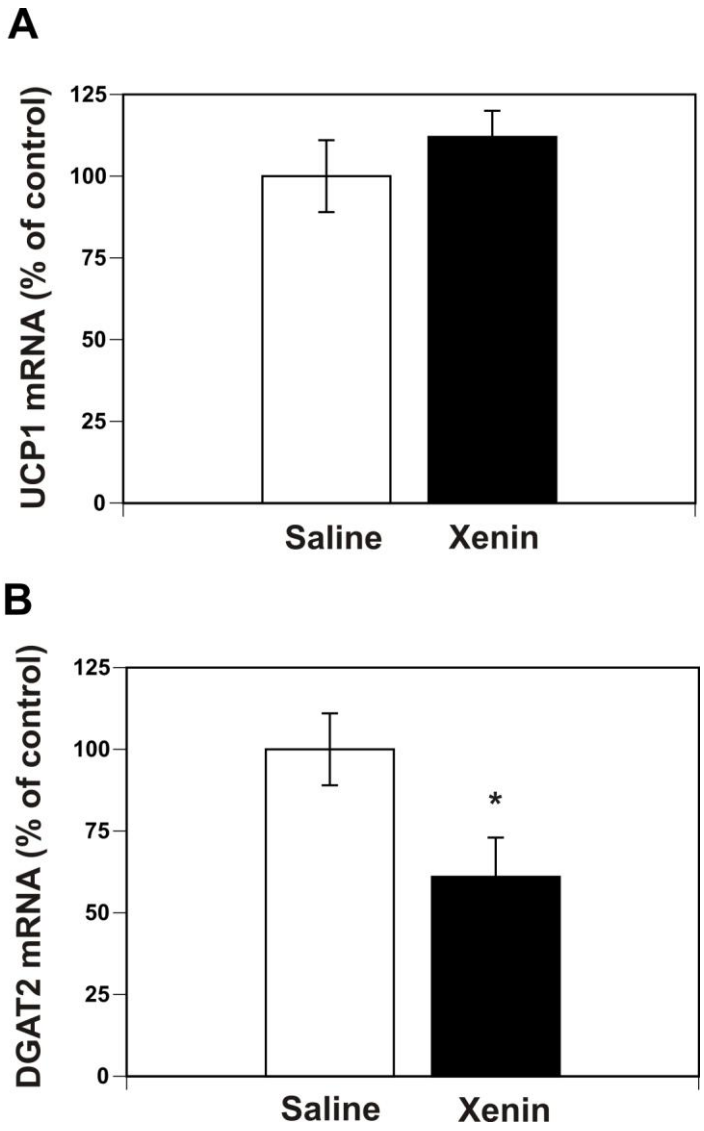


Figure 9. Effects of daily i.p. injection of xenin on the expression of fat metabolism-related genes in mice. Mice received daily i.p. injections of saline or xenin (50 μg/g b.w./day) for 7 days. Mice were sacrificed at the end of the experiment. UCP1 mRNA levels in brown adipose tissue (A) and DGAT2 mRNA levels in white adipose tissue were measured by real-time PCR and were normalized to β-actin mRNA levels. Values in saline-treated mice were set to 100%. Data are means ± SEM (n = 7-9/group). *: $P < 0.05$ (vs. saline-injected control group, Student's t-test).

4. Effects of daily i.c.v. injection of xenin on the expression of fat metabolism-related genes in mice

Daily i.c.v. injection of xenin significantly increased the expression level of HSL mRNA in WAT by 55% compared to the control aCSF treatment (Fig. 10A). DGAT2 mRNA levels in WAT were not significantly different between control and xenin-treated mice (Fig. 10B). Daily i.c.v. injection of xenin did not cause significant changes in mRNA levels of UCP1 and PGC1α in BAT and UCP3 and CPT1b in skeletal muscle (Fig. 10C-F).

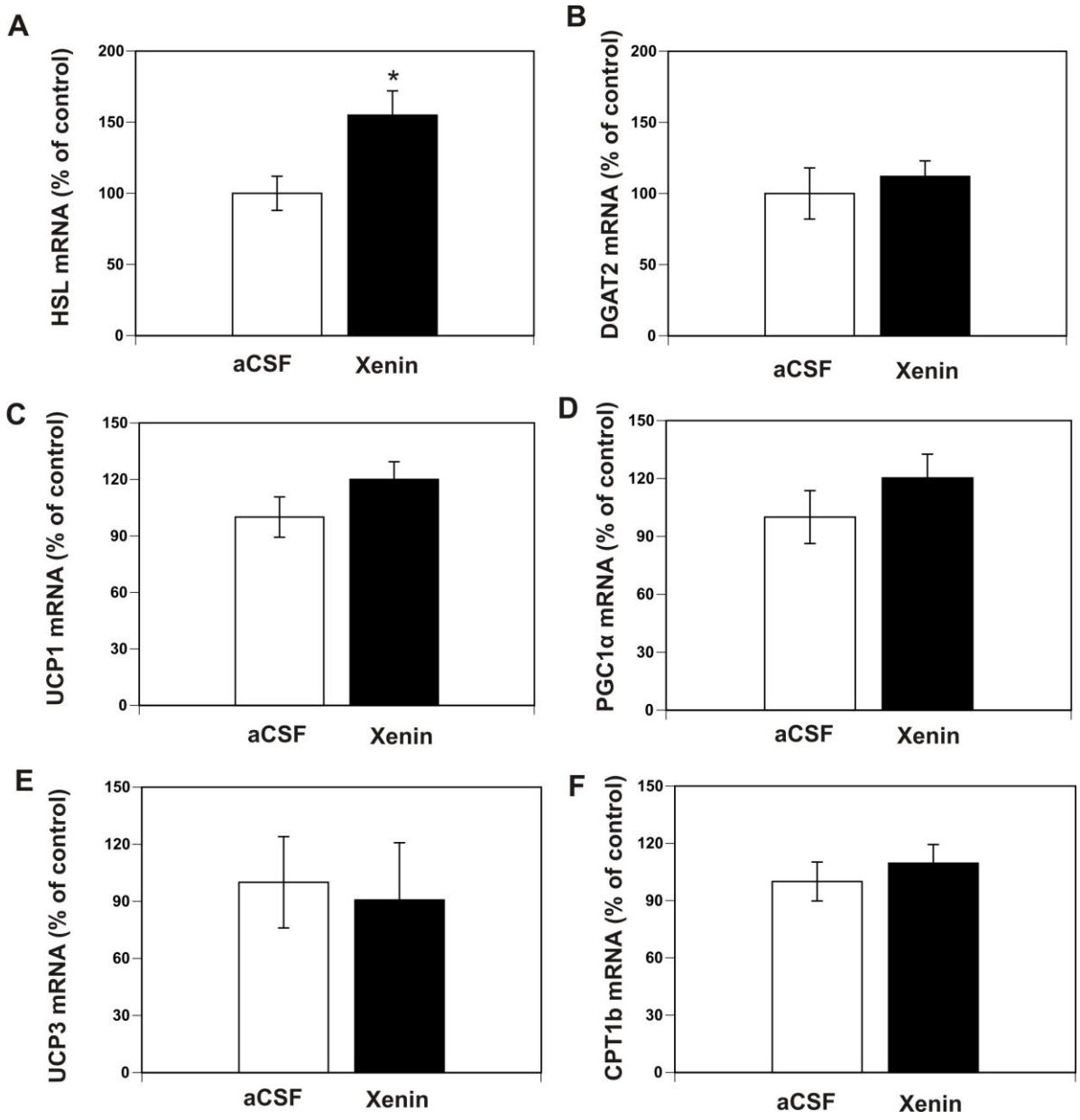

A
HSL mRNA (% of control)
200
150
100
50
0
*
aCSF
Xenin
B
DGAT2 mRNA (% of control)
200
150
100
50
0
aCSF
Xenin
C
UCP1 mRNA (% of control)
150
120
90
60
30
0
aCSF
Xenin
D
PGC1α mRNA (% of control)
150
120
90
60
30
0
aCSF
Xenin
E
UCP3 mRNA (% of control)
150
120
90
60
30
0
aCSF
Xenin
F
CPT1b mRNA (% of control)
150
120
90
60
30
0
aCSF
Xenin

Figure 10. Effects of daily i.c.v. injection of xenin on the expression of fat metabolism-related genes in mice. Mice received daily i.c.v. injections of aCSF or xenin (5 μg) for 10 days. Mice were sacrificed at the end of the experiment. mRNA levels of HSL (A) and DGAT2 (B) in WAT and UCP1 and PGC1α in BAT and UCP3 and CPT1b in skeletal muscle were measured by real-time PCR and were normalized to β-actin mRNA levels. Values in aCSF-treated mice were set to 100%. Data are means ± SEM (n = 5-7/group). *: $P < 0.05$ (vs. aCSF by Student's t -test).

5. Effects of i.p. injection of xenin on conditioned taste aversion in mice

To determine whether the anorectic effect of xenin is associated with nonspecific toxic or aversive effects, we performed conditioned taste aversion tests in mice. I.p. injection of LiCl significantly reduced the intake of saccharin solution compared with that in saline-injected control mice (Fig. 11A). I.p. injection of xenin, at a dose of 50 μg/g b.w. which causes a significant reduction in food intake, did not cause a significant reduction in saccharin intake (P = 0.32, Dunnett's test, Fig. 11A). There were no significant differences in total fluid intake between the groups (P = 0.66, one-way ANOVA, Fig. 11B).

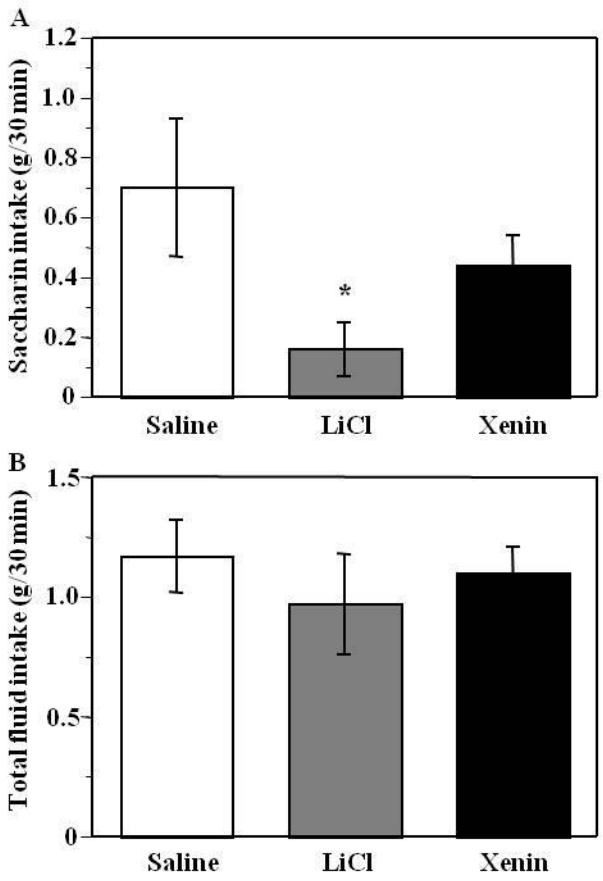


Figure 11. I.p. injection of xenin does not cause a significant conditioned taste aversion in mice. Mice received saccharin solution paired with i.p. injection of saline, LiCl (0.3 mol/l, 2% of b.w.), or xenin (50 μg/g b.w.). Amount of saccharin solution (A) and water ingested during the 30-min drinking session was measured. Total fluid intake (B) was a sum of water and saccharin solution. Data are means ± SEM (n = 7-8/group). *: $P < 0.05$ vs. saline (Dunnett's test).

6. Effects of i.p. injection of xenin on gastric emptying rate in mice

To address the hypothesis that the anorectic effect of xenin is mediated by delayed gastric emptying, we compared 2-h gastric emptying rate between xenin-injected mice and saline-injected control mice. Mice ate similar amounts of food during the 1-h re-feeding period prior to i.p. injection in all 3 groups (P = 0.39, one-way ANOVA, Fig. 12A). Stomach weight was not distinguishable between the groups (P = 0.24, one-way ANOVA, Fig. 12B). There were no significant differences in 1-h food intake per gram of stomach weight between xenin-treated mice and saline-treated mice (P = 0.89, one-way ANOVA, Fig. 12C). I.p. injection of xenin significantly reduced the rate of gastric emptying by about 93% compared to the saline-treated control group (Fig. 13). Urocortin significantly slowed gastric emptying compared to saline treatment (Fig. 13). There was no statistical difference in gastric emptying rate between xenin-treated mice and urocortin-treated mice (P = 0.16, Tukey-Kramer test, Fig. 13).

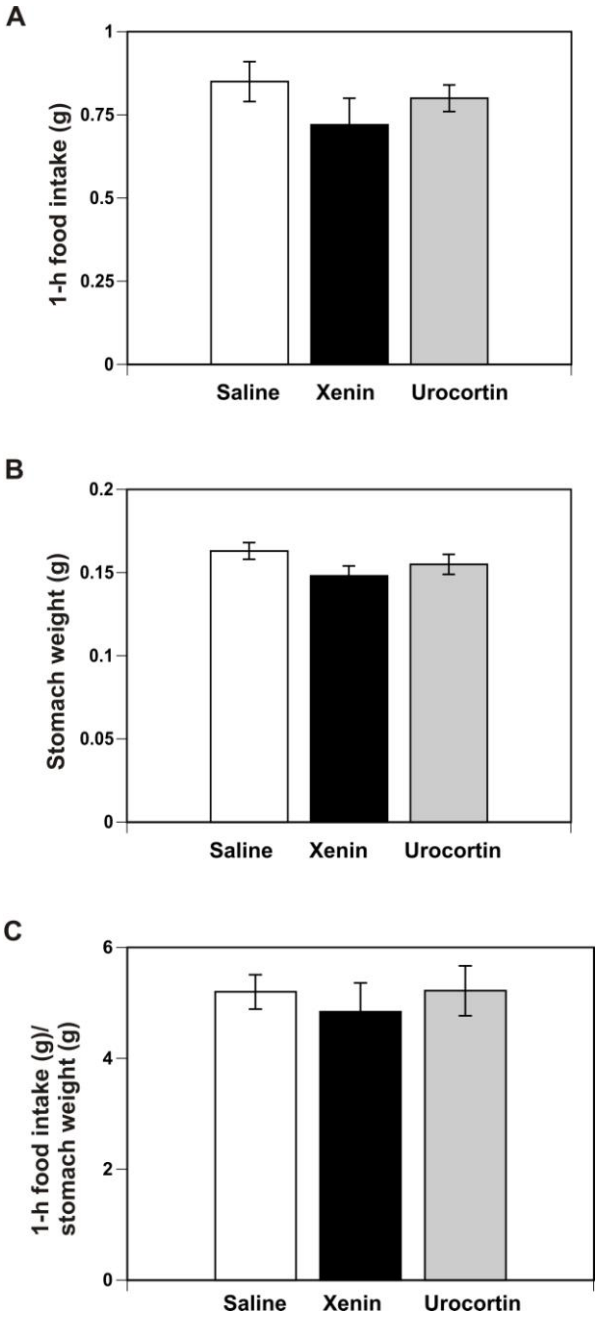


Figure 12. Estimation of gastric distention before i.p. injection of saline, xenin, or urocortin. Mice were fasted overnight, refed for 1 h, and injected i.p. with saline, xenin (50 μg/g b.w.), or urocortin (3 nmol/mouse). Food intake during the 1 h re-feeding period (A) and stomach weight (B) were measured. One hour food intake was normalized to the stomach weight (C). Data are means ± SEM (n = 4–6/group).

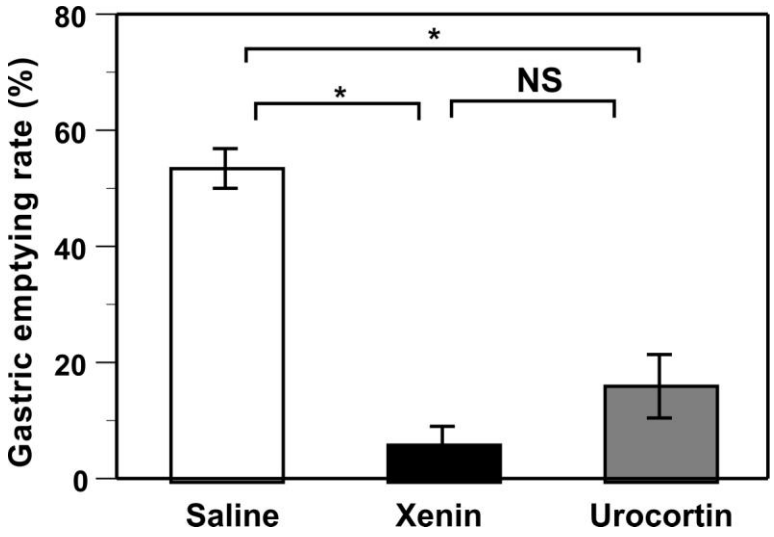


Figure 13. Delayed gastric emptying in mice injected i.p. with xenin. Mice were fasted overnight, refed for 1 h, and injected i.p. with saline, xenin (50 μg/g b.w.), or urocortin (3 nmol/mouse). Gastric emptying rates were measured 2 h after injection. Data are means ± SEM (n = 4–6/group). *: $P < 0.05$ (Tukey-Kramer test). NS: Not significantly different.

7. Effects of i.p. injection of xenin on *c-fos* mRNA and protein expression levels in the mouse brain

To determine whether the effect of xenin on food intake is associated with the activation of CNS cells, *c-fos* mRNA and protein levels were measured in the mouse brain. Real-time PCR analysis revealed a significant 175% increase in the hypothalamic *c-fos* mRNA levels 30 min after i.p. injection of xenin (50 μg/g b.w.) compared with saline injection (Fig. 14A). Hypothalamic *c-fos* mRNA levels were not different between control and xenin-injected mice 90 min after injection (saline: 100.0 ± 10.8%, xenin: 121.8 ± 10.8%, P = 0.22, Student's *t*-test, Fig. 14B).To determine specific regions of the hypothalamus which were activated by xenin, Fos protein-expressing cells were visualized in mouse brain sections by immunohistochemistry. I.p. injection of xenin (50 μg/g b.w.) increased the number of Fos-positive cells in several hypothalamic areas including the PVN, ARC, DMN, SON and VMN (Fig. 15). Cell counting analysis of immunohistochemically-stained sections revealed that the number of Fos-immunoreactive cells was significantly increased in these hypothalamic regions compared with control saline injection (PVN: 404%, ARC: 375%, DMN: 225%, SON: 439%, VMN: 259%, Fig. 16). A small number of Fos-immunoreactive cells was present in the LHA, and the number of Fos-immunoreactive cells in the LHA was not different between the xenin-treated group (8 ± 1 cells) and the saline-treated group (11 ± 3 cells, P = 0.24, Student's *t*-test, Fig. 16).

To determine whether xenin activates the brainstem cells, Fos expression in the brainstem was compared between xenin-treated mice and saline-treated control mice. I.p. injection of xenin (50 μg/g b.w.) significantly increased the number of Fos-immunoreactive cells in the NTS by 530% compared with control saline injection (Fig. 17A-E). Xenin-induced Fos-positive cells were found in the ventrolateral part of the NTS and were not found in the intermediate NTS. Only a very limited number of Fos-immunoreactive cells were found in the DMV without a significant effect of xenin treatment (saline: 1 ± 1, xenin: 4 ± 2, $P = 0.24$, Student's *t*-test, Fig 17A and B). No Fos-positive cells were found in the AP regardless of the treatment (Fig. 17A and B).

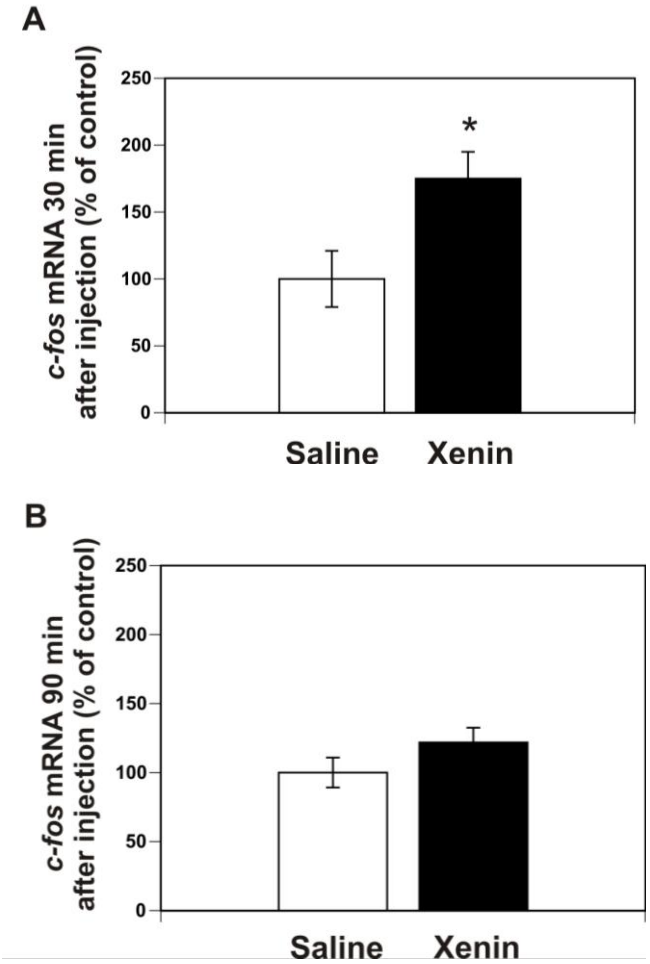


Figure 14. Stimulatory effect of xenin on *c-fos* mRNA expression in the hypothalamus. Mice were fasted overnight, and injected i.p. with saline or xenin (50 μg/g b.w.) and were sacrificed 30 (A) or 90 (B) min later. Relative expression levels of *c-fos* mRNA were measured by real-time PCR analysis and were normalized to cyclophilin mRNA levels. Values in saline-treated mice were set to 100%. Data are means ± SEM (n = 3-7/group). *: $P < 0.05$ vs. saline (Student's t-test).

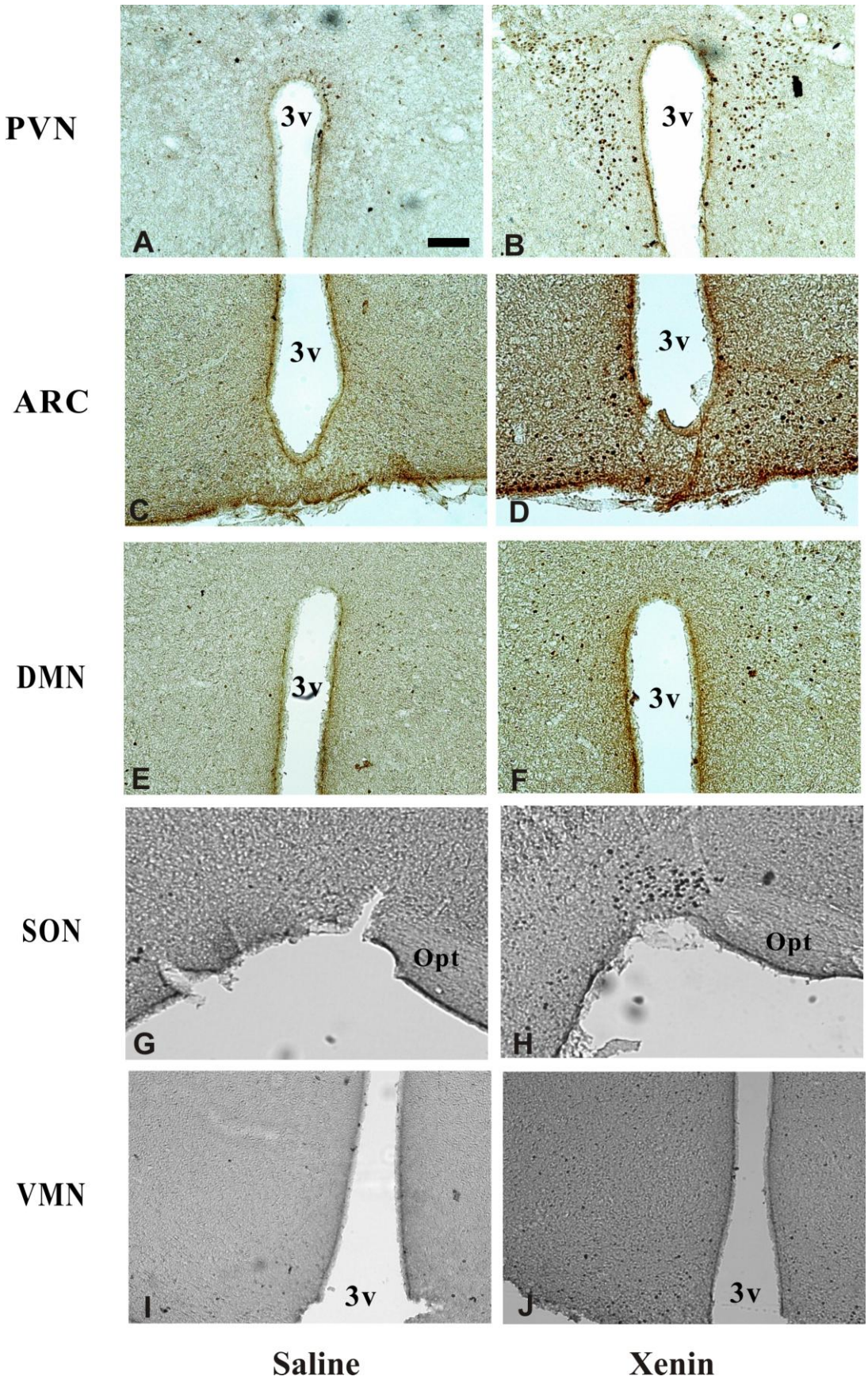

PVN
ARC
DMN
SON
VMN
3v
Opt
A
B
C
D
E
F
G
H
I
J
Saline
Xenin

Figure 15. Stimulatory effect of xenin on Fos expression in the mouse hypothalamus. Mice were fasted for 6 h, injected i.p. with saline or xenin (50 μg/g b.w.), and perfused 2 h post-injection. Fos-immunoreactive cells were visualized by immunohistochemistry in 30-μm coronal sections. Representative photomicrographs of Fos-immunoreactive cells (black and dark brown nuclei) in the PVN (A, B), ARC (C, D), DMN (E, F), SON (G, H) and VMN (I, J) of mice. Scale bar = 100 μm. 3v, third ventricle; Opt, optic tract.

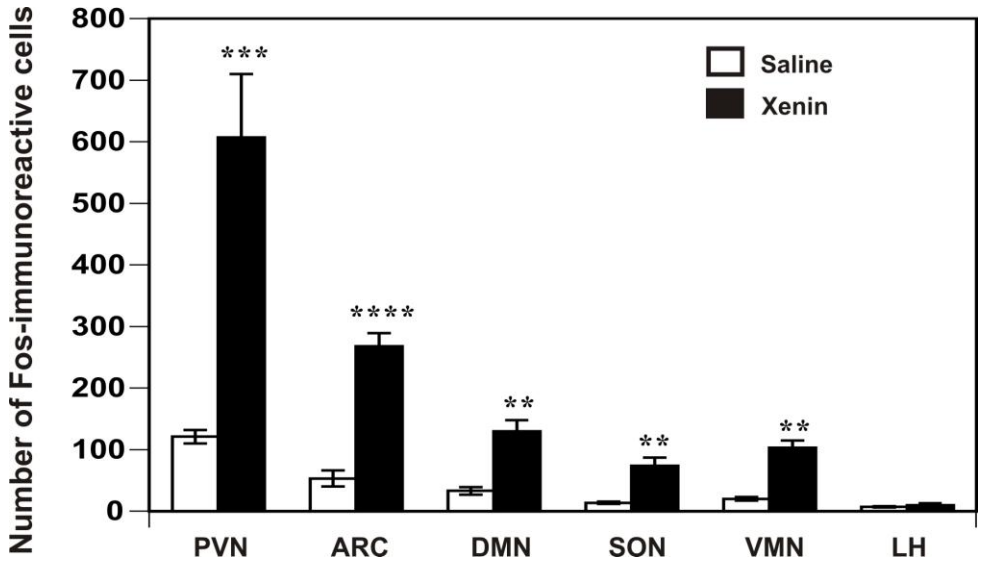


Figure 16. Xenin-induced increase in the number of Fos-immunoreactive cells in hypothalamic regions. Mice were fasted for 6 h, injected i.p. with saline or xenin (50 μg/g b.w.), and perfused 2 h post-injection. Fos-immunoreactive cells were visualized by immunohistochemistry and the numbers of Fos-immunoreactive cells were counted in 2-6 tissue sections per brain region. The sum of the number of Fos-immunoreactive cells on both sides of the brain was calculated in each animal. Data are means ± SEM (n = 6-7/group). **: $P < 0.001$; ***: $P < 0.0005$; ****: $P < 0.0001$ vs. saline (Student's *t*-test).

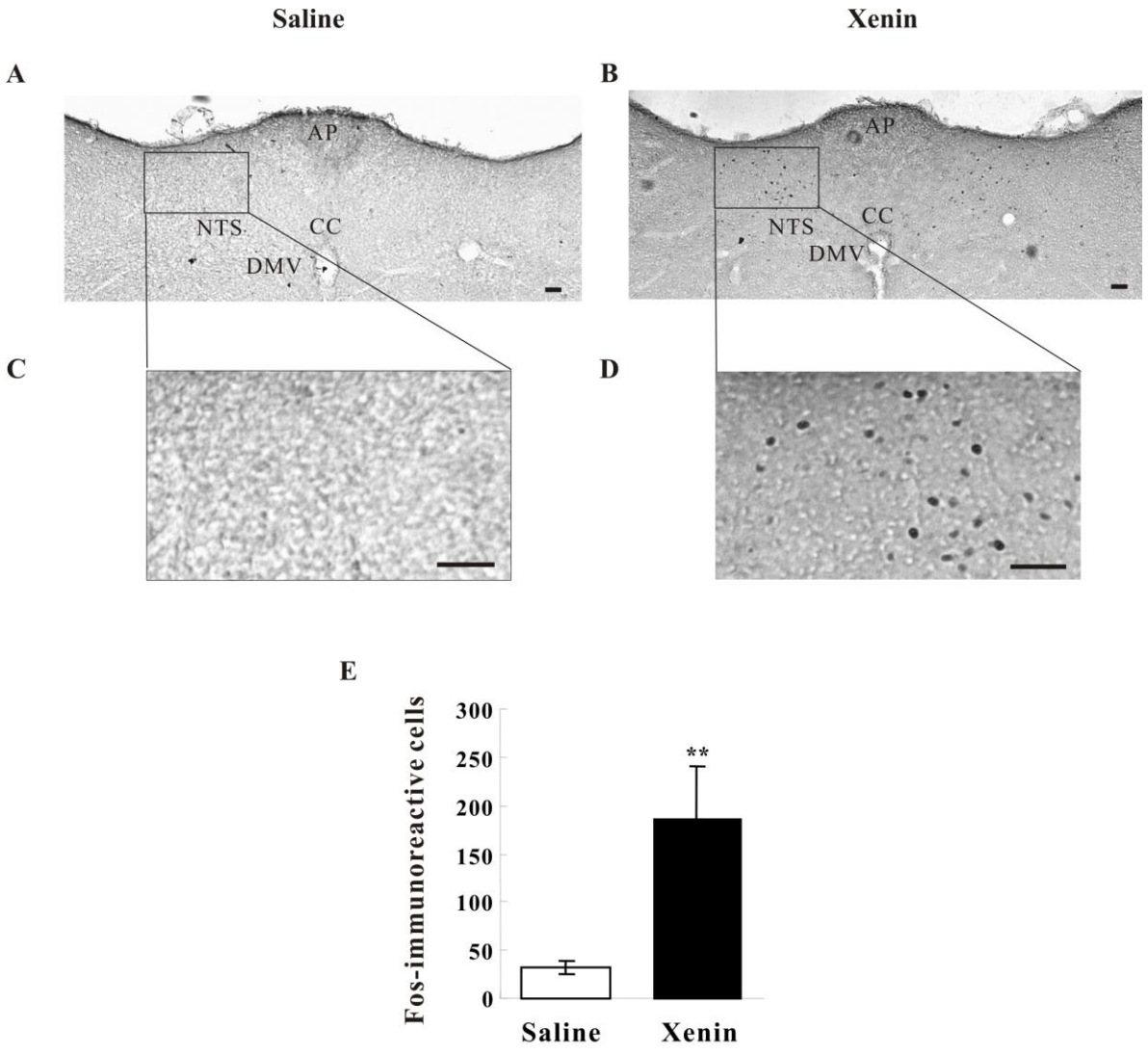

Saline
Xenin
A
B
AP
NTS
CC
DMV
C
D
E
Fos-immunoreactive cells
300
250
200
150
100
50
0
**
Saline
Xenin

Figure 17. Stimulatory effect of xenin on Fos expression in the mouse brainstem. Mice were fasted for 6 h, injected i.p. with saline or xenin (50 µg/g b.w.), and perfused 2 h later. A and B: Representative photomicrographs of Fos-immunoreactive cells in the brainstem (approximately 7.5 mm posterior from the bregma) of mice treated with saline (A) or xenin (B). C and D: Enlarged view of the boxed areas in A and B, respectively. E: The number of Fos-immunoreactive cells in the NTS of mice treated with saline or xenin. Data are means ± SEM (n = 3/group). **: $P < 0.001$ (vs. saline by Student's *t* -test). Scale bar = 50 µm. AP, area postrema; CC, central canal; NTS, nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus.

8. Effects of i.p. injection of xenin on the expression of metabolism-related genes in the mouse hypothalamus

To identify downstream mediators of xenin action in the hypothalamus, we compared expression levels of 84 genes in the hypothalamus between saline-treated control and xenin-treated mice using a commercially available PCR array kit (Table 1 and Table 5). There were 22 up-regulated genes (> 2-fold increase) and only one down-regulated gene by xenin treatment (50 μg/g b.w., i.p., Table 8). IL-1β mRNA was one of the up-regulated genes by xenin treatment (7.8-fold increase, Fig. 18 and Table 8). Xenin treatment did not cause major changes in POMC (1.1-fold increase) and AGRP (1.3-fold increase) mRNA levels (Fig. 18).

Because the PCR array analysis was performed by using pooled cDNA samples (7 individual cDNA samples were pooled for each treatment group) without replication, intra-group variations might be large and might have outweighed inter-group variations. Therefore, we performed conventional real-time PCR using larger number of individual unpooled cDNA samples (n = 7/group) to verify that pooling of cDNA samples did not cause artifacts in the PCR array analysis. Consistent with the PCR array results, xenin treatment significantly increased hypothalamic IL-1β mRNA levels by 79% compared to saline treatment, while POMC and AGRP mRNA levels were not altered by xenin treatment (Fig. 19A-C). Although both analyses consistently demonstrated the up-regulation of IL-1β mRNA by xenin treatment, magnitude of up-regulation was different

between the two methodologies. As described above, PCR array data were based on the analysis using a single pooled sample per group without replication. Therefore, it is likely that the conventional real-time PCR analysis with larger sample size provides more representative and reliable quantitative data than PCR array analysis in the present study.

Table 8. Up-regulated and down-regulated hypothalamic genes by xenin treatment

Up-regulated genes		
Accession No.	Gene	Fold change
NM_009633	Adrenergic receptor, alpha 2b (Adra2b)	10.74
NM_007468	Apolipoprotein A-IV (Apoa4)	2.90
NM_007587	Calcitonin/calcitonin-related polypeptide, alpha (Calca)	3.35
NM_025469	Colipase, pancreatic (Clps)	8.31
NM_008100	Glucagon (Gcg)	3.47
NM_008101	Glucagon receptor (Gcgr)	6.75
NM_008117	Growth hormone (Gh)	5.72
NM_021332	Glucagon-like peptide 1 receptor (Glp1r)	2.06
NM_008177	Gastrin releasing peptide receptor (Grpr)	2.26
NM_198959	Hypocretin (orexin) receptor 1 (Hcrtr1)	2.18
NM_010554	Interleukin 1 alpha (Il1a)	5.30
NM_008361	Interleukin 1 beta (Il1b)	7.81
NM_031168	Interleukin 6 (Il6)	5.48
NM_008386	Insulin I (Ins1)	3.45
NM_008493	Leptin (Lep)	5.22
NM_010704	Leptin receptor (Lepr)	2.27
NM_010341	Neuromedin U receptor 1 (Nmur1)	2.69
NM_010934	Neuropeptide Y receptor Y1 (Npy1r)	2.26
NM_145435	Peptide YY (Pyy)	2.42
NM_013693	Tumor necrosis factor (Tnf)	7.97
NM_021290	Urocortin (Ucn)	3.75
NM_009463	Uncoupling protein 1 (Ucp1)	3.99
Down-regulated gene		
Accession No.	Gene	
NM_008082	Galanin receptor 1 (Galr1)	0.25

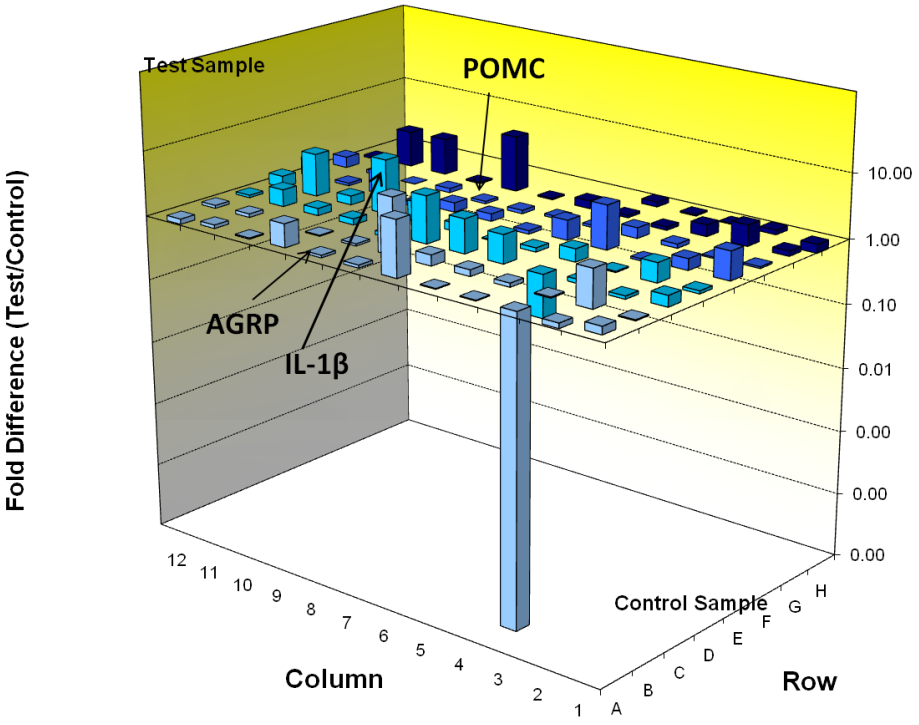


Figure 18. Effects of i.p. injection of xenin on hypothalamic gene expression as assessed by PCR array. Hypothalamic total RNA was prepared from the hypothalamic *c-fos* mRNA expression study. Expression levels of 84 genes (Table 5) in the hypothalamus were compared between saline-treated and xenin-treated groups by using a commercial PCR array kit (RT^{2}Profiler^{TM}PCR Array-Mouse obesity, PAMM-017C). Data were analyzed using ΔΔCt method. The bars represent fold changes of the expression levels of genes in xenin-treated group relative to the control group.

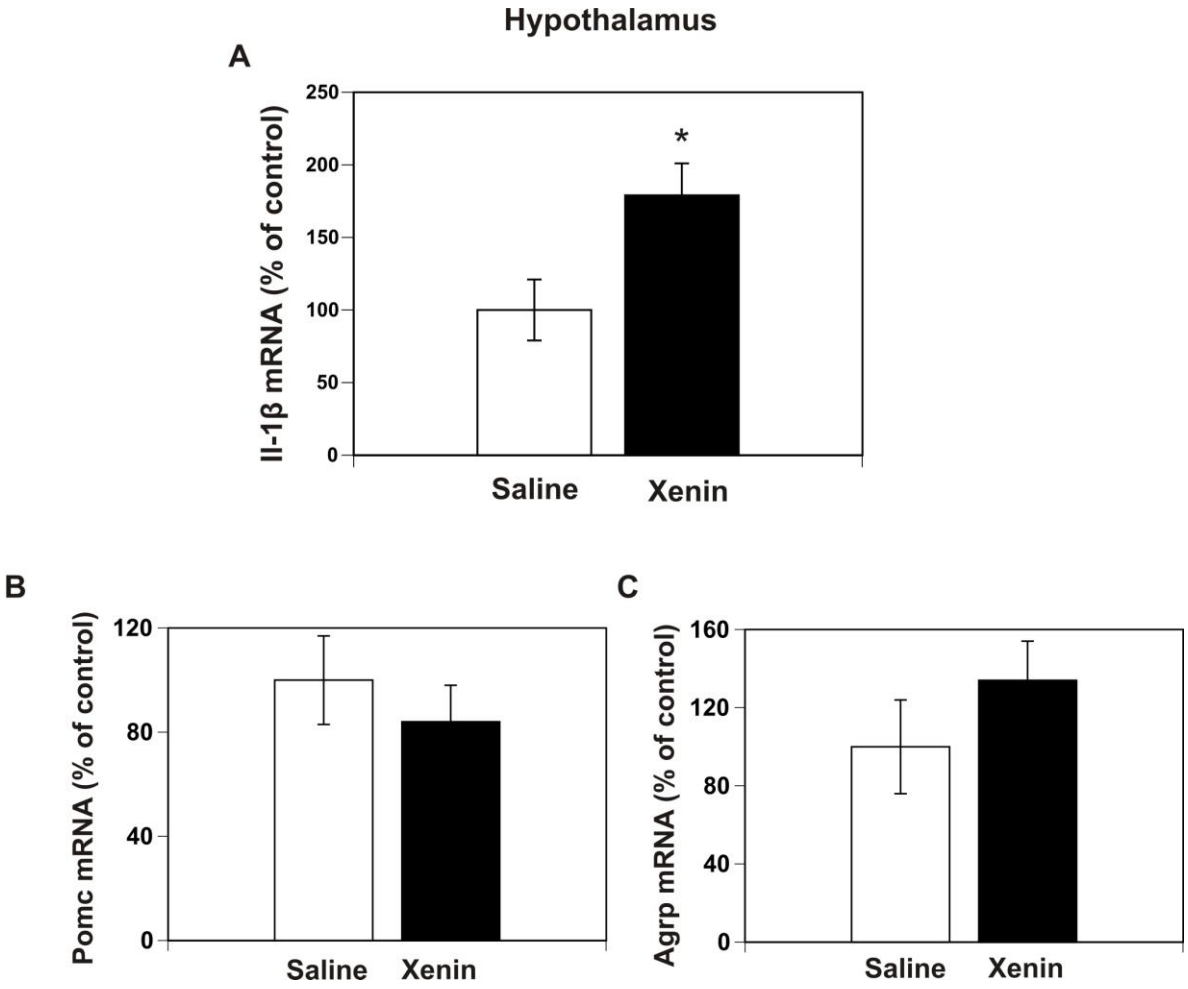


Figure 19. Effects of i.p. injection of xenin on hypothalamic gene expression. Mice were fasted overnight, and injected i.p. with saline or xenin (50 μg/g b.w.) and were sacrificed 30 min later. Relative expression levels of IL-1β (A), POMC (B) and AGRP (C) mRNA were measured by real-time PCR analysis and were normalized to cyclophilin mRNA levels. Values in saline-treated mice were set to 100%. Data are means ± SEM (*n* = 7/group). *: $P < 0.05$ vs. saline (Student's *t*-test).

9. Effects of Ntsr1-deficiency on xenin-induced feeding suppression and weight reduction in mice

Cumulative food intake was significantly reduced by i.c.v. injection of neurotensin up to 8 h after injection in wild-type (*Ntsr1*$^{+/+}$) mice (Fig. 20A). There was no significant difference in cumulative food intake between aCSF and neurotensin injection at any time point examined in Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice (Fig. 20B). I.c.v. injection of xenin caused significant reductions in cumulative food intake up to 24 h post-injection in wild-type mice (Fig. 20A). Xenin treatment failed to reduce cumulative food intake at any time point examined in Ntsr1-deficient mice (Fig. 20B). I.c.v. injection of NMU significantly reduced cumulative food intake up to 24 h after injection in both wild-type and Ntsr1-deficient mice (Fig. 21A and B). Body weight gain (% increase compared to the pre-injection body weight) over a 24-h period following i.c.v. neurotensin injection did not significantly differ from that after i.c.v. aCSF injection in both wild-type and Ntsr1-deficient mice (Fig. 22A). The i.c.v. injection of xenin significantly reduced 24-h body weight gain compared to the control aCSF injection in wild-type mice, but it did not cause a significant change in body weight gain in Ntsr1-deficient mice (Fig. 22A). The i.c.v. administration of NMU significantly reduced 24-h body weight gain in both wild-type and Ntsr1-deficient mice (Fig. 22B)

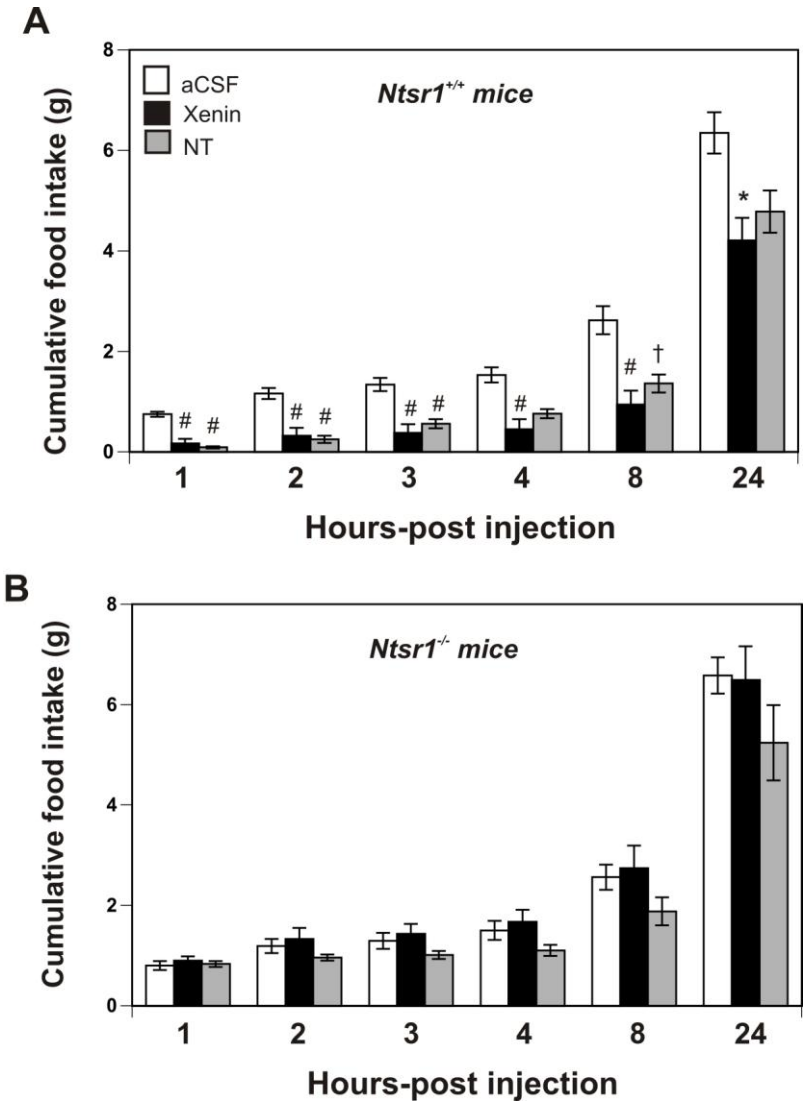


Figure 20. Effect of i.c.v. injection of xenin and neurotensin on food intake in wild-type and Ntsr1-deficient mice. Wild-type (*Ntsr1*$^{+/+}$**,** A) and Ntsr1-deficient (*Ntsr1*$^{-/-}$**,** B) mice were fasted overnight and injected i.c.v. with aCSF, xenin (1 μg), or neurotensin (NT, 0.56 μg, equal molar dose to 1 μg xenin). Cumulative food intake was measured up to 24 h after injection. Data are means ± SEM (n = 8-12/group). *: $P < 0.05$, †: $P < 0.005$, #: $P < 0.0005$ (vs. control at same time point, Dunnett's test).

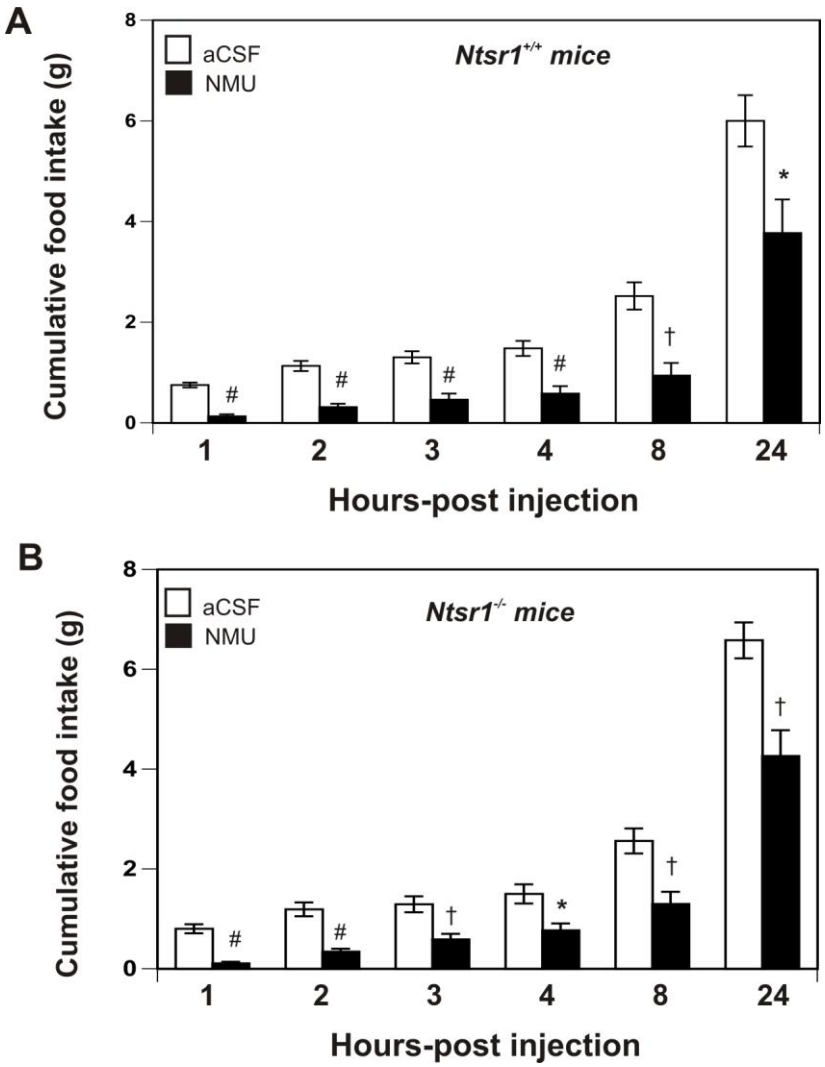


Figure 21. Effect of i.c.v. injection of neuromedin U on food intake in wild-type and Ntsr1-deficient mice. Wild-type (*Ntsr1*$^{+/+}$**,** A) and Ntsr1-deficient (*Ntsr1*$^{-/-}$**,** B) mice were fasted overnight and injected i.c.v. with aCSF or neuromedin U (NMU, 3 nmol). Cumulative food intake was measured up to 24 h after injection. Data are means ± SEM (n = 9-11/group). *: $P < 0.05$, †: $P < 0.005$, #: $P < 0.0005$ (vs. control by Student's *t*-test).

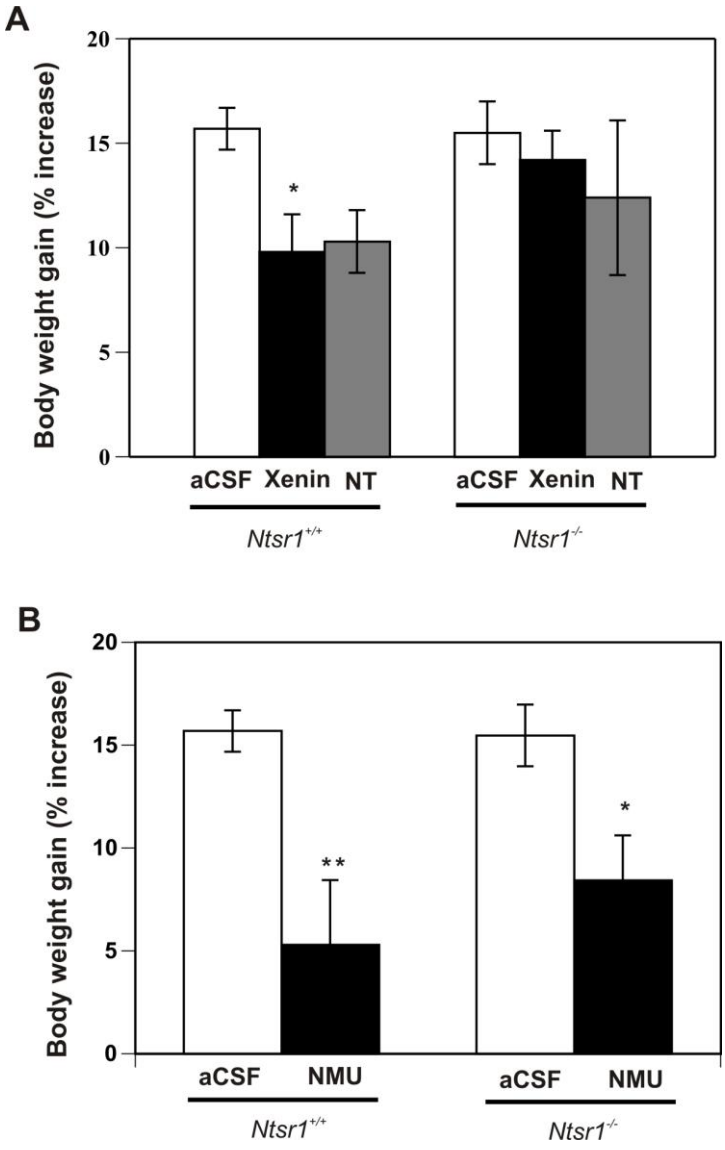


Figure 22. Effect of i.c.v. injection of xenin, neurotensin, and neuromedin U on body weight in wild-type and Ntsr1-deficient mice. Wild-type (*Ntsr1*$^{+/+}$) and Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice were fasted overnight and injected i.c.v. with aCSF, xenin (1 μg, A), neurotensin (NT, 0.56 μg, equal molar dose to 1 μg xenin, A), or NMU (3 nmol, B). Mice were fed *ad libitum* after injection and cumulative body weight gain was measured 24 h after injection. Data are means ± SEM (n = 8-12/group). *: $P < 0.05$, **: $P < 0.001$ (vs. control by Dunnett's test in A and Student's *t*-test in B).

10. Effects of Ntsr1-deficiency on leptin-induced feeding suppression and weight reduction in mice

Cumulative food intake was significantly reduced by 60% and 57% at 16-h and 24-h time point, respectively, in wild-type (*Ntsr1*$^{+/+}$) mice received i.c.v. leptin injection compared to those received i.c.v. aCSF injection (Fig. 23A and B). Leptin significantly reduced body weight compared to the control aCSF injection in wild-type mice (Fig. 23C and D). In Ntsr1-deficient (*Ntsr1*$^{-/-}$) mice, although leptin injection reduced cumulative food intake by 31% and 34% at 16-h and 24-h time point, respectively, compared to the control aCSF injection, these effects were not statistically significant (16-h: $P = 0.28$, 24-h: $P = 0.11$, Tukey-Kramer test, Fig. 23A and B). Similarly, the i.c.v. leptin injection did not cause significant changes in body weight compared to the aCSF injection in Ntsr1-deficient mice (16-h: $P = 0.67$, 24-h: $P = 0.19$, Tukey-Kramer test, Fig. 23C and D).

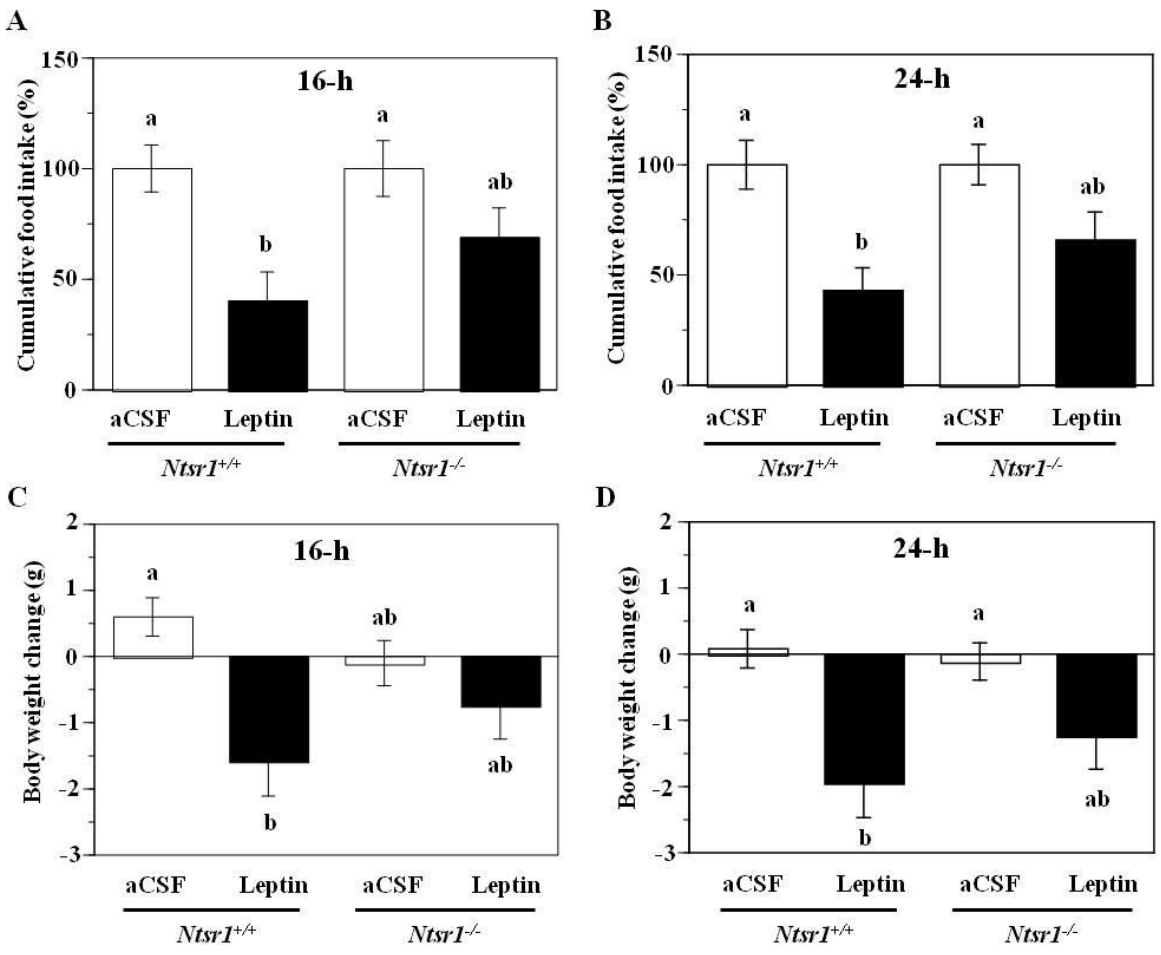
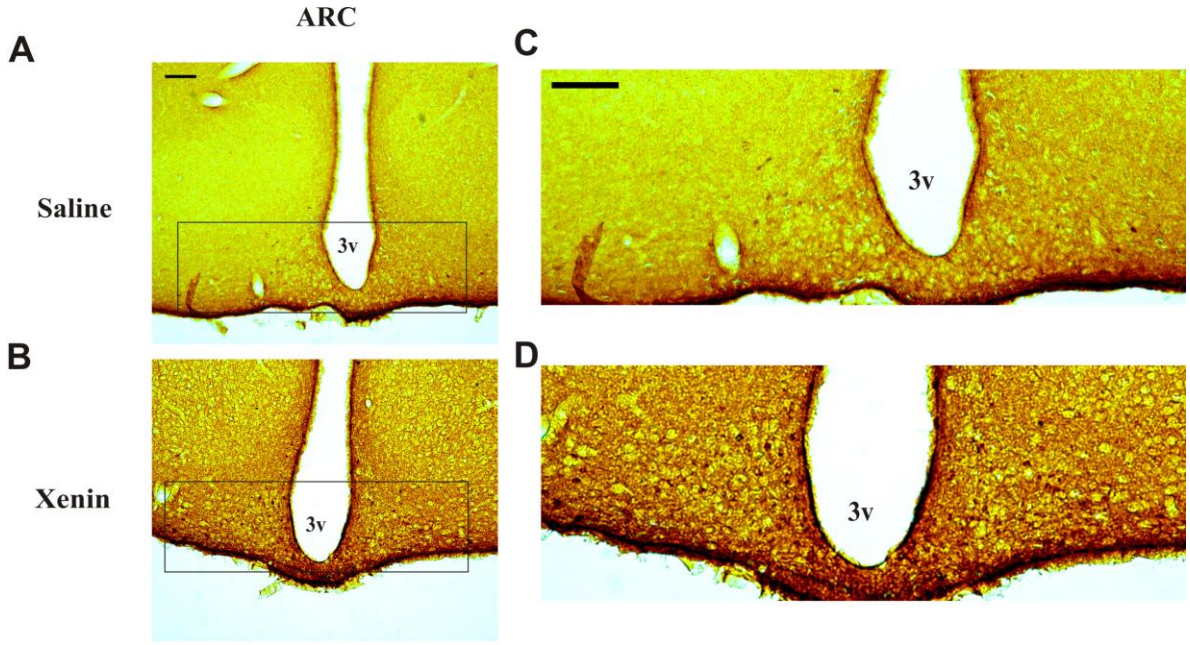


Figure 23. Effect of i.c.v. injection of leptin on food intake and body weight change in wild-type and Ntsr1-deficient mice. Mice were fed *ad libitum* and injected i.c.v. with aCSF or leptin (5 μg) just before light out (1800 h). Cumulative food intake (A and B) and body weight change (C and D) were measured 16 h (A and C) and 24 h (B and D) after injection. Cumulative food intake of the control aCSF-treated mice was set to 100%. Data are means ± SEM (n = 5-8 per group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

11. Effects of i.p. injection of xenin on the phosphorylation of extracellular signal-regulated kinases (ERK) in the mouse hypothalamus

We compared levels of phosphorylation of ERK1/2 in the hypothalamus between xenin-treated mice and saline-treated control mice. Cells expressing pERK1/2 were visualized in the mouse brain sections by immunohistochemistry. I.p. injection of xenin (50 μg/g b.w.) significantly increased the number of pERK1/2-immunoreactive cells by 51% in the ARC compared with control saline injection (Fig. 24A-E). Only a few cells per section of the VMN were stained for pERK1/2. There was no difference in the number of pERK1/2-positive cells in the VMN between saline-treated and xenin-treated groups (saline: 1.9 ± 0.8, xenin: 2.9 ± 1.9, $P = 0.66$, Student's *t*-test).


ARC
A
C
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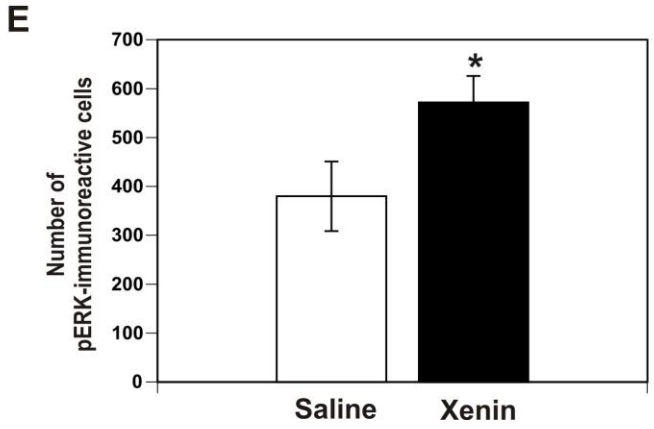

E
Number of pERK-immunoreactive cells
700
600
500
400
300
200
100
0
*
Saline
Xenin

Figure 24. Stimulatory effect of xenin on phosphorylation of ERK1/2 in the mouse ARC. Mice were fasted for 6 h, injected i.p. with saline or xenin (50 μg/g b.w.), and perfused 15 min later. Phosphorylation of ERK1/2 was assessed by immunohistochemically visualizing pERK1/2-immunoreactive cells in 30-μm coronal sections. A and B: Representative photomicrographs of pERK1/2-immunoreactive cells in the ARC of mice treated with saline or xenin. C and D: Enlarged view of the boxed areas in A and B, respectively. E: The number of pERK1/2-immunoreactive cells in the ARC of mice treated with saline or xenin. Data are means ± SEM (n = 7-9/group). *:$P < 0.05$ (vs. saline by Student's *t* -test). Scale bar = 100 μm. 3v, third ventricle.

12. Effects of IL-1RI deficiency on xenin-induced feeding suppression in mice

A single i.p. injection of xenin at a high dose (50 μg/g b.w.) significantly reduced cumulative food intake in wild-type (*IL-1RI*$^{+/+}$) mice up to 8 h post-injection (Fig. 25A). The same dose of xenin also caused significant reductions in cumulative food intake up to 8 h after injection in IL-1RI knockout (*IL-1RI*$^{-/-}$) mice (Fig. 25B).

An intermediate dose of xenin (15 μg/g b.w., i.p.) caused significant reductions in cumulative food intake up to 5 h post-injection in both wild-type and IL-1RI knockout mice (Fig. 26A and B). I.p. injection of leptin (1.2 μg/g b.w.) significantly reduced cumulative food intake up to 6 h and 1 h after injection in wild-type mice and IL-1RI knockout mice, respectively (Fig. 26A and B). The i.p. injection of xenin and leptin did not cause significant changes in 24-h cumulative food intake (Fig. 26A and B) and body weight change over a 24-h period following injection (Fig. 27A and B) in both genotypes.

I.p. injection of xenin at a low dose (7.5 μg/g b.w.) significantly reduced cumulative food intake up to 8 h after injection in wild-type mice (Fig. 28A). In marked contrast, the same dose of xenin did not cause significant changes in cumulative food intake at any time point examined in IL-1RI knockout mice (Fig. 28B). The low dose of leptin (0.85 μg/g b.w.) significantly reduced cumulative food intake from 4 to 8 h post-injection in wild-type mice (Fig. 28A). The same dose of leptin did not cause significant changes in cumulative food intake at any time point examined in IL-1RI knockout mice (Fig. 28B). The i.p.

injection of xenin and leptin at these doses did not cause significant changes in 24-h cumulative food intake (Fig. 28A and B) and body weight change over a 24-h period after injection (Fig. 29A and B) in both genotypes.

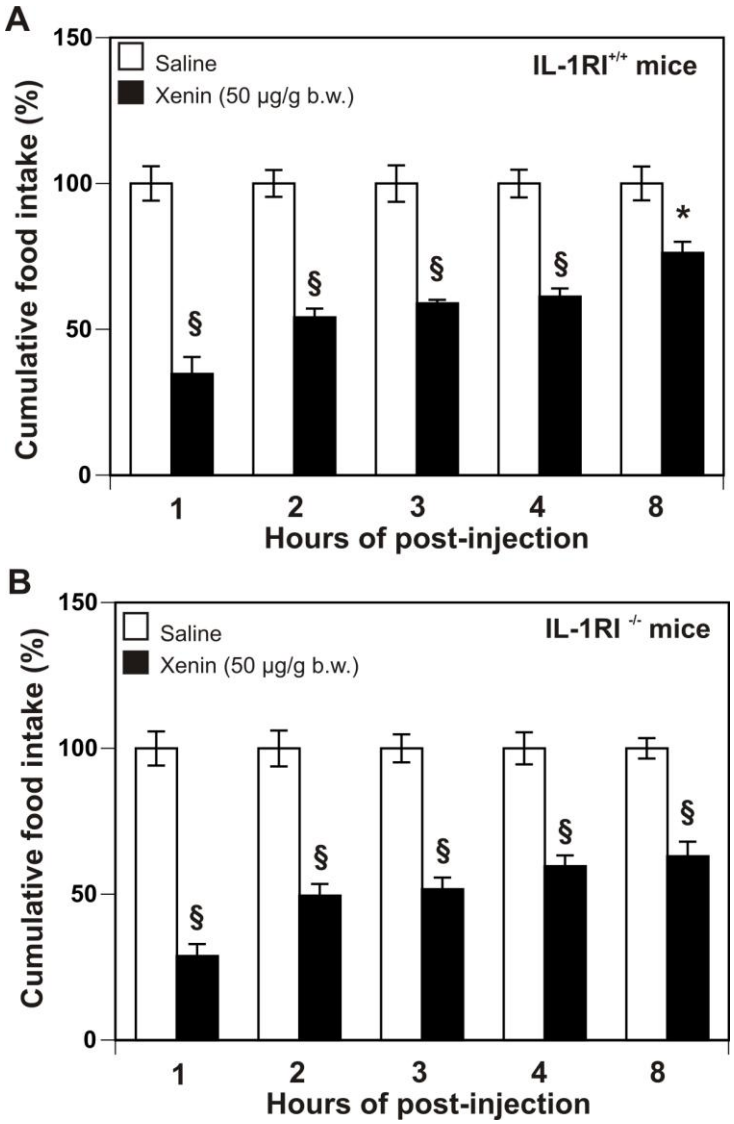
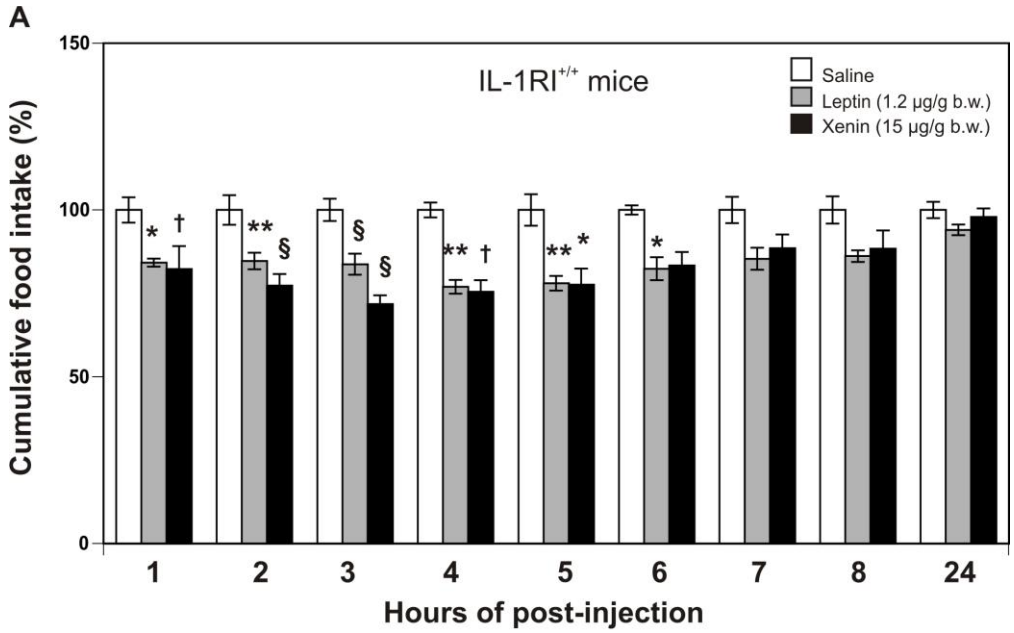


Figure 25. Effect of i.p. injection of high dose of xenin on food intake in wild-type and IL-1RI-deficient mice. Wild-type (IL-1RI$^{+/+}$**,** A) and IL-1RI-deficient (IL-1RI$^{-/-}$**,** B) mice were fasted overnight and injected i.p. with saline or xenin (50 µg/g b.w.). Cumulative food intake was measured up to 8 h after injection. Data are means (% of the control saline-injected mice) ± SEM (n = 8/group). *: $P < 0.05$, §: $P < 0.0001$ (vs. control at same time point by Student's t-test).


A
IL-1RI+/+ mice
Saline
Leptin (1.2 µg/g b.w.)
Xenin (15 µg/g b.w.)
Cumulative food intake (%)
150
100
50
0
1
2
3
4
5
6
7
8
24
Hours of post-injection

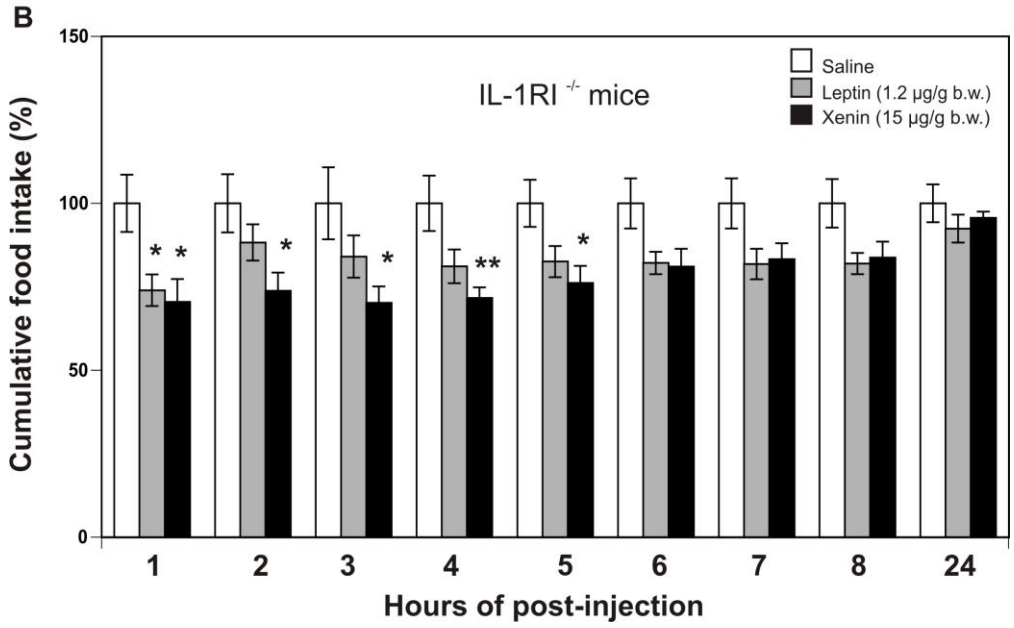

B
IL-1RI -/- mice
Saline
Leptin (1.2 µg/g b.w.)
Xenin (15 µg/g b.w.)
Cumulative food intake (%)
150
100
50
0
1
2
3
4
5
6
7
8
24
Hours of post-injection

Figure 26. Effect of i.p. injection of intermediate dose of xenin and leptin on food intake in wild-type and IL-1RI-deficient mice. Wild-type (IL-1RI$^{+/+}$**,** A) and IL-1RI-deficient (IL-1RI$^{-/-}$**,** B) mice were fasted overnight and injected i.p. with saline, leptin (1.2 μg/g b.w.) or xenin (15 μg/g b.w.). Cumulative food intake was measured up to 24 h after injection. Data are means (% of the control saline-injected mice) ± SEM (n = 8/group). *: $P < 0.05$, ** $P < 0.01$, † $P < 0.005$, § < 0.0001 (vs. control at same time point by Tukey-Kramer test).

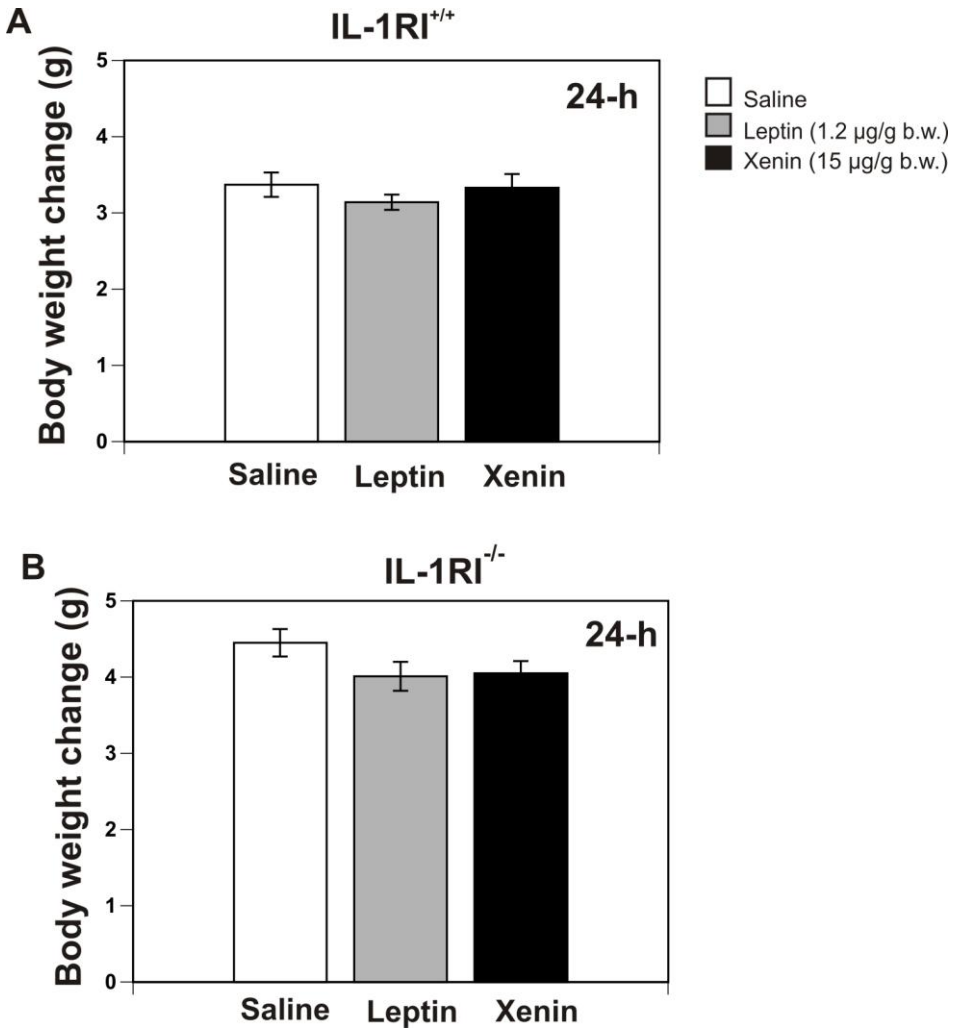


Figure 27. Effect of i.p. injection of intermediate dose of xenin and leptin on body weight in wild-type and IL-1RI-deficient mice. Wild-type (IL-1RI$^{+/+}$**,** A) and IL-1RI-deficient (IL-1RI$^{-/-}$**,** B) mice were fasted overnight and injected i.p. with saline, leptin (1.2. µg/g b.w.) or xenin (15 µg/g b.w.). Body weight change was measured 24 h after injection. Data are means ± SEM (*n* = 8/group).

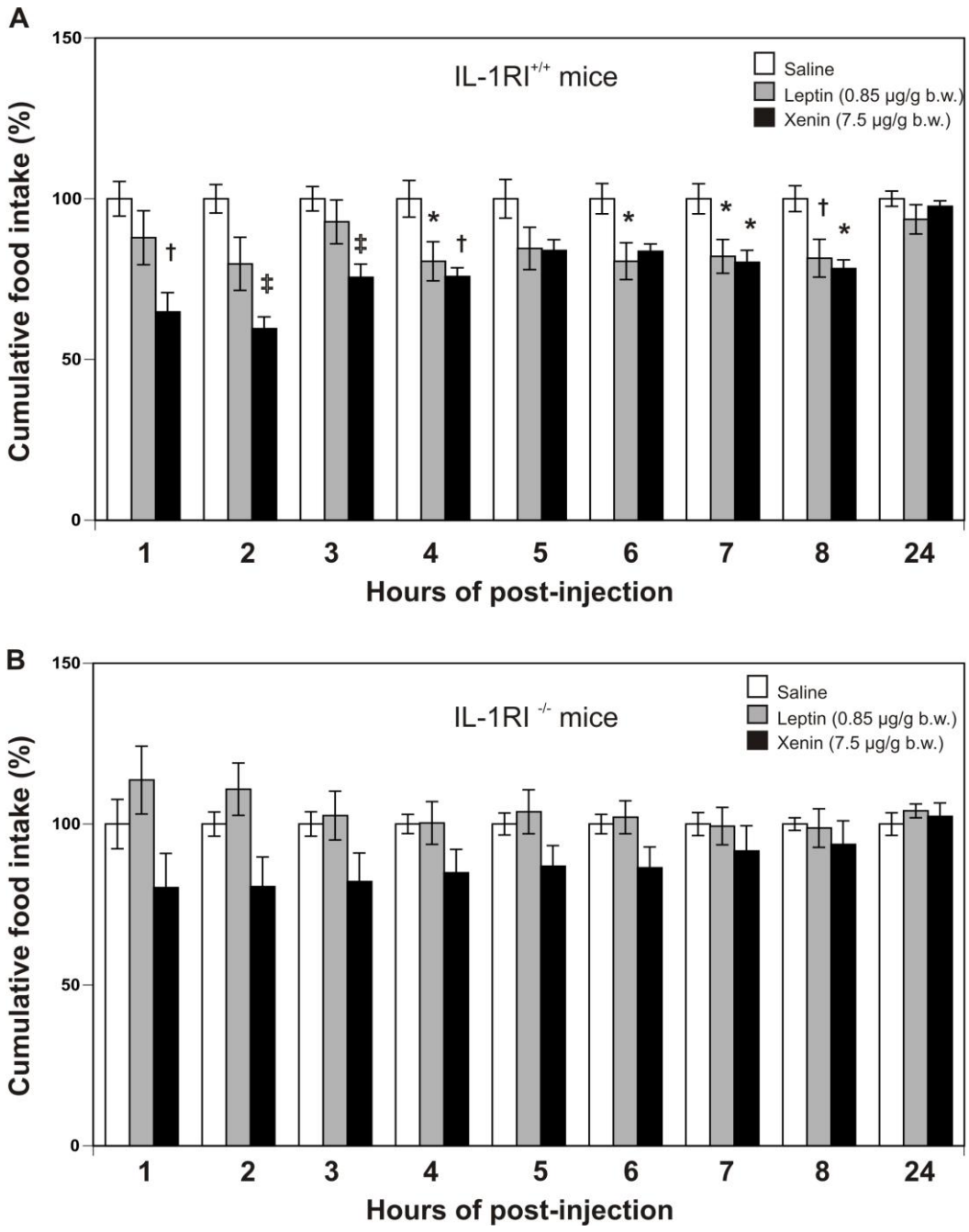

A
IL-1RI+/+ mice
Saline
Leptin (0.85 µg/g b.w.)
Xenin (7.5 µg/g b.w.)
Cumulative food intake (%)
150
100
50
0
1
2
3
4
5
6
7
8
24
Hours of post-injection
B
IL-1RI -/- mice
Saline
Leptin (0.85 µg/g b.w.)
Xenin (7.5 µg/g b.w.)
Cumulative food intake (%)
150
100
50
0
1
2
3
4
5
6
7
8
24
Hours of post-injection

Figure 28. Effect of i.p. injection of low dose of xenin and leptin on food intake in wild-type and IL-1RI-deficient mice. Wild-type (IL-1RI$^{+/+}$**,** A) and IL-1RI-deficient (IL-1RI$^{-/-}$**,** B) mice were fasted overnight and injected i.p. with saline, leptin (0.85. μg/g b.w.) or xenin (7.5 μg/g b.w.). Cumulative food intake was measured up to 24 h after injection. Data (% of the control saline-injected mice) are means ± SEM (*n* = 8/group). *: $P < 0.05$, † $P < 0.005$, ‡ < 0.001(vs. control at same time point by Tukey-Kramer test).

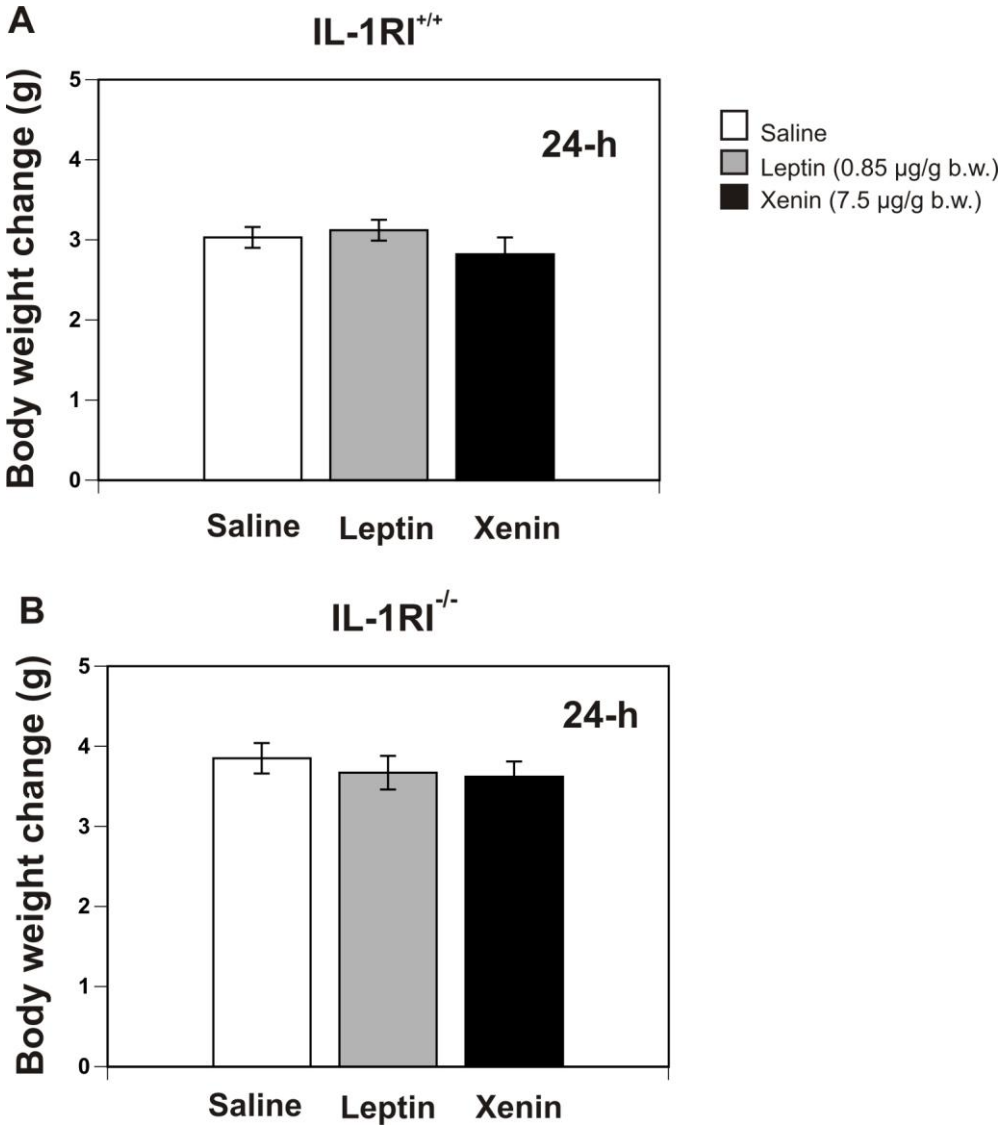


Figure 29. Effect of i.p. injection of low dose of xenin and leptin on body weight in wild-type and IL-1RI-deficient mice. Wild-type (IL-1RI$^{+/+}$**,** A) and IL-1RI-deficient (IL-1RI$^{-/-}$**,** B) mice were fasted overnight and injected i.p. with saline, leptin (0.85. µg/g b.w.) or xenin (7.5 µg/g b.w.). Body weight change was measured 24 h after injection. Data are means ± SEM (*n* = 6-7/group).

13. Effects of co-treatment with xenin and leptin on food intake and body weight in mice

To determine the interaction between xenin and leptin in the regulation of food intake, mice were given i.p. injection of a combination of xenin and leptin and food intake was measured. I.p. injection of a low dose of leptin (0.25 μg/g b.w.) alone did not cause any significant changes in cumulative food intake compare to control saline injection at any time point examined (Fig. 30). Cumulative food intake was significantly reduced by i.p. injection of a low dose of xenin (2.5 μg/g b.w.) alone compared to saline injection during the first two hours (Fig. 30). The i.p. injection of xenin alone did not cause significant reductions in cumulative food intake after 3-h time point. I.p. co-injection of xenin (2.5 μg/g b.w.) and leptin (0.25 μg/g b.w.) significantly reduced cumulative food intake compared to saline injection up to 5 h after injection (Fig. 30). There was no difference in cumulative food intake between the mice receiving xenin alone and those receiving xenin plus leptin at any time point examined.

To further determine the interaction of xenin and leptin in body weight regulation, mice received a 2-day xenin-leptin co-treatment. Daily food intake was not significantly changed by saline, leptin and xenin treatments compared to the pre-treatment levels (Table 9 and Fig. 31A). Xenin-leptin co-treatment caused a significant reduction in 24-h food intake on day 2 compared with the baseline food intake ($P < 0.05$ by Dunnett's test, Table 9). Reduction of daily food intake on days 2 was significantly greater in xenin-leptin co-

treatment group (32.7% reduction) compared to those in saline- (9% reduction), leptin- (9% reduction) and xenin-treated (9% reduction) mice ($P < 0.05$ by Tukey-Kramer test, Fig. 31A). Treatment with xenin alone, leptin alone, and xenin plus leptin had no significant effect on cumulative body weight change over the first 24 h of treatment compared to mice administered saline (Fig. 31B). Cumulative body weight reduction over the 2-day treatment period was significantly greater in xenin-leptin co-treated group compared with all other groups (Fig. 31B). Neither xenin alone nor leptin alone altered cumulative body weight change on day 2 compared with saline-treated control group (Fig. 31B).

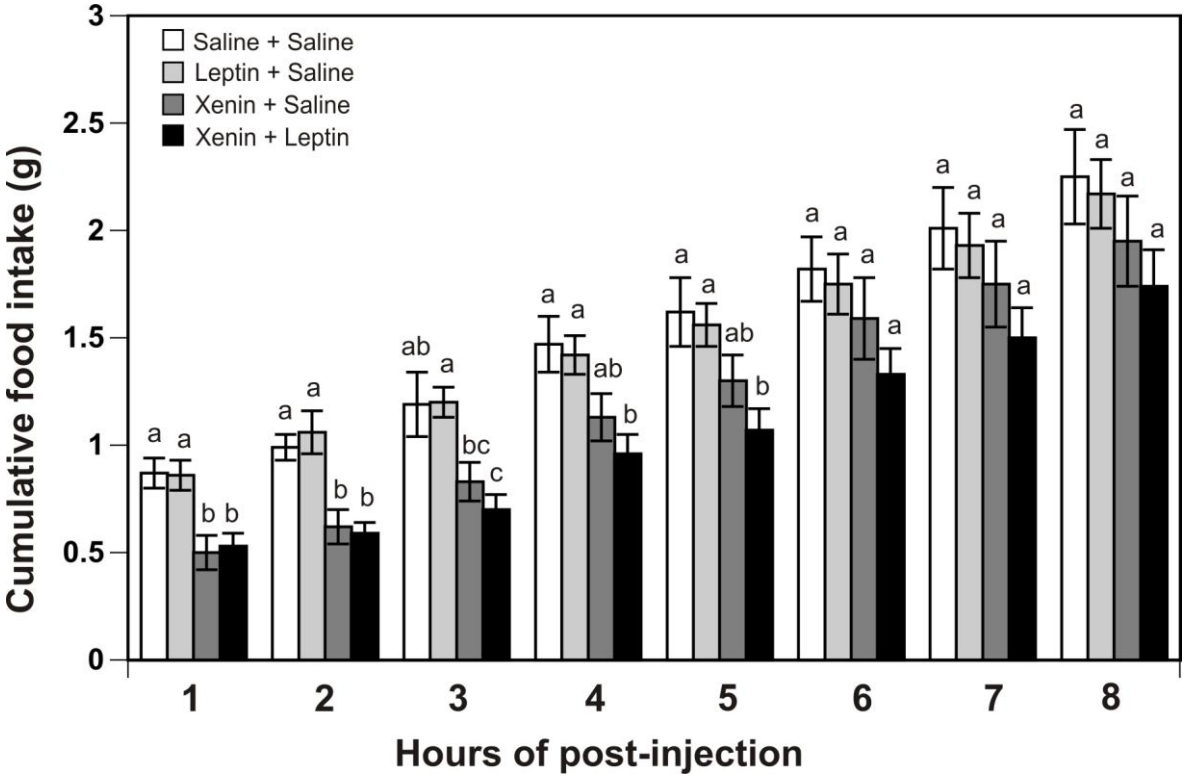


Figure 30. Effect of i.p. co-injection of xenin and leptin on food intake in mice. Mice were fasted overnight and injected i.p. with saline, leptin (0.25. μg/g b.w.), xenin (2.5 μg/g b.w.) or xenin (2.5 μg/g b.w.) plus leptin (0.25. μg/g b.w.). Cumulative food intake was measured up to 8 h after injection. Data are means ± SEM (n = 7-8/group). Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey-Kramer test).

Table 9. Effect of co-treatment with xenin and leptin on daily food intake in mice

	Day 0 (g)	Day 1 (g)	Day 2 (g)	*P**
Saline	3.99 ± 0.16	4.04 ± 0.29	3.63 ± 0.23	0.4102
Leptin	4.00 ± 0.16	3.47 ± 0.15	3.61 ± 0.19	0.1053
Xenin	4.29 ± 0.20	3.84 ± 0.24	3.86 ± 0.22	0.2928
Xenin + Leptin	4.26 ± 0.19[a]	3.73 ± 0.32[a]	2.91 ± 0.36[b]	0.0108
*P***	0.4987	0.4773	0.0869	

Values are means ± SEM (n = 7/group).

*: *P* values by one-way ANOVA within each treatment group.

**: *P* values by one-way ANOVA within each time point.

[a, b]: Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

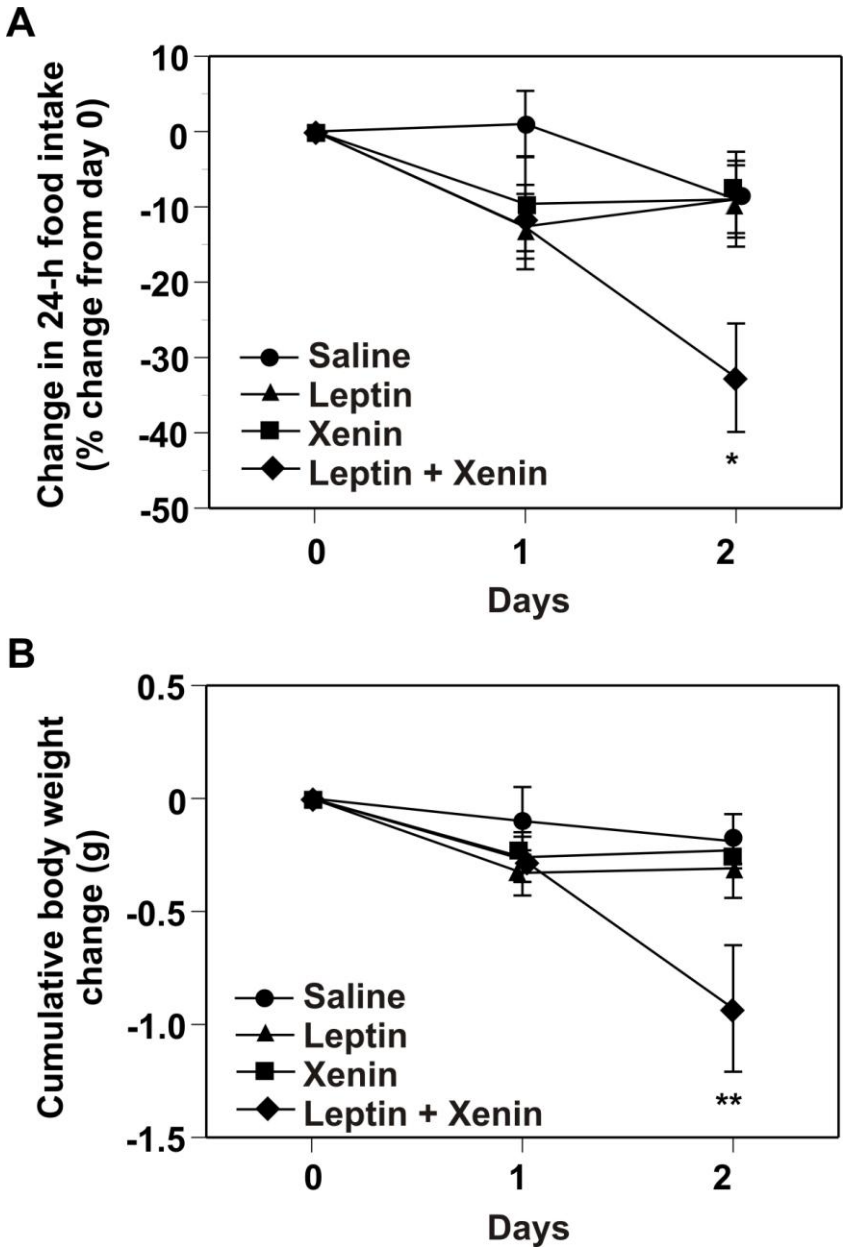

A
Change in 24-h food intake
(% change from day 0)
10
0
-10
-20
-30
-40
-50
Saline
Leptin
Xenin
Leptin + Xenin
*
0
1
2
Days
B
Cumulative body weight
change (g)
0.5
0
-0.5
-1.0
-1.5
Saline
Leptin
Xenin
Leptin + Xenin
**
0
1
2
Days

Figure 31. Effect of i.p. co-treatment with xenin and leptin on food intake and body weight in mice. *Ad libitum* fed mice received a twice daily i.p. injection of saline, leptin (0.25. μg/g b.w.), xenin (2.5 μg/g b.w.) or xenin (2.5 μg/g b.w.) plus leptin (0.25. μg/g b.w.) for 2 days. Food intake and body weight were measured 24 and 48 h after the first injection. Percentage changes in 24-h food intake from day 0 (A) and cumulative body weight changes (B) were calculated. Data are means ± SEM (n = 7/group). *: $P < 0.05$ (vs. saline, leptin and xenin at same time point, Tukey-Kramer test). **: $P < 0.05$ (vs. saline and xenin at same time point, Tukey-Kramer test).

VII. Discussion

1. Main findings

Xenin was identified almost 15 years ago and its anorexic effect was reported more than 10 years ago [243, 252]. However, since then little research has been conducted to investigate the role of xenin in the regulation of energy homeostasis. The present study was aimed at determining the effect of long-term xenin treatment on food intake, body weight, and energy expenditure. In the second part of the study, we sought to determine how xenin regulates food intake through its association with signalling pathways in the CNS. We found that the long-term treatment of xenin reduced food intake and body weight in mice. Xenin treatment decreased respiratory quotient (RQ) and altered the diurnal fluctuation pattern of RQ without causing alterations in energy expenditure or physical activity levels. We also demonstrated that xenin activated cells in the CNS including the hypothalamus and the brainstem. In particular, xenin treatment increased hypothalamic IL-1β mRNA expression. As well, the anorexic effect of xenin was abolished in Ntsr1-deficient mice and was attenuated in IL-1RI knockout mice. Lastly, the present study showed that co-treatment with xenin and the adipocyte hormone leptin extended the duration of xenin-induced feeding suppression. Furthermore, xenin-leptin co-treatment resulted in a greater reduction in body weight compared to that produced by xenin alone or leptin alone. Overall, these findings help further our understanding of the role of xenin in the regulation

of energy homeostasis and underlying mechanisms which were previously unexplored. These findings are discussed in more detail in the following sections.

2. Prolonged treatment with xenin causes sustained weight reduction.

Whole body energy balance is regulated by a balance between energy intake and energy expenditure. We sought to determine whether xenin decreases body weight by reducing energy intake, increasing energy expenditure, or both. In the present study, daily i.p. injection of xenin reduced food intake and body weight gain in normal lean mice, suggesting that xenin-induced reduction in body weight gain is at least partly due to reduced food intake. Similar changes in food intake and body weight were observed in mice receiving once a daily i.c.v. injection of xenin. Although we and others have shown that xenin reduces short-term food intake [243, 280, 286-288], the present findings are the first to demonstrate that xenin treatment for the extended period can produce sustained weight reduction in normal mice. It is important to note that our group found a similar weight-reducing effect with daily i.c.v. injections of xenin in a mouse model of obesity in a subsequent study (Leckstrom & Mizuno, unpublished data). Xenin reduces body weight by maximum 2.4% and 5.73% to saline after daily i.p and i.c.v. injections, respectively. This reduction is significant, because of that modest weight loss is associated with beneficial effect on health. Initial body weight reduction between 5-10% improves cardiovascular risk, hypertension and incidence of type 2 diabetes [289]. One group reported that chronic xenin treatment either by continuous subcutaneous infusion (for 6 days) or twice daily i.p.

injection (for 5 days) did not cause any significant changes in daily food intake and body weight throughout the experimental period in mice [287]. The reason for this discrepancy is unknown at present. The lack of sustained anorectic effect of xenin in these studies may be due to the short half-life of xenin in the circulation or tachyphylaxis [264]. It is also possible that these studies used lower doses (3.7 and 10.7 μg/g b.w.) than the dose (50 μg/g b.w.) used in the present study. Continuous infusion protocol may increase circulating xenin level above basal line, but this level may not be high enough to produce the weight-reducing effect. Note that i.p. injection of a lower dose of xenin (1 nmol per mouse) increased plasma xenin levels (> 2,000 pg/ml) 5 min post-injection, while plasma immunoreactive xenin was below the detection limit of the assay (40 pg/ml) under fasted condition or after oral glucose challenge in mice [277]. Thus, the dose which we used in the present study most likely represents a pharmacological dose. Taken together, these data suggest that although the anorectic effect of xenin is short-lived, xenin can decrease the long-term body weight, at least when administered at high doses. It is often found that prolonged treatment with gut hormones by repeated administration or continuous infusion fails to cause sustained reductions in food intake and body weight [163-165, 233]. These characteristics make it unlikely that the manipulation of gut hormone' signalling is a promising therapeutic strategy for the treatment of obesity. The present findings that daily xenin treatment maintained lower body weight gain for at least 10 days raise the possibility that xenin treatment over extended periods may be an effective obesity therapy. Nevertheless, further studies are necessary to establish longer term body weight regulation by xenin and to improve the delivery method. It is also important to develop small

molecule mimetics or breakdown-resistant analogues, which retain all of biological actions of xenin with the prolonged duration of action.

3. Prolonged treatment with xenin increases fat utilization and reduces adiposity.

Interestingly, xenin treatment maintained significantly decreased body weight gain even after the effect of xenin on daily food intake was no longer significant. Note that cumulative food intake during the entire treatment period was also not altered by xenin treatment. These findings suggest that xenin reduces body weight not only by reducing energy intake but also by increasing energy expenditure. To directly address this hypothesis, we examined the effect of xenin treatment on energy expenditure by using indirect calorimetry. Contrary to our prediction, daily i.p. xenin treatment did not cause any significant changes in oxygen consumption, carbon dioxide production, heat production or horizontal activity level in normal lean mice. On the other hand, RQ was significantly lower in xenin-treated mice compared to the control mice. RQ is an indirect measure of the ratio of carbohydrates and fats being oxidized as an energy source, and a low RQ indicates increased utilization of fats relative to carbohydrates [290]. It has been suggested that a low ratio of fat to carbohydrate oxidation, a high RQ, is associated with accelerated body weight gain [291, 292]. Consistent with reduced RQ, epididymal fat pad weight was reduced by daily xenin treatment. These data suggest that xenin reduces body weight partly

by increasing mobilization of fat from the adipose tissue to other tissues such as skeletal muscle and thereby increasing the oxidization of fatty acids.

To correlate metabolism-related gene expression levels with metabolic consequences of xenin treatment, we measured expression levels of metabolism-related genes in the BAT, WAT and skeletal muscle. We found no differences in UCP1 and PGC1α in BAT and UCP3 and CPT1b in skeletal muscle between control and xenin-treated mice. Increased levels of these genes are associated with increased thermogenesis and energy expenditure, thus these data are consistent with the finding that xenin treatment did not cause any changes in energy expenditure. Only mRNA levels were measured in the present study. Follow-up studies to measure protein levels will be beneficial to further determine the association of these molecules to the metabolic effects of xenin. We found that the expression of lipogenic DGAT2 mRNA was reduced in WAT and the expression of lipolytic HSL mRNA was increased in WAT of xenin-treated mice compared with vehicle-treated control mice. In addition to the xenin-induced reduction in RQ, these changes in gene expression suggest that xenin reduces adiposity and body weight by promoting fat oxidation and limiting fat storage. However, skeletal muscle may not be involved in the fat oxidation by xenin.

4. Prolonged treatment with xenin alters diurnal variation of RQ.

As discussed above, low RQ may contribute to xenin-induced reduction in body weight and adiposity. Although RQ was significantly lower at time points earlier than day 3 of the 7-day treatment, there was no difference in RQ thereafter. Thus sustained reduction of body weight during xenin treatment cannot be explained simply by reduced RQ. Growing evidence suggests that biological rhythms profoundly influence energy homeostasis and that disruption of daily metabolic rhythms is an exacerbating factor in metabolic syndrome [293]. RQ during a 24-h period exhibited a diurnal fluctuation with higher levels during the dark cycle than during the light cycle [294, 295]. Rate of adipocyte lipolysis increases during the light cycle, resulting in increased fatty acids release into the circulation. Conversely, during the dark cycle, rate of adipocyte lipolysis decreases, with a concomitant increase in lipogenesis. Diurnal variation of skeletal muscle lipoprotein lipase (LPL) activity is completely antiphase with the diurnal variation of RQ, while LPL activity in the adipose tissue positively correlates with RQ [296, 297]. Thus, higher RQ during the dark cycle is associated with higher LPL activity in the adipose tissue (i.e. increased uptake of fatty acids by adipose tissue) and lower LPL activity in the skeletal muscle (i.e. reduced uptake of fatty acids by skeletal muscle). These changes in LPL activity result in accelerated fat accumulation and decreased fat oxidation during the dark cycle. A recent study demonstrated that the timing of food ingestion affects diurnal rhythm of metabolic function, leading to the alterations in whole body energy balance [298]. In that study, mice were provided a high-fat diet either at the beginning or the end of the dark cycle and metabolic variables were measured while total daily caloric intake as well as the same proportion of calories from fat, carbohydrate, and protein were kept identical between the groups. Mice fed a high-fat diet at the end of the dark cycle had increased body weight and

adiposity compared with mice fed the same high-fat diet at the beginning of the dark cycle. RQ during the dark cycle was higher in mice given high-fat diet at the end of the dark cycle compared to mice given high-fat diet at the beginning of the dark cycle, resulting in an increased amplitude of the nocturnal peak of RQ in the former mice. Higher RQ during the dark cycle in the mice fed high-fat diet at the end of the dark cycle suggests that these mice failed to activate fat oxidation, causing increased fat accumulation. In that study, there was no difference in energy expenditure and activity levels between the groups [298]. This is clear indication that alterations in diurnal variation of RQ lead to changes in whole body energy homeostasis independent of total caloric intake and whole body energy expenditure. Therefore, the present finding that blunted nocturnal peak of RQ without significant changes in energy expenditure and activity levels in xenin-treated mice suggests that xenin reduces body weight by altering diurnal rhythm of nutrient mobilization independent of energy expenditure. More specifically, xenin reduces body weight by increasing fat oxidation during the dark cycle. It is of interest to determine whether xenin treatment causes similar changes in diurnal variation of lipid metabolism (e.g. circulating NEFA levels and lipolytic activity in adipose tissue) in future studies.

5. Xenin does not cause taste aversion.

Suppression of food intake can occur for many reasons, including malaise, stress and motor impairments. Many appetite-suppressing substances, including gut hormones, inhibit feeding partly by causing nausea and taste aversion [91, 174, 299]. Therefore, it is

possible that the feeding-suppressing effect of xenin is due to nonspecific effect of xenin as it has been observed for several anorexigenic substances. Behavioral pattern changes occur in sick animals. For example, increased resting behaviour occurs as a result of sickness. In the present study, daily xenin treatment did not cause significant changes in activity levels throughout the 7-day treatment period, suggesting that resting behaviour was not altered by xenin treatment. Two other studies examined the effect of xenin on behaviour in animals. Neither resting behaviour nor anxiety-related behaviours such as locomotion in chicks were altered following i.c.v. injection of xenin [300]. Although both i.c.v. and i.p. injection of xenin reduced activity levels and increased resting behaviour during the first hour post-injection in rats and mice, respectively, no obvious aversive behaviours such as hunched posture and tremors were observed in these xenin-injected animals [287]. Therefore, xenin-induced feeding suppression is not due to aversive response to xenin. To further determine whether xenin causes an aversive effect or not, we examined the effect of xenin on conditioned taste aversion in mice. The i.p. injection of xenin at a dose that produces the robust anorexic effect did not cause a significant taste aversion, whereas, i.p. injection of LiCl caused severe taste aversion [288]. These data support the hypothesis that xenin-induced anorexia is not attributable to an aversive response to xenin.

Strong taste aversive stimuli activate specific neuronal populations in the brainstem. Specifically LiCl increases Fos expression in the AP and intermediate NTS of the brainstem [301-303]. A very similar Fos expression pattern in these brainstem regions was observed following an i.p. injection of CCK and PYY_{3-36} at doses that cause taste aversion [174]. Thus, activation of cells in the AP and intermediate NTS may serve as a marker of the establishment of taste aversion. In the present study, i.p. injection of the anorexic dose

of xenin did not increase Fos expression in the AP. Xenin increased the number of Fos-immunoreactive cells in the ventrolateral NTS, but it did not induce Fos in the intermediate NTS [304]. These findings further support the view that xenin-induced anorexia is not due to the production of aversive responses.

6. Xenin delays gastric emptying.

It has been well demonstrated that ingestion of food affects gastrointestinal motility and the rate of gastric emptying, in turn, affects food intake [222]. The rate of gastric emptying is accelerated in animal models of obesity, indicating that rapid gastric emptying contributes to hyperphagia and increased body weight gain [226, 227]. The anorexic effects of a number of gastrointestinal peptides such as CCK and GLP-1 are associated with delayed gastric emptying [91, 155, 198]. Conversely, feeding-stimulatory effect of ghrelin is associated with accelerated gastric emptying rate [208]. In the present study, we found that i.p. injection of xenin significantly reduced the 2-h rate of gastric emptying in mice at the dose which produces a robust suppression of feeding during the first 1-2 hours after injection in mice [304]. The temporal correlation between the anorectic effect of xenin and the inhibitory effect of xenin on gastric emptying suggests that xenin reduces food intake, at least partly, by slowing gastric emptying. In agreement with our findings, earlier studies demonstrated that xenin affects the motility of gastrointestinal tract [255, 256, 270, 271]. Furthermore, i.c.v. administration of xenin increased gastrointestinal transit time in chicks

[286]. Taken together, it is likely that delayed gastric emptying contributes to xenin-induced feeding suppression.

Gastric distension strongly stimulates liquefaction of foodstuffs and causes the relaxation of the pylorus, and hence expels gastric contents into the duodenum. In the present study, the mice were given 1-h feeding period prior to measurement of gastric emptying rather than gavaging the same % body weight diet directly into the stomach. Variation in the amount of spontaneous food intake might cause variation of gastric distension among animals, affecting gastric emptying. Both 1-h food intake and stomach weight were not different between the groups. To estimate gastric distension before i.p. injection of drugs, food intake (g) during the 1-h feeding period was normalized to stomach weight (g). There were no significant differences in 1-h food intake per gram of stomach weight between xenin-treated mice and saline-treated mice. These data suggest that the degree of gastric distension was not different between the groups before i.p. injection of saline or xenin. Thus, gastric distension is not a major contributing factor to xenin-induced delay in gastric emptying in the present study.

Xenin delayed gastric emptying and thereby increased gastric contents remaining after food ingestion. It is assumed that the reduction in gastric emptying rate also reduced the amount of nutrients entering the small intestine. Furthermore, delayed gastric emptying results in greater gastric distension which leads to secretion of other anorectic peptides [305, 306]. Thus, it is possible that increased secretion of other anorectic peptides mediate the feeding-suppressing effect of xenin. Therefore, the present study cannot determine whether xenin-induced feeding suppression is due to reduced stimulation of intestine by nutrients, increased secretion of other anorectic peptides, or delayed gastric emptying *per se*. Further

investigations are necessary to determine the detailed mechanism for xenin-induced delay in gastric emptying and reduction in food intake. Overall, our data support the hypothesis that delayed gastric emptying contributes to the ability of xenin to inhibit food intake.

7. Xenin activates hypothalamic cells.

Although the main site of xenin production is gastrointestinal tract, i.c.v. administration of xenin reduces food intake, indicating that the CNS cells mediate the anorectic action of xenin. However, the exact site(s) of xenin action was unknown. Within the CNS, the hypothalamus plays a major role in the regulation of food intake. A number of anorectic gastrointestinal hormones reduce food intake partly by activating the hypothalamus [91], leading to the hypothesis that xenin reduces food intake through the activation of hypothalamic neurons. In the present study, we found that the levels of hypothalamic *c-fos* mRNA, a marker of cell activation, were increased following i.p. injection of xenin in normal mice. However, levels of *c-fos* mRNA in the cortex were not altered by i.p. injection of xenin [288]. These data support the hypothesis that xenin reduces food intake by specifically activating hypothalamic neurons.

To further determine which hypothalamic area is activated by xenin, xenin-induced Fos expression was assessed by immunohistochemistry in the present study. The i.p. injection of xenin increased the number of Fos-immunoreactive cells in the PVN, ARC, VMN, DMN, and SON but not in the LHA [288]. Similarly, both i.p. and i.c.v. injection of xenin increased the number of Fos-immunoreactive cells in the VMH of chicks [286].

However, the same dose of i.p. or i.c.v. xenin did not cause significant changes in the number of Fos-immunoreactive cells in the LHA. These data suggest that the anorectic effect of xenin is mediated through the activation of subpopulation of hypothalamic neurons. The present study also cannot determine neurochemical characteristics of the Fos-positive cells. These issues need to be addressed in the future. Consistent with xenin-induced Fos expression pattern, injection of xenin directly into the VMH recapitulated the feeding-suppressing effect of i.c.v. xenin in chicks, while direct injection of xenin into the LHA failed to cause a reduction in food intake [243, 300]. These findings suggest that xenin specifically activates the hypothalamus and that a subset of hypothalamic cells mediate the feeding-suppressing effect of xenin.

8. Xenin activates brainstem cells.

In addition to the hypothalamus, the brainstem participates in the regulation of food intake partly by mediating gastrointestinal signal-induced feeding termination [91, 307]. Vagal afferent fibres convey signals important for the termination of feeding primarily to the NTS within the dorsal vagal complex (DVC) of the brainstem. Activation of NTS cells by gut distension and ingestion of food via the vagal afferent neurons contributes to the termination of feeding [307]. Vagotomy blocks or attenuates the effect of gut hormones on food intake and DVC activation, indicating that gut hormones affect feeding by signalling to the brainstem via the vagal afferent neurons [91, 307]. Strong evidence exists to suggest that food intake is regulated specifically by the NTS. Brainstem lesions including the NTS,

attenuate the anorectic action of several gastrointestinal peptides [308-310]. In the present study, we showed that the peripherally administered xenin activates a subset of NTS cells as represented by increased Fos expression [304]. In addition to the NTS, other DVC areas may also play a role in gut hormone-induced satiety [310], but we did not observe any significant effect of xenin treatment on the number of Fos-immunoreactive cells in the AP and the DMV. As discussed later, the feeding-suppressing effect of xenin is partly mediated through Ntsr1 and neurotensin binding sites are present in the vagal afferent neurons, suggesting the possibility that xenin-induced NTS activation and feeding suppression are mediated through the direct action of xenin on the vagal afferent fibres [311]. Taken together, our data argue for the possibility that peripherally administered xenin delays gastric emptying and reduces food intake at least partly through the activation of the NTS cells possibly by a direct action of xenin on vagal afferent neurons.

There are reciprocal neural connections between the hypothalamus and the brainstem, and therefore the brainstem regulates food intake possibly by interacting with the hypothalamus. Because the i.p. injection of xenin induces Fos expression both in the hypothalamus and the brainstem, the effect of xenin on gastric emptying and food intake may be mediated through the hypothalamus-brainstem connection [286, 288, 304]. However, there is also evidence indicating that the brainstem plays a major role in mediating the effect of gastrointestinal peptides on gastric emptying and food intake. Injection of GLP-1 receptor agonist, exendin-4 (Ex-4) reduces gastric emptying rate and food intake in chronically supracollicular decerebrate rats to a similar extent as in control rats, indicating that the hypothalamus-brainstem connection is not necessary for the Ex-4-induced delay in gastric emptying and feeding suppression [312]. It has also been

demonstrated that many dendrites of vagal gastric motor neurons extend from the DMV into the NTS and make synaptic contacts with vagal gastric primary sensory neurons in the NTS [118, 119]. These findings support the possibility that the brainstem is sufficient to mediate the effect of gastrointestinal peptides on gastric emptying and food intake, and that the hypothalamus does not play a major role in this regulation. Thus, it is possible that xenin-induced delay of gastric emptying is mediated via the vagal afferent-brainstem-vagal efferent circuit. Although further studies are necessary to determine whether the brainstem alone is sufficient or both the brainstem and the hypothalamus are required for xenin-induced reductions in gastric emptying rate and food intake, our data strongly suggest that the brainstem plays a key role in these xenin actions.

9. CNS Ntsr1 mediates metabolic action of xenin.

As mentioned earlier, central administration of xenin reduces food intake, reduces body weight, and induces Fos expression in a number of CNS regions. This suggests that the metabolic effects of xenin are mediated through CNS cells. However, the neurochemical identity of these potential mediators of CNS xenin action is unknown. The CNS neurotensin system plays a role in the regulation of food intake and metabolism. Although the specific receptors for xenin have not yet been identified, it has been suggested that the effect of xenin is mediated, at least partly, through neurotensin receptor (Ntsr) 1. Xenin has a strong sequence homology to the C-terminal of neurotensin and binds to the Ntsr1 receptor with an affinity comparable to that of neurotensin [252, 313]. Xenin-

induced changes in gastrointestinal motility and pancreatic secretion are attenuated by an Ntsr1 antagonist [255, 271, 274]. We found that the i.c.v. injection of xenin did not inhibit fasting-induced hyperphagic response in Ntsr1-deficient mice [314]. It is likely that CNS Ntsr1 mediates the anorectic effect of xenin since xenin was injected i.c.v. in the present study. Peripherally-administered xenin also reduces food intake [286-288], however, it is unknown whether or not xenin passes the blood-brain barrier to activate the central Ntsr1 receptors. Neurotensin binding sites are present in the vagal afferent neurons [311]. These findings raise the possibility that xenin-induced feeding suppression is mediated through the direct and/or indirect action of xenin on Ntsr1 in the CNS and the direct action of xenin on the vagal afferent fibres.

To determine whether Ntsr1-deficieny specifically blocks the feeding-supressing effects of neurotensin and xenin, we examined the effect of NMU on food intake in Ntsr1-deficient mice. It has been demonstrated that central administration of NMU suppresses food intake and reduces body weight [315, 316]. Although the feeding-suppressing effect of NMU is predominantly mediated via NMU receptor 2 (NMUR2), it was also suggested that Ntsr1 mediates some effects of NMU [315-317]. We found that the i.c.v. administration of NMU significantly reduced cumulative food intake and body weight gain in both wild-type and Ntsr1-deficient mice [314]. These findings indicate that Ntsr1 does not play a role in NMU-induced feeding suppression and body weight reduction and that the absence of Ntsr1 specifically blocks the anorectic effects of xenin and neurotensin. Taken together, these data suggest that Ntsr1 plays a role in mediating the anorectic effect of xenin and that xenin and neurotensin share the common CNS signalling pathways involving the Ntsr1 in the regulation of food intake.

10. Xenin and leptin converge on CNS Ntsr1.

Metabolic effects of leptin are mediated through a wide variety of signalling pathways in the hypothalamus [101]. Although the leptin-melanocortin pathway is considered as a major mechanism of metabolic regulation by leptin [16], it has been suggested that the metabolic effect of leptin is at least partly mediated by a neurotensin-Ntsr signalling pathway. Levels of neurotensin protein and mRNA are decreased in leptin-deficient *ob/ob* mice and i.c.v. administration of leptin stimulates expression of neurotensin mRNA in the hypothalamus [136, 137]. Chronic i.c.v. infusion of leptin results in the lack of leptin-induced feeding suppression and increase in hypothalamic neurotensin expression [318]. These findings suggest that hypothalamic neurotensin mediates leptin action and that the blunted response of hypothalamic neurotensin gene expression to leptin contributes to the development of leptin resistance. In agreement with the proposed role for CNS neurotensin-Ntsr pathway in the mediation of metabolic action of leptin, the anorectic effect of leptin is blocked by a potent Ntsr1 antagonist, SR 48692 [138]. However, SR 48692 is also a potent agonist for Ntsr2, and therefore, these pharmacological studies cannot rule out the Ntsr2 receptor's role in the anorectic action of leptin [319-321]. In the present study, leptin-induced reductions in food intake and body weight were attenuated in Ntsr1-deficient mice [322], suggesting that CNS Ntsr1 plays a role in mediating the metabolic effect of leptin. It should be noted that the absence of Ntsr1 only partially attenuated the anorectic and weight-reducing effects of leptin. Thus, it is likely that CNS

Ntsr1 mediates some but not all actions of leptin. Furthermore, the anorectic effect of xenin is abolished in Ntsr1-deficient mice, suggesting that xenin and leptin converge in the CNS signalling pathway involving Ntsr1 to regulate food intake and body weight.

11. Xenin activates hypothalamic MAPK signalling pathway.

Similarities in the biological effects of xenin and leptin (i.e. feeding-suppressing and weight-reducing effects) and CNS actions of xenin and leptin (i.e. action via Ntsr1) suggest that these two hormonal signals converge on key central intracellular signalling pathways. As a first step towards the identification of downstream mediators of xenin action, we decided to determine the effect of xenin on hypothalamic signalling molecules which mediate leptin action. Although hypothalamic STAT3 pathway is a major component of intracellular leptin signalling pathway, several non-STAT3 pathways can also mediate leptin action in the hypothalamus [101]. CNS MAPK signalling participates in the regulation of energy balance by mediating the effect of peripheral nutrient and hormonal signals including gastrointestinal hormones [323-327]. More specifically, leptin increases phosphorylation of ERK1/2 in the hypothalamus *in vivo* and in hypothalamic cell lines *in vitro* [99, 139, 328]. ERK1/2 is also phosphorylated in response to the activation of Ntsr1 by neurotensin in several cell types including neuronal cells [329-331]. A recent study also demonstrated that xenin increases phosphorylation of ERK1/2 in exocrine pancreatic cells [277]. These findings led us to hypothesize that xenin regulates food intake through the activation of hypothalamic MAPK signalling pathway. In the present study, we

showed that xenin increased the number of pERK1/2-immunoreactive cells in the hypothalamic ARC, suggesting that the metabolic effect of xenin is, at least partly, mediated through the activation of MAPK signalling pathway.

The hypothalamic ARC contains two distinct neuronal populations expressing either orexigenic NPY/AGRP or anorexigenic POMC/CART. Fasting increases phosphorylation of ERK1/2 in the hypothalamus and increases in phospho-ERK1/2 (pERK1/2) levels are associated with fasting-induced NYP expression in the ARC [323, 324]. Leptin increases pERK1/2 expression specifically in the ARC POMC-expressing neurons, but not in NPY/AGRP-producing neurons [99]. The effect of leptin on food intake, body weight, and sympathetic outflow is attenuated or abolished by i.c.v. pre-treatment with inhibitors of MEK1/2, an upstream effector of ERK1/2 [99]. Furthermore, leptin increased the expression of anorexigenic neurotensin gene in hypothalamic cell lines and this effect was negated by the MEK1/2 inhibitor [139]. These data suggest that increased MAPK signalling in ARC POMC/CART neurons and neurotensin neurons promotes satiety, while MAPK signalling in NPY/AGRP neurons stimulates feeding. Therefore, we speculate that xenin increases MAPK signalling in anorexigenic ARC neurons, thereby reduces food intake. Further studies are necessary to identify specific ARC neurons, in which phosphorylation of ERK1/2 is stimulated by xenin, and other molecules in the MAPK signaling pathway. Collectively, these findings support the hypothesis that xenin regulates food intake, at least partly, by activating hypothalamic MAPK signalling pathway. Also, the hypothalamic MAPK signalling pathway is a possible location for xenin and leptin signalling convergence.

12. CNS IL-1 signalling mediates xenin action.

Although the anorectic effect of xenin is at least partly mediated centrally, CNS mediators of xenin action are unknown. The hypothalamic melanocortin system plays a critical role in the regulation of energy homeostasis by integrating nutrient and hormonal signals including gut hormones [16]. Xenin activated cells in the ARC and PVH where POMC-, AGRP- and MC4R-expressing neurons are present, suggesting the possibility that CNS melanocortin signalling mediates the effect of xenin on food intake. Contrary to this proposal, xenin reduced food intake to a similar extent both in wild-type and *agouti* mice which have impaired signalling through MC4R [288]. Xenin-induced anorexia is not attenuated by i.c.v. injection of a melanocortin receptor antagonist in mice [288]. Furthermore, xenin treatment does not affect hypothalamic POMC and AGRP mRNA levels in mice [288] (also present study). Consistent with the results of these *in vivo* studies, xenin did not influence the release of α-MSH, an anorectic POMC-derived neuropeptide, from the hypothalamic explants cultured *ex vivo* [287]. Another anorectic neuropeptide, CART is expressed in hypothalamic POMC neurons [332]. Xenin did not affect CART release from *ex vivo* hypothalamic explants [287]. In contrast, xenin treatment increased the release of the anorexigenic neuropeptide, corticotropin-releasing hormone (CRH) from *ex vivo* hypothalamic explants, and i.c.v. pre-treatment with a CRH antagonist attenuated the feeding-suppressing effect of i.c.v. xenin in rats [287]. Furthermore, i.c.v. injection of naloxone, an opioid receptor antagonist, potentiated the anorectic effect of xenin in rats

[244]. Although low doses of xenin or naloxone alone did not affect food intake, i.c.v. co-injection of xenin and naloxone reduced food intake in rats and chicks [244, 300]. These findings suggest that the anorectic action of xenin is mediated, at least partly, though CNS signalling used by CRH and opioids, but not melanocortins. Although these neuropeptides are candidates for the CNS mediator of xenin action, further investigations are needed to determine the entire picture of CNS action of xenin.

To further search for CNS mediators of xenin action, we performed PCR array analysis using hypothalamic cDNA from control and xenin-treated mice. We found that hypothalamic IL-1β mRNA levels were increased by xenin treatment, suggesting that hypothalamic IL-1β mediates xenin action. In addition to the role in the regulation of inflammation, IL-1 participates in the regulation of metabolism. Central administration of IL-1β reduces food intake and body weight in rats [142, 143]. IL-1 binding sites are present in the hypothalamus and IL-1RI is expressed in the hypothalamic ARC and VMN [333-335]. These findings suggest that central IL-1β-IL-1RI signalling plays a role in the regulation of energy balance.

Hypothalamic IL-1β expression is reduced in leptin-resistant obese animals and is increased by leptin treatment [144, 336, 337]. Impaired signalling through hypothalamic IL-1RI abolishes anorectic and weight-reducing effects of leptin [141, 145, 146, 338]. In the present study, we confirmed the previous finding that the anorectic effect of leptin is attenuated in IL-1RI-deficient mice. More importantly, our study demonstrated that feeding-suppressing effect of xenin was also attenuated in IL-1RI-deficient mice. These findings support the hypothesis that hypothalamic signalling through IL-1RI regulates

energy homeostasis at least partly by mediating the metabolic effects of xenin and leptin. Based on these findings, we propose that xenin regulates food intake by interacting with leptin through the activation of hypothalamic IL-1β-IL-1RI signalling pathway. In addition to IL-1β, several other genes were up-regulated and one gene was down-regulated by xenin in the present PCR array study. Although we decided to further investigate the role of IL-1β in the mediation of xenin action in the present study, these other genes may also mediate the metabolic effects of xenin as well as leptin.

IL-1Ra KO mice showed reduced adiposity, increased lipase activity and moderate hypophagia without significant changes in energy expenditure [140]. Young pre-obese IL-1RI KO mice showed increased respiratory quotient (RQ) and increased locomotor activity without significant changes in food intake and oxygen consumption [141, 338]. These phenotypes suggest that signalling via IL-1RI regulates body weight through alterations in lipid metabolism and physical activity levels. Of particular interest, we found that xenin treatment reduced body weight gain, fat pad weight, lipogenic gene expression and RQ (i.e. increased fat oxidation) and increased lipolytic gene expression without significant changes in energy expenditure in normal mice. Similarities between the metabolic phenotype of IL-1Ra KO mice and the consequence of xenin treatment in normal mice are consistent with the hypothesis that the metabolic effects of xenin are partly mediated through the IL-1β-IL-1RI signalling pathways.

13. Leptin prolongs the anorexic effect of xenin.

The present study demonstrated that although daily xenin treatment led to alterations in diurnal fluctuation of RQ and sustained weight reduction, it reduced food intake only transiently. These characteristics may limit its possible benefit for anti-obesity treatment. A number of gut-derived hormones regulate food intake and body weight by interacting with leptin [204, 228-231, 233, 339, 340]. Of particular interest, the present study showed that the anorectic effect of xenin is mediated via CNS signalling pathways involving Ntsr1, MAPK, and IL-1β. These signalling pathways also mediate some effects of leptin on metabolism, leading to the hypothesis that xenin and leptin converge onto common signalling pathways in the CNS to regulate energy homeostasis. It was further hypothesized that combination treatment with xenin and leptin may produce a greater effect on food intake and body weight compared to that produced by xenin or leptin monotherapy, and overcome limited efficacy of xenin monotherapy or leptin resistance. In the present study, we showed that a single i.p. injection of xenin plus leptin did not cause significantly greater reductions in food intake compared to xenin alone. Moreover, the same combination treatment causes a longer duration feeding-suppression than that caused by xenin alone. Note that the low dose of leptin alone did not influence food intake in this study. These results are similar to changes in food intake when PYY_{3-36} was given to the rats together with leptin [233]. It is likely that leptin extends the duration of the anorectic action of xenin.

The 2-day treatment study demonstrated that although neither xenin alone nor leptin alone altered food intake and body weight, combined treatment with xenin and leptin caused significantly greater reductions in food intake and body weight compared to saline, xenin alone, or leptin alone treatment. These data suggest the possibility that xenin and

leptin reduce food intake and body weight, at least partly, through the synergistic interaction. A similar synergistic interaction has been reported between CCK and leptin [228-230]. Further studies including dose-response curves for both peptides are required to determine the precise nature of xenin-leptin interaction.

How does the interaction between xenin and leptin occur? Although low dose of CCK or leptin alone does not alter hypothalamic Fos expression, co-administration of CCK and leptin causes a robust increase in Fos expression in the hypothalamus, suggesting that synergistic interaction between CCK and leptin occurs at hypothalamic level [228, 240]. Because both anorectic effects of xenin and leptin are mediated through the CNS, in particular the hypothalamus, we propose that the xenin-leptin interaction occurs in the CNS. Leptin activates a subset of neurons in hypothalamic and brainstem nuclei; PVN, ARC, VMN, DMN, and NTS [129, 341]. Furthermore, both xenin and leptin increase phosphorylation of ERK1/2 in the hypothalamic ARC and anorectic effects of xenin and leptin are mediated via Ntsr1 and IL-1RI [314]. These data suggest the possibility that leptin enhances the action of xenin in these brain regions by activating common signalling pathways. Alternatively, xenin potentiates the effect of leptin on metabolism. It is also possible that the interaction between xenin and leptin occurs through the activation of separate and independent CNS signalling pathways. To support this possibility, Fos expression in the hypothalamic SON is increased in response to xenin injection, but it is not increased following leptin injection [342, 343]. Although the melanocortin system is a major CNS mediator of leptin action, metabolic effects of xenin are not mediated through CNS melanocortin signalling [288]. It was also shown that the restoration of leptin signalling in the hypothalamus of leptin receptor-deficient rats enhanced CCK-induced

satiety and activation of brainstem NTS and AP [344]. Thus, we cannot rule out the possibility that forebrain leptin signalling limits food intake by regulating the hindbrain response to gut hormones including xenin.

Gut hormones may enhance responsiveness to leptin in hypothalamic and brainstem neurons. Diet-induced obese (DIO) animals are resistant to the weight-reducing effect of leptin. Co-administration of amylin and leptin caused a significantly greater reduction in food intake and body weight and a greater increase in energy expenditure compared to the treatment with leptin alone or amylin alone in DIO rats [234, 345]. Amylin pre-treatment restored leptin-induced activation of hypothalamic signalling, as assessed by phosphorylation of STAT3, in the obese rats [234]. Conversely, leptin-induced activation of STAT3 was attenuated in amylin-deficient mice [242]. Collectively, these data suggest that amylin treatment reverses leptin resistance. Time-course of feeding-suppressing effect of xenin and that of leptin are different. A robust suppression of feeding occurs immediately after injection of xenin, whereas reductions in food intake become significant 3-4 hours after leptin injection when low doses of leptin are injected [228]. This raises the possibility that xenin potentiates the anorectic action of leptin rather than leptin extends the duration of the anorectic effect of xenin. If this is true, co-treatment with xenin and leptin may be beneficial in reversing leptin resistance. This possibility requires further investigations.

The present findings support the hypothesis that xenin regulates food intake and body weight at least partly through interacting with leptin. These findings raise the possibility that leptin extends xenin-induced satiety or xenin potentiates the anorectic effect of leptin. Limited efficacy of gut hormone treatment in reducing food intake and body

weight plus the development of tolerance to the repeated treatment with gut hormones are major limitations of the gut hormones as an anti-obesity drug. In addition, leptin resistance is the most commonly observed feature of human obesity, and therefore any strategy that can enhance leptin sensitivity is beneficial in the treatment of obesity. Further studies are warranted to determine the precise of interaction between xenin and leptin and to elucidate the mechanisms through which xenin and leptin interact and regulate metabolism. It is also important to determine the efficacy of xenin/leptin co-treatment on body weight in animal models of leptin resistant obesity in future studies.

Overall the present findings suggest that long-term xenin treatment reduces body weight by reducing food intake and increasing fat oxidization. Xenin reduces food intake possibly by delaying gastric emptying without causing taste aversion. The anorectic effect of xenin is mediated by the activation of CNS signalling pathways involving Ntsr1, MAPK, and IL-1 possibly through the interaction with leptin (Fig. 32). These findings implicate xenin and its downstream mediators as potential targets for anti-obesity drugs.

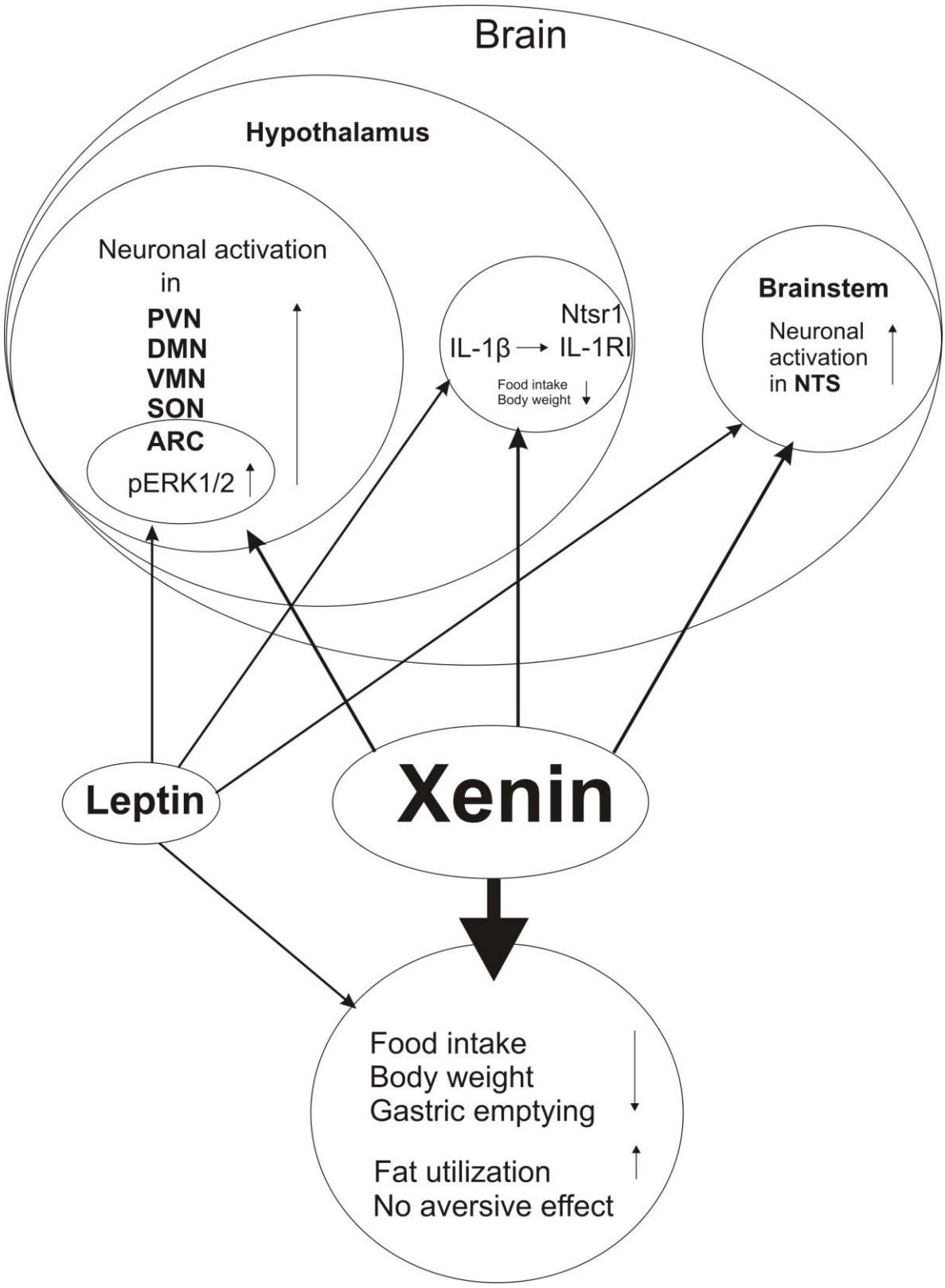

Brain
Hypothalamus
Neuronal activation
in
PVN
DMN
VMN
SON
ARC
pERK1/2
Ntsr1
IL-1β → IL-1RI
Food intake
Body weight
Brainstem
Neuronal
activation
in NTS
Leptin
Xenin
Food intake
Body weight
Gastric emptying
Fat utilization
No aversive effect

Figure 32. Metabolic effect of xenin and the CNS pathways by which xenin regulates energy homeostasis. Xenin reduces food intake and body weight and increases fat oxidation without causing a taste aversion. Delayed gastric emptying may mediate the anorectic action of xenin. Xenin induces neuronal activation in the hypothalamic ARC MAPK signalling and the brainstem NTS. Anorectic effect of xenin is mediated via CNS Ntsr1 and IL-1β-IL-1RI signalling pathways. CNS: central nervous system, PVN: paraventricular nucleus; DMN: dorsomedial nucleus; VMN: ventromedial nucleus; SON: supraoptic nucleus; ARC: arcuate nucleus; NTS: nucleus of the solitary tract; Ntsr1: neurotensin receptor 1; IL-1β: interleukin 1β; IL-1RI: interleukin 1 type 1 receptor; pERK1/2: phosphorylated extracellular signal-regulated kinases 1/2.

VIII. Summary

1. Prolonged xenin treatment (once daily i.p. or i.c.v. injection) caused sustained reductions in body weight and adiposity with a transient reduction in food intake in mice.

2. Xenin treatment (once daily i.p. injection) caused a reduction in RQ without changes in energy expenditure and activity levels in mice. Xenin-induced reduction of RQ occurred during the dark cycle, causing an alteration in the diurnal rhythm of RQ with a blunted nocturnal increase.

3. Xenin treatment (once daily i.p. or i.c.v. injection) caused a reduction in lipogenic DGAT2 mRNA expression and an increase in lipolytic HSL mRNA in the white adipose tissue in mice.

4. Xenin (single i.p. injection) did not cause a significant taste aversion in mice at a dose that produced a robust suppression of feeding.

5. Xenin (single i.p. injection) significantly reduced the rate of gastric emptying in mice at a dose which produced a robust suppression of feeding.

6. Xenin (single i.p. injection) increased the levels of *c-fos* mRNA in the hypothalamus and the number of Fos-immunoreactive cells in the hypothalamic PVN, ARC, VMN, DMN, and SON, and the brainstem NTS. Number of Fos-immunoreactive cells in the LHA, AP, and DMV were not changed by xenin treatment.

7. In Ntsr1-deficient mice, the anorectic effects of xenin (single i.c.v. injection) and leptin (single i.c.v. injection) were abolished or attenuated.

8. Xenin (single i.p. injection) increased the number of pERK1/2-immunoreactive cells in the hypothalamic ARC in mice.

9. Xenin (single i.p. injection) increased the levels of IL-1β mRNA in the hypothalamus.

10. In Il-1RI-deficient mice, the anorectic effects of xenin (single i.p. injection) and leptin (single i.p. injection) were attenuated.

11. Co-injection of xenin (single i.p. injection) and leptin (single i.p. injection) at low doses caused a reduction in food intake with a longer duration compared to the injection of xenin alone in mice. Injection of leptin alone did not affect food intake.

12. Co-injection (four doses over 2 days) of xenin (i.p. injection) and leptin (i.p. injection) at low doses caused reductions in food intake and body weight in mice. Neither leptin alone nor xenin alone altered food intake and body weight.

IX. Conclusions

1. Xenin participates in the regulation of energy homeostasis. Xenin treatment reduces body weight partly by reducing energy intake without alterations in energy expenditure. Xenin also reduces body weight by increasing fat oxidation during the dark cycle (the active period) with a concomitant alteration in the diurnal rhythm of lipid metabolism.

2. Xenin reduces food intake partly by delaying gastric emptying without causing taste aversion.

3. Xenin exerts its metabolic effects through the activation of hypothalamic and brainstem signalling pathways involving neurotensin receptor 1 (Ntsr1), mitogen-activated protein kinase (MAPK), and interleukin 1 (IL-1) possibly through the interaction with leptin.

4. The present thesis established the long-term effect of xenin on energy balance and identified possible neural and molecular mechanisms by which xenin regulates energy balance; indicating that the xenin signalling pathway is an attractive therapeutic target for the treatment of obesity.

X. Future Perspectives

1. Further determination of the effect of prolonged xenin treatment on metabolism

In the present study, daily injections of xenin inhibited body weight gain and reduced adiposity in mice. The same xenin treatment caused a reduction in RQ and an alteration in diurnal fluctuation of RQ with a blunted nocturnal peak of RQ. Therefore, I propose that xenin reduces body weight both by reducing food intake and increasing fat oxidation. Both reduced food intake and increased fat oxidation are contributing factors to reduced body weight. Since, low calorie intake stimulates utilization of fat followed by a decrease in body weight [346, 347], the present study cannot determine the extent to which each of these factors contributes to reduction in body weight. To determine food intake-independent effect of xenin on body weight, I propose to compare the effects of xenin treatment and pair-feeding on body weight and fat metabolism in the future study. Mice will receive once a daily i.p. injection of xenin or saline, or will be pair-fed to xenin-treated group for 1 week. Food intake, body weight, energy expenditure and RQ will be measured throughout the treatment period. At the end of the treatment period, blood and tissue samples will be collected and used for blood chemistry, biochemical, and gene and protein expression analyses which are relevant to lipid metabolism.

If xenin-treated mice show greater reductions in body weight and RQ compared to pair-fed mice, these findings will suggest that xenin reduces body weight by increasing fat oxidation through the mechanism that is independent of food intake. If both groups show similar reductions in body weight and RQ, these results will suggest that xenin reduces

body weight by increasing fat oxidation, but reduced RQ is secondary to the reduced food intake. Although the pair-feeding protocol can make an equal food intake in a given time period (for example, 24-h period) between the two groups (xenin-treated group and pair-fed group), the timing of food intake cannot be identical between the groups. To completely eliminate the effect of food intake on RQ and body weight, I further propose to examine the effect of xenin treatment on RQ and body weight in the absence of food. The same variables as stated above will be measured in this study.

Our data also demonstrated that xenin treatment reduces RQ during the dark cycle, suggesting that xenin treatment causes alterations in diurnal rhythm of lipid metabolism. To address this possibility, I propose to examine the effect of xenin treatment on diurnal fluctuation of variables representing lipid metabolism. Mice will receive once a daily i.p. injection of xenin or saline for 1 week. Food intake, body weight, energy expenditure and RQ will be measured throughout the treatment period. To assess diurnal variation of metabolic variables, the animals will be euthanized every 4 h on the last day of the treatment (2:00, 6:00, 10:00, 14:00, 18:00, and 22:00). Blood and tissue samples will be collected and used for blood chemistry, biochemical, and gene and protein expression analyses which are relevant to lipid metabolism. The variables to be measured include serum NEFA levels and LPL activities in white adipose tissue and skeletal muscle which are known to exhibit diurnal fluctuation [296, 297].

These studies will provide useful information for the mechanism of body weight regulation by xenin.

2. Determination of the role of vagus nerve in the regulation of energy balance by xenin

In the present study, the robust anorexic effect was observed during the first 2-3 h after injection of xenin (i.p. and i.c.v.), and i.p. injection of xenin significantly delayed gastric emptying at the 2-h time point, suggesting that the delayed gastric emptying contributes to the immediate reduction in food intake following xenin injection. We also found that the i.p. injection of xenin activated neurons in the NTS of the brainstem. Vagal afferent fibres are known to convey signals, such as gut distension and gut hormones, important for terminating food intake primarily to the NTS within the DVC [91, 307]. These findings suggest the possibility that an afferent vagus nerve is required to convey xenin signals to the CNS and to promote satiety. To address this possibility, firstly, I propose to perform an electrophysiology study, in which neuronal discharge in gastric vagal afferent fibres will be recorded extracellularly with or without xenin treatment. This technique has been used to examine the effect of gut hormones on the activity of vagal afferent fibres [178]. If xenin increases the activity of vagal afferent fibres, this will suggest that the effect of xenin is transmitted to the CNS via the afferent vagus nerve. Secondly, I propose to determine the effect of the selective denervation of vagal afferent nerve [348] on xenin-induced changes in food intake, gastric emptying, and NTS activation. If the vagal afferent nerve is required for xenin action, vagotomy should attenuate or abolish xenin-induced reduction in food intake, delay in gastric emptying, and increase in Fos expression in the NTS. Lastly, there are reciprocal neural connections between the hypothalamus and the brainstem. Electrical stimulation of vagal afferent nerve increases the expression of IL-1β mRNA and protein in the hypothalamus [349]. The present study

demonstrated that peripherally administered xenin activated hypothalamic neurons and increased the expression of IL-1β mRNA in the hypothalamus. Therefore, I propose to determine the role of vagal afferent nerve in the mediation of xenin-induced activation of hypothalamic neurons. Number of Fos-immunoreactive cell will be counted and levels of IL-1β mRNA will be measured in the hypothalamus after i.p. injection of xenin in control sham operated and vagotomised mice. If the ascending vagus nerve is responsible for hypothalamic activation by i.p. injection of xenin, the hypothalamic neural responses to xenin will be attenuated or abolished in vagotomised mice.

These studies will provide useful information about the role of vagus nerve in the regulation of energy balance by xenin.

3. Determination of the beneficial interaction between xenin and leptin in the treatment of obesity

In the present study, xenin (2.5 μg/g b.w.) reduced food intake and its anorexic effect was prolonged when administered with a low dose of leptin (0.25 μg/g b.w.) which did not cause any significant changes in food intake on its own. Furthermore, 2-day co-treatment with the same dose of xenin and leptin reduced food intake and body weight, while neither xenin alone nor leptin alone altered food intake and body weight. These findings suggest that xenin reduces food intake partly through the synergistic interaction with leptin and that co-treatment with xenin and leptin may produce long-lived and greater effects on food intake, body weight and fat oxidation. To determine the precise nature of the xenin-leptin interaction in the regulation of food intake, future studies will include dose-

response curves for both peptides. If combination of the two peptides causes a reduction in food intake which is same as the sum of reductions in food intake by xenin alone and leptin alone, this will suggest that the interaction between the two peptides is additive. If the combination treatment reduces food intake greater than predicted by the sum of the treatments with each peptide alone, the interaction between the two peptides is synergistic.

Once I determine the most effective combination dose of xenin and leptin, I propose to determine the effect of the combination treatment of xenin and leptin on food intake, body weight and fat oxidation in the mouse model of obesity. I propose to use high-fat diet-induced obese (DIO) mice as a model of human obesity, because these mice develop leptin resistance which is a most commonly observed feature in human obesity. Body weight, food intake, energy expenditure and RQ will be measured throughout the treatment period. At the end of the experiment, blood and tissue samples will be collected and used for blood chemistry analysis and gene expression analysis as described above. This study will provide useful information for the feasibility of the use of xenin-leptin co-administration in the treatment of obesity.

The present study demonstrated that xenin activates specific hypothalamic signalling pathways involving MAPK and IL-1 which are also activated by leptin. Therefore, I hypothesize that these signalling pathways mediate the interaction between xenin and leptin. It is also possible that xenin and leptin interact through independent pathways. For example, central melanocortin system is the major signalling pathway mediating leptin action, whereas xenin reduces food intake independent of the melanocortin system. To determine the neural mechanism of interaction between xenin and leptin in the regulation of energy balance, I propose to examine the effect of xenin-leptin co-treatment

on hypothalamic activity. Firstly, I propose to examine the effect of co-treatment on hypothalamic Fos and IL-1β expression and activation of ERK1/2. Because hypothalamic STAT3 serves as a major signalling pathway mediating leptin action on metabolism, I also propose to assess the effect of co-treatment on hypothalamic STAT3 activity. Secondly, I propose to measure expression levels of hypothalamic metabolism-related genes by PCR array in this study. Last, I plan to determine the effect of combined xenin and leptin treatment on the activity of hypothalamic neurons using electrophysiology. The effect of xenin alone, leptin alone and combination of xenin and leptin on the neuronal activity will be assessed in hypothalamic slice preparation. Although this study will determine whether the interaction between xenin and leptin occurs at single neuron level, the electrophysiology data itself cannot provide the information of neurochemical characteristic of the recorded neurons. To determine the neurochemical characteristic, each neuron will be collected after recording and used for gene expression analysis. Once the neurochemical characteristic of neurons responsive to xenin-leptin co-treatment is determined, the finding will be confirmed by using an appropriate mouse model in subsequent studies. For example, if hypothalamic neurons, whose activity show either additive or synergistic effect of xenin and leptin on their electrical activity, express POMC, hypothalamic slices will be prepared from mice expression green fluorescent protein (GFP) under the control of POMC promoter (POMC-GFP mice). The response of POMC-GFP neurons to xenin alone, leptin alone, and xenin plus leptin will be recorded. These studies will identify specific hypothalamic neurons which mediate the interaction between xenin and leptin.

Overall, these future studies will provide useful information on the feasibility of the use of xenin-leptin co-administration in the treatment of obesity and the neural mechanism of the interaction between xenin and leptin.

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