Nutritional Regulation of Central Fat Mass and Obesity-Associated (FTO) Expression, and its Association with the Central Melanocortin Signaling in the Regulation of Energy Homeostasis

By

Nicole Joanna Poritsanos

A Thesis Submitted to the Faculty of Graduate Studies
The University of Manitoba
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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THE UNIVERSITY OF MANITOBA
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Nutritional Regulation of Central Fat Mass and Obesity-Associated (FTO) Expression, and its Association with the Central Melanocortin Signaling in the Regulation of Energy Homeostasis

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ABSTRACT

The central nervous system (CNS) melanocortin signaling pathway plays a critical role in the regulation of metabolism. However, the role of the CNS melanocortin signaling in the regulation of hepatic lipid metabolism and fatty liver disease has not been well established. Although the activity of the CNS melanocortin system is regulated by metabolic signals, the mechanism for this regulation is not fully understood. Variants of the *FTO* (fat mass and obesity-associated) gene are associated with obesity and FTO is expressed in the hypothalamic neurons including proopiomelanocortin (POMC) neurons. Therefore, it is hypothesized that hypothalamic FTO plays a role in the regulation of metabolism by mediating the effect of metabolic signals on hypothalamic melanocortinergic neurons, and impairments in this regulation may cause metabolic impairments including obesity and fatty liver disease.

Intracerebroventricular (i.c.v.) treatment with SHU9119, a melanocortin antagonist, increased hepatic lipid accumulation and the expression of genes encoding lipogenic enzymes in lean mice. Conversely, i.c.v. treatment with MTII, a melanocortin agonist, reduced the expression of hepatic lipogenic genes in association with reduction in body weight in *ob/ob* mice, a mouse model of fatty liver disease.

Immunohistochemical analysis demonstrated that Fto is co-expressed in both POMC and agouti-related protein (AgRP) neurons in the mouse hypothalamus. *Fto* mRNA and protein expression was reduced by fasting and increased by glucose treatment in nutritionally important hypothalamic nuclei. Fasting-induced reduction in hypothalamic Fto expression was observed in both lean wild-type and obese *ob/ob* mice, while the stimulatory effect of glucose on hypothalamic Fto expression was absent in *ob/ob* mice

These findings support the hypothesis that central melanocortin signaling regulates hepatic lipid metabolism at least partly by regulating *de novo* lipogenesis. Impairments in the central melanocortin signaling lead to the development of hepatic steatosis, while enhanced melanocortin signaling may be beneficial in reversing abnormal hepatic lipid metabolism in fatty liver disease (Poritsanos et al., 2008). These findings also support the hypothesis that Fto is expressed in the hypothalamic melanocortinergic neurons and is regulated by metabolic signals, at least partly by changes in CNS glucose availability and/or glucose action. Impairments in this regulation may cause metabolic impairments including obesity and fatty liver disease.

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Επουράνιε Θεέ, υμνουμέν σε, ευλογοΰμέν σε, προσκυνοΰμέν σε, δοξολογοΰμέν σε, ευχαριστοΰμέν σοι, διά τήν μεγάλην σου δόξαν.-

DEDICATION

To my parents
Angelos Poritsanos and Diana Robinson
with love

ABBREVIATIONS

2-DG: 2-deoxyglucose

3V: Third ventricle

ACC1: acetyl-CoA carboxylase

acetyl-CoA: Acetyl-coenzyme A

ACO: Acyl-CoA oxidase

aCSF: artificial cerebrospinal fluid

ACTH: Adrenocorticotropic hormone

ADRP: Adipose differentiation-related protein

AgRP: Agouti-related peptide

ALT: Alanine aminotransferase

AMPK: AMP-activated protein kinase

αMSH: Alpha melanocyte-stimulating hormone

ANOVA: Analysis of variance

ARC: Arcuate nucleus

BAT: Brown adipose tissue

BBB: Blood brain barrier

BDNF: Brain-derived neurotrophic factor

β-endorphin: Beta-endorphin

BMI: Body mass index

βMSH: Beta melanocyte-stimulating hormone

cAMP : cyclic AMP

CART: Cocaine-amphetamine-regulated transcript

CCK: Cholesystokinin

ChAT: Choline acetyltransferase

ChREBP: carbohydrate responsive element binding protein

CNS: Central nervous system

Cpe: Carboxypeptidase E

CPT1: Carnitine palmitoyltransferase I

CRH: Corticotropin-releasing hormone

CRH: Cytokine receptor homology

Cy3: Cyanine dye 3

DAPI: 4',6-diamidino-2-phenylindole

DGAT: acyl-CoA:diacylglycerol acyltransferase

DIO: Diet-induced obesity

DMN: Dorsomedial nucleus; dorsal motor nucleus of the vagus

Elovl6: elongation of very long chain fatty acids protein 6

ER: Endoplasmic reticulum

ERK: Extracellular signal–regulated kinase

FAS: Fatty acid synthase

FAT/CD36: Fatty acid translocase/Cluster of Differentiation 36

FDG: Fluorodeoxyglucose

FFA: Free fatty acids

FITC: Fluorescein isothiocyanate

fMRI: Functional magnetic resonance imaging

FoxO1: Forkhead box-containing protein of the O subfamily 1

FTO: Fat mass and obesity associated gene (human)

Fto: Fat mass and obesity associated gene (murine)

FTO: Fat mass and obesity associated protein (human)

Fto: Fat mass and obesity associated protein (murine)

G6Pase: Glucose-6-phosphatase

GAPDH: Glyceraldehydes 3-phosphate dehydrogenase

GK: Glucokinase

GLUT: Glucose transporter

γMSH: Gamma melanocyte-stimulating hormone

GPAT: Glycerol-3-phosphate acyltransferase

GSK-3: Glycogen synthase kinase 3

GTG: Gold-thioglucose

GWAS: Genome-wide association study

H&E: Hematoxylin and Eosin

HDL: High density lipoprotein

HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase

HMGCS: 3-hydroxy-3-methylglutaryl-CoA synthase

HPA: Hypothalamus-pituitary-adrenal

HSL: Hormone sensitive lipase

I.c.v.: Intracerebroventricular

IML: Intermediolateral column of the spinal cord

I.p.: Intraperitoneal

IR: Insulin receptor

IRS: Insulin receptor substrate

JAK: Jak kinase family

KATP: ATP-sensitive K+

LC: Locus coeruleus

LC-CoA: Long-chain fatty acyl-CoA

LCFA: Long-chain fatty acid

LDH: Lactate dehydrogenase

LDLR: Low density lipoprotein receptor

LepR: Leptin receptors

LHA: Lateral hypothalamic area

LIPC: hepatic lipase gene

L-PK: Liver-type pyruvate kinase

MAPK: Mitogen activated protein kinase

MC3R: Melanocortin receptor 3

MC4R: Melanocortin receptor 4

MCAD: Medium chain acyl-CoA dehydrogenase

MCD: Malonyl-CoA decarboxylase

MCH: Melanin-concentrating hormone

MCR: Melanocortin receptor

ME: Median eminence

MPO: Medial preoptic area

MTII: Melanotan-II

mTORC1; Mammalian target of rapamycin complex 1

MTP: Microsomal triglyceride transfer protein

N/A: Not applicable

N: number

NAFLD: Nonalcoholic fatty liver disease

NASH: Nonalcoholic steatohepatitis

NCM: Non-caloric mash of cellulose

ND: Not detectable

NEFA: Nonesterified fatty acids

NIRKO: Neuron-specific IR knockout

NPY: Neuropeptide Y

NTS: Nucleus of the solitary tract

PC: Prohormone convertases

PCOS: Polycystic ovarian disease

PDH: Pyruvate dehydrogenase

PDK-1: PI-dependent kinase 1

PEPCK: Phosphoenolpyruvate carboxykinase

PET: Positron emission tomography

PI3K: Phosphatidylinositol 3-kinase

PKA: Protein-kinas A

PKC: Protein-kinas C

PMV: Ventral premammillary nucleus

POA: preoptic area

POMC: Pro-opiomelanocortin

PPARα: Peroxisome proliferators-activated receptor-alpha

PPARγ: Peroxisome proliferating activating receptor-γ

PtdIns: 3-phosphorylated phosphoinositides

PTP1B: Protein tyrosine phosphatase 1B

PVN: Paraventricular nucleus

RCh: Retrochiasmatic area

S.E.M.: Standard error mean

S6: S6 ribosomal protein

S6K1: S6 kinase 1

SCD1: stearoyl-CoA desaturase-1

SCN: Suprachiasmatic nucleus

SF-1: Steroidogenic factor 1

Shp2: Src homology-containing tyrosine phosphatase 2

SHU9119: synthetic melanocortin antagonist

SN: Substantia nigra

SOCS3: Suppressor of cytokine signaling 3

SREBP-1c: Sterol regulatory element binding protein-1c

STAT: Signal transducer and activator of transcription

STZ: Streptozotocin

TCA: Tricarboxylic acid

TRH: Thyrotropin-releasing hormone

UCP2: Uncoupling protein 2

VLCAD: Very long-chain acyl-coenzyme A dehydrogenase

VLDL: Very-low-density lipoprotein

VMH: Ventromedial hypothalamus

VMN: Ventromedial nucleus

VMNc: VMN central part

VMNdm: VMN dorsomedial part

VMNvl: VMN ventrolateral part

VTA: Ventral tegmental area

WAT: White adipose tissue

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GENERAL INTRODUCTION

Obesity and metabolic syndrome

The incidence of metabolic syndrome in industrialized countries has increased dramatically in recent years. In particular, the incidence of obesity has reached epidemic proportions where more than half of the world's population is overweight (Field et al., 2001). The 2004 Canadian Community Health Survey reported that 23.1% of Canadians corresponding to an estimated 5.5 million adults (18 years and older) are obese with a body mass index exceeding 30 kg/m^2 (Tjepkema, 2005). The World Health Organization (WHO) has defined five screening criteria that determine the presence of metabolic syndrome. These include an increase in waist-to-hip ratio, circulating levels of triacylglycerols, fasting glycemia and blood pressure, and a decrease in circulating levels of high density lipoprotein (HDL)-cholesterol (Alberti et al., 2005; Alberti & Zimmet, 1998). The development of metabolic syndrome contributes to increases in the incidence of type 2 diabetes and cardiovascular disease (Galassi et al., 2006; Schrezenmeir, 1996). The WHO estimates proposed that in 2005 1.1 million people died of type 2 diabetes, and this may further increase by up to 50% by 2030 (www.who.int/mediacentre/factsheets/fs312/en/index.html).

1. Metabolic syndrome and fatty liver disease

1.1. Non-alcoholic fatty liver disease (NAFLD)

Metabolic syndrome and nonalcoholic fatty liver disease (NAFLD) are increasing in the rate of occurrence both in adults and children (Adams et al., 2005; Angulo, 2007; Gholam et al., 2007; Hamaguchi et al., 2005; Pacifico et al., 2007; Rashid & Roberts, 2000; Sagi et al., 2007; Savage et al., 2003; Schwimmer et al., 2006). NAFLD is increasingly recognized as a pathophysiological feature of the metabolic syndrome, which includes visceral obesity, insulin resistance and hyperlipidemia. NAFLD is characterized by the progressive accumulation of fat in the liver that can progress to nonalcoholic steatohepatitis (NASH). Inflammation and hepatocytic liver injury are characteristic of NASH that can later progress to fibrosis and cirrhosis (Lee, 1989). Visceral obesity, insulin resistance and oxidative stress are contributing factors to the development and progression of NAFLD. Although, obesity is the most common metabolic disturbance associated with NAFLD, recent human studies have shown that NAFLD is also found in non-obese or lean non-diabetic subjects having normal glucose tolerance (Kim et al., 2004; Marchesini et al., 1999; Seppala-Lindroos et al., 2002) and that fatty liver can be correlated independently of intra-abdominal obesity in subjects having hyperlipidemia and insulin resistance (Sung et al., 2007; Tiikkainen et al., 2002). This variability in the factors associated with NAFLD suggests that in addition to metabolic syndrome, type 2 diabetes and obesity, there may be a genetic basis for the development of NAFLD.

1.2. Hepatic lipid metabolism and NAFLD

The liver is the main organ responsible for the conversion of excess dietary carbohydrate into triglycerides. Glucose is metabolized (glycolysis) in the liver to form acetyl CoA for the formation of polyunsaturated fatty acids (*de novo* lipogenesis). Lipogenesis in the liver and adipose tissue is mainly under the control of transcription factors sterol regulatory element binding protein-1c (SREBP-1c), carbohydrate regulatory element binding protein (ChREBP) and peroxisome proliferating activating receptor-γ (PPARγ) (Shi & Burn, 2004).

Fatty liver disease is primarily dependent on the abnormal accumulation of lipids that ultimately leads to an increase in fat deposition and liver size (hepatomegaly). The etiology of this abnormal lipid accumulation may be due to changes in the pathways of hepatic lipid metabolism, including lipid synthesis (lipogenesis) and lipid degradation (lipolysis and β-oxidation). In the liver, hepatocytes coordinate glucose and lipid metabolism through the use of several regulatory proteins. Mice overexpressing insulin regulated transcription factor SREBP-1c in the liver have elevated mRNA expression levels of lipogenic genes and exhibit fatty liver, both of which defects are reversed in a SREBP-1c mutant background (Foretz et al., 1999; Foufelle & Ferre, 2002; Shimano, Horton et al., 1997; Shimomura et al., 1998). Furthermore, it has been shown that although insulin promotes hepatic lipogenesis, at least in part through the activation of SREBP-1c, carbohydrates also contribute to the regulation of carbohydrate and fat metabolism in liver by an insulin-dependent mechanism. Another important transcription factor is ChREBP which induces glycolytic and lipogenic gene expression in a glucose-

dependent manner (Dentin et al., 2005). Ablation of ChREBP results in reduced levels of hepatic triglyceride content and fatty acid synthesis (Iizuka et al., 2004). It is of particular interest that the levels of both SREBP-1c mRNA and protein are not different between wild-type and ChREBP knockout mice (Iizuka et al., 2004). These data suggest that ChREBP regulates hepatic lipogenic gene expression independent of SREBP-1c. Thus, SREBP-1c and ChREBP appear to be key transcription factors required for the coordinated regulation of hepatic lipid metabolism and impairments in hepatic lipid metabolism by these transcription factors may contribute to the increased lipogenesis and subsequent development of fatty liver disease.

1.3. Genetic factors for NAFLD

In humans the development of fatty liver and later liver cirrhosis has been found in monogenic disorders such as Prader-Willi syndrome, Dunnigan lipodystrophy and abetalipoproteinemia (Merriman et al., 2006). The genetic contribution to NASH can be explained by the "two-hit" hypothesis (Day & James, 1998). The "first-hit" is the development of hepatic steatosis due to either increased *de novo* lipogenesis, peripheral adipocyte lipolysis and free fatty acid flux into the liver, or defective secretion of triglycerides and VLDL from the liver. In humans having abetalipoproteinemia due to mutations in the microsomal triglyceride transfer protein (MTP) in the liver has been linked to NASH and cirrhosis (Partin, 1975; Partin et al., 1974). It has been shown that single nucleotide polymorphisms (SNPs) in the PPARγ and MTP genes are associated with NAFLD susceptibility (Bernard et al., 2000; Hui et al., 2008). Furthermore, a genetic variant of peroxisome proliferators-activated receptor-alpha (PPARα), a

transcriptional regulator of fatty acid oxidation in the liver, was found in subjects with NAFLD (Chen et al., 2008).

As outlined above, obesity and insulin resistance are the two significant contributing factors for the abnormal lipid metabolism and the development of fatty liver disease. Therefore, it is important to understand the mechanism for maintaining body energy stores and insulin sensitivity within the normal range. One of the important organs controlling energy balance and glucose homeostasis is the brain. Within the central nervous system (CNS), hypothalamic neuronal pathways play a critical role in the regulation of metabolism. Therefore, the following sections will review the literature and discuss the role of the hypothalamus in the regulation of energy homeostasis.

2. Neuroanatomy of the central nervous system (CNS)

2.1. Hypothalamus and the limbic system

The limbic system is responsible for regulating the behavioral patterns that support the survival of an individual organism (Luiten et al., 1987). Specifically, the limbic system functions in support of survival and fitness of individual mammalian species, by regulating feeding, drinking, reproductive, defensive, and agonistic behaviors in response to environmental changes. Based on the information acquired from the internal and external environment, the limbic system mediates its influence through two effector pathways in the brain, the true motor systems and the autonomic-neuroendocrine outflow.

The limbic system organization is complex consisting of several brain regions, including the diencephalon, telencephalon and mesencephalon. In the diencephalon, the hypothalamus is a center for integrating and relaying information in relation to the information acquired from the internal environment of the mammalian body. The hypothalamus functions as the first point of entry for certain inputs, and a major point of outflow of information targeted to and from 'higher' limbic centers (Markakis & Swanson, 1997; Risold et al., 1997; Swanson, 2007; Swanson et al., 2007; Thompson & Swanson, 1998, 2003).

2.2. Hypothalamic regulation of feeding and metabolism

The type and the amount of macronutrients being consumed must be tightly regulated in order of achieving the plasma levels of nutrients and metabolites that are required for cellular needs (Luiten et al., 1987). Consumption of diets containing carbohydrates, fats and proteins, will meet the needs for glucose, free fatty acids and amino acids, respectively. The hypothalamus plays a critical role in maintaining energy homeostasis. One of the most important aspects of energy homeostasis relies upon the regulation of food intake. Environmental and hedonic factors can influence food intake. In addition and perhaps more importantly, food intake is regulated by promoting hunger or satiety, as well as, controlling duration of and frequency of the meal being consumed. The hypothalamus plays a critical role in regulating these processes for the achievement of energy homeostasis. The importance of the hypothalamus in regulating feeding and metabolism was demonstrated by lesion studies showing that different regions in the

hypothalamus are responsible for causing either an increase (hyperphagia) or decrease (hypophagia or aphagia) in food intake and/or body weight.

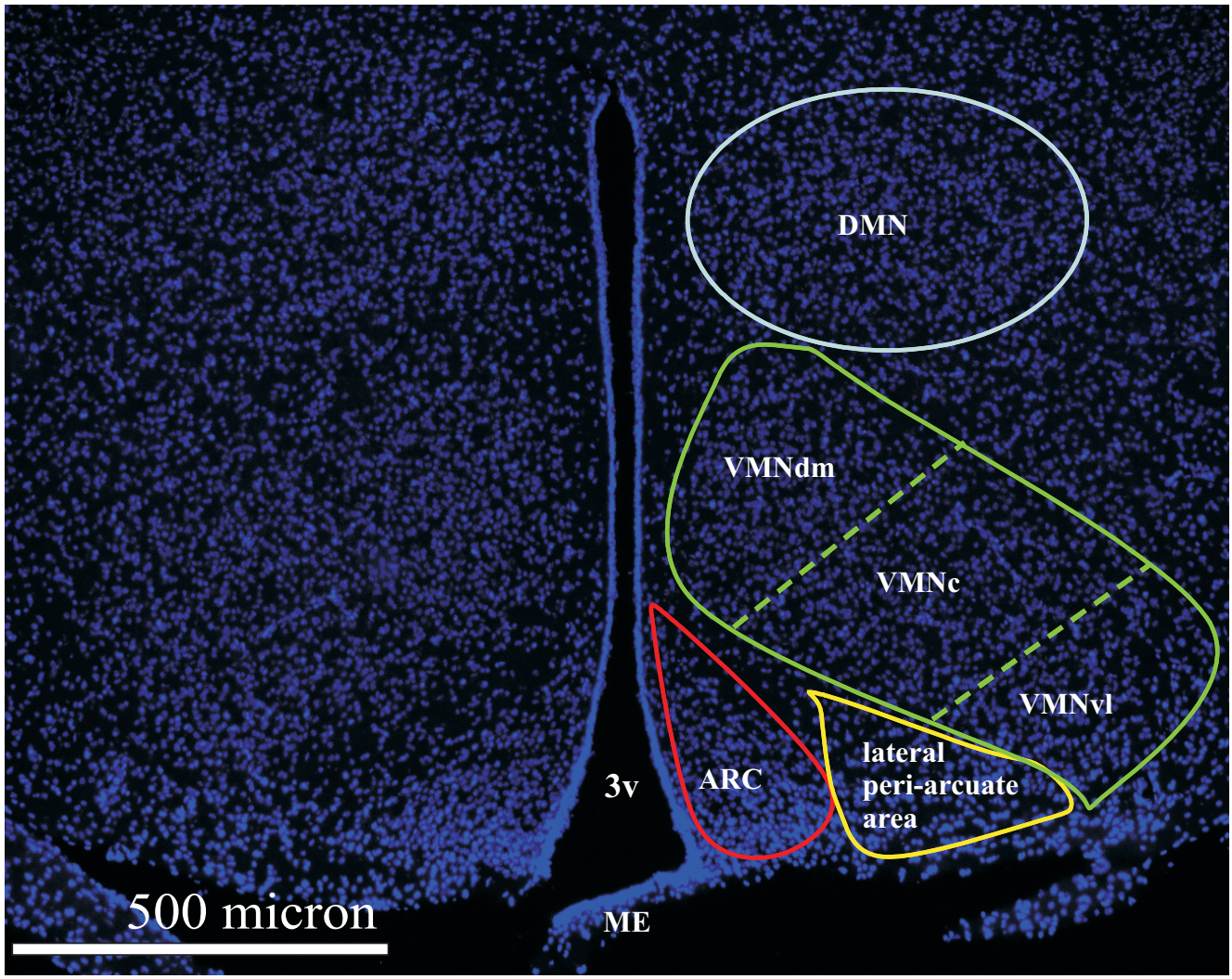


Figure 1: Hypothalamic map depicting the various nuclei and their subdivisions (DAPI staining). Magnification 5X. Dorsomedial nucleus (DMN), ventromedial nucleus (VMN), VMN dorsomedial part (VMNdm), VMN central part (VMNc), VMN ventrolateral part (VMNvl), arcuate nucleus (ARC), and median eminence (ME). Third ventricle (3V).

2.3. Hypothalamic regions and metabolic control

2.3.1. Ventromedial nucleus (VMN)

In the late 1930s, it was found that clinical damage of the ventral diencephalon was associated with changes in food intake and adiposity. In 1939, hypothalamic obesity was described based on the observation that discrete electrolytic lesions targeted at the ventral region of the hypothalamus result in an increase in food intake and body weight (Hetherington & Ranson, 1939). Several subsequent studies demonstrated that lesions in the ventromedial nucleus (VMN) and the destruction of neurons surrounding the ventromedial hypothalamic (VMH) area, result in increased food intake and adiposity (Brobeck, 1946; Brobeck et al., 1943; Hetherington, 1941; Hetherington & Ranson, 1942; Hetherington, 1944; Hetherington & Ranson, 1942). Conversely, electrical stimulation of the VMN showed that the feeding response to hunger was terminated in animals (Anand & Dua, 1955). With the use of gross manipulations, involving lesions and stimulation, the VMN was defined as the 'satiety center', where a variety of neural inputs related to feeding are integrated. This was further validated when bilateral lesion experiments at the VMN indicated that the main disturbance was an increase in meal size (Becker & Kissileff, 1974; Tepperman et al., 1943). VMH lesion also causes hyperinsulinemia and augmented insulin-mediated increase in lipogenesis and inhibition of lipolysis (Frohman et al., 1969; Frohman et al., 1972a, 1972b; Goldman, Bernardis et al., 1972; Goldman, Schnatz et al., 1972; Lowell et al., 1980). Despite the VMN being identified as a satiety center by the lesion and stimulation studies, the molecular events causing obesity in

VMN lesioned rodents are not fully understood. The VMN is divided into three structures, dorsomedial VMN, central VNM, and **ventrolateral VMN** (Paxinos & Franklin, 2001) (Fig. 1). Although the exact role that each sub-division plays in the regulation of metabolism is unclear, some data suggest that they play different roles based on the genes expressed in these sub-divisions. Steroidogenic factor 1 (SF-1) is expressed in the VMN mainly in the dorsomedial and central divisions, while brain-derived neurotrophic factor (BDNF) is expressed mainly in the ventrolateral VMN (Dhillon et al., 2006; B. Xu et al., 2003) (Fig. 2). Ablation of SF-1 or BDNF in mice causes obesity, implicating these VNM sub-divisions are involved in the regulation of energy balance through distinct molecular mechanisms (Majdic et al., 2002; Rios et al., 2001).

2.3.2. Lateral hypothalamic area (LHA)

The lateral hypothalamic area (LHA) is a region situated within the ventral hypothalamus at the lateral side of the VMN. Bilateral electrolytic lesion experiments have indicated that LHA is responsible for eating and drinking behavior (Anand & Brobeck, 1951). This was further proven by the observation that electrical stimulation of the LHA induces a feeding response (Smith, 1961). Based on these findings, the LHA was defined as the 'hunger center'. Some LHA neurons project to the brainstem regions that are associated with the control of autonomic functions (Broberger et al., 1998; Peyron et al., 1998).

Overall, until the late 1970s, the neural control of feeding behavior was thought to be largely dependent on the "dual-center model" consisting of the LHA (hunger center) and the VMH (satiety center)".

2.3.3. Paraventricular nucleus (PVN)

Electrolytic lesions targeted at the PVN increase feeding and body weight, an outcome that resembles the syndrome caused by VMN lesions (Heinbecker et al., 1944; Leibowitz et al., 1981). PVN regulation of food intake was also proven by stimulation experiments (Bray et al., 1990). PVN neurons innervate the autonomic regions of the brainstem and the spinal cord (Blevins et al., 2009; Sawchenko & Swanson, 1982, 1985). These projections are hypothesized to be involved in the mediation of the effect of hormones and nutrients on autonomic function (Caro et al., 1996; Swanson & Sawchenko, 1983). Other hypothalamic nuclei, the medial preoptic area (MPO), and the VMN send neuroendocrine inputs to the PVN. The suprachiasmatic nucleus (SCN) sends both autonomic and neuroendocrine inputs to the PVN, indicating that the PVN is the hypothalamic center for hormonal and autonomic regulation (Buijs, van Eden et al., 2003).

2.3.4. Dorsomedial nucleus (DMN)

Several studies have shown that the DMN is innervated by neurons from the LHA and VMH (Luiten & Room, 1980; Luiten et al., 1987; ter Horst & Luiten, 1986). DMN neurons project to the PVN. These neural connections suggest that DMN may play a role in processing neural inputs from the VMH and LHA conveying hormonal and nutrient information, and a modulatory role in the PVN neuroendocrine output (Bernardis & Bellinger, 1993; Ter Horst & Luiten, 1987). Lesion studies performed on the DMN have

shown that its regulatory role on food intake and body weight is complex (Bellinger & Bernardis, 1984; Bernardis, 1973, 1985; Bernardis & Bellinger, 1987; Dalton et al., 1981), which can be explained by the findings that DMN is innervated by neural circuits originating from hypothalamic nuclei (a satiety center VMN and a hunger center LHA) having opposing roles in the regulation of food intake and metabolism.

2.3.5. Arcuate nucleus (ARC)

As indicated for the VMN, lesion and chemical damage at the arcuate nucleus (ARC) results in an obese phenotype (Rohner-Jeanrenaud, 1995; Sawchenko, 1998). In the hypothalamus, the median eminence (ME) is located at the floor of the third ventricle. The location of the ARC in the brain suggests that it is not completely protected by the blood-brain barrier (BBB) and that the ARC may directly monitor changes in the levels of blood-borne molecules. However, the existence of the BBB in the ARC remains a matter of continuing debate. Several anatomical studies have demonstrated that there are numerous tight junctions between endothelial cells in the ARC, indicating that it is protected by the BBB (Fry & Ferguson). While, other studies demonstrated the expression of the marker of the fenestrated capillary in the ventromedial ARC (vmARC) and the lack of the BBB markers in the vmARC (Ciofi et al., 2009; Norsted et al., 2008). Thus, the mechanism by which the blood-borne substances cause rapid changes in ARC neuronal activity remains unclear.

It was also demonstrated that lesions specific to the hypothalamic area that extend ventrally from the VMN, but not lesions specific to the VMN or ARC, increase food intake and body weight (Gold, 1973). A single intraperitoneal (i.p.) injection of gold-

thioglucose (GTG) produces a lesion in the hypothalamic area that covers the lateral ARC and the cell-sparse area lateral to the ARC and ventral to the VMN (Bergen et al., 1996). Thus, although GTG causes a lesion mainly in the VMN, the lesioned area is extended to the outside of the VMN. These findings suggest that this particular area of the hypothalamus, "lateral peri-arcuate area", plays a role in the regulation of energy balance. Neuronal activities in the lateral peri-arcuate area change in response to a variety of nutritional signals (Shu et al., 2003) **(**Fig. 1**)**.

2.3.6. Suprachiasmatic nucleus (SCN)

SCN regulates the sympathetic and parasympathetic output leading to peripheral organs in relation to its control of the circadian rhythms in a variety of physiological functions. SCN lesion abolishes circadian rhythms of oxygen consumption and respiratory quotient (Nagai et al., 1985). SCN lesion also results in the attenuation of feeding and lipolysis in response to 2-deoxyglucose (2-DG), a synthetic glucose analogue that disrupts glucose metabolism (Yamamoto et al., 1984). Thus, SCN may participate in the regulation of energy balance by controlling energy substrates usage and sensitivity to nutrients.

2.3.7. Medial preoptic area (MPO)

Electrolytic lesion of hypothalamic MPO causes changes in the circadian rhythm of body temperature, but it does not affect food intake (McGowan et al., 1988; Osborne & Refinetti, 1995). The SCN interacts with MPO which is involved in the regulation of

temperature and reproduction. Thus, MPO may participate in the regulation of energy balance by controlling circadian rhythm of body temperature.

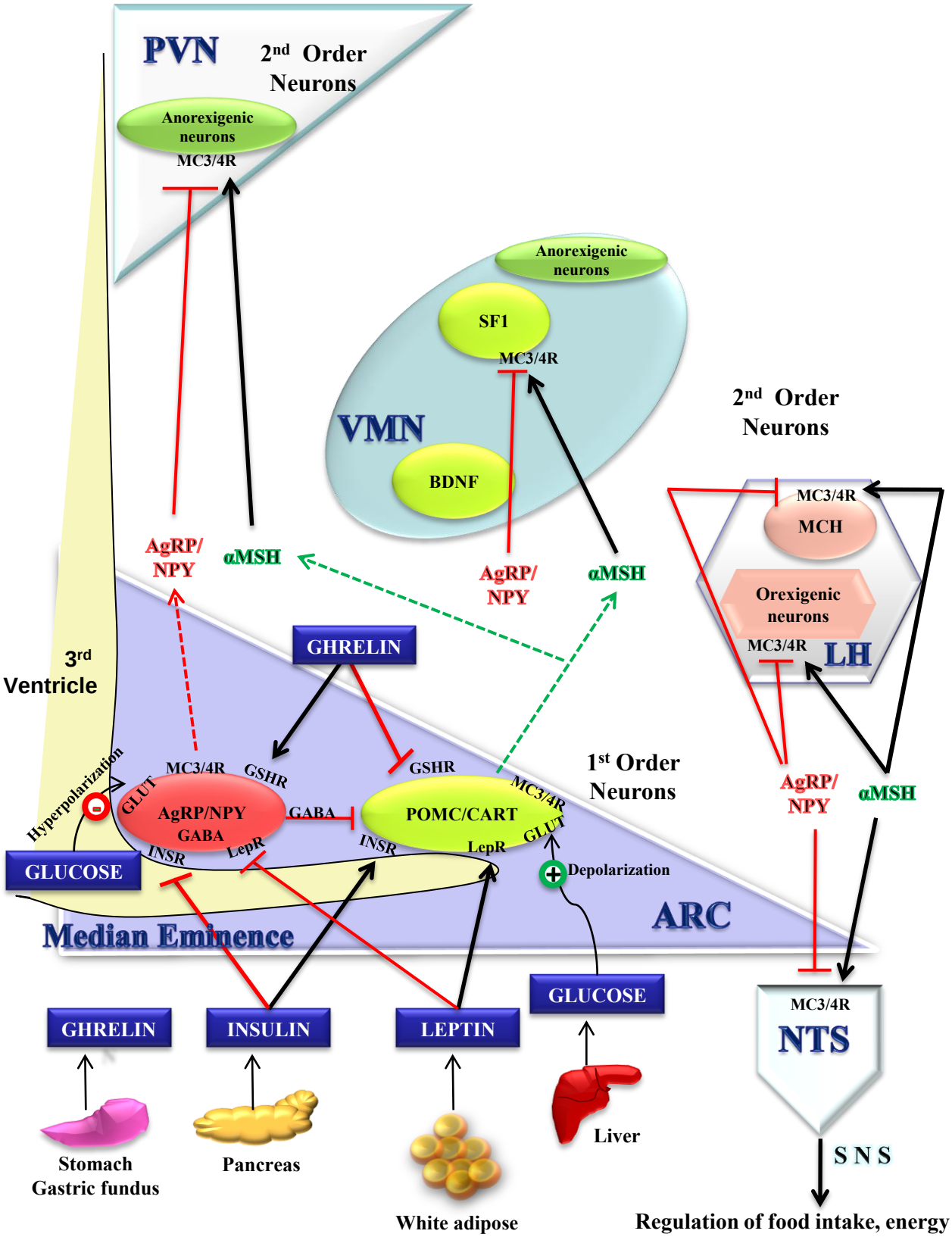

PVN
2nd Order Neurons
Anorexigenic neurons
MC3/4R
Anorexigenic neurons
SF1
MC3/4R
VMN
BDNF
2nd Order Neurons
MC3/4R
MCH
Orexigenic neurons
MC3/4R
LH
AgRP/ NPY
αMSH
AgRP/ NPY
αMSH
3rd Ventricle
GHRELIN
1st Order Neurons
Hyperpolarization
GLUT
MC3/4R
GSHR
AgRP/NPY GABA
GABA
GSHR
MC3/4R
POMC/CART
GLUT
INSR
LepR
INSR
LepR
Depolarization
GLUCOSE
ARC
Median Eminence
AgRP/ NPY
αMSH
MC3/4R
NTS
SNS
GHRELIN
INSULIN
LEPTIN
GLUCOSE
Stomach Gastric fundus
Pancreas
White adipose
Liver
Regulation of food intake, energy expenditure (white and brown adipose), hepatic glucose production and adipose lipolysis.

Figure 2: Hypothalamic neuronal circuitry of the central melanocortin system.
The arcuate nucleus (ARC) contains AgRP/NPY and POMC consisting of the first order neurons. AgRP neurons send inhibitory inputs to POMC neurons via the neurotransmitter γ-aminobutyric acid (GABA). The ventromedial nucleus (VMN) receives inputs from the ARC and sends inputs to the paraventricular nucleus (PVN). Both POMC and AgRP/NPY send neuronal projections to the second order neurons located at the paraventricular nucleus (PVN) and the lateral hypothalamus (LHA). POMC exerts inhibitory effects on food intake through the production of αMSH, while AgRP/NPY exerts stimulatory effects on food intake through the action of AgRP on melanocortin receptors (MC3/4R). Descending neuronal signals from these hypothalamic nuclei send efferent inputs to the nucleus tractus solitarius (NTS) of the brainstem which activates the sympathetic nervous system (SNS). Metabolic effects of leptin, insulin, ghrelin and glucose are partly mediated through POMC and AgRP/NPY neurons. Ghrelin has stimulatory effects on AgRP neurons and inhibitory effects on POMC neurons via its actions on GSHR. Neuronal glucose uptake is mediated by glucose transporters (GLUT) followed by the metabolism of glucose and increase in ATP/ADP. Glucose induces depolarization and hyperpolarization of POMC and AgRP/NPY neurons, respectively.

3. CNS melanocortin system

During the past 20 years, the melanocortin system has been recognized as a critical CNS signaling pathway involved in the regulation of energy homeostasis. The CNS melanocortin system consists of neurons producing melanocortin peptides and neurons expressing receptors for these melanocortin peptides (Fig. 2).

3.1. Agouti

Mutations in the agouti gene promote the development of a yellow coat color, but more interestingly the A^y allele also promotes obesity and insulin resistance (Bultman et al., 1992; Yen et al., 1994). These phenotypes of the agouti mice are due to a dominantly inherited promoter rearrangement responsible for the ectopic overexpression of the agouti peptide in tissues which do not normally express it (Bultman et al., 1992; Miller et al., 1993). Agouti peptide acts as a high-affinity competitive antagonist for at least two receptor subtypes for alpha melanocyte-stimulating hormone (αMSH). These receptors are melanocortin 1 and 4 receptors (MC1R and MC4R) (Lu et al., 1994). Unlike other members of the αMSH receptor family, MC4R and MC3R receptors are expressed primarily in the brain (Mountjoy et al., 1994). These findings led to the hypothesis that central signaling through the MC4R plays a critical role in the regulation of energy balance.

3.2. Melanocortin peptides

The central melanocortin system consists of the melanocortin peptides and receptors for these peptides. Several bioactive peptides are produced from the precursor pro-opiomelanocortin (POMC). Agouti-related protein (AgRP) is another melanocortin peptide. The melanocortin peptides regulate energy balance by acting through the MC3R and MC4R receptors which are mainly expressed in the CNS.

3.2.1. Pro-opiomelanocortin (POMC)

POMC was discovered in 1977 by Herbert, Roberts, and Ling and Mains (Mains et al., 1977; Roberts & Herbert, 1977a, 1977b). POMC is expressed in the anterior and intermediate lobe of the pituitary. In the CNS, expression of POMC is restricted in the hypothalamic ARC and the nucleus of the solitary tract (NTS) of the brainstem (Gee et al., 1983; Jacobowitz & O'Donohue, 1978). Several bioactive peptides are produced from the precursor pro-opiomelanocortin (POMC) encoded by the *Pomc* gene. The POMC protein consists of 241 amino acids in humans, and 209 amino acids in mouse and rat (Drouin & Goodman, 1980; Takahashi et al., 1981; Uhler & Herbert, 1983). POMC protein contains 3 main domains: the central highly conserved ACTH (1-39) sequence, with αMSH at its N-terminus; the C-terminal β-lipotropin, which can be cleaved to generate β-endorphin and γ-lipotropin; and the N-terminus region which contains γMSH. γ-lipotropin is further cleaved to βMSH. POMC gene products undergo posttranslational modifications which include proteolytic cleavage via prohormone convertases (PC1 and PC2) and carboxypeptidase E (Cpe). PC1 cleaves POMC into N-terminal peptide, ACTH (1-39) and β-lipotropin together with low concentrations of β-endorphin, while PC2

cleaves PC2 cleaves ACTH to generate ACTH (1–14) followed by αMSH (Benjannet et al., 1991; Miller et al., 2003). PC2 also cleaves β-lipotropin to produce β-MSH and β-endorphin (Benjannet et al., 1991). The biological activity of POMC-derived peptides is changed based on N-acetylation. For example, N-acetylation of αMSH makes its anorectic effect stronger, while N-acetylation of β-endorphin eliminates its effect on food intake (Tsujii & Bray, 1989).

3.2.2. Adrenocorticotropic hormone (ACTH)

ACTH is a straight-chain peptide with 39 amino acids with its N-terminal 1 to 24 amino acid sequence being responsible for its full biological activity. ACTH from the anterior pituitary plays a pivotal role in stress response by controlling the activity of the hypothalamus-pituitary-adrenal axis. ACTH stimulates steroidogenesis through its receptor, melanocortin 2 receptor (MC2R). ACTH action in the CNS is involved in the regulation of grooming behavior and learning. Although it was reported that intrecerebroventricular (i.c.v.) injection of ACTH (1-24) reduces food intake, its role in feeding regulation has not been studied (Poggioli et al., 1986). Therefore, it is unclear whether or not ACTH plays a physiological role in the regulation of food intake.

3.2.3. Alpha melanocyte-stimulating hormone (αMSH)

In the melanocytes of skin cells, αMSH binds to MC1R, increases cAMP, activates tyrosinase, and converts L-tyrosine to melanin. αMSH functions in an autocrine manner to stimulate melanin formation (Burchill et al., 1989; Hunt, et al., 1994a; Hunt, et al.,

1994b; Suzuki et al., 1996). In addition to the role in melanin formation in the skin, αMSH plays a critical role in the regulation of energy balance through its action in the CNS. Central injection (i.c.v.) of αMSH reduces food intake (Abbott et al., 2000; Kask et al., 2000; Poggioli et al., 1986; Tung et al., 2006). Conversely, i.c.v. injection of SHU9119, a synthetic MC3R/MC4R antagonist, or AgRP, a natural MC3R/MC4R antagonist, blocks the effect of αMSH on food intake (Rossi et al., 1998). Treatment with αMSH reduces body weight in POMC-deficient mice, but βMSH or γMSH treatment failes to show this effect (Tung et al., 2006). Thus, αMSH plays a major role in regulating food intake and body weight among the POMC-derived peptides and this anorectic effect of αMSH is mediated through the MC3R and MC4R in the CNS.

3.2.4. Beta melanocyte-stimulating hormone (βMSH)

Central administration (i.c.v.) of βMSH suppresses food intake in rats and mice (Abbott et al., 2000; Kask et al., 2000; Tung et al., 2006). One study failed to show a significant anorectic effect of βMSH in rats (Millington et al., 2001). A missense mutation within the coding region of the POMC-derived peptide β-MSH is associated with early-onset human obesity (Biebermann et al., 2006; Lee et al., 2006). These data suggest that in addition to αMSH, βMSH plays a role in the regulation of energy balance in humans.

3.2.5. Gamma melanocyte-stimulating hormone (γMSH)

The melanocortin peptide γMSH plays a role in the regulation of natriuresis, sodium metabolism, and cardiovascular function. The effect of i.c.v. γMSH injection on food

intake in rodents is not consistent. Some studies reported a significant anorectic effect, while other studies showed no significant effect on food intake (Abbott et al., 2000; Kask et al., 2000; Millington et al., 2001; Tung et al., 2006). Absence of a functional PC2 has resulted in γMSH deficiency in mice, but these mice did not show any metabolic abnormalities except for lower blood glucose levels probably due to the lack of glucagon (Furuta et al., 1997; Ni et al., 2003). Thus, it is unclear whether or not γMSH plays a role in the regulation of food intake under the physiological condition.

3.2.6. Beta-endorphin (β-endorphin)

Beta-endorphin (β-endorphin) functions as an endogenous opioid through opioid receptors. Similar to other opioid peptides, i.c.v. injection of β-endorphin increases food intake (Tsujii & Bray, 1989). In contrast to its acute orexigenic effect, β-endorphin KO mice have increased food intake, body weight, and adiposity compared with wild-type mice (Appleyard et al., 2003). Interestingly, i.c.v. administration of β-endorphin was equally effective in increasing food intake both in wild-type and β-endorphin KO mice (Appleyard et al., 2003). These studies indicate that β-endorphin plays a role in the regulation of food intake and body weight through a complicated interaction with other POMC-derived peptides.

3.2.7. Agouti-related protein (AgRP)

Since, agouti is not normally expressed in the hypothalamus, it was assumed that hypothalamic neurons produce a protein which can function as an endogenous

melanocortin receptor antagonist. This protein was discovered to be the agouti related peptide (AgRP; also known as agouti related transcript, ART) which is nearly identical in size and structure to the agouti gene product. AgRP is expressed primarily in the hypothalamus and the adrenal gland (Ollmann et al., 1997; Shutter et al., 1997). AgRP was found to be a specific high-affinity competitive antagonist of the MC3R and MC4R (Fong et al., 1997). The discovery of an endogenous antagonist for MC3R/MC4R suggested that the hypothalamic melanocortin system might sense orexigenic signals (AgRP), as well as, anorexigenic signals (αMSH) under normal conditions, and the balance between these signals may produce an important signal to determine metabolic status. Transgenic overexpression of AgRP in mice results in obese phenotypes similar to those in agouti mice and MC4R knockout mice (Graham et al., 1997; Ollmann et al., 1997). Ablation of AgRP neurons in adult mice results in reduction in food intake (Bewick et al., 2005; Gropp et al., 2005). Reducing hypothalamic AgRP expression leads to an increase in energy expenditure and a reduction in body weight (Makimura et al., 2002). It was reported that AgRP polymorphisms are associated with inherited leanness in humans (Marks et al., 2004). These observations indicate that AgRP is a neuropeptide implicated in the central control of energy balance.

3.3. Melanocortin Receptors

There are at least 5 melanocortin receptors (MC1R-MC5R) for melanocortin peptides (Getting, 2006). MCRs belong to the family of the seven transmembrane G protein-coupled receptors. MC1R is the αMSH receptor with weaker affinity to ACTH, βMSH, and γMSH. MCIR expression is found mainly in melanocytes, but also in immune cells

and brain cells. MC2R is the ACTH receptor and is expressed in the adrenal cortex. MC3R binds to γMSH with a higher affinity as compared with αMSH, βMSH, or ACTH. MC3R is expressed in the CNS, as well as, the peripheral tissues including the heart. MC4R binds to all MSHs and ACTH with βMSH having the highest affinity and γMSH having the lowest affinity. MC4R is expressed in the CNS. The MC5R binding pattern is similar to that of the MC1R and MC5R, and is expressed in many peripheral tissues, but not in the CNS.

Binding of the melanocortin ligands to MCR activates adenylyl cyclase with subsequent elevation in intracellular cAMP levels which acts as a second messenger to activate downstream signaling molecules including nuclear factor kappa beta (NF-$_K$B), protein-kinas A (PKA), PKC, and mitogen activated protein kinase (MAPK).

3.3.1. Melanocortin 4 receptor (MC4R)

Based upon the finding that the agouti peptide functions as an antagonist of the MC4R, it was hypothesized that MC4R plays a role in the regulation of energy balance (Lu et al., 1994). MC4R being widely expressed in the CNS including the hypothalamus and brainstem, suggests that signaling through the MC4R in these brain regions contributes to the centrally mediated melanocortin regulation of energy balance (Kishi et al., 2003; Liu et al., 2003; Mountjoy et al., 1994). Central administration of natural or synthetic ligands of MC4R reduces food intake (Benoit et al., 2000; Fan et al., 1997; Poggioli et al., 1986). Conversely, central administration of MC4R antagonists increases food intake and body weight (Fan et al., 1997). Ablation of MC4R in mice results in obesity, hyperphagia and hyperinsulinemia (Huszar et al., 1997). Also, oxygen consumption and activity levels are

reduced in even younger non-obese MC4R knockout mice (Ste Marie et al., 2000). Importantly, mutations in the MC4R gene are associated with severe obesity in humans and about 5-6% of human obesity is accounted for by the MC4R mutation (Coll et al., 2004; Vaisse et al., 1998; Yeo et al., 1998). Intranasal administration of the melanocortin agonist reduces body fat and body weight in normal non-obese humans (Fehm et al., 2001). It has been demonstrated that restoration of the central melanocortin signaling reverses metabolic impairments including obesity and insulin resistance in obese rodents. This suggests that restoration of impaired melanocortin signaling would be beneficial for the treatment of human obesity and obesity-associated impairments. Thus, CNS signaling through MC4R plays a critical role in the regulation of energy balance by altering both energy intake and energy expenditure.

3.3.2. Melanocortin 3 receptor (MC3R)

MC3R is widely expressed in the CNS including the hypothalamus (Jegou et al., 2000; Mounien et al., 2005; Mountjoy et al., 1994). It was demonstrated that ablation of MC3R in mice results in increased body weight and fat mass without hyperphagia (Butler et al., 2000; Chen et al., 2000). Although energy expenditure is normal in MC3R-deficient mice, respiratory quotient is increased in these mice, suggesting that MC3R is associated with substrate preference (Butler et al., 2000; Chen et al., 2000). Furthermore, mice lacking both MC3R and MC4R are more obese than those having MC4R deficiency alone (Chen et al., 2000). Although leptin administration reduces food intake in MC4R-deficient mice, MC3R-deficient mice do not show an anorexic response to leptin, suggesting that the ability of leptin to reduce food intake depends more upon MC3R

rather than MC4R (Zhang et al., 2005). MC3R is specifically expressed in ARC POMC neurons and a specific MC3R agonist reduces the frequency of action potentials in POMC neurons, suggesting that MC3R mediates an auto-feedback mechanism in the ARC (Cowley et al., 2001; Jegou et al., 2000). These data suggest that MC3R also plays a role in the regulation of energy balance through the mechanism that is distinct form the mechanism used by MC4R.

3.4. CNS melanocortin pathway

The central melanocortin system is defined as the central neuronal circuits that consist of (i) hypothalamic neurons expressing POMC, or AgRP, (ii) brainstem neurons expressing POMC, and (iii) downstream targets expressing MC3R or MC4R in the CNS (Fig. 2). The POMC and AgRP neurons are two distinct neuronal populations in the hypothalamic ARC. POMC and AgRP neurons co-express cocaine-amphetamine-regulated transcript (CART) and neuropeptide Y (NPY), respectively (Hahn et al., 1998; Vrang et al., 1999). Cell bodies of AgRP neurons are mainly localized in the medial part of the ARC, whereas those of POMC neurons are distributed throughout the ARC but also in the lateral peri-arcuate area which is known to be a cell-sparse area, located laterally to the ARC and ventrally to the VMN (Shu et al., 2003).

Both POMC/CART and AgRP/NPY neurons in ARC project to the PVN and LHA where MC4R is expressed (Bagnol et al., 1999; Broberger et al., 1998; Jhanwar-Uniyal et al., 1993). The overlapping synaptic inputs between the AgRP and POMC nerve fibers in the PVN neurons expressing MC4R, is considered to have a great impact on the regulation of metabolism. Additionally, POMC neurons project to the other

hypothalamic regions including the DMH and VMN, as well as, extra-hypothalamic sites including nucleus accumbens and amygdala (Bagnol et al., 1999). There are also POMC connections within the ARC where MC3R is highly expressed (Bagnol et al., 1999; Cowley et al., 2001; Pinto et al., 2004). Surgical disruption of the ARC- PVN connection in rats results in increased food intake and weight gain (Bell et al., 2000). These findings indicate that the ARC-PVN connection involving POMC/CART and AgRP/NPY neurons plays a critical role in the regulation of energy balance, and that the anorexigenic effects of POMC/CART neurons are greater than the orexigenic effects of the AgRP/NPY neurons.

In the hindbrain, MC4R is expressed in the dorsal vagal complex (DMC) including the dorsal motor nucleus of the vagus nerve (DMV) and NTS, and the intermediolateral column of the spinal cord (IML) (Liu et al., 2003). Retrograde tracing experiments showed that a small percentage of ARC POMC neurons project to the DMC (Zheng et al., 2005). POMC neurons are also found in the NTS (Fan et al., 2004; Palkovits et al., 1987).

3.5. Regulation of metabolism by CNS melanocortin system

3.5.1. Regulation of food intake, energy expenditure, and body weight

Central pharmacological treatment (i.c.v.) of mice with MC4R/MC3R agonists decreases food intake, increases energy expenditure, and decreases serum insulin levels (Cowley et al., 1999; Fan et al., 1997; Fan et al., 2000; Hohmann et al., 2000; Pierroz et al., 2002;

Poggioli et al., 1986; Thiele et al., 1998). Conversely, central injection of MC4R/MC3R antagonists increases food intake, decreases energy expenditure, and reduces insulin sensitivity (Adage et al., 2001; Obici et al., 2001; Raposinho et al., 2000). These injection studies support the role for CNS melanocortin signaling in the regulation of energy balance.

Impairments in melanocortin signaling are associated with metabolic impairments including obesity and insulin resistance. Reduced synthesis (POMC mutation), processing (PC1 mutation) or sensitivity (MC4R mutation) to αMSH, a key product of POMC, causes obesity in humans (Jackson et al., 1997; Krude et al., 1998; Vaisse et al., 1998; Yeo et al., 1998). Similarly, ablation of POMC, MC4R or MC3R, or overexpression of agouti protein (agouti mouse) or AgRP results in the development of obesity in mice (Butler et al., 2000; Challis et al., 2004; Chen et al., 2000; Graham et al., 1997; Huszar et al., 1997; Ollmann et al., 1997; Xu et al., 2005; Yaswen et al., 1999; Yen et al., 1994). Ablation of AgRP neurons in adult mice results in a reduction in food intake (Bewick et al., 2005; Gropp et al., 2005). Knockdown of hypothalamic AgRP expression increases metabolic rate and decreases body weight in mice (Makimura et al., 2002). Furthermore, enhancing central POMC expression or αMSH production effectively reverses the metabolic impairments including the presence of obesity and insulin resistance in obese animals (Li et al., 2003; Mizuno et al., 2003; Savontaus et al., 2004). These observations support the idea that CNS melanocortin signaling regulates energy balance and impairments in CNS melanocortin signaling cause metabolic abnormalities including obesity and insulin resistance.

Diet-induced thermogenesis is an evolutionarily ancient metabolic pathway that is known to function in counter excessive caloric intake. In response to moderately increased consumption of dietary fat content, MC4R-deficient mice exhibited hyperphagia and accelerated weight gain, as compared with wild-type mice (Butler et al., 2001). Wild-type mice rapidly increased diet-induced thermogenesis and physical activity in response to an increase in the fat content present in the diet, but these responses were not observed in MC4R-deficient mice (Butler et al., 2001). These data suggest that MC4R is involved in the acute regulation of energy expenditure in response to energy consumption, and that enhanced signaling through the MC4R protects against high-fat diet-induced obesity by increasing energy expenditure. In light of both ARC POMC and AgRP neurons sending projections to the PVN, and MC4R being highly expressed in the PVN, it was assumed that PVN is a major CNS site of the central melanocortin signaling pathway. Central injection (i.c.v.) of α-MSH or melanotan II (MTII), a synthetic MC4R/MC3R agonist, has been shown to inhibit feeding (Fan et al., 1997). Conversely, i.c.v. administration of SHU9119, the synthetic MC4R/MC3R antagonist, induces an increase in food intake (Fan et al., 1997). These effects were recapitulated by injection of MTII, SHU9119, or HS014, a MC4R selective antagonist, directly into the PVN (Giraudo et al., 1998; Kask & Schioth, 2000; Wirth et al., 2001). Consistent with these findings, re-expression of MC4R only in the PVN reduced food intake, but it did not affect energy expenditure in MC4R null mice (Balthasar et al., 2005). These data suggest that MC4R signaling in the PVN plays a critical role in the regulation of food intake and that melanocortin regulation of energy expenditure may be mediated via MC4R in other areas of the brain.

The PVN also receives neural input from ARC POMC neurons via DMN (Chen et al., 2004; Singru et al., 2005). Injection of MTII into the DMN reduces food intake, whereas it attenuates suckling-induced NPY expression in the DMN, suggesting that reduced melanocortin signaling is responsible for the induction of NPY expression in the DMN (Chen et al., 2004). NPY expression is up-regulated in the DMN of both the agouti mouse and MC4R knockout mouse (Kesterson et al., 1997). These findings suggest that ARC POMC neurons regulate energy balance by influencing PVN neuronal activity by regulating the expression of NPY in the DMN of the hypothalamus.

3.5.2. Regulation of glucose metabolism

The central melanocortin system has been proven to regulate pancreatic β-cell function. Both agouti mice and MC4R knockout mice have hyperinsulinemia (Huszar et al., 1997; Warbritton et al., 1994). MC4R null mutation in young lean mice results in hyperinsulinemia and impaired insulin tolerance prior to the onset of obesity (Fan et al., 2000). Central administration of the MC3/4R agonist MTII reduces serum insulin levels and this inhibitory effect is prevented by the administration of the α-adrenoreceptor antagonist phentolamine, indicating that MTII/αMSH signaling mediates an increase in sympathetic input to the endocrine pancreas while simultaneously inhibiting parasympathetic tone (Fan et al., 2000). The dorsal motor nucleus of the vagus (DMV) has been proven to express MC4R (Kishi et al., 2003; Mountjoy et al., 1994). Furthermore, MC4R is expressed in cells expressing choline acetyltransferase (ChAT) mRNA, a marker for autonomic preganglionic neurons, in the DMV (Liu et al., 2003). Collectively, these findings support the role for DMV in mediating the effect of

melanocortins on pancreatic function, as well as, explain the mechanisms responsible for the hyperinsulinemia found in the MC4R knockout mice (Huszar et al., 1997; Warbritton et al., 1994).

Central injection (i.c.v.) of αMSH enhanced the actions of insulin on both glucose uptake and production, while SHU9119, a MC4R/MC3R antagonist, exerted the opposite effects (Obici et al., 2001). These findings suggest that CNS melanocortin signaling participates in the regulation of insulin sensitivity. Thus, CNS melanocortin signaling regulates glucose metabolism by regulating insulin secretion and insulin sensitivity.

3.5.3. Regulation of lipid metabolism

Impairments in the central melanocortin signaling result in the development of obesity and insulin resistance, in both humans and rodents. Mutations in human *POMC* result in loss of POMC-derived anorectic peptide αMSH, and the development of severe early-onset obesity (Krude et al., 1998). The actions of the melanocortin peptides on energy homeostasis are mediated through the activation of hypothalamic neurons and their downstream target sites expressing MC3R and MC4R (Cone, 2005). One of the most common forms of human monogenic obesity is the *MC4R* deficiency (Farooqi et al., 2003; Vaisse et al., 2000), that is characteristic of hyperphagia, increase in fat mass and lean mass, and severe hyperinsulinemia (Vaisse et al., 1998; Yeo et al., 1998). Consistent with this, mouse models with mutations in genes of the central melanocortin system, including *Mc4r, Pomc* and *agouti*, and those overexpressing the endogenous MC4R antagonist AgRP, recapitulated the defects seen in humans with MC4R mutations (Huszar et al., 1997; Ste Marie et al., 2000; Yaswen et al., 1999).

Defective signaling in the central melanocortin system is associated with fatty liver disease. Hepatic lipogenic gene expression is elevated in *agouti* mice and MC4R-deficient mice (Albarado et al., 2004; Jones et al., 1996). Enhanced central POMC gene expression largely normalizes hepatic triglyceride levels, serum FFA levels and hepatic stearoyl-CoA desaturate (SCD1) mRNA levels in animal models of fatty liver disease (Li et al., 2007; Mizuno et al., 2003). Central administration of melanocortin agonist reduces hepatic lipogenic gene expression (Lin et al., 2003). In contrast, i.c.v. treatment with melanocortin antagonists stimulates lipogenic gene expression and increases lipid content in the liver (Adage et al., 2001; Nogueiras et al., 2007). These data suggest that defects in CNS melanocortin signaling are associated with increased hepatic lipogenesis and restoration of impaired CNS melanocortin signaling may be beneficial in reversing obesity and obesity-associated impairments including fatty liver disease.

3.5.4. Downstream targets of hypothalamic melanocortin signaling (second-order signaling)

Studies performed in 1940s and 1950s have indicated that hypothalamic PVN and LHA are involved in energy homeostasis. Pharmacological studies in more recent years have shown that anorexigenic and orexigenic signaling molecules can be produced by neurons present in the PVN and LHA, respectively. Both PVN and LHA are densely innervated by ARC neurons expressing AgRP/NPY and POMC/CART, making it possible for the ARC melanocortin system to activate second-order neurons residing in the PVN catabolic and LHA anabolic hypothalamic areas (Elmquist et al., 1999). MC4R is expressed in

corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) neurons in the PVN (Harris et al., 2001; Liu et al., 2003; Lu et al., 2003). Terminals of αMSH-containing neurons make synaptic contacts with TRH neurons in the PVN (Fekete et al., 2000). Both TRH and CRH function as anorexigenic factors (Spina et al., 1996; Steward et al., 2003). Thus, these PVN neurons may mediate the effects of melanocortins on food intake. In the LHA, both POMC and AgRP neurons innervate melanin-concentrating hormone (MCH) and orexin neurons (Broberger et al., 1998). MCH and hypocretin/orexin function as orexigenic neuropeptides (Hagan et al., 1999; Qu et al., 1996; Sakurai et al., 1998). There is evidence duggeting that MCH interacts with the melanocortin system. The findings that anti-MSH antibodies cross-react with MCH neurons suggest that MCH and MSH may share a common epitope (Cechetto & Saper, 1988). Furthermore, in reptiles and amphibians, high levels of MCH show MSH-like actions, implicated to be the result of MCH interacting with a melanocortin receptor(s) (Hadley et al., 1987). MCH antagonized the anorectic effect of αMSH, while αMSH blocked the orexigenic properties of MCH (Ludwig et al., 1998). These data suggest that MCH and αMSH exert opposing and antagonistic influences in a coordinate manner through a novel mechanism.

Finally, MC4R is co-expressed in cells expressing ChAT mRNA in DMV of the brainstem, indicating that autonomic preganglionic neurons are a direct target of melanocortins (Liu et al., 2003). Recent data also suggest that brain-derived neurotrophic factor (BDNF) is a possible downstream effector of MC4R signaling in the hypothalamus and the brainstem (Bariohay et al., 2009; Nicholson et al., 2007).

3.5.5. Brainstem melanocortin signaling and metabolic regulation

As described above, MC4R is expressed in the DMV and NTS, and the intermediolateral column of the spinal cord (IML) (Liu et al., 2003). In the DMV, MC4R is co-expressed in cells expressing ChAT, indicating that autonomic preganglionic neurons are a direct target of melanocortins (Liu et al., 2003). There are reciprocal neural connections between the hypothalamus and the brainstem, and therefore the brainstem may regulate energy balance by interacting with the hypothalamus (Blevins & Baskin, 2010). To assess the contribution of hindbrain MC4R to the control of food intake, the MC3R/MC4R antagonist SHU9119 was delivered to the fourth ventricle. Intra-4th ventricle SHU9119 blocked the anorectic action of the gut-derived hormone cholecystokinin (CCK), indicating that melanocortin action in the hindbrain mediates CCK-induced feeding suppression (Fan et al., 2004; Sutton et al., 2005). Due to the fact that POMC neurons in the NTS are activated by CCK, it is likely that the POMC being produced in the brainstem regulates food intake by acting on the brainstem MC4R. Fourth ventricle injection of MTII increased core temperature and interscapular brown adipose tissue temperature in control intact rats. Response to MTII was greater in chronic decerebrate rats than in control intact rats (Skibicka & Grill, 2008). Furthermore, transgenic overexpression of POMC in the NTS, but not in the hypothalamic ARC, attenuates obesity and insulin resistance in diet-induced obese rats (Zhang et al., 2010). These findings support the idea that brainstem signaling via MC4R plays a role in the regulation of energy homeostasis and that the hypothalamus-brainstem connection is not necessary for the melanocortin regulation of energy balance. NTS receives dendritic contacts from DMV neurons, making it possible for NTS produced αMSH to mediate

melanocortin signaling through DMV without communicating with the hypothalamus (Fox & Powley, 1992; Rinaman et al., 1989; Shapiro & Miselis, 1985).

3.5.6. Amygdala melanocortin signaling and feeding

Rats acquiring lesions in the amygdala exhibit hyperphagia and increased body weight (King et al., 1999). MC4R is expressed in the amygdala and hypothalamic POMC neurons project to the amygdala, suggesting a possible role for amygdala melanocortin signaling in the regulation of energy balance (Bagnol et al., 1999; Kishi et al., 2003). Bilateral injections of HS014, a MC4R specific antagonist, into the central nucleus of the amygdala resulted in a dose-dependent increase in food intake (Kask & Schioth, 2000). These results are consistent with the hypothesis that amygdala melanocortin signaling plays a role in the regulation of energy homeostasis.

3.5.7. Regulation of hypothalamic POMC and AgRP gene expression by leptin and insulin

Hypothalamic POMC and AgRP gene expression is partly under the control of hormonal signals. Two major hormones regulating POMC and AgRP expression are the adipocyte hormone leptin and the pancreatic hormone insulin (Fig. 2).

Hypothalamic *POMC* mRNA is reduced in leptin-deficient *ob/ob* mice and leptin-resistant *db/db* mice, as compared with wild-type mice (Mizuno et al., 1998; Schwartz et al., 1997). Conversely, *AgRP* mRNA is elevated in *ob/ob* and *db/db* mice, as compared with wild-type mice (Mizuno & Mobbs, 1999). *POMC* mRNA is reduced and *AgRP*

mRNA is increased in the hypothalamus of the animal model of type 1 diabetes (Havel et al., 2000; Mizuno et al., 1999). Fasting causes a reduction in hypothalamic *POMC* mRNA and an increase in *AgRP* mRNA (Hahn et al., 1998; Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997). Fasting results in reductions in serum insulin and leptin along with changes in circulating levels of other hormones and nutrients. Both leptin receptors and insulin receptors are expressed in POMC and AgRP neurons (Benoit et al., 2002; Briski et al., 2010; Cheung et al., 1997; Mercer, et al., 1996). Both leptin treatment and insulin treatment increases *POMC* mRNA levels and reduced *AgRP* mRNA levels, supporting the hypothesis that leptin and insulin regulates hypothalamic *POMC* and *AgRP* gene expression (Benoit et al., 2002; Cheung et al., 1997; Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997).

Fasting reduces *POMC* mRNA and increases *AgRP* mRNA in leptin-resistant *db/db* mice (Mizuno et al., 1998; Mizuno & Mobbs, 1999). Furthermore, *POMC* mRNA is reduced and *AgRP* mRNA is increased in the animal model of type 1 diabetes without significant changes in serum insulin and leptin (Mizuno et al., 1999). These data indicate that hypothalamic POMC and AgRP gene expression is regulated by both leptin-/insulin-dependent and –independent mechanisms.

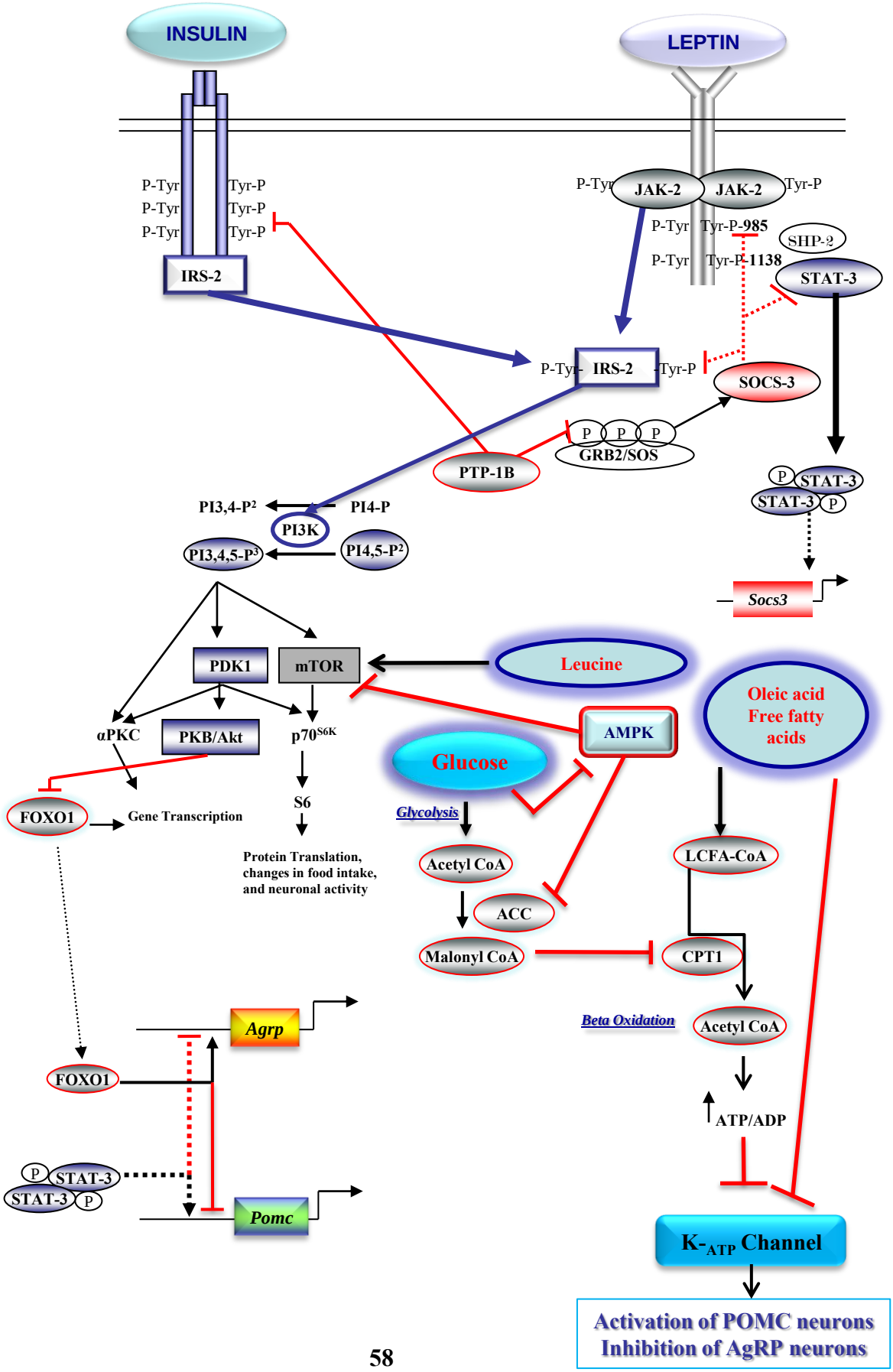

INSULIN
LEPTIN
P-Tyr
Tyr-P
IRS-2
JAK-2
Tyr-P-985
Tyr-P-1138
SHP-2
STAT-3
SOCS-3
GRB2/SOS
PTP-1B
PI3,4-P2
PI4-P
PI3K
PI3,4,5-P3
PI4,5-P2
Socs3
PDK1
mTOR
Leucine
Oleic acid
Free fatty
acids
αPKC
PKB/Akt
p70S6K
AMPK
Glucose
FOXO1
Gene Transcription
S6
Protein Translation,
changes in food intake,
and neuronal activity
Glycolysis
Acetyl CoA
ACC
Malonyl CoA
LCFA-CoA
CPT1
Beta Oxidation
Acetyl CoA
ATP/ADP
Agrp
Pomc
K-ATP Channel
Activation of POMC neurons
Inhibition of AgRP neurons

Figure 3: Central nervous system insulin and leptin signaling. The binding of leptin to its receptor activates JAK2 via the phosphorylation of its tyrosine residues. Phosphorylation-dependent activation of JAK2 induces tyrosine phosphorylation of STAT3. STAT3 functions as a transcription factor by forming dimmers in its phosphorylated state. Actions of leptin and insulin converge within the hypothalamus. Binding of either leptin or insulin to their respective receptors mediates the phosphorylation and activation of insulin receptor substrate (IRS) protein, followed by the activation of phosphatidylinositol-3-hydroxy kinase (PI(3)K). PDK is activated by the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3) by PI3K. PIP3-mediated activation of 3-phosphoinositide-dependent kinase-1 (PDK1) results in the activation of cascade events including the PKC, protein kinase B (PKB/Akt). Akt activates the nutrient sensing enzyme mammalian target of rapamycin (mTOR) and inhibits the transcription factor FOXO1. FOXO1 and STAT3 have reciprocal regulatory effects on *Pomc* and *Agrp* transcription. Intracellular accumulation of either glucose or long chain fatty acyl-CoA (LCFA-CoA) represents an excess of nutrients. Metabolism of glucose and LCFA-CoA results in the inhibition of K-ATP channels due to the increase in intracellular ATP levels. AMP-activated protein kinase (AMPK) is inhibited by insulin and leptin, while activated by ghrelin and low ATP levels. AMPK inhibits the oxidation of LCFA-CoA and metabolism of glucose, both of which result in a reduction in ATP levels (nutrient insufficient state).

3.5.8. Interaction between insulin, leptin, glucose, and CNS melanocortin signaling

Both hypothalamic POMC and AgRP neurons are a direct target of insulin and leptin actions, leading to the hypothesis that hypothalamic melanocortin signaling mediates the metabolic effects of insulin and leptin. An increase in circulating insulin and leptin stimulates the POMC neurons and inhibits the AgRP/NPY neurons (Abizaid & Horvath, 2008; Porte et al., 2005). The anorectic effect of insulin was blocked by melanocortin antagonist SHU9119, (Benoit et al., 2002). Similarly, feeding suppressing- and weight reducing-effects of leptin were negated by pre-treatment with SHU9119 (Seeley et al., 1997). These results support the role for CNS melanocortin signaling in the mediation of CNS insulin and leptin actions.

Although ablation of insulin receptor in AgRP neurons did not cause any significant changes in food intake and body weight, insulin failed to normally suppress hepatic glucose production in AgRP-restricted insulin receptor knockout mice (Konner et al., 2007). There were no significant changes in food intake, body weight, and hepatic glucose production in POMC neuron-specific insulin receptor knockout mice (Konner et al., 2007). These data suggest that insulin action in AgRP neurons is required for insulin-induced suppression of hepatic glucose production.

Mice lacking leptin receptors (a long-form functional LepRb receptor) only in POMC neurons are mildly obese and hyperleptinemic, suggesting that leptin signaling in POMC neurons is required for leptin's regulation of energy homeostasis (Balthasar et al., 2004). Inactivation of downstream targets of leptin, signal transducer and activator of

transcription 3 (STAT3), in POMC neurons resultes in the development of obesity and hyperphagia, while increasing STAT3 activity only in AgRP neurons causes reduction in body weight in mice mainly due to increased locomotor activity (Mesaros et al., 2008; Xu et al., 2007). Furthermore, suppressor of cytokine signaling 3 (SOCS3) negatively regulates both insulin and leptin signaling and ablation of SOCS3 only in the POMC neurons in mice enhances leptin sensitivity and protects against diet-induced obesity and insulin resistance (Kievit et al., 2006). These findings suggest that leptin signaling via STAT3 plays an important role in melanocortinergic regulation of energy homeostasis.

Intracellular signaling pathways of insulin and leptin converge at the level of the phosphatidylinositol 3-kinase (PI3K) (Niswender et al., 2003). The role of PI3K signaling in melanocortinergic regulation of energy balance is slightly complicated as it may affect multiple downstream pathways. Disruption of constitutive activation of PI3K signaling in POMC neurons causes hyperphagia, obesity, and impaired feeding response to leptin in mice through alterations in the activity of ATP-sensitive K^+ (K_{ATP}) channels or forkhead box protein O1 (FoxO1) (Belgardt et al., 2008; Hill et al., 2008; Plum et al., 2006). Disruption of K_{ATP} channels in POMC neurons caused the lack of depolarization response of POMC neurons to increased glucose and impairments in glucose tolerance in mice (Parton et al., 2007). Ablation of FoxO1 only in POMC neurons decreased food intake and protects against diet-induced obesity in mice (Plum et al., 2009). These findings support the hypothesis that the insulin/leptin-PI3K signaling is important for normal metabolic regulation by hypothalamic POMC neurons.

In addition to hormones such as insulin and leptin, nutrients also act on hypothalamic melanocortin neurons and regulate metabolism. Hypothalamic AMP-

activated protein kinase (AMPK) has been suggested to act as a key sensing mechanism, responding to hormones and nutrients in the regulation of energy homeostasis (Minokoshi et al., 2004). Ablation of AMPKα2 only in POMC neurons or AgRP neurons in mice resulted in increased body weight, adiposity, and reduced energy expenditure, or reduced body weight, respectively (Claret et al., 2007). These mice remained to be sensitive to the anorectic and weight-reducing effects of leptin, suggesting that the effect of leptin can be exerted through other signaling pathways. POMC neurons lacking AMPKα2 showed normal responses to insulin or leptin. Reducing glucose hyperpolarized the POMC and AgRP neurons having intact AMPK, but it did not alter the electrophysiological activities of POMC and AgRP neurons lacking AMPKα2 (Claret et al., 2007). These data suggest that hypothalamic AMPK plays a role in CNS melanocortin mediation of glucose action, but not insulin or leptin action.

Collectively, hypothalamic melanocortin pathway mediates the metabolic effects of insulin, leptin, and glucose. Therefore, impairments in these signaling pathways lead to abnormal melanocortin activities and consequently metabolic impairments.

4. Regulation of hypothalamic activity and energy balance by metabolic signals

4.1. Regulation of hypothalamic activity and energy balance by hormones

4.1.1. Role of CNS insulin signalling in the regulation of energy balance

CNS regulation of energy expenditure, food intake and metabolism are under the control of the adiposity signals insulin and leptin. In 1953 Kennedy postulated that peripheral adipocyte related humoral factors function as a negative feedback mechanism to the hypothalamus to inhibit food intake (Kennedy, 1953). Such factors include insulin and leptin which circulate in the blood in proportion to body fat stores (Caro et al., 1996; Considine et al., 1996).

Insulin was first discovered by Banting and Best as the humoral factor aiding diabetic patients to normalize their blood glucose levels and consequently contribute to their survival (Banting, 1937; Banting & Best, 1990; Banting et al., 1991). Insulin is produced and secreted by pancreatic beta cells and functions as an adiposity signal conveying signals to the brain for the regulation of energy homeostasis (Schwartz et al., 1992). Insulin resistance is a pathological symptom observed in visceral obesity, hyperglycemia, hypertriglyceridemia, type 2 diabetes, polycystic ovarian disease (PCOS), cardiovascular disease and nonalcoholic fatty liver disease. The molecular mechanisms and signaling pathways involved in insulin resistance are not entirely understood as there are multiple factors coming into play. Scientific investigation using tissue-specific knockout approaches, and *in vivo* and *in vitro* models have demonstrated how insulin resistance can develop in specific tissues or pathways while not in others.

Insulin secretion from the pancreatic beta-cells (islets of Langerhans) controls nutrient substrate metabolism in skeletal muscle, liver and adipose tissue (anabolic action). During the time periods of nutrient excess and the development of obesity, insulin secretion is proportional to adipose tissue mass. Under these conditions insulin functions as a feedback mechanism in the CNS in inducing anorexigenic responses and inhibitory effects on body weight gain (catabolic action) (Woods et al., 1979). The involvement and critical role of insulin in CNS control of food intake and body weight is supported by studies showing insulin transport across the blood brain barrier, the decrease in food intake as a result of direct injection of insulin to the brain, and that insulin receptors are expressed in areas involved in energy homeostasis and metabolism (Reviewed in: Biddinger & Kahn, 2006; Schwartz et al., 2000).

4.1.2. CNS insulin resistance in obesity and systemic insulin resistance

In the presence of increased circulating levels of insulin, obese individuals may have reduced insulin sensitivity due to peripheral insulin resistance, reduced insulin levels in the brain, and/or reduced insulin binding to its receptor in brain capillaries as detected in obese and hyperinsulinemic rats (Schwartz et al., 1990). Obese men are resistant to the metabolic effects of insulin, while their responses to the effect of insulin on cognition/memory are not affected (Hallschmid, Benedict, Schultes, Born et al., 2008). Intranasal insulin administration improved memory and reduced the hypothalamus-pituitary-adrenal (HPA) activity as assessed by the reduction in the circulating levels of ACTH and cortisol in both lean and obese men, indicating that CNS action of insulin in these functions is not impaired in obese individuals (Hallschmid, et al., 2008a;

Hallschmid, et al., 2008b). Conversely, the weight-reducing and adiposity-reducing effects of insulin treatment were observed only in lean subjects, and not in obese subjects. Similarly, these findings indicate that obesity is associated with CNS resistance to the catabolic effects of insulin on body weight and adiposity.

The inhibitory effects of centrally administered insulin on body weight and food intake are not present in obese rats (Ikeda et al., 1986; Woods et al., 2004). Insulin infusion increases whole-brain metabolic rate of glucose by about 17% in healthy human subjects who showed normal systemic insulin sensitivity, but the effect of insulin on brain metabolism is attenuated in individuals who show systemic insulin resistance (Anthony et al., 2006). These data suggest that insulin resistance exists not only in peripheral insulin-sensitive tissues but also in the CNS. However, this study did not examine whether or not response to insulin was reduced in the hypothalamus.

Hypothalamic insulin resistance has been demonstrated in animal models of obesity. The feeding-suppressing effect of i.c.v. insulin is blunted in DIO rats (Clegg et al., 2005; Posey et al., 2009). Insulin-induced phosphorylation of PKB/Akt is also reduced in the hypothalamus of DIO rats (Benoit et al., 2009; Posey et al., 2009). Injection of insulin into the amygdala results in a reduction in food intake in rats, however the feeding-suppressing effect of amygdala insulin is attenuated in rats fed with a high-fat diet (Boghossian et al., 2009).

Reduced availability of insulin in the CNS is one possible cause of CNS insulin resistance. CSF levels of insulin are lower in obese humans compared with healthy non-obese individuals (Kern et al., 2006). Insulin binding to its receptor in brain capillaries is impaired in obese hyperinsulinemic rats (Schwartz et al., 1990). Diet induced obesity is

associated with a reduction in insulin transport into the brain (Israel et al., 1993; Kaiyala et al., 2000). These findings support the possibility that reduced insulin availability in the CNS is a contributing factor in the development of CNS insulin resistance and subsequent whole body insulin resistance.

Another possible mechanism causing CNS insulin resistance is impairments in CNS insulin signaling. Phosphorylation of IRS1 at Ser307 in the hypothalamus is higher in ob/ob mice when compared with that in wild-type mice (Komori et al., 2005). High-fat diet feeding causes insulin resistance and is associated with increased palmitic acid levels in the hypothalamus in mice (Kimes et al., 1985). Increased neuronal lipid levels and the subsequent lipotoxicity-induced inflammation results in hypothalamic insulin resistance (Arase et al., 1988; Clegg et al., 2005; De Souza et al., 2005; Levin et al., 2005; Posey et al., 2009). Central injection (i.c.v.) of palmitic acid results in elevated PKC-theta localization to the cell membrane of rat hypothalamic neurons in the ARC (specifically NPY/AgRP neurons) and DMN (Benoit et al., 2009). The increase in PKC-theta membrane localization was associated with reduced insulin signalling in the hypothalamus and knockdown of PKC-theta expression in the hypothalamus resulted in attenuated response to high-fat diet-induced obesity and insulin resistance in mice (Benoit et al., 2009). Consistent with these *in vivo* experiments, palmitic acid treatment attenuates insulin-induced Akt phosphorylation in immortalized hypothalamic neuronal cells (Mayer & Belsham, 2010b). Attenuated insulin signaling is associated with the induction of endoplasmic reticulum (ER) stress and cellular apoptosis. These effects of palmitic acid are blocked by pre-treatment with AMPK activator (Mayer & Belsham, 2010b). These data suggest that prolonged exposure of lipids, in particular palmitic acid,

to hypothalamic neurons causes impairments in hypothalamic insulin signaling, leading to peripheral insulin resistance.

Lastly, prolonged exposure of high levels of insulin to hypothalamic neurons may cause impairments in hypothalamic insulin signaling. By using immortalized hypothalamic cells, a recent study demonstrated that prolonged insulin exposure to hypothalamic neurons causes an impaired response to insulin in these cells (Mayer & Belsham, 2010a). The attenuated insulin signaling is mediated by the inactivation of IRS1 by phosphorylation of Ser1101, proteasomal degradation of IRS1, and lysosomal degradation of IR (Mayer & Belsham, 2010a). These data suggest that direct prolonged insulin exposure to CNS contributes to the development of insulin resistance in these cells.

Collectively, these findings suggest that insulin resistance in the CNS, in particular hypothalamus and amygdala, is associated with metabolic impairments including obesity and systemic insulin resistance and that reduced insulin transportation into the brain and impairments in CNS insulin signaling contributes to the development of CNS insulin resistance.

4.1.3. Role of CNS leptin signalling in the regulation of energy balance

The existence of circulating factors that control energy homeostasis was suggested by parabiosis (common blood supply through the union of two bodies) experiments (Coleman, 1978). These experiments suggested that *db/db* mice produce, but do not respond to, a satiety factor which circulates in the blood stream. In contrast, although *ob/ob* mice respond to the satiety factor, they do not produce it. However, genes which

are responsible for these phenotypes have not been identified until the mid 1990's. In 1994, Friedman's group identified leptin as an adipocyte hormone, encoded by the *obese* gene, *ob*, by positional cloning (Zhang et al., 1994). Soon after the cloning of leptin, leptin receptors (LepR), encoded by the *lepr* gene, *db*, were identified (Tartaglia et al., 1995). Thus, either a functional leptin or LepR was lacking in the obese mice used in the earlier parabiosis studies. These findings clearly demonstrated that the *obese* gene and *lepr* gene are involved in the regulation of energy balance, and mutations in these genes result in obesity in mice. Mutations in the *obese* gene in mice (*ob/ob* mice) result in severe obesity with hyperphagia and decreased energy expenditure. Treatment of *ob/ob* mice with leptin reverses these impairments, suggesting that leptin physiologically regulates energy homeostasis (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Weigle et al., 1995). Although the incident rate is very low, mutations in these genes are also associated with human obesity (Montague et al., 1997; Strobel et al., 1998). The leptin gene is abundantly expressed in adipose tissue, but it is also expressed in gastric epithelium and placenta (Bado et al., 1998; Masuzaki et al., 1997; Y. Zhang et al., 1994). Expression of leptin mRNA is elevated in several mouse models of obesity (Frederich et al., 1995; Maffei et al., 1995; Mizuno et al., 1996). Serum leptin levels are elevated in human obese and decreased in anorexia nervosa (Grinspoon et al., 1996; Maffei et al., 1995). The circulating levels of leptin correlate with the amount of adipose tissue, body weight, or BMI (Considine et al., 1996). Thus, it is apparent that leptin is highly correlated with total fat mass and obesity is associated with elevated, rather than reduced, leptin levels in both rodents and humans, indicating that increased adiposity is associated with leptin resistance.

LepRb is widely expressed in the CNS of the rodent brain with a particularly high expression in the hypothalamus including the areas VMN, ARC, DMN, PVN, and LHA (Elmquist, Bjorbaek et al., 1998; Fei et al., 1997; Mercer, Hoggard, Williams, Lawrence, Hannah, & Trayhurn, 1996; Schwartz, Seeley et al., 1996; Tartaglia et al., 1995). In addition, LepRb is found in several thalamic nuclei including the mediodorsal, ventral anterior, ventral medial, submedial, ventral posterior, and lateral dorsal thalamic nuclei, and in the Purkinje and granular cell layers of the cerebellum (Elmquist, Bjorbaek et al., 1998). Systemic or central administration of leptin induces Fos (early gene marker) expression in specific hypothalamic regions including the retrochiasmatic area (RCh), PVN, ARC, VMN, DMN, and the ventral premammillary nucleus (PMV) (Elmquist, Ahima et al., 1998; Elmquist et al., 1997; Van Dijk et al., 1996; A. J. Woods & Stock, 1996). Leptin-induced Fos-immunoreactive cells are also found in extra-hypothalamic sites including the superior lateral and external lateral subdivisions of the parabrachial nucleus, the supragenual nucleus, and the nucleus of the solitary tract (NTS) in the brainstem (Elias et al., 2000). Fos immunoreactivity is also induced in the central amygdala following i.c.v. leptin injection (Van Dijk et al., 1996). Furthermore, leptin induces activation of signaling pathways including STAT and MAPK in immortalized mouse hypothalamic cells (Cui et al., 2006). These observations clearly indicate that leptin exerts some of its effects by engaging directly at distinct sites within the CNS.

Injection of leptin into the VMN increases glucose uptake by skeletal muscle, brown adipose tissue, heart, and spleen, but injection of leptin into the LH has little effect on glucose uptake in these tissues (Minokoshi et al., 1999). Direct application of leptin to the ARC, VMN, or LH causes reductions in food intake and body weight with a rank

order of potency; ARC > VMH = LH (Satoh et al., 1997). A direct microinjection of leptin into VMN increases renal sympathetic nerve activity (Tanida et al., 2003). Injection of leptin into the VTA reduces food intake and knockdown of VTA LepRb causes a reduction in food intake (Hommel et al., 2006). Although, i.c.v. injection of leptin reduces body weight in leptin-deficient *ob/ob* mice, leptin does not reduce body weight in ARC-lesioned *ob/ob* mice (Takeda et al., 2002). Enhanced LepRb expression specifically in ARC attenuates obesity and improves insulin sensitivity in rats lacking functional leptin receptors (Morton et al., 2005; Morton et al., 2003). Furthermore, restoration of LepRb specifically in ARC partially or completely reverses obesity, hyperphagia, hyperglycemia, hyperinsulinemia, and reduces the activity levels in mice lacking functional LepRb (Coppari et al., 2005). Selective loss of LepRb in VMN results in obesity and increased susceptibility to high-fat diet-induced obesity, suggesting that signaling through VMN LepRb plays a role in the regulation of energy balance (Bingham et al., 2008; Dhillon et al., 2006). Finally, electrophysiological studies demonstrated that leptin induces changes in the activity of POMC and AgRP neurons in ARC, SF-1-positive neurons in VMN, and dopaminergic neurons in VTA (Cowley et al., 2001; Dhillon et al., 2006; Hommel et al., 2006; K. A. Takahashi & Cone, 2005; van den Top et al., 2004).

These findings indicate that circulating leptin acts primarily through hypothalamic ARC and VMN involved in regulating feeding, body weight, and neuroendocrine function. The localization of LepRb and leptin-induced Fos in extra-hypothalamic sites in the thalamus, brainstem, and cerebellum suggests that leptin may act on specific sensory and motor systems.

4.1.4. Leptin resistance and obesity

Identification of leptin in *ob/ob* mouse indicated that leptin deficiency is a cause of obesity. However, leptin deficiency is very rare in human obesity and soon it became clear that human obesity is associated with leptin resistance. Although the identity of the crucial mediator(s) of leptin resistance still remains unclear, there are some possibilities including the failure of circulating leptin to reach its targets in the brain and inhibition of the intracellular LepRb signaling cascade.

One possible mechanism for leptin resistance is a low availability of leptin in the CNS. This could be due to defective leptin transport across the BBB. Transportation of leptin into the brain is mediated via a specific transport mechanism across the BBB and/or via the circumventricular organs (Banks et al., 1996; Zlokovic et al., 2000). In rats lacking all leptin receptor isoforms, there is a marked decrease in leptin transport rate from the circulation into the brain. Normal transport rate of leptin is maintained in *db/db* mice in which only LepRb is missing, but other isoforms are intact, suggesting that the short form of leptin receptor, LepRe, partly mediates leptin transportation across the BBB. Obese humans have a decreased CSF-to-serum ratio for leptin (Caro et al., 1996; Schwartz, Peskind et al., 1996). Obese animals do not respond to peripherally administered leptin, but they partially respond to central injection of leptin, suggesting that a defect in leptin transport is one of the causes for leptin resistance (Halaas et al., 1997). Leptin transport rate is also reduced in DIO rats (Burguera et al., 2000; Levin et al., 2004). In addition, hypothalamic *LepRb* mRNA levels are reduced in diet-induced obese rats (Wilsey et al., 2003). These findings suggest that reduced leptin availability in

the hypothalamus limits the ceiling of LepRb-mediated metabolic effect of leptin, leading to the development of CNS leptin resistance.

Another possible mechanism for leptin resistance involves the impairments in intracellular LepRb signaling system. For example, leptin-induced phosphorylation of STAT3 is reduced in the hypothalamus of DIO animals (Levin et al., 2004). Leptin-induced activation of PI3K and mTORC1 and inhibition of AMPK is reduced in DIO animals (Cota et al., 2008; Martin et al., 2006; Metlakunta et al., 2008). Furthermore, expression of PTP1B and SOCS3 is increased and AMPK activity is reduced in the hypothalamus of diet-induced leptin-resistant obese animals (Martin et al., 2006; Munzberg et al., 2004; White et al., 2009). These data indicate that impairments in LepRb signaling contribute to the development of leptin resistance.

4.2. Regulation of hypothalamic activity and energy balance by nutrients

4.2.1. Fasting- and feeding- induced alterations in hypothalamic activity

Fasting causes dramatic changes in hypothalamic activity and metabolism. Of particular importance, activity of the hypothalamic melanocortin system is affected by feeding condition. Fasting increases AgRP and NPY expression and reduces POMC and CART expression in the hypothalamic ARC, and re-feeding reverses the fasting-induced changes in the expression of these genes as represented by mRNA or protein expression (Brady et al., 1990; Hahn et al., 1998; Kristensen et al., 1998; McAlister & Van Vugt, 2004; Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997). Fasting also induces Fos expression in the ARC where AgRP/NPY neurons are located, and re-

feeding reverses this fasting-induced Fos expression (Morikawa et al., 2004; Ueyama et al., 2004). In contrast, feeding induces Fos expression in the lateral peri-arcuate area of the hypothalamus where POMC/CART neurons are located (Shu et al., 2003). Feeding also increases the number of POMC-producing cells expressing Fos in the hypothalamus (Shu et al., 2003). Thus, fasting increases the hypothalamic activity of NPY/AgRP neurons and decreases the hypothalamic activity of POMC/CART neurons, while feeding produces the opposite direction of changes. Similar regulations by fasting/feeding can be seen in other hypothalamic neuronal systems such as MCH, orexin, and CRH.

4.2.2. Lipids

Everyone has a set-point for body fat, and deviations from this lead to compensatory adjustments in food intake. Based on this concept, in 1953, Kennedy first proposed the "lipostatic hypothesis" that inhibitory signals released into the circulation in proportion to body adipose stores act directly in the hypothalamus to adjust food intake and maintain the normal adipose store (Kennedy, 1953). According to this theory, hormones such as insulin and leptin function as an adiposity signal that can be delivered to the hypothalamus. In addition, fatty acids are also proportional to adiposity and hypothalamus senses changes in levels of fatty acids. Upon entry into cells of the CNS, a fatty acid is esterified to a fatty acyl-CoA. Carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2) are required for the transfer of long-chain fatty acyl-CoA (LC-CoA) to the mitochondria and their subsequent oxidation. Central injection (i.c.v.) of long-chain fatty acid (LCFA) and oleic acid reduces food intake and hepatic glucose production (Obici et al., 2003). In contrast, injection of octanoic acid, which does not require CPT1 for entry

into mitochondria, does not affect food intake (Obici et al., 2002). Decreasing CPT1 activity within the hypothalamus increases LCFA-CoA levels in the hypothalamus and inhibits food intake and hepatic glucose production (Obici et al., 2003). Thus, the availability of LCFA in the hypothalamus is a key signal which regulates food intake and glucose homeostasis by altering CPT1 activity.

4.2.3. Amino acids

It has been demonstrated that changes in dietary protein influence feeding behavior, supporting the existence of neural mechanisms which sense and respond to changes in protein or amino acid availability (Anderson & Moore, 2004; Bensaid et al., 2003; Morrison et al., 2007; Westerterp-Plantenga, 2003). Hypothalamic neurons participate in this amino acid sensing system through the serine/threonine kinase mammalian target of rapamycin (mTOR) signaling. Central administration (i.c.v.) of leucine causes reductions in food intake and body weight, and this effect is negated by rapamycin, a mTOR inhibitor (Cota et al., 2006). Phosphorylated S6 kinase 1 (S6K1), a downstream mTOR target, is expressed in the hypothalamic AgRP/NPY and POMC/CART neurons, suggesting that mTOR signaling in these hypothalamic neurons is necessary for the leucine-induced suppression of food intake (Cota et al., 2006). Decreasing amino acid availability increases *AgRP* mRNA levels in hypothalamic cell line, and this effect is attenuated by replacement of leucine (Morrison et al., 2007). Acute exposure to elevated amino acid concentrations increases S6K1 phosphorylation via a rapamycin-sensitive mechanism, suggesting that amino acids directly stimulates mTOR signaling. Furthermore, rapamycin treatment increases *AgRP* mRNA levels in hypothalamic cells

exposed to high amino acids (Morrison et al., 2007). These findings indicate that amino acids can regulate food intake and body weight by directly acting on hypothalamic neurons including AgRP/NPY neurons through the mTOR signaling.

4.3. Role of glucose in the regulation of hypothalamic activity and energy balance

4.3.1. Glucostatic theory

In one of the earliest studies to address the role of glucose in the regulation of food intake, subcutaneous injection of glucose reduced food intake in rats, whereas injection of calorically equivalent fat emulsion did not affect food intake (Mayer & Bates, 1952). Based on this finding, Mayer first proposed the glucostatic theory of hunger, which suggested that reduced glucose utilization in key brain regions leads to perception and expression of hunger and that increased glucose utilization in these brain sites constitutes a physiological signal for the cessation of eating (Mayer, 1952, 1953, 1955). In this theory, blood glucose concentration and/or its arteriovenous difference were used to infer the rates of glucose utilization. Thus, hypoglycemia and decreased glucose utilization leads to initiation of feeding, whereas postprandial hyperglycemia and increased glucose utilization results in cessation of feeding.

4.3.2. Glucose uptake by CNS cells

Glucose is the major metabolic substrate of the brain cells and is transported into the brain across blood-brain barrier (BBB) by a saturable carrier-mediated mechanism (Pappenheimer & Setchell, 1973). Subsequently, this carrier was shown to be glucose transporter 1 (GLUT1) which is abundantly expressed in the brain capillary endothelium (Dick et al., 1984). GLUT1 is asymmetrically distributed on the brain capillary endothelial lumenal and ablumenal membranes, and the cytoplasmic compartment, suggesting that glucose uptake across brain capillary luminal membrane may be a rate-limiting step for glucose transport into the brain across BBB (Farrell & Pardridge, 1991).

GLUT2 is required for glucose-stimulated insulin secretion in pancreatic β-cells and has been proposed as a glucose sensor. GLUT2 is expressed widely in the CNS with relatively higher expression in the hypothalamus (Leloup et al., 1994; B. Li et al., 2003). GLUT2 knockout mice exhibit hyperglycemia and hypoinsulinemia, and die within 3 weeks after the birth (Guillam et al., 1997). Transgenic expression of GLUT1 in β-cells (RIP*Glut1;Glut2*$^{-/-}$ mice) is sufficient to rescue GLUT2 knockout mice from lethality (Thorens et al., 2000). RIP*Glut1;Glut2*$^{-/-}$ mice showed increased food intake and loss of feeding responses to i.c.v. injection of glucose and 2-DG, respectively (Bady et al., 2006). Central administration (i.c.v.) of glucose decreases *AgRP* and *NPY* mRNA and increases *POMC* and *CART* mRNA in the hypothalamus of wild-type mice, however this effect is absent in RIP*Glut1;Glut2*$^{-/-}$ mice (Bady et al., 2006). Consistent with this phenotype, reduced GLUT2 expression by i.c.v. injection of GLUT2 antisense oligonucleotides abolishes the 2-DG-induced stimulation of feeding (Wan et al., 1998). In addition, GLUT2 overexpression results in enhanced glucose inhibition of AgRP expression and

attenuates the 2-DG stimulation of *AgRP* expression in neuronal cell line (Li et al., 2006). These findings suggest that brain GLUT2 plays a role in the CNS glucose sensing and regulation of food intake.

GLUT3 is expressed widely in the CNS including the hypothalamus (Brant et al., 1993; Gerhart et al., 1992; Nagamatsu et al., 1992). Insulin signalling in the VMH mediates neuronal glucose uptake by inducing the translocation of GLUT3 to neuronal membranes in the hypothalamus (Uemura & Greenlee, 2006). The low *K*m (1-3 mM) of GLUT3 may be suited for the low levels of glucose in the brain compared with the high *K*m of GLUT2. There was no difference in food intake, body weight and feeding responses to the i.c.v. treatments of either glucose or 2-DG between wild-type and heterozygous GLUT3 knockout mice (Schmidt et al., 2008). This suggests that a single allele of the GLUT3 gene is sufficient for normal hypothalamic glucose sensing and regulation of energy balance.

GLUT4 is expressed in the hypothalamus, in particular in hypothalamic glucose-sensing neurons (Brant et al., 1993; El Messari et al., 1998; Fioramonti et al., 2004). Similar to GLUT3, insulin signalling in the VMH mediates neuronal glucose uptake by inducing the translocation of GLUT4 to neuronal membranes in the hypothalamus (Grillo et al., 2007). Little is known for the role of brain GLUT4 in hypothalamic glucose sensing and metabolic regulation.

It is likely that glucose is transported into the brain across the BBB via GLUT1. However, currently the literature does not suggest a conclusive mechanism by which hypothalamic cells uptake glucose. This may be due to a possible diversity in hypothalamic glucose-sensing mechanism as outlined in later sections.

4.3.3. Direct action of glucose in the hypothalamus

Subsets of hypothalamic neurons exhibit specific responses to changes in extracellular glucose levels. These glucose-sensing neurons are electrically excited (glucose-excited neurons) or inhibited (glucose-inhibited neurons) when extracellular glucose levels are elevated (Anand et al., 1964; Oomura et al., 1969; Oomura et al., 1974; Song et al., 2001). Within the VMH (which includes VMN and lateral ARC), the densest population of glucose-sensing neurons is glucose-excited neurons, with glucose-inhibited neurons consisting a very small proportion (<1%) (Cotero & Routh, 2009). The electrophysiological properties of the hypothalamic glucose-sensing neurons clearly indicate that some hypothalamic neurons are a direct target of glucose action.

Central administration (i.c.v.) of glucose causes reduction in blood glucose levels and suppression of hepatic glucose production. These effects are recapitulated by a direct injection of glucose into the hypothalamus (Lam et al., 2005). Glucose also causes changes in gene expression in immortalized hypothalamic cell lines and hypothalamic tissues cultured *ex vivo*, indicating that glucose directly acts on hypothalamic cells (Cai et al., 2007; Cheng et al., 2008; Lee et al., 2005). These data support the hypothesis that glucose regulates energy homeostasis through its direct action in the hypothalamus.

4.3.4. CNS glucose metabolism and whole body energy balance

Central administration of glucose causes changes in a variety of metabolic and endocrine functions, suggesting that central glucose action or glucose metabolism plays a role in regulating metabolic and endocrine functions.

Glucose i.c.v. treatment reduces food intake and body weight, and increases oxygen consumption (Davis et al., 1981; Le Feuvre et al., 1991). Conversely, neuroglucopenia induced by i.c.v. injection of 2-DG caused a significant increase in food intake (Berthoud & Mogenson, 1977; Granneman & Friedman, 1983). The anorectic effect of i.p. glucose was abolished by i.c.v. injection of 2-DG, indicating that the effect of peripherally administered glucose is mediated by CNS glucose metabolism (Wolfgang et al., 2007). A single i.p. injection of gold-thioglucose (GTG) produces a lesion in the hypothalamic area covering the lateral ARC, and the cell-sparse area lateral to the ARC and ventral to the VMN (Bergen et al., 1996). These hypothalamic lesioned mice do not have alterations in food intake in response to i.p. injection of glucose or 2-DG, suggesting that intact hypothalamic cells are necessary for glucose-induced suppression of feeding and neuroglucopenia-induced feeding (Bergen et al., 1996). In contrast, glucose-induced increase in oxygen consumption is not impaired in VMH-lesioned rats, indicating that the defective diet-induced thermogenesis associated with VMH is not due to an inability to respond to changes in extracellular glucose concentrations (Le Feuvre et al., 1991). These findings suggest that central action of glucose and/or glucose metabolism in the CNS mediates the anorectic effect of glucose.

Furthermore, i.c.v. injection of glucose results in decreased circulating levels of glucose and insulin (Lam et al., 2005). I.c.v. glucose-induced reduction in blood glucose levels is

associated with reduced expression of a gluconeogenic enzyme-encoding gene, glucose-6-phosphatase (G6Pase) mRNA in the liver and suppression of hepatic glucose production (Lam et al., 2005). Hypothalamic glucose concentration is increased after i.c.v. glucose injection and intra-hypothalamic glucose injection recapitulates the blood glucose-lowering effect of i.c.v. glucose injection (Lam et al., 2005). Conversely, both blood glucose levels and hepatic glucose production are increased after i.c.v. 2-DG injection (Molina et al., 1993). The synthetic glucose analogue, 2-DG, interferes with glucose metabolism and induces glucopenia, as a result mimicking a hypoglycaemic condition. These findings indicate that increased glucose availability and/or glucose metabolism in the hypothalamus is a key signal to cause reductions in hepatic glucose production and blood glucose levels. Hypoglycemia-induced secretion of glucagon and catecholamines is attenuated in VMH-lesioned rats, whereas LHA-lesioned rats show normal glucagon and catecholamine responses (Borg et al., 1994). Intra-VMH infusion of 2-DG result in an increase in plasma glucose in association with elevations in plasma glucagon and epinephrine in rats (Borg et al., 1995). Furthermore, during systemic hypoglycemia, glucose infusion directly into the VMH inhibited hypoglycemia-induced secretion of glucagon, epinephrine and norepinephrine. These findings are consistent with the hypothesis that glucopenia localized to the VMH triggers the release of counter-regulatory hormones that defend against hypoglycemia, and that VMH cells a key sensor for hypoglycemia.

Glucose i.c.v. treatment reduces plasma triglyceride levels through the inhibition of very low-density lipoprotein (VLDL) secretion by the liver, indicating that CNS glucose action and/or glucose metabolism plays a role in the regulation of lipid

metabolism (Lam et al., 2007). This highlights the importance of an intacthypothalamic glucose sensing for the normal regulation of lipid metabolism in the liver.

4.3.5. Regulation of hypothalamic gene and protein expression by glucose

Fasting causes alterations in a variety of metabolic and neuroendocrine functions and exerts metabolic adaptation. Fasting also caused changes in expression levels of hypothalamic neuropeptide genes involved in the regulation of energy balance. Of particular importance, hypothalamic *POMC* mRNA is reduced and *AgRP* mRNA is increased by fasting, and feeding produces opposite effects on the expression of these genes (Hahn et al., 1998; Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997). Because circulating levels of a number of hormones and nutrients are changed during fasting, each of these changes possibly contribute to the fasting- and feeding-induced changes in both hypothalamic gene expression and activity. For example, fasting reduces plasma leptin levels and increases hypothalamic *AgRP* and *NPY* mRNA and reduces *POMC* mRNA, and leptin treatment reverses the fasting-induced changes in expression levels of these genes (Ahima et al., 1996; Fekete et al., 2006). Similarly, insulin treatment reverses the fasting-induced changes in hypothalamic *AgRP* and *NPY* expression (Fekete et al., 2006). These findings suggest that reduction in leptin and insulin, at least partly, contributes to the changes in hypothalamic gene expression during fasting. Similar to leptin and insulin, plasma glucose levels drop during fasting. Thus, glucose may also play a role in the regulation of hypothalamic gene expression.

Fasting induces Fos expression, a widely-used marker of cell activation, in the ARC, a hypothalamic area where AgRP/NPY and POMC cells are located, while 2-h

refeeding with regular rodent chow reduces the number of Fos-positive cells to the level observed in *ad libitum* fed animals (Becskei et al., 2009; Morikawa et al., 2004; Ueyama et al., 2004). Because circulating levels of glucose, insulin and leptin are returned to the *ad libitum* fed levels after re-feeding, the extent to which each hormone and nutrient contributes to the reduction in Fos expression is unknown. When mice were fed with a non-caloric mash of cellulose (NCM) alone or NCM supplemented with protein (calcium caseinate) for 2 hours following a 14-h fast, protein supplementation causes a significantly greater effect on Fos expression (significantly less Fos-positive cells compared with NCM alone) (Becskei et al., 2009). Re-feeding with protein supplemented NCM causes a significant elevation in blood glucose without any significant changes in serum insulin and leptin compared with re-feeding with NCM alone. Consequently, glucose may reduce Fos expression in the ARC independent of changes in insulin and leptin under this experimental condition. Furthermore, the number of Fos-immunopositive cells is reduced 2 h after the i.p. glucose treatment and re-feeding in 12-h fasted mice. Under these conditions, there is no significant difference in Fos-immunopositive cell number between glucose-injected group and re-fed group (Becskei et al., 2008). Taken together, these findings suggest that glucose plays a major role in the mediation of fasting- and feeding-induced changes in ARC Fos expression (ARC activity).

Fasting increases *AgRP* and *NPY* mRNA and reduces *POMC* mRNA in both normal non-diabetic and streptozotocin-induced diabetic mice (Mizuno et al., 1999). Fasting reduces blood glucose levels without any significant changes in serum insulin or leptin in diabetic mice, suggesting that glucose regulates hypothalamic gene expression

independent of changes in insulin and leptin (Mizuno et al., 1999). More specifically, i.c.v. injection of glucose partially reverses the fasting-induced increase in hypothalamic *NPY* mRNA without significant changes in serum glucose levels, indicating that a reduction in blood glucose contributes to the fasting-induced elevation in *NPY* mRNA (Fekete et al., 2006). It should be noted that i.c.v. injection of leptin or insulin also produces similar effects on *NPY* mRNA levels. In addition, although i.c.v. leptin partially reverses the fasting-induced changes in *POMC* and *AgRP* mRNA, in this study i.c.v. glucose does not have any significant effect on *POMC* and *AgRP* expression (Fekete et al., 2006). Thus, it is likely that glucose, insulin, and leptin differentially contribute to fasting-induced changes in hypothalamic gene expression.

Hypothalamic *POMC* mRNA is increased and *AgRP* mRNA is reduced by i.p. glucose injection. The effect of glucose on *POMC* and *AgRP* mRNAs is negated by i.c.v. injection of 2-DG, a non-metabolizable glucose analog (Wolfgang et al., 2007). *POMC* mRNA is increased and *NPY* mRNA is reduced after i.c.v. glucose injection (Bady et al., 2006). Central injection (i.c.v.) of 2-DG increases *AgRP* and *NPY* mRNA levels in ARC (Fraley et al., 2002; Sergeyev et al., 2000). Glucose stimulates *POMC* expression and inhibits *AgRP* and *NPY* expression in the hypothalamic tissue cultured *ex vivo* or in immortalized hypothalamic cell lines (Cai et al., 2007; H. Cheng et al., 2008; K. Lee et al., 2005). These data are consistent with the hypothesis that glucose can regulate hypothalamic gene expression through its direct action on hypothalamic cells and its metabolism in the hypothalamus.

4.3.6. Hypothalamic glucose sensing mechanism

Based on the i.c.v. and intra-hypothalamic glucose injection studies, it is clear that glucose action in the hypothalamus plays a role in the regulation of hypothalamic gene expression, neuronal activity, and whole body metabolism. Although, the molecular mechanisms by which hypothalamic neurons sense changes in extracellular glucose levels are still under investigation, several mechanisms have been proposed. Included among these are ATP-sensitive K^+ channel (K_{ATP} channel), AMPK, glucose metabolism, and malonyl-CoA.

4.3.7. Role of K_{ATP} channel

ATP-sensitive K^+ (K_{ATP}) channels play a significant role in the regulation of hypothalamic glucose sensing. 2-DG-induced feeding and glucagon secretion is attenuated in mice deficient in Kir6.2, one of the four pore-forming units of the K_{ATP} channel (Miki et al., 2001). Central injection (i.c.v.) of a K_{ATP} channels activator reduces blood glucose levels by suppressing hepatic glucose production (Pocai et al., 2005). Direct injection of glucose into the hypothalamus reduces plasma glucose levels and triglyceride levels, and this effect is blocked by co-infusion of the K_{ATP} channels blocker (Lam et al., 2007; Lam et al., 2005). These data suggest that hypothalamic glucose sensing mechanisms involve K_{ATP} channels.

In glucose-excited VMH neurons, an increase in extracellular glucose inhibits K^+ channels and increases neuronal action current activity, while ATP induces the closure of this K^+ channel (Ashford et al., 1990). Decreasing glucose from 2.5 to 0.5 mM increases K_{ATP} single-channel currents in ARC glucose-excited neurons (Wang et al., 2004). These

data lead to the hypothesis that glucose-induced activation of glucose-excited neurons is mediated by closure of K_{ATP} channels. This hypothesis was confirmed by the study using a mouse model lacking Kir6.2, one of the four pore-forming units of the K_{ATP} channel. VMH glucose-excited neurons show an increase in firing rate in response to an increase in glucose from 2.5 (or 5) mM to 25 (or 20) mM (Miki et al., 2001; Yang et al., 1999). A similar change is found in VMH neurons in response to a smaller elevation of glucose (2.4 mM to 4.2 mM) (Silver & Erecinska, 1998). These glucose-excited neurons express Kir6.2 and sulfonylurea receptor 1 (SUR1), but not Kir6.1, SUR2A, or SUR2B (Miki et al., 2001). VMH glucose-excited neurons from Kir6.2 knockout mice show higher spontaneous firing rate at low glucose concentration (2.5 mM) without further increase in response to high glucose concentration (25 mM) (Miki et al., 2001). These data suggest that Kir6.2 is the pore-forming subunit of the plasma membrane K_{ATP} channels in VMH neurons and that Kir6.2-containing K_{ATP} channels are essential for glucose sensing in VMH glucose-excited neurons.

The K_{ATP} channel-dependent mechanism for glucose-induced activation of hypothalamic glucose-excited neurons is similar to glucose-sensing mechanism for pancreatic β-cells. In pancreatic β-cells, glucose enters the cell via GLUT2, is phosphorylated by glucokinase (GK) and is metabolized to produce ATP (Schuit et al., 2001). Increased cytosolic ATP:ADP ratio closes the K_{ATP} channels, resulting in depolarization of the β-cells. In addition to Kir6.2, expression of GLUT2 and GK is found in hypothalamic glucose-excited neurons, supporting the hypothesis that some of hypothalamic glucose-excited neurons sense changes in extracellular glucose levels and translate it into an increase in electrical activity (Kang et al., 2004).

The activity of VMH glucose-excited neurons is also increased by galactose and glycerol, but neither of these can stimulate pancreatic β-cells (Dean et al., 1975; Noel et al., 1997). This indicates that hypothalamic glucose-excited neurons sense glucose via a mechanism which is distinct from that used by pancreatic β-cells. Some of neurons in the ARC also increase firing rates in response to an increase in extracellular glucose (from 5 to 20 mM) (Fioramonti et al., 2004). The same response to glucose was also found in ARC neurons from Kir6.2 knockout mice (Fioramonti et al., 2004). Additionally, it should be noted that Kir6.2 is expressed in some, but not all, hypothalamic glucose-sensitive neurons (Ibrahim et al., 2003; Kang et al., 2004). Collectively, glucose can activate hypothalamic glucose-excited neurons through K_{ATP} channel-dependent and -independent mechanisms.

4.3.8. Role of AMPK

A subpopulation of ARC-NPY neurons are glucose-inhibited neurons. During fasting, a smaller decrease in glucose depolarizes glucose-inhibited NPY neurons and this effect is blocked by AMPK inhibitor (Murphy et al., 2009). These data suggest that during fasting, low glucose level induces the activation of AMPK in glucose-inhibited NPY neurons, leading to the depolarization of glucose-inhibited NPY neurons.

AgRP gene expression is inhibited by glucose and glucose-induced reduction in AgRP expression is attenuated in the presence of 2-DG in neuronal cell lines, as well as, in hypothalamic tissues cultured *ex vivo* (Lee et al., 2005). Central glucose treatment (i.c.v.) suppresses AMPKα2 activity in the hypothalamus, specifically in the ARC, PVN, and LHA, while glucose does not alter AMPKα2 activity in the cortex (Minokoshi et al.,

2004). Glucose-induced reduction in *AgRP* expression is correlated with increased ATP production and reduced phosphorylation of AMPK. Treatment with AMPK activators increases *AgRP* mRNA levels and overexpression of a dominant negative mutant of AMPK blocks the inhibitory effect of low glucose on *AgRP* expression (Lee et al., 2005). A similar effect of glucose on AMPK phosphorylation is observed in immortalized hypothalamic neuronal cells in which POMC is endogenously expressed. In this cell line, increasing glucose concentrations decreases phosphorylation of AMPK and increases *POMC* mRNA expression levels (Cai et al., 2007).

Contrary to these findings, one study showed that knockdown of AMPKα2 does not block the stimulatory effect of low glucose on *AgRP* expression, when another hypothalamic neuronal cell line was used (Cheng et al., 2008). In this study, high glucose-induced suppression of *AgRP* expression is not blocked by adding an AMPK activator (Cheng et al., 2008). These discrepancies may arise from differences in cell lines used in each study.

Overall, the majority of the published reports support the role for AMPK in mediating the effect of glucose on hypothalamic neuronal activity, neuropeptide gene expression, and feeding.

4.3.9. Role of lactate and pyruvate

Similar to glucose, injection of pyruvate and lactate decreases food intake (Lam et al., 2008; Langhans et al., 1985). Central administration (i.c.v.) of pyruvate blocks the orexigenic effect of 2-DG in rats (Lee et al., 2005). Furthermore, pyruvate suppresses 2-DG-induced *AgRP* expression both in neuronal cell line and hypothalamic tissue cultured

ex vivo (Lee et al., 2005). These data suggest that downstream metabolites of the glycolysis pathway beyond pyruvate are responsible for glucose-induced changes in *AgRP* expression and food intake. It is possible that blood lactate and pyruvate enter the brain and act on hypothalamic neurons, especially since lactate and pyruvate can be transported into the brain across the BBB (Cremer et al., 1979). Another possibility is that lactate and pyruvate are locally produced in the hypothalamus and act on hypothalamic neurons. Central administration of glucose increases the formation of lactate in astrocytes to provide extracellular lactate to neighboring neuronal cells, which in turn convert it to pyruvate by lactate dehydrogenase (LDH) (astrocyte-neuron lactate shuttle hypothesis) (Ainscow et al., 2002; Bittar et al., 1996; Pellerin & Magistretti, 2004; Tsacopoulos & Magistretti, 1996). Thus, it has been hypothesized that astrocytes serve as a primary sensor for changes in extracellular glucose levels and glucose-sensing neurons act as a downstream effector. To support this hypothesis, i.c.v. lactate-induced anorexia was attenuated by a LDH inhibitor (Lam et al., 2008). A more direct evidence for the direct action of lactate on hypothalamic neurons was provided by an electrophysiological study. In this study, lactate has excitatory influences on VMN glucose-excited neurons, but not on non-glucose-sensing neurons (Song & Routh, 2005).

Glucose i.c.v. treatment lowers blood glucose and triglyceride levels by suppressing hepatic glucose production and hepatic VLDL secretion, respectively (Lam et al., 2007; Lam et al., 2005). Blood glucose–lowering effects of i.c.v. glucose are blocked by co-injection of LDH inhibitor and i.c.v. injection of lactate mimicks the effect of i.c.v. glucose on blood glucose concentration and hepatic glucose production (Lam et al., 2005). Similarly, the effect of i.c.v. glucose on plasma triglycerides and hepatic

VLDL secretion is mimicked by i.c.v. lactate injection and is blocked by the LDH inhibitor (Lam et al., 2007). These findings indicate that conversion of lactate to pyruvate mediates the effect of i.c.v. glucose on hepatic glucose and lipid metabolism.

Pyruvate can be further metabolized in the neurons. The entry of pyruvate in the tricarboxylic acid (TCA) cycle is governed by its conversion to acetyl-coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH). Intra-hypothalamic injection of dichloroacetate, a PDH activator, recapitulated the effects of i.c.v. glucose or lactate on hepatic glucose production and blood glucose levels (Lam et al., 2005). If PDH is activated in astrocytes, increased PDH activity should decrease lactate formation. In contrast, activation of PDH in neurons should increase conversion of pyruvate to acetyl-CoA. Therefore, these results suggest that increased flux into the TCA cycle is the key step in sensing local glucose availability and regulating peripheral carbohydrate and lipid metabolism. In light of the glucose-sensing responses of hypothalamic neurons being observed in isolated cell preparations, it is probable that increased glucose availability can also cause changes in the activity of glucose-sensing neurons and the subsequent alterations in metabolic function, in the absence of lactate supply from astrocytes.

4.3.10. Role of NADH

It has been suggested that glycolysis, in particular the cytoplasmic conversion of NAD^+ to NADH, may play a role for some of the effects mediated glucose. Sodium iodoacetate, a specific inhibitor of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), blocks glycolysis at the step that produces cytoplasmic NADH. Glucose reduces *AgRP* mRNA levels in immortalized hypothalamic cells and the inhibitory effect of glucose on AgRP is

attenuated by sodium iodoacetate (Cheng et al., 2008). Consistent with these findings, activation of VMH glucose-excited neurons by a transition of glucose from 5 to 20 mM is blocked by iodoacetic acid (Yang et al., 1999). These data suggest that the effect of glucose on AgRP expression is mediated by glycolysis, in particular increased NADH formation.

4.3.11. Role of malonyl-CoA

AMPK is a possible glucose sensor by directly sensing ATP:ADP ratio. Activation of AMPK leads to the inhibition of the key regulatory enzyme of fatty acid synthesis, acetyl-CoA carboxylase (ACC) by phosphorylating ACC. ACC adds a carboxyl group to acetyl-CoA to form malonyl-CoA. Subsequently, fatty acid synthase (FAS) converts malonyl-CoA to palmitate by serially adding two-carbon units to the fatty acid chain. In addition to its role in fatty acid synthesis, malonyl-CoA also serves a signaling role as an inhibitor of carnitine palmitoyltransferase 1 (CPT1). CPT1 promotes translocation of long-chain fatty acids into mitochondria and increases fatty acid oxidation. Thus, increased malonyl-CoA may serve as a signal that couple recent carbohydrate ingestion to decreased fatty acid oxidation.

Central injection of FAS inhibitors, such as cerulenin and C75, reduces food intake and treatment with FAS inhibitors is effective in reducing body weight in obese mice by reducing food intake and increasing energy expenditure (Loftus et al., 2000; Makimura et al., 2001; Thupari et al., 2002). These findings led to the hypothesis that hypothalamic malonyl-CoA serves as a signaling molecule that couples body energy status to metabolic regulation. More specifically, it has been hypothesized that malonyl-

CoA serves as a key hypothalamic signaling molecule to convert changes in extracellular glucose levels to hypothalamic neuronal activity, because malonyl-CoA is synthesized *de novo* from glucose. Central injection (i.c.v.) of C75 increases hypothalamic malonyl-CoA levels and pre-treatment (i.c.v.) with an acetyl-CoA carboxylase inhibitor preventes both the C75-induced rise of hypothalamic malonyl-CoA and the C75-induced feeding suppression (Hu et al., 2003; Loftus et al., 2000). Moreover, hypothalamic overexpression of malonyl-CoA decarboxylase (MCD), a malonyl-CoA degrading enzyme, increases food intake and body weight (He et al., 2006; Hu et al., 2005). Enhanced hypothalamic MCD expression also blocked feeding-suppressing effect of i.c.v. C75 injection (Hu et al., 2005). These findings suggest that increased hypothalamic malonyl-CoA is the key signal to trigger feeding suppression.

Peripheral glucose injection causes an increase in hypothalamic malonyl-CoA levels which is accompanied by reductions in food intake, phosphorylation of AMPK and ACC, and *AgRP* mRNA levels in the hypothalamus, and an increase in hypothalamic *POMC* mRNA (Wolfgang et al., 2007). These glucose-induced changes were reversed by i.c.v. injection of 2-DG. In addition, i.c.v. injection of the AMPK activator reduces hypothalamic malonyl-CoA levels and increases food intake (Hu et al., 2005). Taken together, these findings support the hypothesis that increased glucose availability in the hypothalamus leads to increased ATP:ADP ratio, decreased AMPK activity, increased ACC activity, increased malonyl-CoA levels, increased melanocortin tone and reduced food intake. Thus, malonyl-CoA plays an important role in mediating the hypothalamic action of glucose.

4.3.12. Convergence of CNS glucose signaling with insulin and leptin signaling

Both CNS insulin action and leptin action are partly mediated through the common signaling pathways involving AMPK (Minokoshi et al., 2004). Glucose also affects the activity of hypothalamic AMPK (Minokoshi et al., 2004). Glucose-induced reduction in AMPK phosphorylation results in reduced phosphorylation of ACC and subsequent increase in hypothalamic malonyl-CoA levels (Wolfgang et al., 2007). Interestingly, leptin also causes the same changes in the hypothalamus and there is an additive effect of glucose and leptin on malonyl-CoA levels (Wolfgang et al., 2007). Leptin-induced increase in hypothalamic malonyl-CoA is negated by i.c.v. 2-DG (Wolfgang et al., 2007). These data clearly indicate that glucose and leptin (and possibly insulin) signaling converge on the hypothalamic AMPK-ACC-malonyl-CoA pathway.

Hypothalamic glucose-excited neurons increase their firing rates in response to elevated extracellular glucose levels via K_{ATP} channel closure (Ashford et al., 1990; Miki et al., 2001). It has been also demonstrated that both leptin and insulin hyperpolarize rat hypothalamic glucose-sensitive neurons by opening K_{ATP} channels (Spanswick et al., 1997; Spanswick et al., 2000). These findings indicate that glucose, insulin, and leptin convert their hypothalamic action to neuronal activity via the K_{ATP} channels. Consequently, glucose, insulin, and leptin are involved in the hypothalamic regulation of energy balance possibly through mechanisms that involve their independent actions, as well as, interactions among them.

4.3.13. Obesity and hypothalamic glucose sensitivity

Obese individuals with glucose intolerance are often hyperglycaemic, hyperinsulinemic, and hyperleptinemic, suggesting that these individuals are resistant to the anorectic effects of these factors. In fact, obesity is associated with both insulin resistance and leptin resistance as outlined in earlier sections. There are evidences supporting the hypothesis that hypothalamic neuronal responses to glucose are impaired in obesity.

Although, i.c.v. injection of glucose reduces food intake and i.c.v. injection of 2-DG increases food intake in normal lean rats, these feeding responses are absent in obese rats (Tsujii & Bray, 1990). Carotid infusion of glucose increases brain glucose levels and hypothalamic Fos expression without elevating blood glucose levels (Dunn-Meynell et al., 1997; Guillod-Maximin et al., 2009). Glucose-induced hypothalamic Fos expression is reduced in obese-prone rats, as compared with obese-resistant rats (Levin et al., 1998). Glucose-sensing neurons are fewer in number and show abnormal responses to glucose in diet-induced obese rats (Song et al., 2001). Although, the direction of the effect of glucose on hypothalamic neuronal activity is controversial, studies using functional magnetic resonance imaging (fMRI) showed hypothalamic activity to be altered in rats after glucose injection, and in humans after glucose ingestion (Liu et al., 2000; Mahankali et al., 2000). Importantly, the effect of glucose injection on hypothalamic activity is attenuated in obese humans compared with non-obese healthy individuals (Matsuda et al., 1999). Despite of these studies indicating that there is an association between obesity and altered hypothalamic response to glucose, these studies cannot identify whether the altered hypothalamic neuronal responses to glucose are the cause of or the consequence of obesity.

It has been hypothesized that high glucose levels in the brain may cause impairments in hypothalamic sensitivity to other anorectic factors. Leptin resistance is present in many obese individuals with hyperglycemia. Central administration (i..c.v.) of leptin reduces food intake and this anorectic effect of leptin is attenuated by pre-treatment with i.c.v. glucose (Fujita et al., 2003). Central glucose injection (i.c.v.) also negates leptin-induced STAT3 phosphorylation in the hypothalamus (Fujita et al., 2003). These data suggest the possibility that high glucose levels in the brain may cause reduction in hypothalamic leptin sensitivity.

Overall, these data clearly indicate that obesity is associated with impairments in hypothalamic glucose sensing and prolonged exposure to high levels of glucose may impair the actions of other anorectic molecules.

5. Search for genetic factors predisposing obesity – discovery of FTO (fat mass and obesity-associated)

As outlined above, the activity of the CNS melanocortin pathway is regulated by hormonal and nutrient signals. Some molecular mechanisms for the hormonal and nutritional regulation of hypothalamic POMC and AgRP gene expression have been demonstrated over the last two decades. However, it has been extremely difficult to identify single genes which are solely responsible for the determination of body weight in humans, most likely due to the complex nature of body weight regulation which involves both gene-gene interactions and gene-environment interactions. To further identify regulators of POMC and AgRP gene expression, a genome-wide association study (GWAS) is a powerful approach. GWAS identifies common genetic associations with

observed traits (such as obesity and insulin resistance) through the analysis of genetic variations across the entire human genome (Frayling, 2007; Frayling et al., 2007).

Through the search for sequence variants and their association to obesity and type 2 diabetes, a strong association between SNPs in the first intron of the *FTO* (fat mass and obesity-associated) gene and increased adiposity has been identified (Frayling et al., 2007). This association has been consistently observed across multiple populations (Dina et al., 2007; Marvelle et al., 2008; Scuteri et al., 2007). Despite the strong association between *FTO* variants and obesity, the biological function of FTO and the mechanism by which *FTO* variants lead to the development of obesity have not been yet identified. *FTO* variants are associated with higher energy intake and reduced satiety response with one exception in which no significant association was reported (Haupt et al., 2008; Speakman et al., 2008; Stutzmann et al., 2009; Wardle et al., 2008). Recently, it was also shown that *FTO* variants are associated with increased energy intake, but not with changes in the weight of ingested food, suggesting that FTO plays a role in the regulation of food intake and food choice (Cecil et al., 2008). It was also reported that *FTO* variants are associated with reduced resting metabolic rate (Do et al., 2008). However, other studies failed to identify a significant association between *FTO* variants and reduced energy expenditure (Cecil et al., 2008; Haupt et al., 2008; Speakman et al., 2008). Based upon these reports, the available data favor the possibility that increased energy intake contributes to the increased adiposity and body weight in individuals with the FTO risk alleles. Further studies are necessary to clarify the relationship between *FTO* variants and energy intake and energy expenditure.

FTO is expressed in various tissues with its expression level in the hypothalamus being one of the highest (Frayling et al., 2007; Fredriksson et al., 2008; Gerken et al., 2007; Stratigopoulos et al., 2008). Of particular interest, hypothalamic *Fto* mRNA levels are reduced by fasting (Gerken et al., 2007; Stratigopoulos et al., 2008). These findings suggest that hypothalamic *FTO* gene expression is regulated by metabolic states and FTO plays a role in the regulation of metabolism. However, the regulatory mechanism for hypothalamic FTO expression is unknown and further investigations are required to elucidate the detailed mechanism of the regulation of hypothalamic FTO expression. In addition, based on the co-expression of Fto and POMC in the hypothalamus, as well as, the phenotypic similarities between *FTO* variants and *MC4R* variants, it has been suggested that hypothalamic FTO may function as a regulator of CNS melanocortin signaling, such as a regulator of POMC gene expression (Fischer et al., 2009; Loos et al., 2008; Olszewski et al., 2009; Stutzmann et al., 2009). This possibility has not been addressed and requires further investigations.

Summary of literature review and research rational

It is clear that central melanocortin signaling plays a critical role in the regulation of energy homeostasis. The importance of the melanocortin pathways in energy homeostasis has been supported by extensive genetic, biochemical, electrophysiological, and neuroanatomical studies. However, these studies mainly focused on the elucidation of the role for central melanocortin signaling in the regulation of food intake, energy expenditure, and body weight. Little is known about the role for central melanocortins in

the regulation of lipid metabolism, in particular hepatic lipid metabolism with a specific focus on fatty liver disease. Additionally, the central melanocortin system regulates energy homeostasis largely by mediating the effect of peripheral metabolic signals such as insulin, leptin, and glucose. However, the detailed mechanisms by which these metabolic signals regulate the activity of the central melanocortin system have not been fully elucidated. Furthermore, it is not known whether a specific response in the central melanocortin system to these metabolic signals is altered in association with metabolic impairments including obesity and fatty liver disease. Therefore, the present study was designed to investigate (i) the role of central melanocortin signaling in the regulation of hepatic lipid metabolism and (ii) the mechanism by which the hypothalamus mediates the effect of glucose on hypothalamic melanocortin signaling.

THESIS HYPOTHESES

Hypothesis I: Impairments in central melanocortin signaling may contribute to the development of hepatic steatosis and enhanced central melanocortin signaling may be beneficial in reversing abnormal hepatic lipid metabolism in fatty liver disease.

Hypothesis II: Hypothalamic Fto expression is regulated by nutritional signals, such as glucose.

Hypothesis III: Fto function is part of the hypothalamic nutritional sensing mechanisms related to the central melanocortin system

Hypothesis IV: Nutritional regulation of hypothalamic Fto expression is impaired in obesity.

STUDY I: Regulation of Hepatic Lipid Metabolism by Central Melanocortin Signaling

Regulation of hepatic PPARgamma2 and lipogenic gene expression by melanocortin. **Poritsanos NJ**, Wong D, Vrontakis ME, Mizuno TM. Biochem Biophys Res Commun. 2008 Nov 14;376(2):384-8. Epub 2008 Sep 18.

Introduction

Fatty liver disease increases the risk of developing progressive liver injury. It is well known that chronic alcohol consumption causes fatty liver disease. However, there is increased recognition of factors other than chronic alcohol consumption that also increase the risk of fatty liver disease, known as non-alcoholic fatty liver disease (NAFLD). NAFLD is strongly associated with obesity and insulin resistance, and is characterized by increased triglyceride accumulation and steatosis in the liver (Angelico et al., 2005; Gholam et al., 2007; Hamaguchi et al., 2005; Yu & Keeffe, 2002). Accumulation of triglycerides in the liver could be due to increased hepatic *de novo* lipogenesis and increased influx of free fatty acids (FFA) into the liver (Postic & Girard, 2008; Tamura & Shimomura, 2005). The accumulation of triglycerides in the liver could also be a result of decreased oxidation of FFA in the liver and decreased secretion of very low density lipoprotein (VLDL) (Postic & Girard, 2008; Tamura & Shimomura, 2005). However, the precise mechanism causing abnormal hepatic lipid metabolism and fatty liver disease is not well understood.

Similar to human NAFLD, obesity and insulin resistance are often associated with abnormal lipid metabolism, as represented by increased lipogenesis in both the liver and

adipose tissue in rodents (Bray, 1979; Herberg & Coleman, 1977). Consistent with increased lipid synthesis, expression of lipogenic genes including fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) is elevated in adipose tissue and the liver of obese and insulin resistant animals (Ferrante et al., 2001; Soukas et al., 2000). *Agouti* mice have mutations in the *agouti* gene whose gene product, *agouti* peptide, antagonizes the effects of α-MSH at MC4R and causes abnormal body weight gain and insulin resistance (Bultman et al., 1992; Lu et al., 1994; Miller et al., 1993; Yen et al., 1994). Hepatic triglyceride content and hepatic lipogenesis rate are elevated in *agouti* mice (Yen et al., 1976). Hepatic lipogenic gene expression is also elevated in *agouti* mice and MC4R-deficient mice (Albarado et al., 2004; Jones et al., 1996). Enhanced central POMC gene expression largely normalizes hepatic triglyceride levels, serum FFA levels and hepatic *SCD1* mRNA levels in animal models of fatty liver disease (Li et al., 2007; Mizuno et al., 2003). Central administration of Melanotan II (MTII), a MC3R/MC4R agonist, reduces hepatic *SCD1* mRNA (Lin et al., 2003). Conversely, i.c.v. treatment with melanocortin antagonists stimulates lipogenic gene expression and increased lipid content in the liver (Adage et al., 2001; Nogueiras et al., 2007). These data suggest that central melanocortin signaling regulates hepatic lipid metabolism and that reduced central melanocortin signaling increases the activity of lipid biosynthetic pathways in the liver towards the development of fatty liver. However, the mechanism by which central melanocortin signaling regulates hepatic lipogenic gene expression is not fully understood. To further delineate the molecular mechanisms through which the central melanocortin signaling regulates hepatic lipid metabolism, the effect of i.c.v. treatment

with either melanocortin agonist or antagonist was tested on hepatic lipid content and hepatic expression of genes involved in lipid metabolism in mice.

Hepatic lipogenic gene expression is under the control of transcription factors including sterol regulatory element-binding protein 1c (SREBP-1c), carbohydrate responsive element-binding protein (ChREBP), and peroxisome proliferators-activated receptor γ2 (PPARγ2). Recently, it was demonstrated that i.c.v. injection of SHU9119 increased SREBP-1c expression and lipogenic gene expression in the liver, suggesting that the activation of SREBP-1c mediates, at least partly, SHU9119-induced hepatic lipogenic gene expression (Nogueiras et al., 2007). However, it is unclear whether reduced central melanocortin signaling stimulates hepatic lipogenic gene expression through the activation of other transcription factors, ChREBP and PPARγ2. It is also unknown whether these changes in hepatic lipid metabolism occur at the very early stages of the development of obesity after blockade of central melanocortin signaling. To clarify these possibilities, the effect of increased melanocortin signaling, using i.c.v. MTII treatment, on hepatic lipogenic gene expression was investigated in a mouse model of hepatic steatosis (*ob/ob*). Conversely, the effect of reduced melanocortin signaling, using a short-term i.c.v. SHU9119 treatment, on hepatic lipid content and hepatic expression of genes encoding enzymes involved in lipid metabolism, including the transcription factors SREBP-1c, ChREBP, and PPARγ2, was tested in wild-type mice.

Materials and Methods

Animals

Male C57BL/6J mice and male *ob/ob* mice (C57BL/6J background) were obtained from the Jackson Laboratories (Bar Harbor, ME). For the purspose of investigating CNS melanocortin and Fto in relation to peripheral metabolism, the studies presented in this thesis used young (6-8 weeks old) male mice instead of female mice based upon the rational presented below. There are differences in obesity and diabetic phenotypes between male and female mice (Leiter et al., 1999). Estrogens mediate inhibitory effects on food intake and body weight through hypothalamic dependent mechanisms (Asarian & Geary, 2006; Diano et al., 1998). Gender differences are also observed in lipid metabolism (Yang et al., 2009). To avoid the effect of gender and sex steroids on metabolism, only male mice were used in the present study. Mice were individually housed under a 12:12 light/dark cycle (lights on at 0600 h) with free access to standard rodent chow pellets (Prolab RMH 3000, 4.5% fat by weight, Ralston Purina). Water was available throughout the experiment. All studies were approved by the Institutional Animal Care and Use Committee (University of Manitoba).

I.c.v. cannulation

Mice were anesthetized with isoflurane with an induction dose of ketamine (100 mg/kg b.w., i.p.) and xylazine (10 mg/kg b.w., i.p.). A stainless steel guide cannula (o.d.: 0.64 mm, i.d.: 0.33 mm) was stereotaxically implanted into the lateral ventricle with the coordinates 0.4 mm posterior to the bregma, 1.0 mm lateral from the midline and 1.8 mm

deep to the dura in accordance with the atlas of Paxinos (Paxinos & Franklin, 2001). After full recovery from the surgery, the localization of the cannula was verified by assessing drinking behavior in response to the i.c.v. administration of angiotensin II (100 ng in 1 μl, Sigma-Aldrich, St. Louis, MO). Only mice responding with a robust drinking behavior (within 30 sec. post i.c.v. of angiotensin) were used for the experiment.

I.c.v. treatment and tissue collection

SHU9119 and MTII were obtained from BACHEM (King of Prussia, PA) and dissolved in water. Control animals were injected with artificial cerebrospinal fluid (aCSF). The composition of aCSF was as follows; NaCl 124 mM, $NaHCO_3$ 26 mM, KCl 5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2.4 mM, and $_D$-glucose 10 mM. Drugs were injected in a total volume of 1 μl over 30 seconds, and an injection pipe was left inside the cannula for another 30 seconds and removed from the cannula. To examine if enhanced central melanocortin signaling reduces hepatic lipogenic gene expression, MTII (6 nmol) or aCSF was injected i.c.v. every 24 hours for 7 days in *ob/ob* mice. Mice were sacrificed 16 hours after the last injection (between 1000 h and 1200 h). To determine if reduced central melanocortin signaling stimulates hepatic triglycerides accumulation and hepatic lipogenic gene expression, C57BL/6J mice received i.c.v. injections of aCSF or SHU9119 (day 1-3, 1 nmol; day 4, 6 nmol) every 24 hours. These doses of MTII and SHU9119 have been shown to reduce food intake in mice (Fan et al., 1997). On the fourth day of the treatment, mice were sacrificed 6 hours after the last i.c.v. injection (between 1400 h and 1600 h). Food intake and body weight were monitored daily and the weight of liver and epididymal fat pad was measured at sacrifice. Liver and white

adipose tissues were collected and saved for RNA and histological analyses. Blood was collected to measure levels of hormones and metabolites.

Assays

Blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN). Serum insulin and leptin concentrations were determined by ELISA with commercial kits (LINCO Research, St. Charles, MO). Serum nonesterified fatty acid (NEFA) and total serum triglyceride concentrations were determined by colorimetric methods using commercial kits (Wako Chemicals, Richmond, VA). Serum alanine aminotransferase (ALT) activity was determined spectrophotometrically with an ALT assay reagent from the Diagnostic Chemicals Limited (Charlottetown, PE). Hepatic triglyceride contents were determined by a colorimetric method (Wako Chemicals). From each mouse, liver (100 mg) was homogenized in chloroform/methanol mix (2:1, vol/vol). The extracts were dried, re-suspended in ethanol and used for the assay. Final hepatic triglycerides contents were expressed as ng of triglyceride per mg wet tissue.

Histological analysis

Fresh frozen and formalin-fixed liver tissues were sectioned (10 μm) and stained with hematoxylin and eosin (H&E) Gill's method or Oil Red O (Poritsanos et al., 2009).

RNA analysis

Total RNA was extracted from liver or white adipose tissue in TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 5 μg of total RNA, using SuperScriptII RNaseH reverse transcriptase and random primer (Invitrogen), and diluted 1:100. Gene expression levels were measured using the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA). Real-time PCR was performed in a 25-μl reaction containing SYBR green (Power SYBR Green Master Mix, Applied Biosystems), primer mixture (final concentration: 0.2 mmol/L) and 5 μl of cDNA for 40 cycles at 94 ˚C for 15 sec; 60 ˚C for 20 sec and 72 ˚C for 30 sec. All primers (Table 1) were designed using the Primer Express Software (Ver. 3.0, Applied Biosystems) and synthesized by Invitrogen. Data were analyzed by the ΔΔCt method using the ABI 7500 Fast System SDS software package (Ver. 1.3.1, Applied Biosystems) and mRNA levels were normalized to β-actin mRNA levels. To validate the quantitative data, we compared β-actin and cyclophilin as an endogenous control for normalization in our pilot study. Both β-actin and cyclophilin produced similar results in relative expression levels of the target gene. Thus, we decided to use β-actin in all subsequent studies. Data are expressed as means (% of the control group) ± SEM. All reactions were performed in triplicates and the coefficient of variation was less than 2% for each triplicate.

Statistical analysis

Values are presented as means ± SEM. Comparisons between 2 groups were performed using Student's *t*-test. Food intake and body weight data in *ob/ob* mice study were analyzed by two-way analysis of variance (ANOVA). Kruskal-Wallis test was performed

and the Bonferroni-corrected Wilcoxon test was used for post hoc analysis when the data were not normally distributed. Comparisons between two groups were performed by Wilcoxon test when the data were not normally distributed. In all cases, differences were taken to be significant if *P*-values were below 0.05.

Results

The effect of i.c.v. MTII treatment on food intake, body weight, and hepatic SCD1 in *ob/ob* mice

To examine whether enhanced central melanocortin signaling inhibits hepatic lipogenic gene expression, we treated *ob/ob* mice with MTII for 7 days. There was a significant main effect of treatment on cumulative food intake [$F(1, 112) = 15.4$, $P < 0.0005$, Kruskal-Wallis test] and body weight change [$F(1, 109) = 35.1$, $P < 0.0001$, Kruskal-Wallis test] (Fig. 5A and 5B). Due to the wide variation in body weight change in MTII-treated *ob/ob* mice (Fig. 5B), there was also a wide variation in hepatic SCD1 mRNA levels in MTII-treated *ob/ob* mice. SCD1 mRNA levels in the MTII-treated group did not differ significantly from those in the control group ($P = 0.16$, Student's *t*-test, Fig. 6). Similarly, levels of hepatic FAS and ACC1 mRNA were not statistically different between the two groups (Fig. 6). To further examine if MTII-induced weight losses are associated with decreases in SCD1 expression, we performed correlation analysis between MTII-induced body weight change and hepatic SCD1 mRNA levels. There was a significant positive correlation between MTII-induced body weight changes and SCD1 mRNA levels ($r = 0.85$, $P < 0.05$, Fig. 7C). Similarly, there were positive correlations between MTII-induced body weight change and other lipogenic genes (ACC1: $r = 0.93$, $P < 0.005$, FAS: $r = 0.87$, $P < 0.05$, Fig. 7A and B).

The effect of i.c.v. SHU9119 treatment on metabolic parameters

The next experimental pursuits were aimed at testing whether impairments in CNS melanocortin signaling contribute to increased hepatic lipogenic gene expression and lipid accumulation. To minimize the possible contribution by SHU9119-induced changes in body weight and food intake, it was decided to treat animals with SHU9119 for a shorter duration (4 days) compared with the afore-mentioned MTII-treatment study (7 days). A 4-day i.c.v. SHU9119 treatment significantly increased body weight gain ($P < 0.001$, Fig. 8B) and epididymal fat pad weight ($P < 0.05$, Fig. 8C) compared with the control aCSF treatment without changes in food intake ($P = 0.15$, Fig. 8A). There was no difference in blood glucose levels between 2 groups ($P = 0.77$, Table 2). Although SHU9119 treatment increased serum insulin levels by 140% compared with aCSF treatment, the effect did not reach statistical significance ($P = 0.08$, Table 2). Serum leptin levels were significantly increased in SHU9119-treated mice compared with control mice ($P < 0.05$, Table 2).

The effect of i.c.v. SHU9119 treatment on hepatic triglycerides and serum NEFA and triglyceride levels

Although SHU9119 treatment increased liver weight by 10% compared with control aCSF treatment, the effect did not reach statistical significance ($P = 0.051$, Fig. 9A). Hepatic triglyceride content was significantly increased by 65% in SHU9119-treated mice compared with control mice ($P < 0.05$, Fig. 9B). Histological analysis using Hematoxylin (stains nuclei blue) and Eosin (stains cytoplasm pink) histological analysis

demonstrated that the architectural nature of the liver tissue appears to be the same in both SHU9119 and aCSF treated mice (Fig. 10A-10K). Oil Red O staining of lipid droplets (red) demonstrated that SHU9119 i.c.v. treatment resulted in increased lipid accumulation within the hepatocytes (Fig. 10F and 10M) as compared with the aCSF i.c.v. treated control group (Fig. 10E and 10L). Large magnification clearly shows the intracytoplasmic accumulation of small lipid vacuoles (pointed by black arrows) to be localized around the nucleus (blue) in mice treated with SHU9119, but not aCSF (Fig. 10L and 10M). As a model of NAFLD, *ob/ob* hepatic tissue is depicted in Oil Red O stained sections (Fig. 10G and 10N). Qualitative analysis under high magnification (40X and 60X) shows that *ob/ob* mice have an advanced stage of hepatic steatosis as a result of fat droplet accumulation, as well as, an increase in size and number of vacuoles displacing the nucleus to the periphery of the cell (Fig. 10N). These results indicate that the acute effects of SHU9119 i.c.v. treatment are potent in mediating an increase in hepatic lipid accumulation, an early pathophysiological marker of NAFLD, further highlighting the importance of central melanocortin signalling in the hepatic lipid metabolism.

Serum triglyceride levels were significantly increased by 47% with SHU9119 treatment compared with control aCSF treatment ($P < 0.05$), while serum NEFA levels were not changed by SHU9119 treatment ($P = 0.58$, Table 2). There was no difference in serum ALT levels between the groups ($P = 0.43$, Table 2). Although a 4-day SHU9119 treatment did not cause dramatic changes in liver histology, histological examination revealed that SHU9119 treatment induced changes in hepatocyte morphology depicting

hepatocellular rupture and spacing, and increased number of cells positive for Oil Red O staining (Fig. 10).

The effect of i.c.v. SHU9119 treatment on lipid metabolism gene expression in liver

We examined the effect of SHU9119 treatment on the expression of genes involved in hepatic glucose metabolism and lipid metabolism (Fig. 11). Glucokinase (GK) mRNA levels were significantly increased by 121% in SHU9119-treated mice compared with control mice ($P < 0.0001$), while the mRNA levels of liver-type pyruvate kinase (L-PK) were not changed by SHU9119 treatment ($P = 0.65$, Fig. 12). SHU9119 increased the mRNA levels of ACC1, FAS, Elovl6, and SCD1 by 51% ($P < 0.01$), 120% ($P < 0.01$), 96% ($P < 0.05$), and 108% ($P < 0.05$), respectively (Fig. 12). Hepatic mRNA levels of glycerol-3-phosphate acyltransferase 1 (GPAT1, $P = 0.17$), GPAT2 ($P = 0.12$), acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1, $P = 0.35$), and DGAT2 ($P = 0.49$) were not changed by SHU9119 treatment (Fig. 12). Although SHU9119 treatment increased adipose differentiation-related protein (ADRP) mRNA levels by 156%, the effect did not reach statistical significance ($P = 0.06$, Fig. 12). Furthermore, SHU9119 significantly increased hepatic SREBP-1c and peroxisome proliferators-activated receptor γ2 (PPARγ2) mRNA levels by 89% ($P < 0.05$) and 218% ($P < 0.05$), respectively (Fig. 13). Carbohydrate responsive element-binding protein (ChREBP) mRNA levels in SHU9119-treated group did not differ from those in control group ($P = 0.79$, Fig. 13). Western blotting showed no significant difference in hepatic ChREBP protein levels between the groups ($P = 0.18$, Fig. 14). SHU9119 also increased the levels of genes involved in

cholesterol biosynthesis, SREBP-1a and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), by 20% ($P < 0.05$) and 112% ($P < 0.05$), respectively (Fig. 15). The expression levels of 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) and HMGCS2 mRNA were not changed by SHU9119 treatment ($P = 0.053$ and $P = 0.23$, respectively, Fig. 15). SHU9119 did not change the expression levels of hepatic triglyceride lipase (LIPC, $P = 0.45$), fatty acid translocase (FAT/CD36, $P = 0.052$) in liver, while it significantly increased hepatic low density lipoprotein receptor (LDLR) mRNA levels by 59% ($P < 0.005$, Fig. 16A). SHU9119 decreased very long chain acyl CoA dehydrogenase (VLCAD) mRNA levels by 22% compared with control aCSF treatment, but had no significant effect on the mRNA levels of PPARα ($P = 0.54$), carnitine palmitoyl transferase 1a (CPT1a, $P = 0.13$), and medium chain acyl-CoA dehydrogenase (MCAD, $P = 0.14$, Fig. 16B). Hepatic uncoupling protein 2 (UCP2) mRNA levels were not different between the two groups (aCSF: 100.0 ± 5.4%, SHU9119: 114.9 ± 13.8%, $P = 0.32$).

The effect of i.c.v. SHU9119 treatment on lipid metabolism gene expression in adipose tissue

Although there was a trend of increase in the mRNA levels of SDC1 ($P = 0.53$), ADRP ($P = 0.10$), and hormone sensitive lipase (HSL) ($P = 0.17$) in white adipose tissue by SHU9119 treatment, these effects did not reach statistical significances (Fig. 17).

Discussion

Impairments in the central melanocortin signaling cause metabolic abnormalities including obesity and insulin resistance which are associated with NAFLD. The present study was performed to determine the ability of the central melanocortin signaling to regulate hepatic lipid metabolism and to delineate molecular mechanisms for this regulation. Although the precise mechanism which causes NAFLD is unclear, the accumulation of triglycerides in liver could be the result from an imbalance among *de novo* lipogenesis, FFA or triglycerides uptake, FFA oxidation, and VLDL secretion into the circulation. This PhD thesis study reports that reduced central melanocortin signaling stimulates hepatic lipogenic gene expression and promotes hepatic triglycerides accumulation possibly through the activation of transcription factors, SREBP-1c and PPARγ2, independent of hyperphagia and obesity.

The effect of i.c.v. MTII treatment on hepatic lipogenic gene expression in ob/ob mice

In a long-term experiment, we used the synthetic melanocortin agonist MTII to determine the effects of i.c.v. injections in reversing the obese phenotype of leptin-deficient *ob/ob* mice. Central administration of MTII, two times daily for 7 days (9 nmol/day) in *ob/ob* mice significantly suppressed cumulative food intake and circulating glucose levels. The expected weight-reducing effect of MTII in *ob/ob* mice however, was not significant, as reflected by no reduction in cumulative body weight change and epididymal fat pad

weight in MTII-treated mice compare to a-CSF-treated control mice. Although the mRNAs encoding enzymes in the pathway of lipid biosynthesis (SCD-1, FAS, and ACC1) were not significantly decreased after the MTII-treatment, there were significant positive correlations between cumulative body weight change and levels of SCD-1, FAS, or ACC1 levels in MTII-treated mice. These results suggest that enhanced CNS signaling via MC3R/MC4R reduces the expression of lipogenic gene expression in liver partly by reducing food intake and/or body weight. Consequently, impairments in CNS melanocortin signaling may contribute to abnormally increased lipid accumulation in liver and enhanced CNS melanocortin signaling may be beneficial in reversing abnormal hepatic lipid metabolism in fatty liver disease.

SHU9119 induces the early stages of fatty liver

To examine if reduced central melanocortin signaling stimulates hepatic lipid synthesis and accumulation, male C57BL/6J mice were treated with a MC4R/MC3R antagonist, SHU9119 for 4 days. SHU9119 treatment increased hepatic triglyceride content without significant changes in food intake. These findings are consistent with earlier studies by other groups and suggest that SHU9119-induced increase in hepatic triglyceride accumulation is not due to increased food intake and the subsequent increased transport of dietary triglycerides and FFAs to liver (Adage et al., 2001; Nogueiras et al., 2007). Serum levels of ALT, a marker for liver damage, were not different between SHU9119-treated group and control group. Histological examination revealed that SHU9119 treatment induced changes in hepatocyte morphology including rupture and spacing of

the hepatocytes and increased number of cells positive for Oil Red O staining (Fig. 10F and 10M). Although, these changes were not phenotypically dramatic and no quantitative data are available for the 4-day SHU9119 treatment as compared with the advanced stage of steatosis observed in *ob/ob* mice (Fig. 10G and 10N), our data suggest that reduced central melanocortin signaling by the 4-day SHU9119 treatment causes biochemical and morphological changes in liver which might represent early-stage changes towards the development of fatty liver disease.

Obesity is often associated with increased lipid accumulation in the liver. However, it is unknown whether small changes in body weight and/or adiposity are sufficient to stimulate hepatic lipid accumulation before developing massive obesity. Therefore, mice were treated (i.c.v.) with SHU9119 for only 4 days to produce very modest changes in energy balance. As expected, SHU9119 treatment slightly but significantly increased body weight gain and epididymal fat pad weight in the absence of hyperphagia in the present study. These data suggest that the effect of reduced central melanocortin signaling on hepatic lipid metabolism occurs rapidly (i.e. within 4 days) and is not a consequence of massive changes in adiposity or body weight. Although, obesity and insulin resistance are the most common metabolic disturbances associated with NAFLD, it has been reported that NAFLD is also found in non-obese or non-diabetic subjects (Kim et al., 2004; Marchesini et al., 1999; Seppala-Lindroos et al., 2002). Treatment with thiazolidinedione, an insulin sensitizing agent, ameliorates hepatic steatosis in the presence of increased weight gain and adiposity (Promrat et al., 2004; Tiikkainen et al., 2004). Consequently, the central melanocortin signaling may play a

role in regulating hepatic lipid metabolism partly through a mechanism that is independent of body weight regulation.

SHU9119 treatment and *de novo* lipogenesis in the liver

It has been suggested that a large portion (approximately 26%) of triglyceride accumulation in hepatic steatosis results from increased hepatic *de novo* lipogenesis (Diraison et al., 2003; Donnelly et al., 2005). Fatty acid biosynthesis is sequentially catalyzed by the enzymes, ACC1, FAS, Elovl6, and SCD1 in liver (Postic & Girard, 2008). To examine whether the SHU9119-induced increase in hepatic triglyceride content is associated with increased hepatic lipogenic activity, the expression levels of genes involved in fatty acid synthesis in the liver was investigated. Consistent with a recent report by Nogueiras *et al.* mRNA levels of ACC1, FAS, Elovl6, and SCD1 were significantly elevated in SHU9119-treated mice compared with control mice, suggesting that increased *de novo* lipogenesis contributes to SHU9119-induced hepatic triglyceride accumulation (Nogueiras et al., 2007). In contrast to the elevated expression of these genes, this thesis study did not find any changes in GPAT1, DGAT1, DGAT2, or ADRP, which are known to play a role in the biosynthesis of triglycerides or lipid droplet formation (Buhman et al., 2001; Chang et al., 2006; Gonzalez-Baro et al., 2007). It is possible that posttranslational modifications of these enzymes might also contribute to the increased hepatic triglyceride accumulation. Although fat pad weight was already significantly increased by the 4-day SHU9119 treatment, expression of SCD1 and ADRP was not significantly increased in the white adipose tissue yet. These data suggest that

the stimulatory effect of SHU9119 on lipogenic gene expression occurs in the liver prior to white adipose tissue. Overall, these thesis data suggest that reduced central melanocortin signaling increases hepatic triglyceride accumulation by selectively up-regulating a subset of genes encoding lipid biosynthetic enzymes in the liver.

SHU9119 treatment and FFA transportation

Both FFA and lipoprotein particles provide lipids to the liver, and it is estimated that plasma FFA pool accounts for approximately 60% of hepatic triglycerides in patients with NAFLD (Donnelly et al., 2005). Triglycerides which are stored in adipose tissue undergo lipolysis by HSL to produce FFA for export to the circulation. Hepatic LIPC breaks down triglycerides to produce FFAs and FFAs are taken up by a combination of passive diffusion and facilitated transport (Berk & Stump, 1999). Fatty acid transporters including FAT/CD36 facilitates hepatic uptake of FFAs from the circulation and elevated FAT/CD36 levels are associated with increased hepatic triglyceride levels (Lee et al., 2008). Some lipoprotein particles are taken up by receptor-mediated endocytosis (Cooper, 1997; Lambert et al., 2001). Thus, increased FFA influx to the liver may also contribute to SHU9119-induced lipid accumulation in liver. SHU9119 treatment did not alter serum NEFA levels in the present study. Adipose HSL mRNA levels, as well as, hepatic LIPC and FAT/CD36 mRNA levels were not changed by SHU9119 treatment. However, hepatic LDLR mRNA was increased in SHU9119-treated mice. These data suggest that SHU9119-induced increase in hepatic triglyceride accumulation is not due to

increased lipolysis in adipose tissue or increased FFA influx into the liver, but at least partly, due to increased uptake of lipoprotein particles.

SHU9119 treatment and β-oxidation

Oxidation of NEFA occurs via the induction of PPARα regulated oxidative pathway and its target oxidative genes, acyl-CoA oxidase (ACO), carnitine palmitoyl transferase-1 (CPT1), and uncoupling proteins (UCP) (Lee et al., 2002). Mice deficient in MC3R or MC4R are characterized by increased adiposity, increased susceptibility to diet-induced obesity and reduced oxidation of fatty acids (Albarado et al., 2004; Butler, 2006; A. S. Chen et al., 2000; Huszar et al., 1997). Therefore, it is possible that increased hepatic triglyceride accumulation could be due to reduced fatty acids oxidation in liver. To address this possibility, this study investigated the mRNA levels of enzymes involved in fatty acid oxidation, CPT1a, very long chain acyl-CoA dehydrogenase (VLCAD) and medium chain acyl CoA dehydrogenase (MCAD). Although hepatic CTP1a and MCAD mRNA levels were not changed by SHU9119 treatment, hepatic VLCAD mRNA levels were lower in SHU9119-treated group compared with control group. These data suggest that reduced fatty acid oxidation might partially contribute to SHU9119-induced increases in hepatic triglyceride accumulation.

SHU9119 treatment and VLDL secretion

FFAs can be either stored as triglycerides in liver or secreted into the circulation as very low density lipoprotein (VLDL)-triglycerides. Thus, increased hepatic triglyceride accumulation could be due to reduced hepatic VLDL secretion. SHU9119 treatment increased serum triglyceride levels in the present study, reflecting an increased hepatic VLDL production and triglyceride secretion in SHU9119-tretaed group. Therefore, it is unlikely that reduced central melanocortin signaling promotes hepatic triglyceride accumulation by reducing VLDL secretion from liver to the circulation. It was recently demonstrated that i.c.v. SHU9119 treatment increased hepatic levels of microsomal triglyceride transfer protein (MTP) mRNA, reflecting an increased VLDL-triglyceride assembly and secretion (Nogueiras et al., 2007; Tietge et al., 1999). The findings of this thesis study, showing that SHU9119 treatment increased hepatic triglyceride content, serum triglyceride levels, and fat pad mass, suggest that reduced central melanocortin signaling stimulates not only hepatic triglyceride synthesis and deposition but also VLDL assembly and secretion, thereby promoting a transportation of triglycerides from liver to white adipose tissue.

SHU9119 induces *de novo* triglyceride synthesis in PPARγ2- and SREBP1c-dependent pathways

Hepatic lipogenic gene expression is under the control of several transcription factors (Dentin et al., 2005; Postic & Girard, 2008). Transcription factor SREBP-1 plays a crucial role in the regulation of lipid biosynthesis (Tontonoz et al., 1993; Yokoyama et al.,

1993). Levels of both SREBP-1c mRNA and its active nuclear protein are elevated in liver of animal models of fatty liver disease (Shimomura et al., 1998). Overexpression of SREBP-1c in liver results in elevated hepatic triglyceride content, and elevated hepatic lipogenic gene expression levels, while ablation of SREBP-1 in mice results in reduced hepatic lipogenic gene expression and hepatic lipid synthesis (Shimano, Horton et al., 1997; Shimano, Shimomura et al., 1997). The absence of SREBP-1 also attenuated hepatic steatosis and reduced levels of hepatic lipogenic genes in animal models of fatty liver disease without changes in body weight, suggesting that elevated SREBP-1 activity contributes to the development of fatty liver disease independent of obesity (Yahagi et al., 2002). Consistent with a previous observation, i.c.v. SHU9119 treatment increased hepatic SREBP-1c mRNA levels, as well as, lipogenic gene expression levels in mice without changes in food intake and prior to developing massive obesity in the present study (Nogueiras et al., 2007). These data suggest that reduced central melanocortin signaling stimulates hepatic lipogenic gene expression, *de novo* lipogenesis, and lipid deposition through the activation of SREBP-1c. Although it is clear that SREBP-1c plays a critical role in the regulation of hepatic lipogenic gene expression, the deletion of SREBP-1c in mice results in only 50% reduction in fatty acid synthesis (Liang et al., 2002). Thus, SREBP-1c alone does not fully account for hepatic lipogenesis and other factors also may be involved in the regulation of hepatic lipogenesis. ChREBP is required for glucose-induced hepatic lipogenic gene expression by directly promoting the transcription of lipogenic genes (Dentin et al., 2004; Ishii et al., 2004; Yamashita et al., 2001). Ablation or reduction of ChREBP in mice results in reduced levels of hepatic lipogenic gene expression and amelioration of hepatic steatosis (Dentin et al., 2006;

Iizuka et al., 2004; Iizuka et al., 2006). ChREBP transcriptional activity is under the regulatory control of hormone dependent kinase activity and glucose-dependent phosphatase activity. Under fasted conditions, glucagon and epinephrine induce an increase in intracellular cAMP which activates cAMP-activated protein kinase (PKA). PKA inactivates ChREBP by phosphorylation (Kawaguchi et al., 2001). PKA-mediated phosphorylation of ChREBP at the serine residue 196 results in inhibition of its nuclear import, while phosphorylation at the threonine residue 666 inhibits its DNA binding (Kawaguchi et al., 2002). Conversely, the conversion of glucose to xylulose-5-phosphate via the hexose monophosphate shunt results in the activation of ChREBP through the phosphatase 2A delta-mediated dephosphorylation of ChREBP (Kabashima et al., 2003). SHU9119 treatment increased lipogenic gene expression in liver without changes in the mRNA levels of ChREBP and its target L-PK. This thesis study also found that SHU9119 did not cause significant changes in hepatic ChREBP protein levels by Western blotting. Although these data suggest that ChREBP does not mediate the effect of SHU9119 on hepatic lipogenesis, levels of mRNA and protein may not reflect the activity of transcription factors. Future analysis will be required to assess the phosphorylated nature of ChREBP and its localization within the nucleus.

The nuclear receptor peroxisome PPARγ also plays a role in the regulation of hepatic expression of genes including lipogenic genes. Levels of hepatic PPARγ are markedly elevated in animal models of obesity- or insulin resistance-associated fatty liver disease (Bedoucha et al., 2001; Gavrilova et al., 2003; Matsusue et al., 2003; Memon et al., 2000). PPARγ2 stimulates SREBP-1, as well as, its target lipogenic gene expression, lipid synthesis, and lipid accumulation in hepatocytes (Schadinger et al., 2005).

Suppressing endogenous PPARγ2 expression was effective in reversing elevated lipogenic gene expression levels and triglycerides content in liver in animal models of fatty liver disease (Y. L. Zhang et al., 2006). These observations suggest that PPARγ2 promotes the development of hepatic steatosis by stimulating the expression of genes involved in lipid synthesis and accumulation in hepatocytes. We found that SHU9119 treatment significantly increased PPARγ2 mRNA levels in liver, suggesting that reduced central melanocortin signaling promotes hepatic lipogenesis and lipid accumulation through the activation of the pathway which is used by PPARγ2. Since SHU9119 treatment also increased hepatic SREBP-1c mRNA levels, it is possible that SHU9119-induced increase in hepatic PPARγ2 might stimulate lipogenic gene expression through the activation of SREBP-1c. However, reduced PPARγ2 expression suppressed hepatic lipogenic gene expression without changes in SREBP-1c and ChREBP expression levels (Y. L. Zhang et al., 2006). It is also demonstrated that a putative PPAR-responsive element is present in the promoter of a lipogenic gene ACC (C. H. Lee et al., 2006). Therefore, we cannot rule out the possibility that reduced central melanocortin signaling stimulates hepatic PPARγ2 expression which in turn stimulates lipogenic gene expression independent of SREBP-1c and ChREBP. Overall, my data suggest that the central melanocortin signaling regulates hepatic lipogenic gene expression and lipid deposition through the activation of PPARγ2 and SREBP-1c.

Possible involvement of sympathetic nervous system (SNS) in the mediation of melanocortinergic regulation of hepatic lipid metabolism

Studies have shown that the central melanocortin system mediates the cardiovascular, renal and metabolic actions of leptin through the activation of the sympathetic nervous system (SNS) (da Silva et al., 2004; Haynes et al., 1999; Rahmouni et al., 2003; Tallam et al., 2006). Efferent hypothalamic projections are part of the autonomic regulation of hepatic function (Buijs, la Fleur et al., 2003; Shimazu, 1987, 1996). MC4R mRNA is expressed in brain areas involved in lipid mobilization (Song et al., 2005). SNS governing WAT originates in brain regions that include MC4R expressing neurons in the hypothalamus, midbrain, brainstem and spinal cord areas (Giordano et al., 2006; Song et al., 2005; Tsou et al., 1986). Activation of the hypothalamic MC3/4R reduces appetite and increases arterial pressure and heart rate (Kuo et al., 2003). Conversely, blockade of signaling through the MC3/4R reduced heart rate and arterial pressure (Kuo et al., 2003; Tallam et al., 2004) and inhibited the effects of leptin in reducing food intake and increasing arterial pressure and heart rate. Mice carrying a mutation in MC4R are not responsive to the actions of leptin in reducing food intake and raising arterial blood pressure (da Silva et al., 2004). A recent study demonstrated that the central melanocortin system regulates WAT lipid metabolism by altering the SNS outflow to WAT (Nogueiras et al., 2007). Furthermore, destruction of the hepatic sympathetic nerves results in a decrease in cholesterol synthesis and circulating cholesterol levels (Shanygina et al., 1981). Although the present study did not address the possible involvement of the SNS in the melanocortinergic regulation of hepatic lipid metabolism, it is likely that the observed hepatic lipid accumulation and increased lipogenic gene

expression following SHU9119 treatment are likely mediated by reduced SNS outflow to the liver. Further studies are warranted to clarify this possibility.

SHU9119-induced hepatic lipogenesis: role of insulin and leptin

How is hepatic lipogenic gene expression stimulated through the activation of SREBP-1c or PPARγ2? Nutritional factors regulate hepatic lipogenic gene expression and insulin plays a major role in the regulation of SREBP-1c-mediated lipogenic gene expression (Dentin et al., 2005; Postic & Girard, 2008). Serum insulin was elevated 2.4-fold in SHU9119-treated mice, though the effect did not reach statistical significance in the present study. These data cannot draw a definitive conclusion as to whether SHU9119-induced increase in hepatic lipogenesis is associated with insulin action or not. However, generally the 2.4-fold elevation in serum insulin level is considered as a major difference, and i.c.v administration of melanocortin agonists acutely reduce insulin secretion (Fan et al., 2000). Thus, a favorable hypothesis is that reduced central melanocortin signaling stimulates hepatic lipogenic gene expression and triglyceride accumulation through the insulin-induced activation of SREBP-1c. Nevertheless, hepatic lipogenic gene expression is also regulated by insulin-independent manner, as well as, SREBP1-independnet manner (Biddinger et al., 2006; Matsuzaka et al., 2004). Further studies are required to determine whether SHU9119-induced increase in lipogenic gene expression is mediated by insulin action and SREBP1 activity.

Leptin inhibits hepatic lipogenic gene expression and ameliorates hepatic steatosis in animal models of fatty liver disease (Cohen et al., 2002). Although serum leptin levels

were elevated by SHU9119 treatment in the present study, hepatic lipogenic gene expression and triglyceride content were also elevated in SHU9119-treated mice. These data suggest that SHU9119-induced increase in fat pad weight and serum leptin levels may function as a compensatory mechanism against SHU9119-induced increase in hepatic lipogenesis when the animals are still sensitive to the effect of leptin. Subsequent development of leptin resistance may further stimulate lipogenesis and accelerate the development of fatty liver disease. Prolonged or chronic reduction in the central melanocortin signaling is known to cause leptin resistance, consequently leptin resistance possibly augments SHU9119-induced hepatic lipogenesis (da Silva et al., 2004; Halaas et al., 1997; Tallam et al., 2006).

Serum levels of leptin are elevated in patients with NAFLD, and it has been suggested that leptin plays a pivotal role in the progression of hepatic fibrosis in a variety of chronic liver diseases (Leclercq et al., 2002; Uygun et al., 2000). In animals with leptin deficiency or leptin receptor deficiency, the development of fibrosis does not occur during hepatic steatosis or chronic liver injury (Ikejima et al., 2005; Leclercq et al., 2002). In this thesis study, SHU9119 treatment increased serum leptin levels which coincided with induction of hepatocellular changes resembling the tissue rearrangements of fatty liver. Thus, despite having an inhibitory influence on hepatic lipogenic gene expression and lipid accumulation, leptin also functions as a hepatic fibrogenic factor and increased leptin levels contribute to the progression of hepatic fibrosis.

In summary, enhanced central melanocortin signaling reduces hepatic lipogenic gene expression and possibly reduced central melanocortin signaling promotes hepatic

triglyceride accumulation by inducing multiple changes in lipid metabolism including increased *de novo* lipogenesis, increased FFA or lipoprotein uptake, and reduced FFA oxidation. This thesis study also suggests that increased hepatic lipogenic gene expression is mediated through the PPARγ2- and SREBP1-dependent, but ChREBP-independent, pathway. Impairments in the central melanocortin signaling cause biochemical and morphological changes resembling early stage of hepatic steatosis before developing overt obesity and apparent insulin resistance. Therefore, the above thesis findings suggest that the central melanocortin signaling regulates hepatic lipid metabolism independent of the regulation of body weight and insulin sensitivity and that impairments in the central melanocortin signaling alone may be sufficient to cause fatty liver disease.

Table 1. Primer sequences used for real-time PCR

Gene	Accession No.	Primer	Sequences
GK	L41631	Forward	5’-CCCCAACGACCCCTGCTTATCC-3’
		Reverse	5’-GCATCCGGCTCATCACCTTCTTCA-3’
L-PK	NM_013631	Forward	5’-CTTGCTCTACCGTGAGCCTC-3’
		Reverse	5’-ACCACAATCACCAGATCACC-3’
ACC1	NM_133360	Forward	5’-TCTCTGGCTTACAGGATGGTTTG-3’
		Reverse	5’-GAGTCTATTTTCTTTCTGTCTCGACCTT-3'
FAS	NM_007988	Forward	5'-GCTGCGGAAACTTCAGGAAAT-3’
		Reverse	5’-AGAGACGTGTCACTCCTGGACTT-3'
Elovl6	NM_130450	Forward	5’-AAAAGCAGTTCAACGAGAACGAA-3’
		Reverse	5’-GGCTCGCTTGTTCATCAGATG-3’
SCD1	NM_009127	Forward	5'-GTCAAAGAGAAGGGCGGAAAAC-3'
		Reverse	5'-AAGGTGTGGTGGTAGTTGTGGAAG-3'
GPAT1	NM_008149	Forward	5’-ACATCGCCTCGGGC-3’
		Reverse	5’-AAGCCCCCAAGCTT-3’
DGAT1	NM_010046	Forward	5’-GTGCACAAGTGGTGCATCAG-‘3
		Reverse	5’-CAGTGGGACCTGAGCCATCA-3’
DGAT2	NM_026384	Forward	5’-TTCCGAGACTACTTTCCCATCCAG-3’
		Reverse	5’-ACCAGCCAACGTAGCCAAATAGG-3’
ADRP	NM_007408	Forward	5’-TGGCAGCAGCAGTAGTGGAT-3’
		Reverse	5’-CTGACATAAGCGGAGGACACAA-3’
HMGCR	NM_008255	Forward	5’-ACCAAACCCCGTAACCCAAA-3’
		Reverse	5’-CGACTATGAGCGTGAACAAGGA-3’
HMGCS1	NM_145942	Forward	5’-GGCGGCTAGAAGTTGGAACA-3’
		Reverse	5’-CAGGGCCACAGCTCCAACT-3’
HMGCS2	NM_008256	Forward	5’-CTTCAATGCTGCCAACTGGA-3’
		Reverse	5’-CAAAAGGGTGTGTGGAAGATCA-3’
SREBP-1a	NM_011480	Forward	5’-GCGCCATGGACGAGCTGGC-3’
		Reverse	5’-GGCCCGGGAAGTCACTGT-3’
SREBP-1c	NW_001030469	Forward	5’-GGAGCCATGGATTGCACATT-3’
		Reverse	5’-GGCCCGGGAAGTCACTGT-3’
ChREBP	NM_021455	Forward	5’-TTCAAAGGCCTCAAGTTGCT-3’
		Reverse	5’-AGAAGAGCTGTTCGCACCAT-3’
PPARα	X57638	Forward	5’-GTGGCTGCTATAATTTGCTGTG-3’
		Reverse	5’-GAAGGTGTCATCTGGATGGTT-3’
PPARγ2	NM_011146	Forward	5’-TCTGGGAGATTCTCCTGTTGA-3’
		Reverse	5’-GGTGGGCCAGAATGGCATCT-3’
LIPC	NM_008280	Forward	5’-CTGCCACTTCCTGG-3’
		Reverse	5’-ATGGGCACATTTGA-3’
FAT/CD36	BC010262	Forward	5’-GCCAAGCTATTGCGACATGA-3’
		Reverse	5’-GAAAAGAATCTCAATGTCCGAGACT-3’
LDLR	NM_010700	Forward	5’-TGAGGACGCAACGCAGAA-3’
		Reverse	5’-ATTGATTGGACTGACAGGTGACA-3’
CPT1a	NM_013495	Forward	5’-CTTTGGGCCGGTTGCTGATGAC-3’
		Reverse	5’-GCGGTGAGGCCAAACAAGGTGATA-3’
VLCAD	NM_017366	Forward	5’-TCTCTGCCCAGCGACTTTATG-‘3
		Reverse	5’-CCCACAGCAAAGGACTTCGA-‘3

Table 1 (cont.). Primer sequences used for real-time PCR

Gene	Accession No.	Primer	Sequences
MCAD	NM_007382	Forward	5'-TACCCGTTCCCTCTCATCAAAA-'3
		Reverse	5'-TCCAGCAAGAATCACAGGCAT-'3
UCP2	NM_011671	Forward	5'-TCAGCCTGAGACCTCAAAGCA-3'
		Reverse	5'-CATCTGTGGCCTTGAAACCA-3'
β-actin	X03672	Forward	5?-CAGCTTCTTTGCAGCTCCTT-3?
		Reverse	5?-TCACCCACATAGGAGTCCTT-3?

Hypothesis I: Impairments in central melanocortin signaling may contribute to the development of hepatic steatosis and enhanced central melanocortin signaling may be beneficial in reversing abnormal hepatic lipid metabolism in fatty liver disease.

Thesis Study I: Investigate the effect of intracerebroventricular (i.c.v.) treatment of a) MTII in obese mice and b) SHU9119 in lean mice on hepatic lipid metabolism

Animals: Wild-type mice; age 6-8 weeks

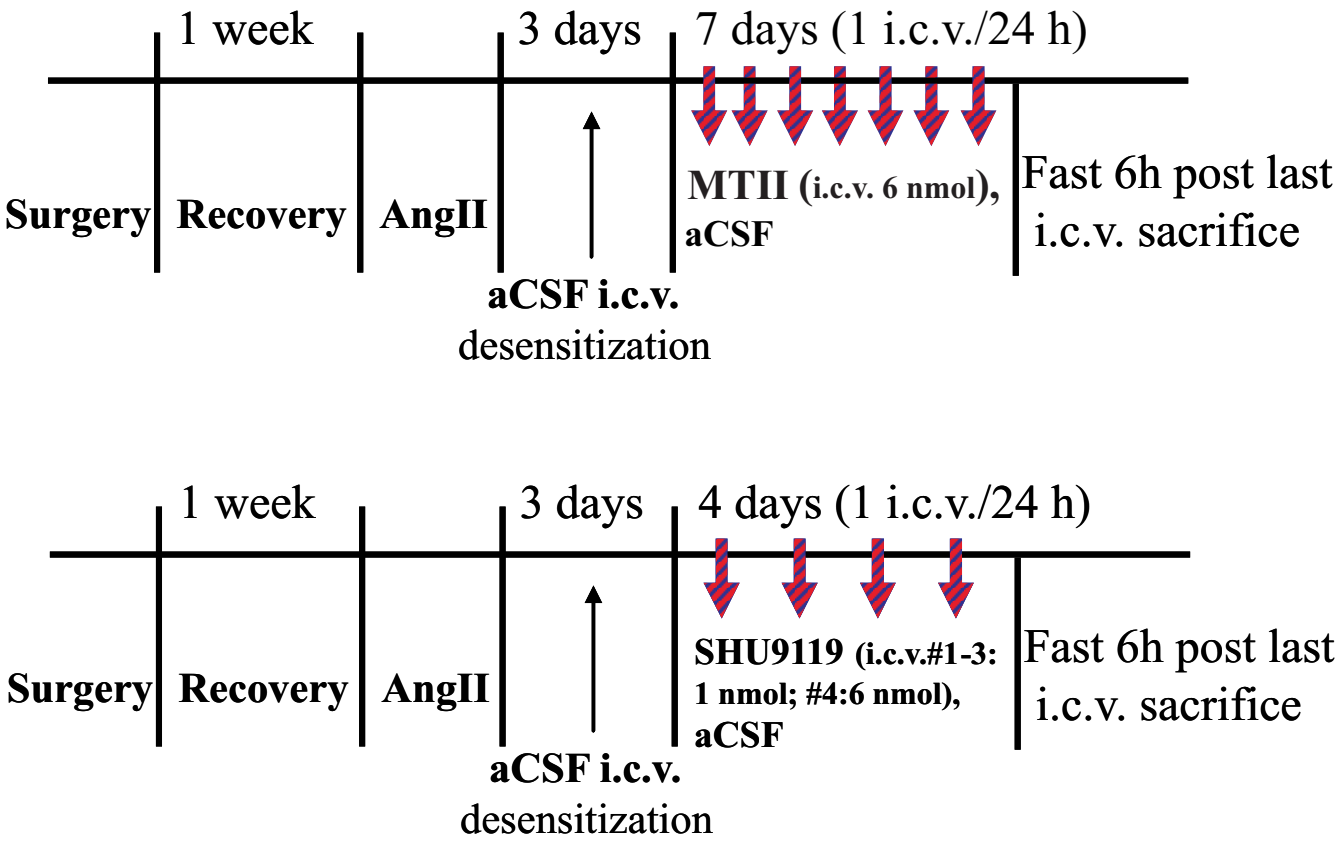


Figure 4: Experimental outline in pursuit of Study I aimed at addressing the Thesis Hypothesis I.

Table 2. The effect of SHU9119 treatment on blood levels of hormones and metabolites

	aCSF (N = 7-9)	SHU9119 (N = 10)	P
Blood glucose (mg/dl)	155.7 ± 10.1	151.7 ± 8.9	0.77
Serum insulin (ng/ml)	0.30 ± 0.03	0.72 ± 0.20	0.08
Serum leptin (ng/ml)	0.57 ± 0.16	2.01 ± 0.49	< 0.05
Serum NEFA (mEq/l)	2.19 ± 0.11	2.08 ± 0.17	0.58
Serum triglycerides (mg/dl)	133.3 ± 11.0	196.1 ± 20.5	< 0.05
Serum ALT (U/l)	21.0 ± 2.4	24.2 ± 2.8	0.43

Values are means ± SEM.
Differences between groups were analyzed by Student's *t*-test.

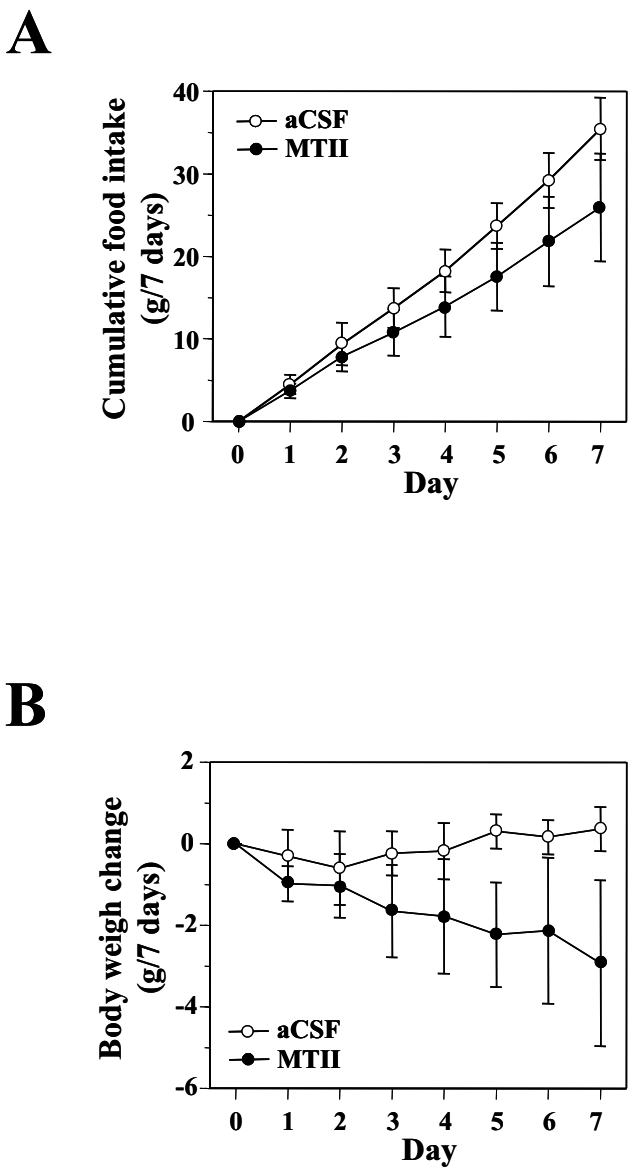


Figure 5: Daily MTII treatment reduces food intake and body weight gain in obese mice. The effect of daily intracerebroventricular (i.c.v.) injections of MTII for 7 days on cumulative food intake (A) and cumulative body weight change (B) was examined in male *ob/ob* mice. Cumulative food intake ($P < 0.0005$) and cumulative body weight change ($P < 0.0001$) were significantly lower in MTII-treated mice compared with aCSF-treated control mice (Kruskal-Wallis test). Data are means ± SEM (N = 9-10 mice/group).

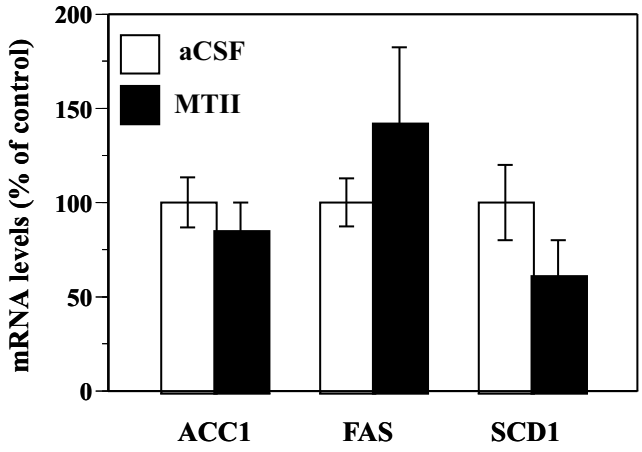


Figure 6: Daily MTII treatment did not reduce hepatic lipogenic gene expression in obese mice. The effect of daily intracerebroventricular (i.c.v.) injections of MTII for 7 days on the hepatic expression of genes involved in triglyceride biosynthesis was examined in male *ob/ob* mice. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). Statistical analysis was performed by Student's *t*-test.

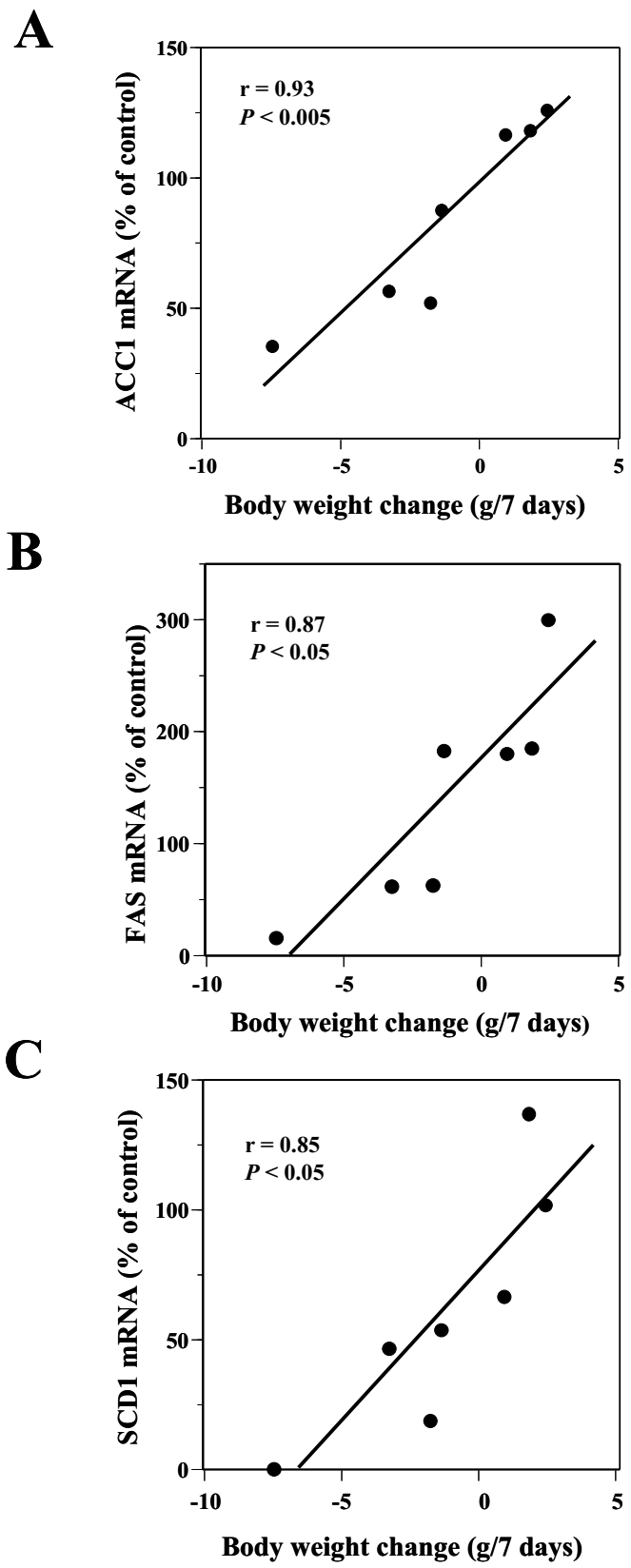


Figure 7: Reduced body weight is associated with reduced hepatic lipogenic gene expression in MTII-treated obese mice. Male ob/ob mice received daily intracerebroventricular (i.c.v.) injections of MTII for 7 days. Correlation analysis showed significant positive correlation between MTII-induced body weight change and hepatic expression of ACC1 (A), FAS (B) or SCD1 (C) mRNA in ob/ob mice ($N = 7$).

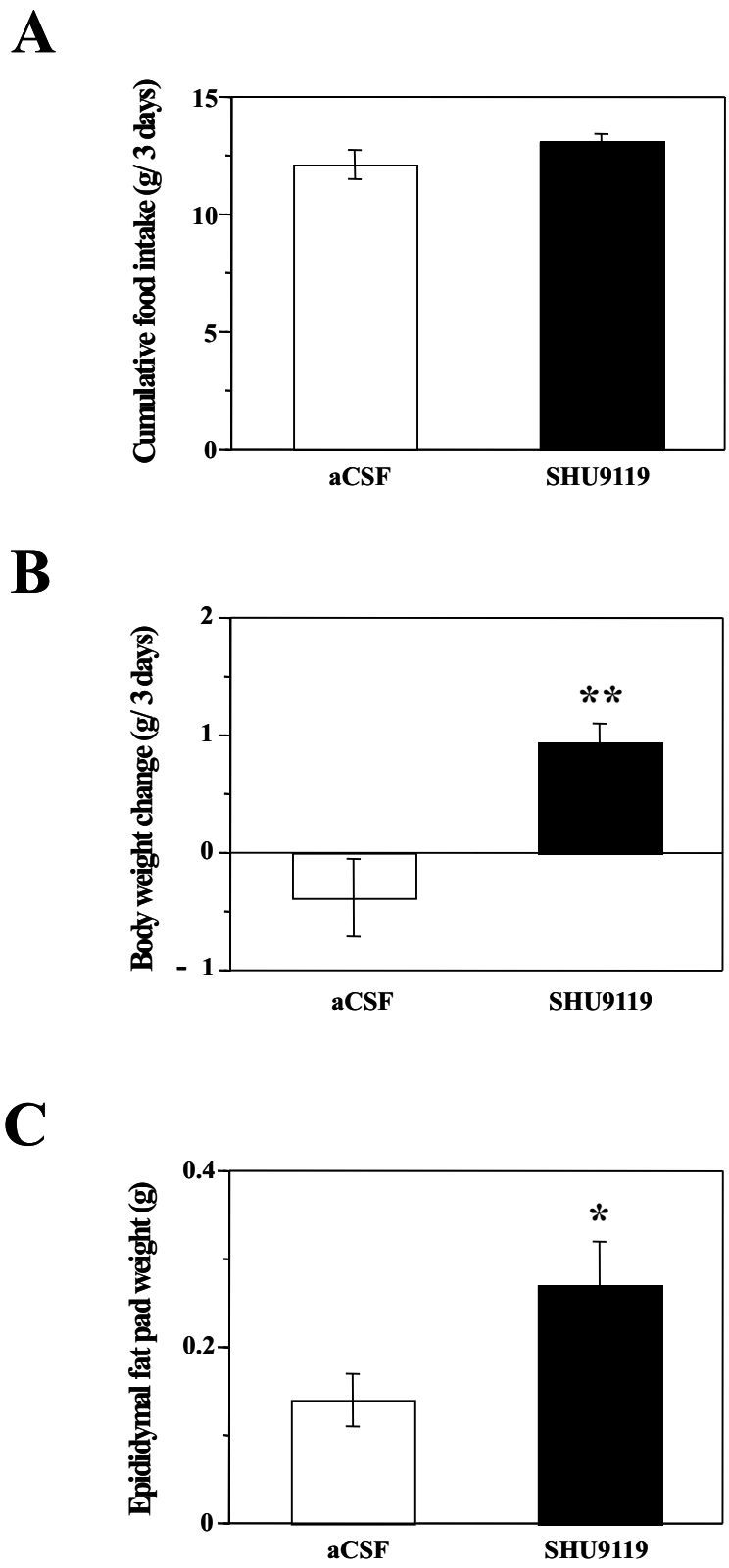


Figure 8: SHU9119 treatment increases body weight change and epididymal fat pat weight without significant changes in food intake in lean mice. The effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on cumulative food intake (A), cumulative body weight change (B), and epididymal fat pad weight (C) were tested in C57BL/6J mice. Data are means ± SEM (N = 9-10 mice/group). *: $P < 0.05$, **: $P < 0.01$ vs. aCSF (Student's t-test).

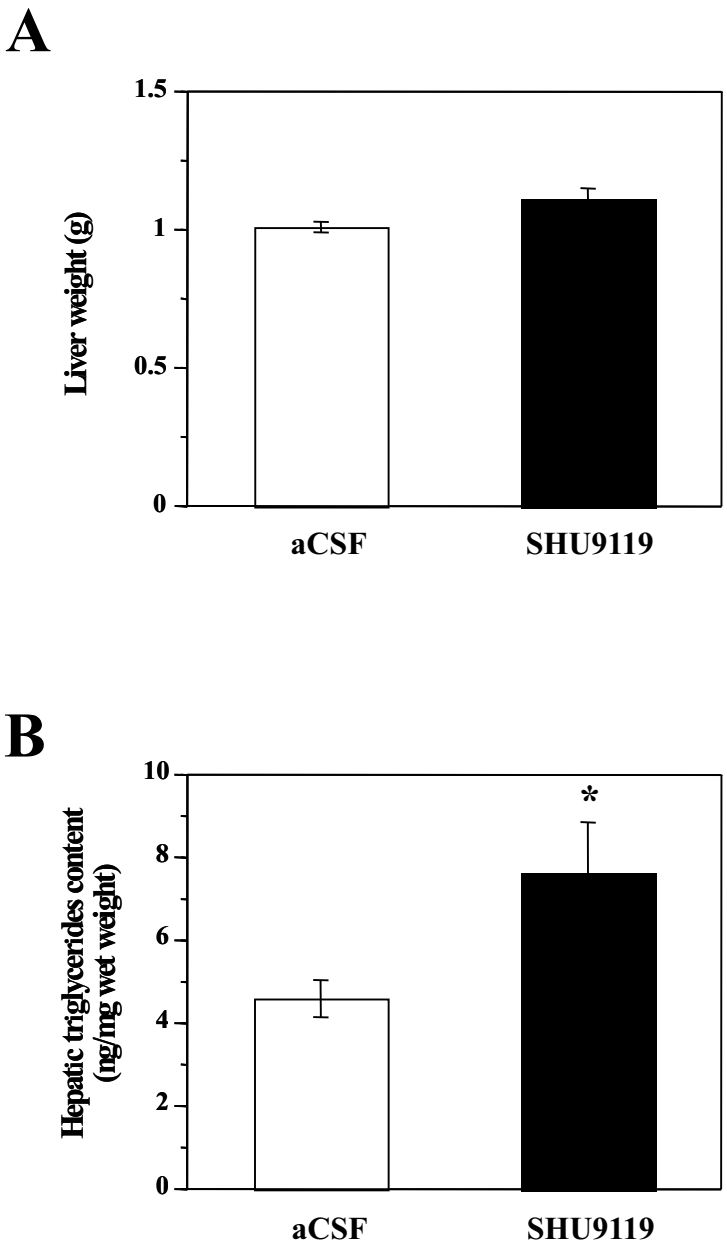


Figure 9: SHU9119 treatment increases hepatic triglyceride levels in lean mice. The effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on liver weight (A) and hepatic triglyceride content (B) were tested in C57BL/6J mice. Data are means ± SEM (N = 9-10 mice/group).
*: $P < 0.05$ vs. aCSF (Student's t-test). There is no significant difference in lever weight between the groups ($P = 0.051$, Student's t-test).

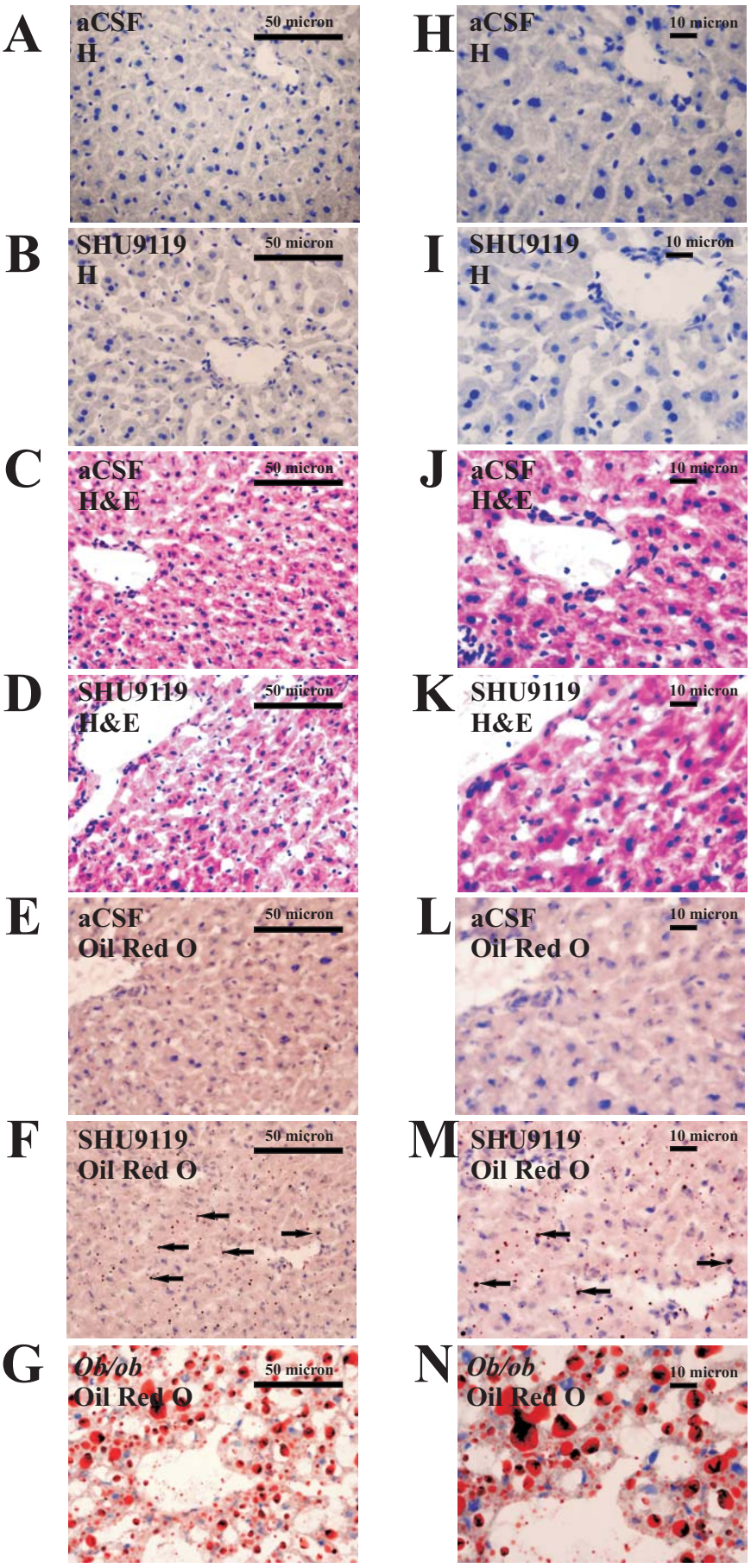


Figure 10: SHU9119 treatment increases lipid accumulation in the hepatocytes of lean mice. The effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on liver histology and hepatic lipid deposition was examined in C57BL/6J mice. A, B, H and I: Hematoxylin (H) staining . C, D, J and K: Hematoxylin and Eosin (H&E) staining. E, F, G, L, M and K: Oil Red O staining. Magnification: 40X (A, B, C, D, E, F and G) and 60X (H, I, J, K, L, M and N).

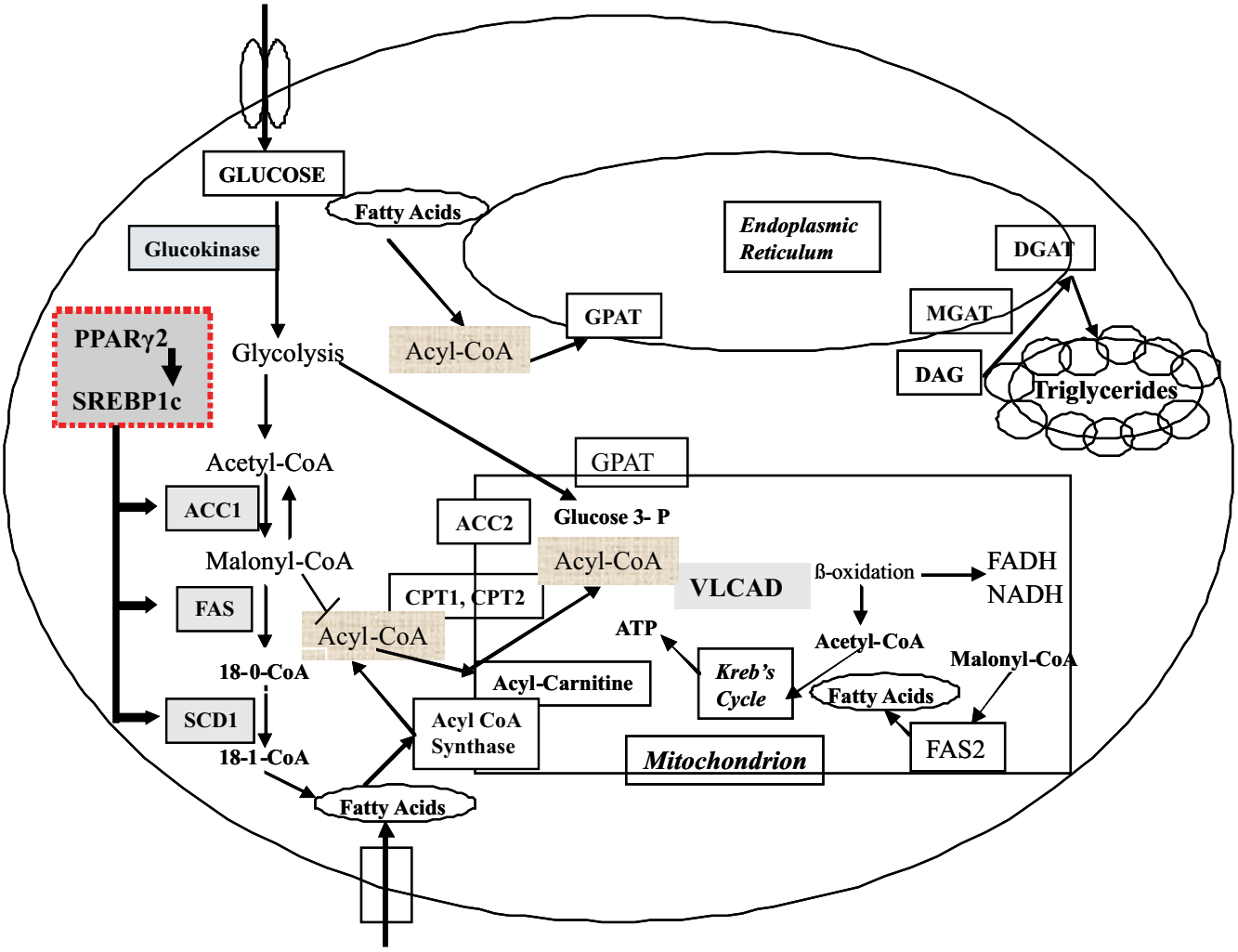


Figure 11: Schematic summary of lipid biosynthetic and fatty acids oxidation pathways in the liver.

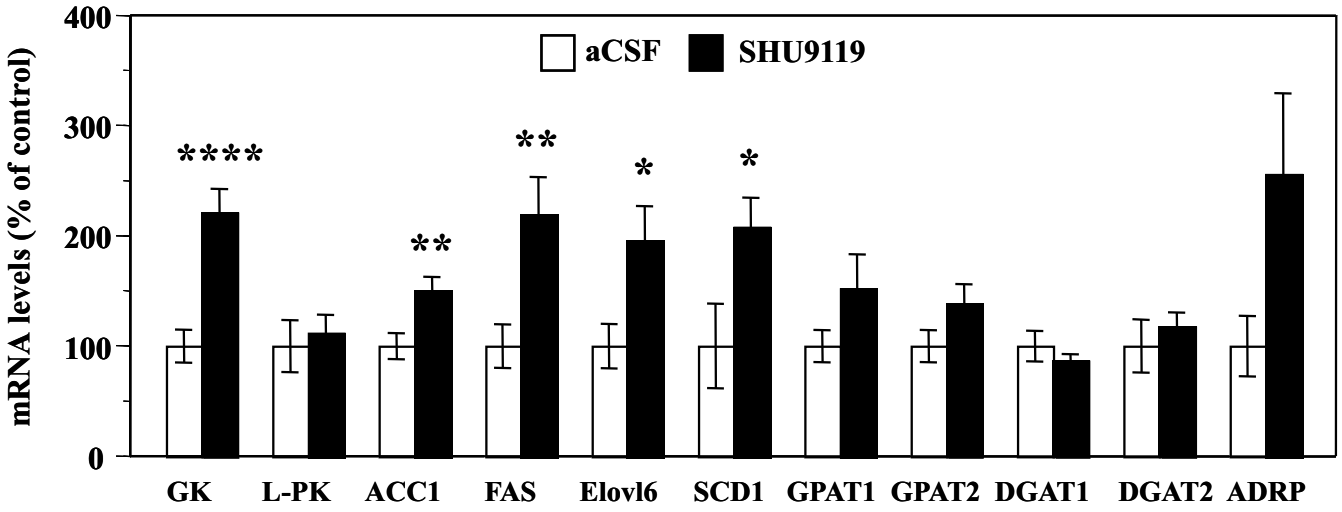


Figure 12: SHU9119 treatment increases the expression of genes involved in *de novo* lipogenesis in the liver. The effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on hepatic expression of genes involved in glycolysis, fatty acid and triglyceride biosynthesis, and lipid droplet formation was tested in C57BL/6J mice. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0005$ vs. aCSF (Student's t-test).

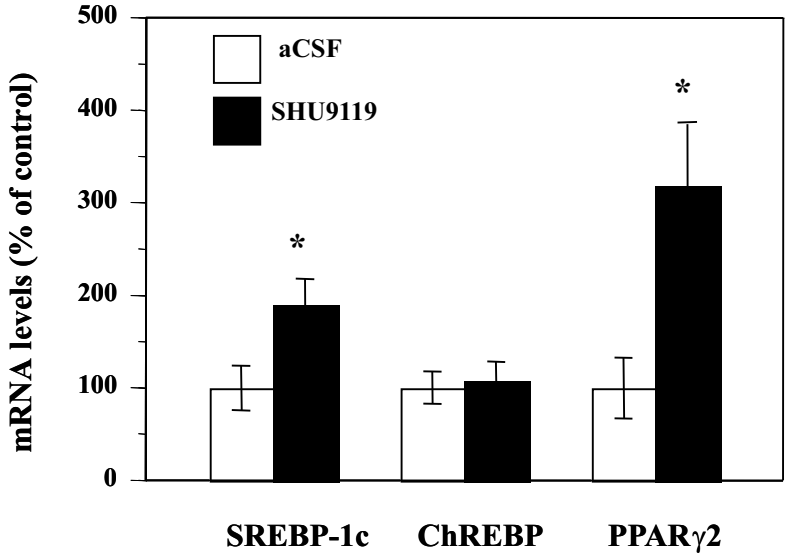


Figure 13: SHU9119 treatment increases the expression of lipogenic transcription factors SREBP-1c and PPARγ2 in the liver of lean mice. Effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on hepatic expression of genes encoding transcription factors which are involved in the regulation of lipid metabolism genes. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). *: $P < 0.05$ vs. aCSF (Student's t-test).

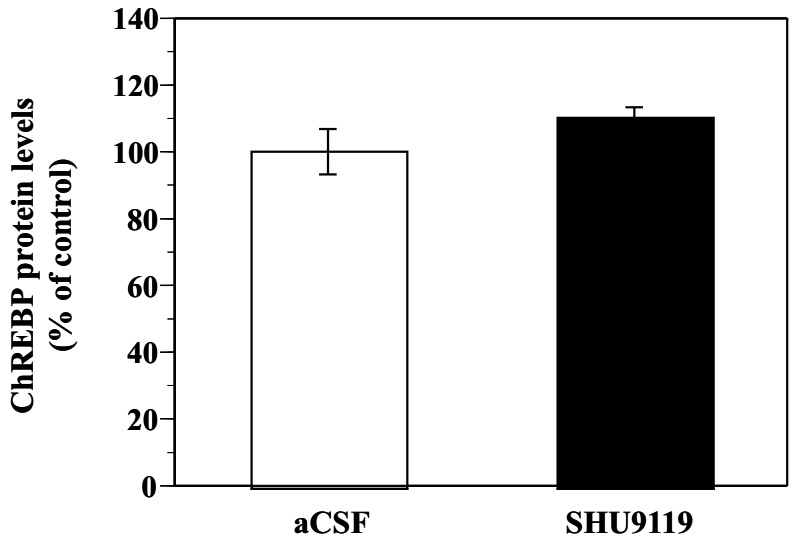


Figure 14: SHU9119 treatment does not alter the protein levels of carbohydrate responsive element-binding protein (ChREBP) in the liver. Relative expression levels of ChREBP protein were measured by Western blot analysis and normalized to α-Tubulin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (*N* = 9-10 mice/group).

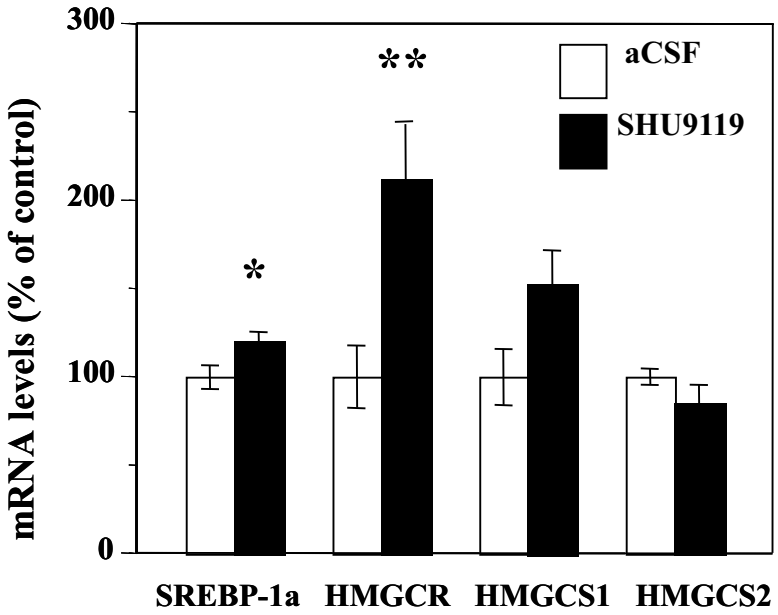


Figure 15: SHU9119 treatment increases the expression of genes involved in cholesterol biosynthesis in the liver of lean mice. Effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on hepatic expression of genes involved in the regulation of cholesterol biosynthesis was examined in C57BL/6J mice. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). *: $P < 0.05$, **: $P < 0.01$ vs. aCSF (Student's t-test).

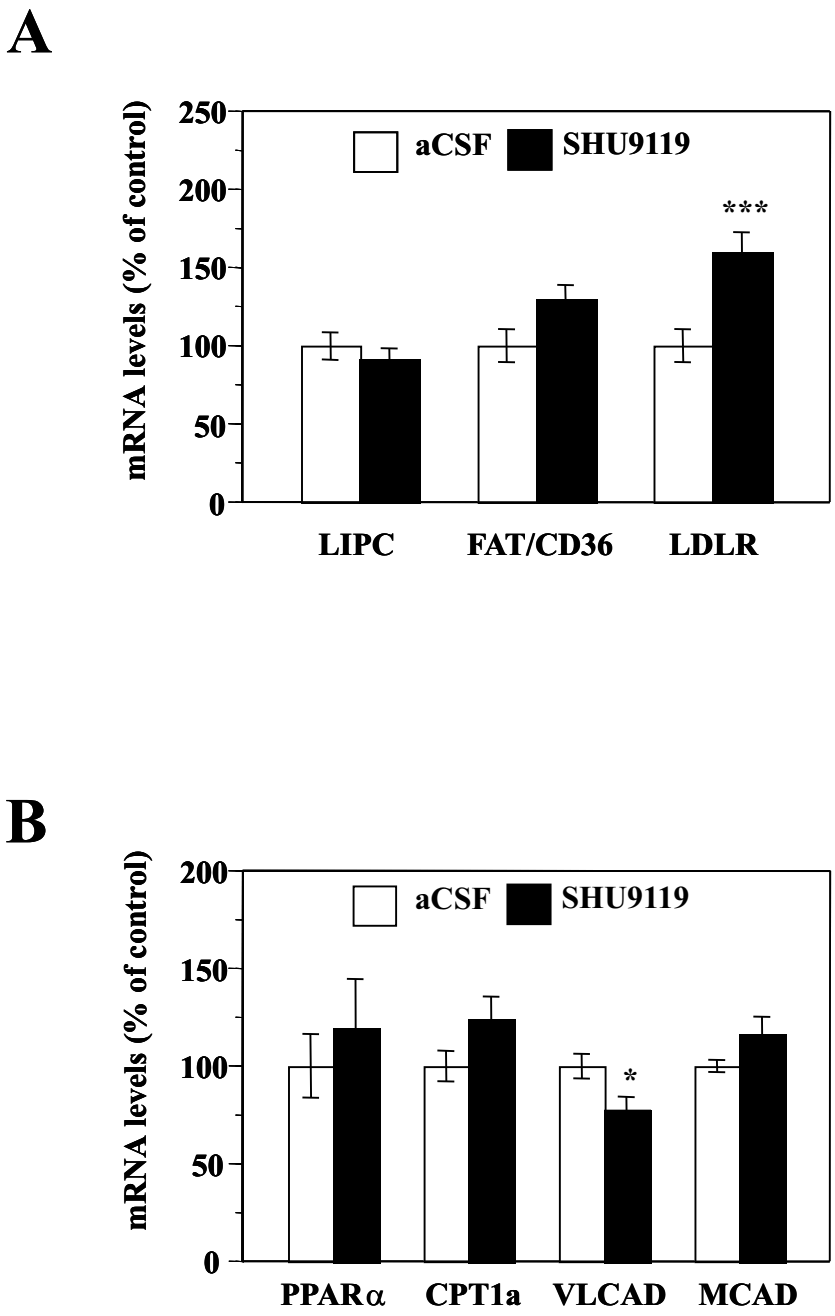


Figure 16: SHU9119 treatment induces changes in the genes involved in lipid transport and fatty acid oxidation in lean mice. The effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on hepatic expression of genes involved in the regulation of fatty acid transport (A) and fatty acid oxidation (B) was tested in C57BL/6J mice. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). *: $P < 0.05$, ***: $P < 0.005$ vs. aCSF (Student's *t*-test).

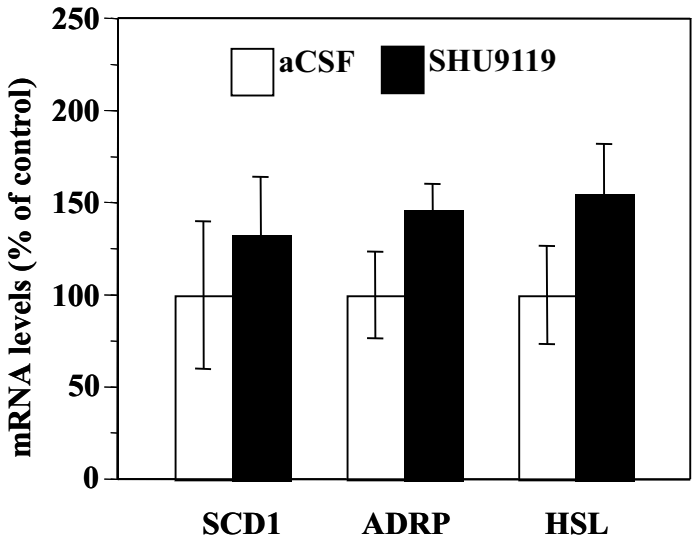


Figure 17: SHU9119 treatment does not alter the expression levels of lipogenic genes in the white adipose tissue. Effects of a 4-day intracerebroventricular (i.c.v.) SHU9119 treatment on expression of genes involved in the regulation of fatty acid biosynthesis, lipid droplet formation, and lipolysis in white adipose tissue in C57BL/6J mice. Relative expression levels of mRNA were measured by real-time PCR analysis and normalized to β-actin expression. Values in aCSF-treated control mice were set to 100%. Data are means ± SEM (N = 9-10 mice/group). There was no significant difference between the groups (Student's t-test).

STUDY II: Nutritional regulation of ChREBP and Fto

Relationship between blood glucose levels and hepatic Fto mRNA expression in mice. **Poritsanos NJ,** Lew PS, Mizuno TM. Biochem Biophys Res Commun. 2010 Oct 1;400(4):713-7. Epub 2010 Sep 9.

Introduction

The discovery of *FTO* (fat mass and obesity-associated) through recent genome-wide association studies demonstrated a strong association between a cluster of SNPs in the *FTO* gene and obesity and type 2 diabetes (Frayling et al., 2007). Most of SNPs have been identified to be located in the first intron of *FTO*, a genetic region known to be highly conserved among species. FTO genomic sequence consists of nine exons (>400 kb) on chromosome 16. In addition to obesity, *FTO* SNPs are also consistently associated with metabolic syndrome-related traits including increased circulating leptin, fat mass, body weight, hip and waist circumference, while no association was established with the presence of increased lean mass or body height across multiple populations (Dina et al., 2007; Frayling et al., 2007; Marvelle et al., 2008; Scuteri et al., 2007). Despite the strong association between *FTO* variants and obesity, the biological function of FTO and the mechanism through which variants in the *FTO* gene lead to excess weight gain have yet to be identified. In general, *FTO* variants are associated with higher energy intake and reduced satiety response, suggesting that the gene plays a role in the regulation of appetite (Haupt et al., 2008; Speakman et al., 2008; Stutzmann et al., 2009; Wardle et al., 2008). It was also shown that *FTO* variants are associated with increased energy intake, but not with changes in the weight of ingested food, suggesting that FTO plays a role in the regulation of food intake and food choice (Cecil et al., 2008). The role of *FTO*

variants in the regulation of resting metabolic rate remains unclear as some studies suggest there is an association while others do not (Cecil et al., 2008; Do et al., 2008; Haupt et al., 2008; Speakman et al., 2008). Collectively, available data favor the possibility that increased energy intake contributes to the increased adiposity and body weight in individuals with the *FTO* risk alleles. The association of *FTO* SNPs with obesity and the development of type 2 diabetes raised great interest into the functional role of FTO in metabolism. To this day, the biological function of FTO in energy homeostasis and whole body metabolism is not known.

Recently generated Fto-deficient mouse models exhibit reduce body weight and adipose tissue on a high-fat diet compared with the wild-type controls (Fischer et al., 2009; Church et al., 2009). The phenotypes in these mice are associated with increased energy expenditure and increased sympathetic activity. These finings clearly indicate that Fto is involved in the regulation of energy homeostasis. Fto is expressed in various tissues, and particularly it exhibits high levels of expression in the hypothalamus (Frayling et al., 2007; Fredriksson et al., 2008; Gerken et al., 2007; Stratigopoulos et al., 2008). Enhanced Fto expression in the hypothalamic ARC reduced food intake, while Fto knockdown in ARC resulted in increased food intake, in mice (Tung et al., 2010). Of particular interest, hypothalamic *Fto* mRNA levels are reduced by fasting, and feeding with a regular rodent chow or a high-fat diet increased *Fto* mRNA expression or activated Fto-expressing cells in the hypothalamus (Gerken et al., 2007; Olszewski et al., 2009; Tung et al., 2010). These findings suggest that Fto functions as a satiety factor and increased Fto expression in the hypothalamus is beneficial in reducing food intake and body weight. Consequently, if Fto plays such a role, its expression levels may be altered

in obesity and regulated by nutritional signals. However, these possibilities have not been addressed.

The hypothalamus plays a critical role in the regulation of energy balance and hypothalamic neurons expressing a variety of neuropeptides are involved in the regulation of energy homeostasis. These neurons are sensitive to nutritional signals including the adiposity signals insulin and leptin. In addition to these adiposity signals, glucose has been proven to be an important nutrient signal in regulating hypothalamic neuronal activity and gene expression. Fasting induces Fos expression in the ARC and re-feeding reverses fasting-induced Fos expression (Morikawa et al., 2004; Ueyama et al., 2004). Glucose injection also reverses the effect of fasting on Fos expression, indicating that glucose regulates hypothalamic activity, and a decline in blood glucose levels mediates fasting-induced changes in hypothalamic activity (Becskei et al., 2008). These findings indicate that glucose is involved in the regulation of hypothalamic gene expression and activity. Thus, hypothalamic glucose sensing plays a critical role in the regulation of metabolism and also plays a role in the regulation of Fto expression (Davis et al., 1981; Lam et al., 2007; Lam et al., 2005; Le Feuvre et al., 1991).

Little is known about the mechanism through which hypothalamic Fto regulates energy balance. Based on the co-expression of Fto and POMC in the hypothalamus and phenotypic similarities between *FTO* variants and *MC4R* variants, it has been suggested that hypothalamic FTO may function as a regulator of CNS melanocortin signaling, such as a regulator of POMC gene expression (Fischer et al., 2009; Loos et al., 2008; Olszewski et al., 2009; Stutzmann et al., 2009). *In vitro* studies have shown that FTO protein functions as an AlkB-like demethylase of 3-metT in single-stranded DNA and 3-

metU in single-stranded RNA (Gerken et al., 2007; Han et al., 2010; Jia et al., 2008). A mutation causing reduced Fto expression results in reduced catalytic activity in the presence of 3-methylthymine oligonucleotide substrate (Church et al., 2009). A recent study showed that both obese and lean humans being carriers for a naturally occurring FTO mutation in Arg96His results in the inability of FTO to function as a demethylase (Meyre et al., 2010). *POMC* expression levels are strongly influenced by the methylation pattern of the promoter region, and hypermethylation of the *POMC* gene is associated with obesity or overweight in both humans and rodents (Coupe et al., 2010; Newell-Price, 2003). These findings led to the assumption that Fto may increase *POMC* expression by promoting demethylation of the (hyper)methylated *Pomc* promoter.

It is of great interest to know whether Fto plays a role in the regulation of energy balance by regulating the activity of hypothalamic melanocortin pathway, and whether glucose is involved in the regulation of hypothalamic Fto expression. This is especially important since such a relationship would indicate that Fto neuronal regulatory mechanisms may be impaired in obesity based upon the following. Glucose-sensing neurons are fewer in number and show abnormal responses to glucose in obesity rats (Song et al., 2001). Central injection of glucose reduces food intake, but this feeding response is absent in obese rats (Tsujii & Bray, 1990). Glucose-induced hypothalamic Fos expression is reduced in obese-prone rats compared with obese-resistant rats (Levin et al., 1998). Consistent with these findings in rats, glucose-induced hypothalamic activation is attenuated in obese humans compared with non-obese healthy individuals (Matsuda et al., 1999). These data clearly indicate that hypothalamic glucose sensing is impaired in obesity.

Based upon these findings, it was hypothesized that (i) hypothalamic Fto expression is regulated by nutritional signals and glucose plays a major role in the regulation of Fto expression, (ii) Fto plays a role in hypothalamic nutrient sensing as part of the central melanocortin system, and (iii) obesity is associated with impairments in nutritional regulation of hypothalamic Fto expression. The present thesis study was designed to address these hypotheses.

Materials and Methods

Animals

Male C57BL/6 mice and male *ob/ob* mice (C57BL/6J background) were obtained from the Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Montreal, QC). Young mice (8-10 weeks of age) were used, because lipid and glucose metabolic states are normal at this age in this strain of mice. Only male mice were used in the present study to avoid the effects of gender and sex steroids on metabolism. *ob/ob* mice on the C57BL/6 background are known to be transiently hyperglycemic as pancreatic β-cells can compensate with increased insulin production (Reviewed in: (A. J. Kennedy et al.). This is in support of our findings that 48-h fasted *ob/ob* mice exhibit reduced glucose levels compared with the hyperglycemic *ad libitum* fed controls (Table 10)

To induce diabetes, mice were injected intraperitoneally (i.p.) with streptozotocin (STZ, 100-125 mg/kg b.w.). The STZ treatment was performed by Dr. Arnold Leckstrom. STZ (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving in citrate buffer (pH 4.5) immediately before administration. Control mice received i.p. injection of citrate buffer. Mice with blood glucose higher than 200 mg/dl by 4 days after STZ treatment were used as diabetic animals, and sacrificed 4 weeks after STZ treatment.

Mice were individually housed under a 12:12 light/dark cycle (lights on at 0600 h) with free access to standard rodent chow pellets (Prolab RMH 3000, 4.5% fat by weight, Ralston Purina). Water was available throughout the experiment. All studies were approved by the Institutional Animal Care and Use Committee (University of Manitoba).

I.c.v. cannulation

Mice were anesthetized with isoflurane with an induction dose of ketamine (100 mg/kg b.w., i.p.) and xylazine (10 mg/kg b.w., i.p.). A stainless steel guide cannula (o.d.: 0.64 mm, i.d.: 0.33 mm) was stereotaxically implanted into the lateral ventricle with the coordinates 0.4 mm posterior to the bregma, 1.0 mm lateral from the midline and 1.8 mm deep to the dura in accordance with the atlas of Paxinos (Paxinos & Franklin, 2001). After full recovery from the surgery, the localization of the cannula was verified by assessing drinking behavior in response to the i.c.v. administration of angiotensin II (100 ng in 1 µl, Sigma-Aldrich, St. Louis, MO). Only mice responding with a robust drinking behavior were used for the experiment.

I.c.v. treatment and tissue collection

Mice fasted for either 30-h or 48-h, received respectively 5 i.c.v. (every 6 h) and 9 i.c.v. (every 5 h and 20 min) injections of either saline or 100 µg of glucose. The 100 µg dose has been shown not to affect circulating glucose levels in the same strain of mice (Minokoshi et al., 2004). Control mice were fed *ad libitum* and treated with saline i.c.v. injections. I.c.v. injections were in a total volume of 1 µl over 30 seconds, and an injection pipe was left inside the cannula for another 30 seconds and removed from the cannula. On the day of sacrifice, mice were sacrificed 1 h after the last i.c.v. injection (between 0900 h and 1100 h).

I.p. treatment

Mice fasted for either 30-h or 48-h, received a single i.p. injection of either saline or glucose (2 mg/g body weight). Control mice were fed *ad libitum* and treated with a saline i.p. injection. I.p. injections were in a volume of 100 µl/g body weight administered over 30 seconds. Mice were sacrificed 2 h after the i.p. injection (between 0900 h and 1100 h). For immunohistochemical analysis, mice were perfused transcardially 2 h after the i.p. injection (between 0900 h and 1100 h).

Assays

A drop of blood was collected from the tail vain prior to anesthesia and blood glucose levels were measured using a glucose meter (ELITE XL, Bayer HealthCare, Mishawaka, IN). Serum insulin concentrations were determined by Mercodia Ultrasensitive Mouse Insulin ELISA immunoassay (Mercodia, AB Sylveniusgatan, Sweden). Performance characteristics were: detection limits 0.025 µg/L to 6.5 µg/L, and 100% specificity to mouse insulin. The intra-assay %Coefficient of Variation (%CV) was 2%-8%. Serum leptin concentrations were determined by Mouse Leptin ELISA immunoassay (R&D Systems, Minneapolis, MN USA). Performance characteristics were: detection limits 22 pg/mL to 4000 pg/mL, and 100% specificity to mouse leptin. The intra-assay % CV was 4%-10%. Standard controls and samples were assayed in duplicates.

RNA analysis

Total RNA was extracted from liver or white adipose tissue in TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 5 µg of total RNA, using

SuperScriptII RNaseH reverse transcriptase and random primer (Invitrogen), and diluted 1:100. Gene expression levels were measured using the ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA). Real-time PCR was performed in a 25-µl reaction containing SYBR green (Power SYBR Green Master Mix, Applied Biosystems), primer mixture (final concentration: 0.2 mmol/L) and 5 µl of cDNA for 40 cycles at 94 ˚C for 15 sec; 60 ˚C for 20 sec and 72 ˚C for 30 sec. All primers were designed using the Primer Express Software (Ver. 3.0, Applied Biosystems) and synthesized by Invitrogen. Data were analyzed by the ΔΔCt method using the ABI 7500 Fast System SDS software package (Ver. 1.3.1, Applied Biosystems) and mRNA levels were normalized to β-actin mRNA levels. Data are expressed as means (% of the control group) ± SEM. All reactions were performed in triplicates and the coefficient of variation was less than 5% for each triplicate.

Perfusion and brain sectioning

Mice were anesthetized with i.p. injection of 40% avertin (0.02 mL/g of body weight). Under deep anesthesia, the thoracic cavity was opened to target the left ventricle. Mice were perfused transcardially (using a blunted 25G5/8 needle on a 10 mL syringe) with 2-3 mL of ice cold (4 °C) of 0.1M phosphate buffer (PBS), pH 7.3 containing 1 Unit/mL of heparin. Next, mice were perfused with 40 mL of freshly prepared ice cold 0.2 M PBS-4% para-formaldehyde, pH 7.3. Mice were decapitated and brain blocks were stored at 4 °C in 2% para-formaldehyde for 3 h. Brain blocks were rinsed twice in 0.1M PBS followed by an overnight incubation in cryoprotectant (0.1M PBS, 10% sucrose, 0.04% sodium azide) at 4 °C. Brain blocks were rapidly frozen using the cold stream of

nitrogen gas immediately prior to sectioning. Coronal brain cryosections of 30 µm thickness were stored in cryoprotectant (0.1M PBS, 30% sucrose, 1% polyvinylpyrollidine and 30% ethylene glycol) at -20 °C until ready for immunofluorescence.

Immunofluorescence

Single immunofluorescence:

Cryosections were washed in 0.01M PBS for 3-h at room temperature and permeabilized with 0.01M PBS containing 0.5% Triton-X-100 for 30 min. at room temperature. Primary antibody incubation was performed overnight (16-h) at 4 °C with polyclonal rabbit Fto (1:2500 dilution; a gift from Dr. Julia Fischer) diluted in 0.01M PBS containing 2% bovine serum albumin, and subsequently washed three times for 1 h in 0.01M PBS. Secondary antibody incubation was performed for 2 h at room temperature in the dark with Cy3-conjugated goat anti-rabbit (1:200 dilution, Jackson ImmunoResearch Laboratories), and subsequently washed three times for 15 min. in 0.01M PBS. Sections were placed on superfrost slides (Fisherbrand, Fisher Scientific, USA) and mounted using an aqueous antifade mounting medium containing DAPI (Fluoro Gell II with DAPI, Electron Microscopy Sciences, Hatfield, PA, USA). Negative control procedures involved the absence of the primary antibody with the inclusion of the secondary antibody for the purpose of showing the absence of inappropriate cross-reaction between the primary and secondary antibodies.

Double immunofluorescence:

Cryosections were washed in 0.01M PBS for 3 h at room temperature and permeabilized with 0.01M PBS containing 0.5% Triton-X-100 for 30 min. at room temperature. Primary antibody incubation was performed overnight (16-h) at 4 °C with polyclonal guinea pig-Fto (1:2500 dilution; a gift from Drs. Julia Fischer and Urlich Rüther) and simultaneously with either polyclonal rabbit cFos (1:5000 dilution; EMD Biosciences, San Diego**,** CA, USA), monoclonal rabbit POMC (1:1000 dilution, POMC (27-52), Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) or monoclonal rabbit AgRP (1:200 dilution, AgRP (82-131), Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) diluted in 0.01M PBS containing 5% normal donkey serum and 5% normal goat serum. Cryosections were subsequently washed three times in 0.01M PBS for 1 h at room temperature. Secondary antibody incubation was performed for 2 h at room temperature in the dark with the following fluorescent dyes. Combination 1: Cy3-conjugated donkey anti-guinea pig (1:200 dilution, Jackson ImmunoResearch Laboratories) against guinea pig Fto, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch Laboratories) for either POMC or AgRP. Combination 2: FITC-conjugated donkey anti-guinea pig (1:200 dilution, Jackson ImmunoResearch Laboratories) against guinea pig Fto, and Cy3-conjugated goat anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch Laboratories) for either POMC or AgRP. Cryosections were subsequently washed three times for 15 min. in 0.01M PBS. Sections were placed on superfrost slides (Fisherbrand, Fisher Scientific, USA) and mounted using an aqueous antifade mounting medium containing DAPI (Fluoro Gell II with DAPI, Electron Microscopy Sciences, Hatfield, PA, USA). Negative control

procedures involved the absence of the primary antibodies with the inclusion of the secondary antibody for the purpose of showing the absence of inappropriate cross-reaction between the primary and secondary antibodies.

Histological quantification

Immunofluorescent images were viewed using a Zeiss Axioskop2 fluorescence microscope with image capture using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, Ontario Canada). The same scanning parameters and exposure intensity were used for each immunofluorochrome for all images viewed on the same day. Images were adjusted for contrast to remove "empty" pixels by applying the same parameters for each chromophore (Photoshop 6.0 Adobe Systems, San Jose, CA, USA).

Counts for immunopositive cells

Quantitative analysis of immunopositive cells for either Fto, POMC, AgRP or cFos was conducted by manually counting cells that were positive for DAPI staining using the NIH ImageJ software (version 1.43). Sections were blindly counted in random order and in duplicate. Colocalization analysis was performed by manually counting double-labelled cells from the double labelled images overlapping the single labelled images. Cells that stained for DAPI and either POMC, AgRP or cFos were analyzed to assess the percent of POMC, AgRP or cFos cells that colocalized with Fto.

Image analysis (semiquantitavive automated analysis)

Immunofluorescent images were analyzed using the NIH ImageJ software (version 1.43). The active image was converted into an 8-bit grayscale image, followed by adjustment for **threshold** to yield the pictures with highest level of contrast between particles of interest (in black) and background (in white). For image processing watershed segmentation was used to automatically separate overlapping particles. Image analysis was conducted for mean gray value, intensity, integrated density, on the selected region of interest. The area of interest was selected using the free-line tool option to surround the region of Fto immunopositive cells. "**Area"** of selection is presented in square pixels. "**Mean Gray Value"** is the sum of gray values of all the pixels found within the selected region divided by the total number of pixels, thus it represents a semiquantitative measure of mean fluorescence intensity. "**Integrated Density"** is the result of the product of Area selected times the Mean Gray Value, thus integrated density is a semiquantitative measure of total protein quantity within the selected region. Surface Plot analysis was used to display a three-dimensional graph of the selected region of interest, visually representing the intensity (height of a pixel), density and distribution of immunopositive cells. This analysis yields a three-dimensional graph of the intensity of pixels, where the y-axis represents the height of individual signal intensities (ie. amount of protein staining per cell) and the x-axis represents the distribution area of the signal intensity (ie. the area protein staining is detected).

Statistical analysis

Values are presented as means ± SEM. Comparisons among three groups were performed using one-way ANOVA followed by Tukey-Kramer test. Food intake and body weight data in *ob/ob* mice study were analyzed by two-way ANOVA. Kruskal-Wallis test was performed and the Bonferroni-corrected Wilcoxon test was used for post hoc analysis when the data were not normally distributed. Comparisons between two groups were performed by Wilcoxon test when the data were not normally distributed. Correlation analyses were performed between blood glucose levels and the number of Fto-immunopositive cells or cFos-immunopositive cells. In all cases, differences were taken to be significant if *P*-values were below 0.05.

Results

Region-specific expression of *Fto* and *ChREBP* mRNA in the mouse brain

Using RT-PCR (Fig. 18A) and Northern blot analysis (Fig. 18B), it was confirmed that *Fto* mRNA expression is present in the brain, white adipose tissue and liver tissue of C57BL/6 mice. Specifically, *Fto* mRNA expression was detected in all brain regions examined including the hypothalamus, thalamus, prefrontal cortex, cortex, hippocampus, brain stem, cerebellum and spinal cord (Fig. 18B). *ChREBP* mRNA expression was also detected with RT-PCR in the liver and hypothalamus (Fig. 18C).

The effect of fasting and glucose on *Fto* and *ChREBP* mRNA expression in the mouse hypothalamus – a pilot study

To determine whether hypothalamic *Fto* and *ChREBP* mRNA levels are regulated by nutritional condition, the effect of glucose treatment was examined on the expression of hypothalamic *Fto* and *ChREBP* mRNA in C57BL/6 mice following an overnight (16-h) fast. A single i.c.v. glucose injection significantly increased hypothalamic *Fto* mRNA expression, as compared with the aCSF-treated control group (Fig. 20). In contrast, under the same conditions, glucose did not affect *ChREBP* mRNA expression (Fig. 20). Both aCSF and glucose treated groups had no difference in body weight and blood glucose levels at the time of sacrifice (Table 4). Based on the findings of this pilot study

(Fig. 20-21), it was decided to pursue further investigation on the nutritional regulation of hypothalamic Fto expression in subsequent studies.

To determine if these effects were dose-dependent, a study was conducted to determine hypothalamic Fto expression in mice following a prolonged (30-h) fast. This experiment resulted in a significant reduction in body weight, blood glucose, and serum insulin levels compared with *ad libitum* fed control group (Table 5). Serum leptin levels were below the detection limit in fasted mice (Table 5). The 30-h fast significantly reduced *Fto* mRNA levels in the hypothalamus by 53% compared with the *ad libitum* fed control group (Fig. 22). *Fto* mRNA levels in the cortex were not affected by fasting (Fig. 22).

The effect of i.p. glucose on hypothalamic and peripheral *Fto* mRNA expression in fasted mice

To determine if reduced blood glucose levels contributes to the fasting-induced reduction in hypothalamic *Fto* mRNA expression, we restored glucose levels with a single i.p. injection of glucose (2 mg/g of body weight) following a 30-h fasting. Compared with the *ad libitum* fed saline-treated control group, both saline and glucose-treated fasted groups had a significant reduction in body weight, WAT weight, and serum insulin levels (Table 6). Serum leptin levels were below the detection limit of the assay in saline- and glucose-treated fasted groups (Table 6). Blood glucose levels were significantly reduced with fasting and restored to fed levels with a single i.p. injection of glucose, as compared with the fasted saline-treated group (Table 6). Fasting-induced reductions in

hypothalamic *Fto* mRNA expression were reversed by 53% as compared with saline-treated *ad libitum* fed mice, when blood glucose levels were restored to the *ad libitum* fed levels (Fig. 23B). Glucose injection (i.p.) significantly increased *Fto* mRNA levels by 50% compared with i.p. saline injection in 30-h fasted mice (Fig. 23B). *Ft* mice have a deletion of 1.6 Mbp in chromosome 8 which results in the loss of *Fto*, as well as, 5 neighbouring genes, *Ftm, Ftl*, and the Iroquois B cluster (*Irx3, Irx5* and *Irx6*) (genetic diagram in Fig. 23A). To determine if the expression of these genes is also regulated by nutritional states, the mRNA expression levels of these genes were also measured in the present study. Although, fasting had no effect on any of the neighbouring genes, glucose had a significant stimulatory effect on *Irx5* mRNA levels (59% increase vs. saline-treated fasted mice, Fig. 23C). In contrast to the hypothalamus, fasting significantly increased *Fto* mRNA expression in the liver and skeletal muscle by 160% and 219%, respectively, while glucose had an inhibitory effect on *Fto* mRNA only in the liver (61% reduction vs. saline-treated fasted mice, Fig. 24). No changes in *Fto* mRNA expression were detected in WAT or BAT by either fasting or glucose treatment (Fig. 24).

The effect of i.c.v. glucose on hypothalamic *Fto* mRNA expression in fasted mice

The effect of multiple (5) i.c.v. injections of glucose on *Fto* mRNA expression was investigated in C57BL/6 mice fasted for 30 hours. Compared with the saline-treated control group, i.c.v. glucose treatment significantly increased the levels in blood glucose without any effect on body weight, or circulating insulin levels (Table 7). Serum leptin levels were again below the detection limit of the assay in both groups following a 30-h

fast (Table 7). Compared with the saline-treated control group, i.c.v. glucose injection (100 μg) significantly increased hypothalamic *Fto* mRNA levels, reaching an expression of 99% compared with saline injection (Fig. 25). The same glucose treatment did not cause any significant changes in *Fto* mRNA levels in the cortex (Fig. 25).

To investigate the dose-response nature of this effect a study was conducted having an extended duration of fasting (48-h) and number of i.c.v. injections (N = 9) of glucose. Compared with the *ad libitum* fed group, fasting significantly reduced body weight and blood glucose levels in both the saline and glucose treated groups (Table 8). Consistent with the results in the 30-h fasting studies (Fig. 23B and 25), hypothalamic *Fto* mRNA levels were significantly reduced by 48-h fast (Fig. 26). In contrast to the study using 30-h of fasting, under 48-h of fasting i.c.v. glucose was unable to restore hypothalamic *Fto* mRNA expression to *ad libitum* fed levels (Fig. 26). Under these conditions, WAT *Fto* mRNA expression levels were significantly reduced by fasting, but were not affected by i.c.v. glucose treatment (Fig. 27). Neither fasting nor glucose treatment caused significant changes in hepatic *Fto* mRNA expression (Fig. 27). The absence of an inhibitory effect of i.c.v. glucose treatment on hypothalamic *Agrp* mRNA expression (Fig. 26) suggests that this i.c.v. glucose treatment was insufficient to overcome the effects of prolonged fasting as seen in the previous studies.

The effect of fasting and glucose on Fto-immunopositive cells in the mouse hypothalamus

Fto expression in the mouse brain

The aforementioned studies analyzed *Fto* gene expression using the whole hypothalamic tissue. To further investigate the effect of nutritional states on Fto expression in specific hypothalamic regions, immunohistochemistry was used in the subsequent studies. To identify Fto-positive cells, three commercially available antibodies against Fto were tested and found to yield less than convincing staining of cells in mouse brain sections. Consequently, we obtained the anti-Fto from Dr. Rüther's group (Heinrich Heine University, Germany) which did not yield any staining in tissue sections from the Fto knockout mice (Fischer et al., 2009). Additionally, the negative control without the primary antibody or secondary antibody (Cy3 or FITC) showed no staining in mouse brain sections (Fig. 28). These results verify that the antibody is specific to Fto and the immunohistochemistry protocol used is optimal.

Immunofluorescence analysis of Fto protein indicated that Fto is specifically localized in the nucleus and is distributed abundantly in the brain (Fig. 29), as indicated by co-localization of Fto-positive staining and DAPI as it has been previously reported. Nuclear localization was confirmed when Fto (Cy3 or FITC) labelled cells co-localized with DAPI stained nuclear regions throughout the brain (Fig. 29). Fto-immunopositive cells were found in several areas of the brain including the hippocampus, dentate gyrus, paraventricular thalamic nucleus, lateral habenular nucleus, medial habenular nucleus. In the hypothalamus, Fto-positive cells were found in the paraventricular nucleus (PVN),

dorsomedial nucleus (DMN), ventromedial nucleus (VMN), the arcuate nucleus (ARC) and the retrochiasmatic area (RCh) (Fig. 29).

Despite Fto-immunopositive cells being abundantly present throughout the brain (Fig. 29), not every neuron expresses Fto as indicated in the hypothalamus by Fto-DAPI co-localization analysis (Fig. 30A). Surface plot analysis of the entire hypothalamic regions clearly showed in a three-dimensional way that the intensity and distribution of Fto-immunopositive cells is less than the DAPI-stained nuclei in the same brain region (Fig. 30B). Quantitative analysis indicated that Fto is present in 62%, 72% and 72% of the neurons within the ARC, VMN and DMN hypothalamic regions (Fig. 30C). This is more clearly demonstrated by using images at a higher magnification to perform surface plot analysis for the ARC, VMN or DMN. Compared with the DAPI staining, Fto-immunopositive cells are less in area of distribution and intensity (Fig. 30D). This suggests that Fto may function in selective neuronal populations.

The effect of fasting and glucose on hypothalamic Fto expression

Number of Fto-positive cells

To test whether hypothalamic Fto protein expression is regulated by nutritional conditions, the effect of fasting (30 h) on hypothalamic Fto expression was examined. Effect of i.p. glucose injection on hypothalamic Fto protein expression was also examined in 30-h fasted mice. Fasting significantly reduced the number of Fto-immunopositve cells in the ARC and VMN as compared with the *ad libitum* fed control group (Fig. 31A and 31B). I.p. glucose injection reversed the fasting-induced reduction

in ARC and VMN Fto expression (Fig. 31A and 31B). Within the ARC, the stimulatory effect of glucose on Fto immunoreactivity is observed in the caudal (Fig. 32B) but not the rostral region (Fig. 32A). There is a significant positive correlation between blood glucose levels and the number of Fto-immunopositive cells in the VMN (Fig. 33B), however, no such correlation exists in the ARC (Fig. 33A).

Fto protein levels (semi-quantitative analysis)

Fto-immunopositive cells exhibited differential cell density and labelling intensity in different nuclei of the hypothalamus in response to fasting and glucose treatments (Fig. 31 and Fig. 34). The area of Fto-immunopositive cell distribution did not differ in response to either fasting or glucose treatment when analyzed in the ARC, VMN, or DMN (Fig. 34B). Analysis of Fto protein levels, as determined by fluorescence intensity, revealed that compared with the *ad libitum* fed saline-treated group, fasting reduced the Fto protein levels only in the VMN but not the ARC. However, glucose treatment increased Fto protein levels in both ARC and VMN, as compared with the saline-treated fasted group (Fig. 34C). Total density of Fto protein was reduced by fasting and reversed by glucose treatment only in the VMN (Fig. 34D).

The effect of fasting and glucose on POMC- and AgRP-immunopositive cells in the mouse hypothalamus

In order to investigate the potential role of Fto in nutritional regulation of metabolism, immunofluorescence analysis of Fto, and the melanocortin neuropeptides POMC and AgRP, was undertaken in *ad libitum* fed mice and 30-h fasted mice subjected to either i.p. saline or glucose (2 mg/g body weight) treatment. As previously known, fasting reduced and glucose induced POMC-immunopositve cell number in the ARC (Fig. 36A and 36B). Under high magnification, it was shown that POMC-Fto co-expressing neurons (Fig. 37B) showed the same pattern of expression of POMC alone (Fig. 36B). In POMC neurons, Fto is localized within the nucleus (Fig 37A). Visual analysis indicated that compared with the *ad libitum* fed conditions, fasting reduced both the number of Fto- and POMC-immunopositive neurons, while glucose reversed the inhibitory effect of fasting (Fig. 36A, and Fig. 37A). Fasting reduced the number of Fto-immunopositive cells throughout the ARC, but is also reduced Fto-positive cells in the lateral peri-arcuate area, the cell-sparse area lateral to the ARC and ventral to the VMN (Fig. 36A). I.p. glucose injection reversed fasting-induced reduction in Fto-positive cells in these areas (Fig. 36A). The effect of fasting and glucose injection on POMC-positive cells was similar to that on Fto-positive cells (Fig. 36B). The number of cells expressing both Fto and POMC was significantly increased by glucose treatment (Fig. 37C). The extent of POMC-immunopositive cells co-expressing Fto was slightly reduced by glucose treatment (Fig. 37C).

When tested for the presence of Fto in AgRP neurons, Fto was also co-localized within the nucleus (Fig. 38A). Similar to previous studies, fasting increased AgRP-

positive cells in the medial part of the ARC. Compared with the *ad libitum* fed conditions, fasting increased the number of AgRP neurons (Fig. 38B) to the same extent as the number of Fto-AgRP co-localized neurons in the ARC (Fig. 39B). Glucose did not have a significant effect in reversing the number of either AgRP- or AgRP-Fto-immunopositive neurons (Fig. 39A and 39B). Furthermore, there were no changes in the level of co-localization of AgRP and Fto under these conditions (Fig. 39C).

The effect of fasting and glucose on Fto-immunopositive cells in the hypothalamus of obese mice

To assess the effects of 48-h fasting and glucose treatment in obese mice the study included wild-type and *ob/ob* mice that were subjected to the treatments of either *ad libitum* fed plus saline, fasting plus saline or fasting plus glucose. In wild-type mice both fasting saline and fasting glucose treated groups were significantly lower in body weight and body weight change, as compared with *ad libitum* fed saline control group (Table 9). Compared with the *ad libitum* fed saline control group, fasted saline-treated group had significantly lower blood glucose levels and i.p. glucose injection reversed blood glucose levels in fasted mice (Table 9). The same trend was observed in *ob/ob* mice, where both fasting saline and fasting glucose treated groups were significantly lower in body weight and body weight change, as compared with *ad libitum* fed saline control group (Table 10). In *ob/ob* mice blood glucose levels were significantly reduced by fasting and i.p. glucose injection partially reversed glucose levels in fasted mice (Table 10). It is important to note that *ob/ob* mice were hyperglycaemic compared with the wild-type mice under both

ad libitum fed and glucose treated conditions (Tables 9-10), suggesting that these *ob/ob* mice are likely glucose intolerant. The 2-day cumulative food intake was significantly higher in *ob/ob* mice (15.6 ± 0.5 g, $N = 10$) than the wild-type mice (10.0 ± 0.5 g, $N = 10$, $P < 0.05$, Student's *t*-test. These results show that the obese mouse model used for this study exhibits hyperphagia, hyperglycaemia and obesity.

Pattern of Fto expression in wild-type mice under 48 hour fasted and glucose treated conditions

In Figure 44A the expression pattern of Fto-immunopositive cells is depicted in the ARC and VMN of wild-type mice. There was a significant reduction in the number of Fto-immunoporitive cells in the ARC and VMN of fasted mice compared with *ad libitum* fed mice (Fig. 44B). Compared with the *ad libitum* fed conditions, there were clear trends of reduction in the area of Fto-immunopositive cells in the ARC and VMN by fasting, but the effect of fasting did not reach statistical significance (Fig. 44A, C). Of particular note is that although fasting reduces Fto expression uniformly throughout the ARC, it is evident that Fto is robustly reduced in the lateral peri-arcuate area and the vetrolateral VMN (Fig. 44A). Similarly, there was a trend of fasting-induced reduction in the integrated density of Fto-immunopositive cells in ARC (Fig. 44D). Fasting did not reduce the number or the area of expression that Fto-immunopositive cells are present in the DMN (Fig. 44A-C)**.** Visual analysis of the Fto-DAPI overlay images indicates that the reduction in Fto-immunopositive cell number is not due the absence of neuronal cells in the ARC or VMN area (Fig. 44A). As indicated above, although the region of Fto

reduction in the ARC appears to be uniformly throughout the nucleus, it is evident by the Fto-DAPI overlay images that Fto is reduced mainly in the area lateral to the ARC and ventral to the VMN (lateral peri-arcuate area) (Fig. 44A). Glucose treatment significantly increased the number, the area, and the integrated density of Fto-immunopositive cells in ARC compared with saline injection (Fig. 44B-D). The number of Fto-immunopositive cells in VMN was significantly increased by glucose treatment (Fig. 44B). I.p. glucose injection did not cause any significant effects on the area of Fto-immunopositive cells in VMN and DMN (Fig. 44C). Interestingly, glucose treatment increased Fto-immunopositive cells both in the medial ARC (near the third ventricle) and in the lateral peri-arcuate area and the vetrolateral VMN (Fig. 44A). Furthermore, the distribution of Fto protein levels (Fto intensity) as depicted by the hight of bars in the surface plot analysis (Fig. 45) follows the same trend as that observed in Fto-immunopositive cell number (Fig. 44B) and distribution (Fig. 44C) in the VMN.

In the VMN, although computational analysis of the total area of Fto-immunopositive cell distribution did not detect any significant differences between the groups (Fig. 44C), visual inspection of Fto-immunoreactivity in VMN indicated that the significant reduction of Fto-immunopositive cell number is mainly in the ventrolateral subnucleus of the VMN (Fig. 44A). This observation is further substantiated by the surface plot analysis which indicates that compared with the *ad libitum* fed control group, fasting reduces the distribution of Fto-immunopositive cells in the VMN, in particular towards the right lower corner of the plot corresponding to the ventrolateral part of the VMN (Fig. 45).

The effect of obesity and diabetes on hypothalamic *Fto* mRNA expression

Streptozotocin (STZ) treatment caused a trend of reduced body weight (Fig. 40A) and diabetic condition with significantly increased circulating glucose and NEFA and reduced insulin and leptin levels (Fig. 40B-E). Hypothalamic *Fto* mRNA levels were not different between non-diabetic control and STZ-induced diabetic mice (Fig. 40F).

Hypothalamic *Fto* mRNA expression was found to be significantly increased in *ob/ob* mice by 156% compared with wild-type mice (Fig. 41). Immunofluorescence analysis showed a similar increase in Fto expression in the hypothalamus of *ob/ob* mice (Fig. 43). The number of Fto-immunopositive cells was significantly increased in VMN and ARC, but not DMN, of *ob/ob* mice compared with wild-type mice under the *ad libitum* fed condition (Fig. 43A). The area of Fto-immunopositive cell was significantly increased in VMN of *ob/ob* mice compared with wild-type mice (Fig. 43B). There was no significant effect of *ob/ob* genotype on the area of Fto-immunopositive cell in ARC and DMN (Fig. 43B). This observation is further substantiated by the surface plot analysis which indicates that Fto expression is more widely distributed within the ARC or VMN in *ob/ob* mice compared with wild-type mice (Fig. 43C). Specifically, a larger number of Fto-immunopositive cells were found in the lateral peri-arcuate area, the dorsomedial ARC, and the ventrolateral VMN in *ob/ob* mice compared with wild-type mice (Fig. 43C).

Pattern of Fto expression in *ob/ob* mice under 48 hour fasted and glucose treated conditions

In Figure 46A, the expression pattern of Fto-immunopositive cells is depicted in the ARC and VMN of *ob/ob* mice. Compared with the *ad libitum* fed conditions, fasting significantly decreased the area of Fto-immunopositive cells without significant changes in the number of Fto-immunopositive cells in the VMN of *ob/ob* mice (Fig. 46B-C). Visual analysis of the Fto-DAPI overlay images indicates that the reduction in Fto-immunopositive cell number is not due to the absence of neuronal cells in the VMN (Fig. 46A). Although, the number and the area of Fto-immunopositive cells were not altered in the ARC under fasted conditions (Fig. 46B-C), the integrated density of Fto-immunopositive cells was significantly reduced in ARC by fasting (Fig. 46D). Fasting did not cause any significant changes in Fto expression in DMN (Fig. 46B-C). The level of Fto protein expression in ARC, as assessed by a surface plot analysis, showed a trend in reduction by fasting (Fig. 47). Glucose treatment had no effect on the number, area, or integrated density of Fto-immunopositive cells in ARC, VMN, and DMN (Fig. 46B-D). Surface plot analysis showed glucose to have a slight increase in Fto protein levels in the ARC and VMN without any great effect on Fto distribution (Fig. 47).

Correlation analysis indicated that there is a significant positive correlation between Fto-immunopositive cell number and blood glucose levels in the ARC, but not in the VMN, of wild-type mice (Fig. 48A and 48B). In *ob/ob* mice, no positive correlation was found between Fto-immunopositive cell number and blood glucose levels in either ARC or VMN (Fig. 48A and 48B).

Fto-colocalization with POMC and AgRP in wild-type and *ob/ob* mice under 48 hour fasted and glucose treated conditions

High magnification imaging (Fig. 49A) and surface plot analysis (Fig. 49B) of the ARC region known to be expressing the majority of POMC and AgRP neurons (near the third ventricle) clearly shows that the inhibitory effects of fasting and stimulatory effects of glucose on Fto-immunopositive cell number found in the wild-type mice is not evident in *ob/ob* mice. Similar to the wild-type mice fasted for 30 hours, the number of POMC-positive neurons and POMC-Fto co-expressing neurons is decreased under 48 hour fasted conditions, and slightly increased after glucose injection (Fig. 50 and Fig. 52B). Analysis of the immunofluorescence staining in *ob/ob* mice reveals the number of POMC-expressing neurons to be reduced when compared with the wild-type mice (Fig. 51 and Fig. 52B), while the number of POMC neurons co-expressing Fto remains the same (Fig. 51). The expression pattern of AgRP in wild-type mice under 48 hour fasted conditions or glucose treated conditions was the same as that in wild-type mice fasted for 30 hours, where compared with the low expression of AgRP under *ad libitum* fed conditions, fasting greatly increased and glucose decreased the number of AgRP-immunopositive cells. Under these conditions almost all AgRP expressing neurons co-expressed Fto (Fig. 53 and Fig. 55A and 55B). As expected, in *ob/ob* mice the number of AgRP neurons is increased compared with the wild-type mice under *ad libitum* fed, fasted and glucose-treated conditions (Fig. 54 and Fig. 55B). Furthermore, image analysis indicated that there was no difference in the intensity of Fto protein in cells co-expressing either POMC or AgRP either in wild-type or *ob/ob* mice (Fig. 52A and 55A).

The effect of fasting and glucose on the activation Fto-immunopositive neurons of normal and obese mice

To investigate whether Fto expressing neurons are activated in response to alterations in metabolic states, the expression of cFos was assessed in the hypothalamuic Fto-expressing neurons of wild-type and *ob/ob* mice that were either subjected to the treatments of *ad libitum* fed saline, fasted saline or fasted glucose (Fig. 56). In wild-type mice, fasting significantly increased the number of cFos-immunopositive cells in the ARC (Fig. 57A). The number of cFos-immunoreactive cells in the ARC was not altered by glucose treatment in wild-type mice. In *ob/ob* mice, the number of cFos-immunopositive cells was significantly increased compated with wild-type mice under *ad libitum* fed condition, and fasting did not cause a further increase in the number of cFos-immunopositive cells in the ARC (Fig. 56 and 57A). Glucose treatment signifcantly reduced the number of cFos-immunopositive cells in the ARC of *ob/ob* mice. Despite a significant reduction in blood glucose levels (Table 10) in response to the fasted condition, fasting did not cause a significant change in the number of cFos-immunopositive cells in *ob/ob* mice. In contrast to the findings in the ARC, there was no significant effect of either fasting or glucose treatment on the number of cFos-immunopositive cells in the VMN of both the wild-type and *ob/ob* mice (Fig. 58A). This suggests that either the effect of the treatment was not adequate enough to reach the threshold of cFos expression in the VMN, or that cFos expression may not serve as an early marker of neuronal activation in the VMN.

The percent of Fto-immunopositive cells that were also positive for cFos showed the same pattern as that of cFos alone in the ARC and VMN of both the wild-type and *ob/ob* mice (Fig. 57B, and Fig. 58B). To assess the effect of blood glucose levels on hypothalamic cFos expression, correlation analyses between blood glucose levels and the number of cFos-immunopositive cells were performed in saline-treatd and glucose-treated fasted groups. A significant positive correlation between cFos-immunoreactive cells and blood glucose levels was seen in the ARC, but not the VMN of wild-type mice (Fig. 59A and 59B). Under these conditions, the *ob/ob* mice lacked any correlation between cFos and blood glucose levels in either the ARC or VMN (Fig. 59A and 59B). In both ARC and VMN all cFos-immunoreactive cells expressed Fto. Collectively, these findings suggest that an increase in circulating glucose levels can have a stimulatory effect in neurons mainly in the ARC of 48 h fasted mice, and this effect is lost in obesity.

Discussion

ChREBP and *Fto* mRNA expression in the mouse hypothalamus

The hypothalamus has become an important regulatory center where multiple nutrient sensing networks converge to regulate whole body energy homeostasis. Within the hypothalamus, several nutrient-sensing neurons have been identified to be responsive to nutrients, such as glucose, fatty acids and amino acids (Morton et al., 2006). ChREBP, is a glucose sensing transcription factor that induces glycolytic and lipogenic gene expression in the liver in a glucose-dependent manner (Dentin et al., 2005). Genetic variants of the *FTO* gene are associated with human obesity and FTO plays a role in DNA demethylation, leading to the hypothesis that FTO plays a role in the regulation of energy homeostasis partly by altering nutrient-sensitive hypothalamic gene expression (Frayling et al., 2007; Gerken et al., 2007). Prior to the pursuit of this study, it was unknown as to whether ChREBP is expressed in the hypothalamus and whether its expression is regulated by nutritional states in the brain. RT-PCR analysis proved that *ChREBP* mRNA is expressed in the hypothalamus for the first time. Consistent with the results by other groups, *Fto* mRNA, as assessed by RT-PCR and Northern blot analysis, is ubiquitously expressed throughout the brain and especially in hypothalamic regions known to have regulatory effects on metabolism (Fig. 18A and 18B) (Dina et al., 2007; Frayling et al., 2007; Gerken et al., 2007; Peters et al., 1999). These data support the possible role for ChREBP and Fto in the hypothalamic regulation of energy balance (Fig. 18B and 18C, respectively). To investigate the nutritional response of hypothalamic expression of these genes, a pilot study was conducted assessing the acute effects of

glucose under fasted conditions. In overnight fasted mice i.c.v. glucose treatment did not yield a significant effect on hypothalamic *ChREBP* mRNA expression (Fig. 20). In contrast, under the same conditions, glucose had a significant stimulatory effect on hypothalamic *Fto* mRNA expression (Fig. 20). These findings support the hypothesis that hypothalamic Fto expression is regulated by nutritional states and that Fto may possibly have a regulatory function in the hypothalamic regulation of energy homeostasis. Thus, these findings geared the course of further investigation to assess the nutritional regulation of hypothalamic Fto expression in the mouse hypothalamus.

Fto expression pattern in the mouse hypothalamus

It has been reported that Fto localizes to the nucleus of neurons and is not expressed in astrocytes or glial cells (Fischer et al., 2009; Fredriksson et al., 2008). Fto is abundantly expressed throughout the brain (Fig. 29). Quantitative analysis of Fto-DAPI overlapping cells showed that Fto is present in 62%, %72 and %72 of the neurons in the ARC, VMN and DMN, respectively (Fig. 30C). Qualitative analysis (surface plot analysis) indicated that the area of distribution of Fto-expressing cells and the intensity of Fto-expressing cells are lower compared with those of the DAPI-stained cells in these hypothalamic regions (Fig. 30D). These findings suggest that Fto is expressed in selective neuronal populations in the hypothalamus and plays specific roles in each neuronal population.

Effect of fasting on hypothalamic *Fto* mRNA expression

To investigate the possibility that Fto has a role in the nutritional pathways of the CNS regulating whole body metabolism, it is important to examine the expression pattern of Fto in the brain in response to changes in nutritional states. To address this hypothesis, *Fto* mRNA expression was examined under fasted conditions in mice. A 30-h fast significantly reduced *Fto* mRNA levels in the hypothalamus (Fig. 22). The lack of a fasting-mediated effect on *Fto* mRNA expression in the cortex (Fig. 22) indicates that the nutritional effects on Fto might be specific to the nutrient sensing neuronal pathways found in the hypothalamus. This further indicates that Fto may be part of hypothalamic neuronal pathways that monitor the amount of nutrient-related signals available in the body.

To further determine whether hypothalamic Fto protein levels behave in a similar pattern as in *Fto* mRNA in response to nutritional cues, Fto-immunopositive cells were visualized using immunofluorescence analysis. Consistent with the reports on *Fto* mRNA expression, Fto-immunopositive cells were found in many areas in the hypothalamus including ARC, VMN, DMN, PVN, and RCh (Fredriksson et al., 2008; Gerken et al., 2007; Olszewski et al., 2009; Stratigopoulos et al., 2008). Similar to the *Fto* mRNA expression, fasting significantly reduced the number of Fto-immunopositive cells in the VMN. Fasting also caused a significant reduction in the number of Fto-immunopositive cells in the caudal ARC, but not in the rostral ARC. It has been demonstrated that cells respond differently to nutritional signals depending on the area (rostral versus caudal) being localized within the ARC (Mizuno et al., 1998; Schwartz et al., 1997). These findings suggest that, both hypothalamic *Fto* mRNA and protein

change their expression levels in response to changes in body's metabolic states. Consistent with the present findings, it was reported that fasting reduces *Fto* mRNA in the ARC and feeding can stimulate Fto-expressing cells in the hypothalamic ARC and PVN (Gerken et al., 2007; Olszewski et al., 2009). Since, fasting causes a variety of neuroendocrine and metabolic changes, such as declines in leptin, insulin, glucose, and elevations in glucocorticoids and FFA, changes in these nutritional and hormonal signals may contribute to the fasting-induced reduction in hypothalamic Fto expression which can also play a role in feeding-induced activation of Fto expression.

Effect of i.p. glucose on hypothalamic Fto expression in fasted mice

It has been proposed that hypothalamic glucose sensing plays a critical role in the regulation of energy homeostasis. To determine if the decline in blood glucose mediates the effect of fasting on hypothalamic Fto expression, the effect of i.p. glucose injection on Fto expression was examined in fasted mice. Fasting significantly reduced the levels of *Fto* mRNA in the hypothalamus and the number of Fto-immunopositive cells in the (caudal) ARC and VMN, but not in DMN, and i.p. glucose treatment reversed this inhibitory effect of fasting (Fig. 23B). These findings indicate that glucose stimulates the mRNA expression levels of *Fto* and the number of hypothalamic neurons expressing Fto in a region-specific manner. To test whether there is a possible relationship of *Fto* glucose sensing response with any of its neighboring genes known to be deleted in the *Ft* mouse, the effect of fasting and glucose treatment was examined in *Fts, Ftm, Irx3, Irx5* and *Irx6* mRNA expression. Fasting had no effect on any of the neighbouring genes;

however glucose did have a stimulatory effect on *Irx5* mRNA (Fig. 23C). Irx5-deficient mice, similar to Fto-deficient mice, have reduced body weight compared with wild-type mice, suggesting a possible role for Irx5 in the regulation of metabolism (Cheng et al., 2005; Fischer et al., 2009).

Neuronal activity in the ARC, as assessed by c-Fos induction, is known to be increased by fasting (Morikawa et al., 2004; Ueyama et al., 2004). Both refeeding and glucose injection reversed the fasting-induced increases in ARC c-Fos expression, suggesting that glucose plays a role in mediating the effect of fasting on hypothalamic activity (Becskei et al., 2008; Shu et al., 2003). In the present study, i.p. glucose injection increased hypothalamic Fto (both mRNA and protein) expression and blood glucose levels. Additionally, there were significant positive correlations between blood glucose levels and hypothalamic *Fto* mRNA or protein levels. These data support the hypothesis that a rise in circulating glucose increases hypothalamic Fto expression and a reduction in circulating glucose levels, at least partly, mediates the inhibitory effect of fasting on hypothalamic Fto expression.

Leptin-independent regulation of hypothalamic Fto expression

Fasting reduced hypothalamic *Fto* mRNA and protein levels in the present study, suggesting that hypothalamic Fto expression is regulated by nutritional states. Fasting is associated with a variety of neuroendocrine changes and one of which is a decline in circulating leptin levels (Ahima et al., 1996). Expression of several hypothalamic neuropeptide-encoding genes is regulated by leptin. Of particular importance,

hypothalamic *Pomc* mRNA is increased and *Agrp* mRNA is reduced by leptin treatment *in vivo* (Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997; Thornton et al., 1997). It has been demonstrated that leptin treatment reverses fasting-induced changes in hypothalamic nutrient-sensitive gene expression, supporting the role for leptin in mediating the effect of fasting on hypothalamic gene expression (Ahima et al., 1996; Fekete et al., 2006). Therefore, one obvious hypothesis was that a decline in circulating leptin mediates the effect of fasting on hypothalamic Fto expression. However, fasting may also regulate hypothalamic gene expression independent of leptin action. Hypothalamic *Pomc* mRNA was reduced and *Agrp* mRNA was increased by fasting in mice lacking leptin receptor, indicating that POMC and AGRP gene expression is at least partly regulated by a mechanism that is independent of signaling through leptin receptors (Mizuno et al., 1998; Mizuno & Mobbs, 1999).

In the present study, hypothalamic *Fto* mRNA and protein (in ARC and VMN) levels were elevated in leptin-deficient *ob/ob* mice compared with wild-type mice. These findings support the hypothesis that leptin plays a role in regulating hypothalamic Fto expression. In the present study, i.p. or i.c.v. glucose treatment was performed in fasted mice. Serum leptin levels in these mice were under the detection limit of the assay, and therefore it is unknown whether these glucose treatments caused any changes in serum leptin levels. It is, however, clear that both i.p. and i.c.v. glucose treatment did not increase leptin levels to the level that is seen in *ad libitum* fed mice. It was reported that leptin treatment failed to reverse fasting-induced reduction in *Fto* mRNA in ARC in mice (Gerken et al., 2007). In agreement with this observation, the present study also demonstrated that fasting reduced Fto expression in VMN of leptin-deficient *ob/ob* mice,

clearly indicating that fasting reduces Fto expression without a functional leptin mechanism. Consequently, it is likely that hypothalamic Fto expression is regulated by both leptin-dependent and leptin-independent mechanisms.

Insulin-independent regulation of hypothalamic Fto expression

Fasting is also associated with a decline in circulating insulin levels. It has been demonstrated that insulin increases *Pomc* mRNA and reduces *Agrp* mRNA and that insulin treatment reverses fasting-induced changes in hypothalamic nutrient-sensitive gene expression, supporting the role for insulin in mediating the effect of fasting on hypothalamic gene expression (Benoit et al., 2002; Fekete et al., 2006). These findings led to the hypothesis that a decline in circulating insulin mediates the effect of fasting on hypothalamic Fto expression. However, fasting also reduced *Pomc* mRNA and increased *Agrp* mRNA without changes in serum insulin levels, suggesting that POMC and AgRP gene expression is at least partly regulated by an insulin-independent mechanism (Mizuno et al., 1999). If insulin plays a major role in the regulation of Fto gene expression, its expression levels should be altered in insulin-deficient animals. In the present study, it was found that hypothalamic *Fto* mRNA levels were indistinguishable between non-diabetic control and streptozotocin (STZ)-induced diabetic mice in which serum insulin levels were markedly reduced. Nevertheless, STZ-induced diabetic mouse model exhibits a number of impairments, such as hyperglycemia, in addition to insulin deficiency (or chronic hypoinsulinemia). Thus, the inhibitory effect of insulin deficiency on hypothalamic Fto expression might be neutralized by enhanced stimulatory effect of

glucose in this hypoinsulinemic/hyperglycemic mouse model. In the present study, both i.p. and i.c.v. glucose injection increased hypothalamic *Fto* mRNA levels without any significant changes in serum insulin levels. Therefore, these findings support the idea that hypothalamic Fto expression is regulated, at least partly, independent of changes in circulating insulin levels and insulin action.

Regulation of hypothalamic Fto expression by CNS glucose

It has been shown that hypothalamic activity is altered by local glucose availability in the CNS without changes in circulating glucose levels (Dunn-Meynell et al., 1997; Minokoshi et al., 2004). Hypothalamic *Pomc* mRNA is reduced and *Agrp* mRNA is increased by i.p. glucose injection, and these effects are blocked by i.c.v. injection of 2-DG, a non-metabolizable glucose analog (Wolfgang et al., 2007). Central (i.c.v.) glucose injection increases *Pomc* mRNA and i.c.v. 2-DG injection reduces *Agrp* mRNA (Bady et al., 2006; Fraley et al., 2002; Sergeyev et al., 2000). Furthermore, glucose stimulates POMC expression and inhibits AgRP expression in the hypothalamic tissues cultured *ex vivo* or in immortalized hypothalamic cell lines (Cai et al., 2007; Cheng et al., 2008; Lee et al., 2005). These data led to the hypothesis that glucose can regulate hypothalamic Fto expression through its direct action on hypothalamic cells.

In order to assess the direct effects of glucose on hypothalamic *Fto* expression, without causing changes in peripheral nutrient related signals, i.c.v. glucose treatment was given in fasted mice. Although it is assumed that glucose injected into the lateral ventricle reaches both hypothalamus and cortex, glucose-induced increase in *Fto* mRNA

was observed in the hypothalamus, but not in the cortex. These results, together with no effect of fasting on cortex *Fto* mRNA, suggest the possibility that increased extracellular glucose levels in the CNS stimulate *Fto* mRNA expression specifically in the hypothalamic nutrient-sensing neurons.

In the present study, compared with the saline-treated control group, i.c.v. glucose treatment caused a significant increase in blood glucose levels (Table 7) implying that it cannot be rule out that there is a possibility of peripheral glucose effects on hypothalamic *Fto* mRNA expression. Peripheral glucose injection (i.p.) caused a 50% increase in hypothalamic *Fto* mRNA with a 42% (45 mg/dl) increase in blood glucose levels compared with i.p. saline injection in the aforementioned study. Whereas, i.c.v. glucose injection caused a 99% increase in hypothalamic *Fto* mRNA with a 28% (25 mg/dl) increase in blood glucose levels compared with i.c.v. aCSF injection. Consequently, the i.c.v. glucose treatment induced a smaller effect (about 0.6-fold) on blood glucose levels, yet induced a greater effect (about 2-fold) on *Fto* mRNA compared with the i.p. glucose treatment. These data support the hypothesis that an increase in local glucose availability triggers induction of hypothalamic *Fto* mRNA. Additionally, similar to the i.p. injection study, i.c.v. glucose treatment did not alter serum insulin levels, suggesting that glucose regulates hypothalamic *Fto* expression at least partly independent of insulin action.

To assess the nutritional effects of glucose on hypothalamic *Fto* mRNA under prolonged fasted conditions, mice were fasted for 48 h and given multiple (9) i.c.v. injections of glucose. Consistent with the previous studies, the 48-h fast had an inhibitory effect on hypothalamic *Fto* mRNA expression. However, i.c.v. glucose treatment did not cause any changes in *Fto* mRNA (Fig. 26). It is possible that in

excessively fasted conditions, the concentration of glucose used for i.c.v. in the 30-h fasting study does not have the same nutritional effect at 48-h fasting. This is further proven by the finding that i.c.v. glucose treatment did not have an expected inhibitory effect on the fasting-induced *Agrp* mRNA expression in 48-h fasted mice (Fig. 26). Collectively, these findings are consistent with the hypothesis that glucose stimulates hypothalamic *Fto* gene expression and reduced glucose availability (by fasting) in the hypothalamus, at least partly, mediates the inhibitory effects of fasting on hypothalamic *Fto* expression.

Fto expression in peripheral tissues

Fto is ubiquitously expressed throughout the body. The effect of obesity on FTO expression in white adipose tissue is controversial. *FTO* gene expression in adipose tissue was negatively correlated to BMI and per cent body fat in humans, and *Fto* mRNA levels were reduced in white adipose tissue of several mouse models of obesity compared with lean mice (Kloting et al., 2008; Stratigopoulos et al., 2008). Therefore, it is assumed that *FTO* gene expression is down-regulated in response to fat accumulation (Kloting et al., 2008). Other studies found that *FTO* mRNA levels are elevated in subcutaneous adipose tissue of obese individuals compared with lean subjects (Villalobos-Comparan et al., 2008; Wahlen et al., 2008; Zabena et al., 2009). Fasting reduced *Fto* mRNA expression in white adipose tissue in wild-type mice, but this effect was not present in *ob/ob* mice (Stratigopoulos et al., 2008). These data suggest that increased fat accumulation is associated with increased FTO expression in the adipose tissue.

Subcutaneous adipose tissue *FTO* mRNA levels were positively correlated with leptin gene expression, suggesting that adipose FTO in the regulation of metabolism (Zabena et al., 2009). In the present study, WAT *Fto* mRNA levels were reduced by fasting supporting the role for adipose Fto in the regulation of metabolism.

Skeletal muscle *FTO* mRNA levels are negatively correlated with both basal and insulin-stimulated fat oxidation rate (Grunnet et al., 2009). The association between FTO expression and fat oxidation remained significant after adjustment for total fat mass, suggesting that FTO regulates fat oxidation independent of adiposity. In the present study, skeletal muscle *Fto* mRNA levels were increased by fasting in mice. It is possible that fasting-induced increase in skeletal muscle *Fto* gene expression reflects an increase in FFA utilization by skeletal muscle during fasting. Because i.p. glucose injection reversed the effect of fasting on Fto expression, a decline in blood glucose levels may contribute to the fasting-induced increase in skeletal muscle *Fto* gene expression.

Little is known about metabolic conditions governing FTO expression in the liver. *Fto* mRNA levels were reduced in the liver of *ob/ob* mice compared with wild-type mice, suggesting that hepatic Fto may be also involved in the regulation of metabolism (Stratigopoulos et al., 2008). In the present study, fasting increased *Fto* mRNA levels in the liver, while glucose reversed the stimulatory effect of fasting on hepatic *Fto* mRNA expression, suggesting that a reduction in circulating glucose, at least partly mediates the fasting-induced increase in hepatic *Fto* mRNA expression. These findings further support the hypothesis that Fto in the liver may have a role in the regulation of metabolism including the metabolic adaptation to fasting (Poritsanos et al., 2010).

Overall, the role of peripheral Fto in the regulation of energy balance is unclear at present. However, studies on *Fto* gene expression in peripheral metabolically active tissues, including the present study, suggest that *Fto* gene expression responds to changes in metabolic states. Collectively, peripheral Fto may play a role in the regulation of energy storage and expenditure.

Hypothalamic Fto expression in obesity

If FTO plays a role in the regulation of metabolism, expression levels of *FTO* gene and protein may be altered in obese and diabetic conditions. To address this possibility, *ob/ob* mice were used as a model of obesity and type 2 diabetes. In the presence of hyperglycaemia and obesity, hypothalamic *Fto* mRNA levels were significantly elevated in *ob/ob* mice compared with wild-type mice. Similarly, the number of Fto-immunopositive cells was significantly increased in the ARC and VMN, but not in DMN, in *ob/ob* mice compared with the wild-type mice (Fig. 43A). These data suggest that obesity is associated with increased basal Fto expression in specific hypothalamic regions including ARC and VMN. Since, hypothalamic Fto appears to be regulated by nutritional factors, it is possible that defects in hypothalamic responses to nutrients and hormones result in dysregulation of *Fto* gene expression leading to the up-regulation of Fto. Alternatively, chronically elevated blood glucose levels in *ob/ob* mice might cause the elevated basal Fto expression is glucose-sensitive hypothalamic neurons. However, hypothalamic *Fto* mRNA levels were not changed in STZ-induced diabetic mice, indicating that hyperglycemia does not necessarily induce Fto expression. It is

speculated that the elevated hypothalamic Fto expression reflects the excess nutritional signals in the hypothalamus and/or altered hypothalamic response to these signals. In any case, the elevated hypothalamic Fto expression reflects metabolic impairments or underlies the mechanism for the metabolic impairments.

Impaired glucose regulation of hypothalamic Fto expression in obesity

Obese individuals with glucose intolerance are often hyperglycaemic, hyperinsulinemic, and hyperleptinemic, and insulin resistance and leptin resistance are often observed in obese individuals. There are also evidences supporting the hypothesis that hypothalamic neuronal response to glucose is impaired in obesity. Glucose-induced feeding suppression is absent in obese animals (Tsujii & Bray, 1990). Glucose-sensing neurons are fewer in number and show abnormal responses to glucose in diet-induced obesity rats (Song et al., 2001). Glucose-induced hypothalamic activity, as assessed by Fos expression, is reduced in obese-prone rats compared with obese-resistant rats (Levin et al., 1998). Furthermore, the effect of glucose injection on hypothalamic activity, as assessed by fMRI, was attenuated in obese humans compared with non-obese healthy individuals (Matsuda et al., 1999). These data indicate a clear association between obesity and altered hypothalamic response to glucose. Based on these earlier studies, it was hypothesized that regulation of hypothalamic Fto expression by nutritional states (i.e. fasting and glucose) is impaired in obesity. To address this hypothesis, effects of fasting and glucose treatment on hypothalamic Fto expression were compared between wild-type and *ob/ob* mice in the present study.

Consistent with the previous study, although it did not reach statistical significance, there was a clear trend of fasting-induced reduction in Fto expression in the hypothalamus of wild-type mice. As expected, i.p. glucose injection has a significant stimulatory effect on Fto expression in ARC and VMN in wild-type mice. Interestingly, fasting reduced Fto expression in VMN and ARC in *ob/ob* mice, indicating that VMN and ARC Fto-expressing neurons are sensitive to fasting-induced neuroendocrine and nutritional signals. In contrast to wild-type mice, glucose treatment was not effective in increasing Fto expression levels in both ARC and VMN in *ob/ob* mice. These findings clearly indicate that hypothalamic Fto-expressing neurons cannot respond to glucose stimulation in obese mice. More specifically, these hypothalamic Fto-expressing neurons remain to be responsive to fasting, but they lost their responsiveness to glucose stimulation. Therefore the present results suggest the possibility that hypothalamic glucose sensing plays a major role in the regulation of energy balance compared with the sensing mechanisms for other nutritional signals. A recent study demonstrated that high-fat diet feeding increases *Fto* mRNA expression in the hypothalamic ARC (Tung et al., 2010). Although this study did not examine Fto expression in VMN, these results, together with the present study, suggest that certain nutritional factors such as fat and glucose have a stimulatory effect on Fto protein expression and function towards a counter-regulatory response for the ultimate prevention of obesity. Thus, the blunted response of hypothalamic Fto-expressing neurons to glucose may cause metabolic impairments in this obese model. Overall, these data clearly indicate that obesity is associated with impairments in hypothalamic glucose sensing. Further investigations are

required to elucidate the mechanism by which impaired glucose-induced Fto expression causes metabolic impairments observed in this obese model.

Possible relationship between Fto and melanocortin pathway

The present study demonstrated that hypothalamic Fto expression is responsive to changes in nutritional condition and that nutritional regulation of Fto expression is specific at least in ARC and VMN. Within the ARC, it was clear that Fto is highly expressed in the medial part of the ARC where AgRP neurons are located. It is interesting to note that Fto immunoreactivity changes more drastically in the lateral peri-arcuate area, the cell-sparse area lateral to the ARC and ventral to the VMN, where some of the POMC neurons have been found (Fig. 34A and Fig. 44A) (Shu et al., 2003). Based on these observations, it was hypothesized that Fto plays a role in the regulation of the activity of hypothalamic AgRP and POMC neurons. In mice, feeding increased the activity of Fto-expressing cells in the hypothalamic ARC and PVN, suggests that Fto may possibly function as a satiety factor by altering the activity of ARC (as well as PVN) neurons including POMC and AgRP neurons (Olszewski et al., 2009). In is not known whether feeding stimulates Fto-expressing neurons in the lateral peri-arcuate area. In order to investigate the potential role of Fto in nutritional regulation of metabolism, immunofluorescence analysis of Fto, and the melanocortin peptides POMC or AgRP, was undertaken in *ad libitum* fed mice and 30-h fasted mice subjected to either saline or glucose i.p. treatments. As previously shown, Fto localizes in the nucleus of POMC neurons (Fischer et al., 2009). Fto-immunopositive cell number followed the same

pattern of POMC expression, where fasting reduces and glucose increases Fto- or POMC-immunopositive cell number in ARC (Fig. 31B and Fig. 36-37, respectively). This pattern of immunoreactivity indicates that Fto and POMC may share a common nutritional regulatory pathway. It has been demonstrated that Fto plays a role in the demethylation of nucleic acids and possibly regulates gene expression (Gerken et al., 2007; Han et al., 2010; Jia et al., 2008). Furthermore, *POMC* expression levels are strongly influenced by the methylation pattern of the 5' promoter region, and hypermethylation of the *POMC* gene is associated with obesity or overweight in both humans and rodents (Coupe et al., 2010; Newell-Price, 2003). These findings led to the assumption that Fto may increase *POMC* expression by promoting demethylation of the (hyper)methylated *Pomc* promoter. Further investigations are necessary to address this hypothesis.

As expected, fasting increased AgRP-immunopositive cell number. Under fasted conditions, glucose had a limited effect in suppressing AgRP immunoreactivity in ARC (Fig. 38A -33B, and Fig. 39A). The nearly 100% co-localization of AgRP and Fto in the ARC (Fig. 39B) suggests that Fto may also have some regulatory function in the AgRP orexigenic pathway. Overall, the present data support the hypothesis that hypothalamic Fto interacts with hypothalamic melanocortin system possibly by regulating POMC and AgRP gene expression.

Studies investigating hypothalamic neuroendocrine cell activation in response to specicific stimuli have generally accepted that the amount of cFos expression is positively correlated with the stimulus intensity and neuronal activity. In *ob/ob* mice, the elevated cFos expression levels and the absence of fasting-induced cFos induction in the

ARC, as compared with the wild–type mice, indicates that in the presence of obesity certain neuronal populations, such as AgRP neurons, are fully activated and are unresponsive to the effects of fasting (Table 10). Similar elevations in basal expression levels are observed in Fto (Fig. 43, Fig. 44 and Fig. 46) and AgRP (Fig. 55) when comparing wild-type and *ob/ob* mice. In the case of Fto expression in *ob/ob* mice, even when the circulating levels of glucose are normalized in response to fasting, the threshold of the nutritional sensing mechanisms, such as those involving the Fto neurons, are heightened to the point that a change in nutritional state will not be adequate enough to reverse neuronal activity back to a normal condition. If Fto functions as a factor that inhibits anabolic processess in the ARC (i.e. AgRP neurons), then it would be expected that the expression pattern of Fto and that of an anabolic target would be inversely related. The observation that glucose treatment of *ob/ob* mice reduced AgRP (Fig. 55) and cFos (Fig. 57A) expression, while the expression of Fto remained high (Fig. 46B) suggests that a nutritionally responsive molecular factor, such as Fto may indeed have an inhibitory effect on the activity of AgRP neurons. The nearly 100% co-localization pattern of Fto with AgRP and cFos ARC neurons does not give a definitive answer but rather an indication in regards to the regulatory function of Fto on AgRP. To address this further, future analysis should include an assessment of the AgRP promoter activity, as well as, the colocalization pattern of AgRP/cFos in response to an increase in Fto (neuronal) activity.

Although, fasting did not induce cFos expression in the ARC of *ob/ob* mice, glucose treatment reduced cFos expression (Fig. 57A), suggesting that in the ARC of *ob/ob* mice glucose sensing mechanism may still be present in subsets of neurons

possibly including Fto-expressing neurons. The observed reduction in cFos expression may represent glucose-induced inhibition of AgRP neurons. In contrast, glucose treatment did not cause a significant change in cFos expression in fasted wild-type mice. This may be due to glucose-induced activation of POMC neurons in the presence of glucose-induced reduction in the activity of AgRP neurons. Consistent with this idea, there was a significant positive correlation between blood glucose levels and cFos immunoreactivity in wild-type mice, but not in *ob/ob* mice (Fig. 59). Thus, a (nutritional) response to glucose is possibly impaired specifically in POMC neurons in *ob/ob* mice without having impairments in glucose sensitivity in AgRP neurons.

Although cFos has been widely used as a marker of neuronal activation, the threshold for cFos induction may be 1) different for specific neuronal populations (i.e. Fto, POMC or AgRP) or 2) a persistent stimulus (i.e. extended fasting period) may result in intracellular mechanisms that inhibit cFos expression. The presence of cFos expression is not reflective of the duration of the stimulatory signal. Specifically, the decline in cFos as a result of its half-life does not always reflect a decrease in the duration of the stimulatory signal (Sharp et al., 1991; Verbalis et al., 1991). Consequently, the 48-h fasted condition may have resulted in the expression of cFos in only a subset of neurons out of a larger population that are capable of expressing cFos under fasted conditions. This may be one explanation for the lack of a suppressive effect on the ARC cFos expression of glucose-treated mice, as compared with the fasted saline-treated group in wild type mice (Fig. 57).

Site-specific nutrient sensing in the hypothalamus

It is of some interest to note that the effect of fasting in reducing Fto expression was more dramatic in the lateral peri-arcuate area and ventrolateral VMN compared with other areas of the ARC and VMN (Fig. 44A). Glucose-induced increase in Fto expression was also more robust in these areas compared with other ARC and VMN areas (Fig. 44A). Furthermore, under the *ad libitum* fed condition, Fto expression was more robustly increased in the lateral peri-arcuate area, the dorsomedial part of the ARC and the ventrolateral VMN in *ob/ob* mice as compared with wild-type mice (Fig. 43A). These results suggest that these specific areas in the basomedial hypothalamus play a major role in glucose-sensing and subsequent regulation of energy homeostasis. POMC neurons are located not only in the ARC but also in the lateral peri-arcuate area (Gee et al., 1983; Shu et al., 2003). Responses to nutrient signals are more robust in POMC neurons located in the lateral peri-arcuate area compared with those in the ARC (Fig. 36, 37) (Shu et al., 2003). These findings support the hypothesis that Fto plays a role in mediating the nutritional regulation of POMC expression in the hypothalamus, in particular in the lateral peri-arcuate area, and that impairments in this regulation leads to metabolic impairments.

The robust changes in Fto expression were also found in the ventrolateral VMN in response to fasting and glucose treatment, while the glucose-induced Fto expression was absent in the ventrolateral VMN of *ob/ob* mice. These findings suggest that the ventrolateral VMN also plays a significant role in glucose-sensing and regulation of metabolism. In agreement with these findings, it has been shown that lesions specific to the hypothalamic area that extend ventrally from the VMN, but not lesions specific to the

VMN or ARC, increases food intake and body weight, supporting the importance of the ventrolateral VMN in the regulation of energy balance (Gold, 1973). Brain-derived neurotrophic factor (BDNF) is known to be an anorexigenic factor, and brain-specific ablation of BDNF in mice causes obesity (Rios et al., 2001). BDNF is highly expressed in the VMN and its expression levels are regulated by metabolic states (Xu et al., 2003). Interestingly, BDNF expression is much higher in the ventrolateral VMN compared with the dorsomedial and central VMN (Xu et al., 2003). These findings raise the possibility that the ventrolateral VMN is particularly sensitive to the changes in glucose availability and Fto may also increase the expression of BDNF. In light of the findings that Fto is highly expressed in the ventrolateral VMN, Fto may directly stimulate BDNF gene expression. Alternatively, Fto may increase BDNF expression through an indirect mechanism. One possible indirect mechanism is mediation by the POMC or AgRP neurons, because Fto is co-expressed in these neurons and both POMC and AgRP neurons innervate to the VMN (Xu et al., 2003). Recent data also suggest that BDNF is a possible downstream effector of MC4R signaling in the hypothalamus and the brainstem (Bariohay et al., 2009; Nicholson et al., 2007).

Importance of the lateral peri-arcuate area and the ventrolateral VMN in glucose sensing and metabolic regulation was supported by the findings that glucose-excited neurons are present in these hypothalamic regions, and many efferent projections to other brain regions involved in metabolic regulation arise from the ventrolateral VMN (Cotero & Routh, 2009; King, 2006). Fto in the lateral peri-arcuate area may function as a hypothalamic site where Fto is part of the melanocortin system, while the ventrolateral VMN may serve as a site where Fto is part of other neuronal networks, such as BDNF.

The site-specific responses in Fto expression to nutritional signals (i.e. glucose), and impairments in these responses in obese mice support the hypothesis that Fto in the lateral peri-arcuate area and the ventrolateral VMN play a major role in hypothalamic glucose sensing and regulation of metabolism.

Possible role for Fto in the regulation of energy homeostasis

Although *FTO* variants are unequivocally associated with obesity, little is known about the biological function of Fto in the regulation of metabolism. Since, the obesity-associated SNPs are mostly intronic, it is unclear whether these variants cause changes in FTO expression or if the regulatory elements within the intron affect expression of other genes. A loss-of-function mutation in *FTO* in humans was recently reported to have effects other than obesity. A single amino acid substitution in *FTO* resulted in post-natal retardation, brain and cardiac defects, and face dysmorphism (Boissel et al., 2009). Another recent study reported that loss-of-function heterozygous mutations in human *FTO* were found in both lean and obese individuals, indicating that partial-loss of function of FTO does not cause alterations in metabolism (Meyre et al., 2010). These studies do not support the role for FTO itself in the regulation of energy balance.

Phenotypic similarities between *FTO* variants and *MC4R* variants suggest that hypothalamic FTO may function as a regulator of CNS melanocortin signaling (Loos et al., 2008; Stutzmann et al., 2009). Interestingly, *FTO* is located approximately 13.6-Mb downstream the *AgRP* coding region on chromosome 16. About 60% of human genome is filled with the protein-non-coding gene-poor region, known as gene deserts. In

comparison to other genomic regions, gene deserts are higher in the density of SNPs (Ovcharenko et al., 2005). It has been shown that some human gene deserts harbor regulatory elements of distant (>1,330 kb) genes (Nobrega et al., 2003). *FTO* SNPs were found around the largest evolutionarily conserved gene-poor region of the human chromosome 16 (Martin et al., 2004). Despite the 42.5-kb region upstream of *Agrp* is required for the hypothalamic expression of *Agrp*, as well as, for the appropriate response to fasting in mice, currently it is not known whether other genomic regions, including the gene desert around the *Fto* gene, play a role in the regulation of *Agrp* expression (Kaelin et al., 2004). In accordance with these studies, it is probable that *FTO* SNPs disrupt regulatory elements (such as repressors) for the expression of *AgRP* or other genes, ultimately leading to increased energy intake and the development of obesity.

To date, there are three Fto-deficient mouse models. *Ft* mice have a deletion of 1.6 Mbp in chromosome 8 which results in the loss of *Fto*, as well as, 5 neighboring (*Ftm, Ftl*, *Irx3*, *Irx5* and *Irx6)*. Mice homozygous for the *Ft* deletion were embryonically lethal in addition to having severe CNS developmental defects and growth retardation (Anselme et al., 2007). Heterozygous mice have fused forelimbdigits and thymic hyperplasia (van der Hoeven et al., 1994). Unlike the other two mouse models of *Fto* deficiency, *Ft* mice did not exhibit any changes in body weight. Interestingly, in humans duplication of a region harbouring the *FTO* gene on chromosome 16 resulted in mental retardation, obesity and digital abnormalities (Stratakis et al., 2000).

The $Fto^{-/-}$ mouse lacks exons 2 and 3 of the *Fto* gene results in the complete absence of Fto protein (Fischer et al., 2009). The Fto^{I36F} mouse model has a point mutation in exon 6 of the *Fto* gene resulting in partial loss-of-function and reduced Fto

protein levels (Church et al., 2009). Both *Fto*$^{-/-}$ and *Fto*^{I36F} mouse models exhibit reduce body weight and adipose tissue on a high-fat diet compared with the wild-type controls (Church et al., 2009; Fischer et al., 2009), suggesting that disrupting Fto activity may have beneficial effects against the development of obesity. These phenotypes are associated with increased energy expenditure and increased sympathetic activity. Additionally, a reduction in hypothalamic *Npy* expression in *Fto*$^{-/-}$ mice under fasted conditions, and in the *Fto*^{I36F} mice under fed conditions suggests that there might be a regulatory role of Fto on *Npy* expression in the hypothalamus (Church et al., 2009; Fischer et al., 2009). It is possible that the regulatory effect of Fto on any hypothalamic gene expression may involve nucleic acid demethylation. These latter two animal models suggest that reducing Fto expression may be beneficial in attenuating obesity.

However, these suggestions based on the Fto knockout mouse models are not consistent with the findings of the present study and another recent study by others. Since, hypothalamic glucose action causes reductions in food intake, circulating glucose and triglyceride levels, hepatic glucose production, and hepatic VLDL secretion, it is hypothesized that glucose-induced increase in hypothalamic Fto mediates these beneficial effects of glucose. To support this idea, a recent study demonstrated that high-fat diet feeding induces *Fto* expression in the ARC of rats, suggesting that certain nutritional factors such as fat have a stimulatory effect on hypothalamic Fto expression and function towards a counter-regulatory response for the ultimate prevention of obesity (Tung et al., 2010). Consistent with this idea, site-directed overexpression of *Fto* in the ARC of rats decreases food intake, while reducing ARC Fto expression increases food intake, suggesting Fto to function as a satiety molecule (Tung et al., 2010). Furthermore, the

present study clearly demonstrated that hypothalamic Fto expression is increased by glucose and this response was blunted in obese mice. These findings support the idea that hypothalamic Fto plays a beneficial effect in protecting against or reversing obesity. The answer to the question as to whether FTO has beneficial or adverse roles in obesity requires further investigation.

Table 3. Primer sequences used for real-time PCR

Gene	Accession No.	Primer	Sequences	Exon
Fts	NM_010241	Forward	5'-TGGAATATTCGCTTCTTGCAGAA-5''	3
		Reverse	5'-GCGGTAAGATGGCTGTACGTAGA-5'	4
Ftm	AJ344253	Forward	5'-AGTTCTCCAGAAGTGGGACCAA-3'	20
		Reverse	5'-TCTACCTTAGCCACACCGCTTT-3'	21
*Fto**	NM_011936	Forward	5'-GACATCGAGACACCAGGATTAACA-3'	4
		Reverse	5'-GTGAGCCAGCCAAAACACAGT-3'	5
*Fto**	NM_011936	Forward	5'-TCTGAGGATGAAAGTGAGGACGAG-3	4
		Reverse	5'-CCACACGGTGAGTGGAACTAAAC-3'	5
Irx3	NM_008393	Forward	5'-CGCAGCCGCCTATGCT-3'	2
		Reverse	5'-GCTGGAAAGCTGTCTTGAGTAACTT-3'	3
Irx5	NM_018826	Forward	5'-GCCGCTGCCTTCTCTTACG-3'	1
		Reverse	5'-GCCCAGAGGTGCTGCATAA-3'	2
Irx6	AJ271055	Forward	5'-CTCTGGGATGCCTGAATGGTGACA-3'	4
		Reverse	5'-CCGCTGCCTCCTCCTCTGCTTCTT-3'	5
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
Cyclophilin	X52803	Forward	5'-AAGCATACAGGTCCTGGCATCT-3?	4
		Reverse	5'-TGCCATCCAGCCATTCAGT-3?	4-5

*: Two sets of primers were used for *Fto* mRNA measurement and both primer sets produced equivalent results.

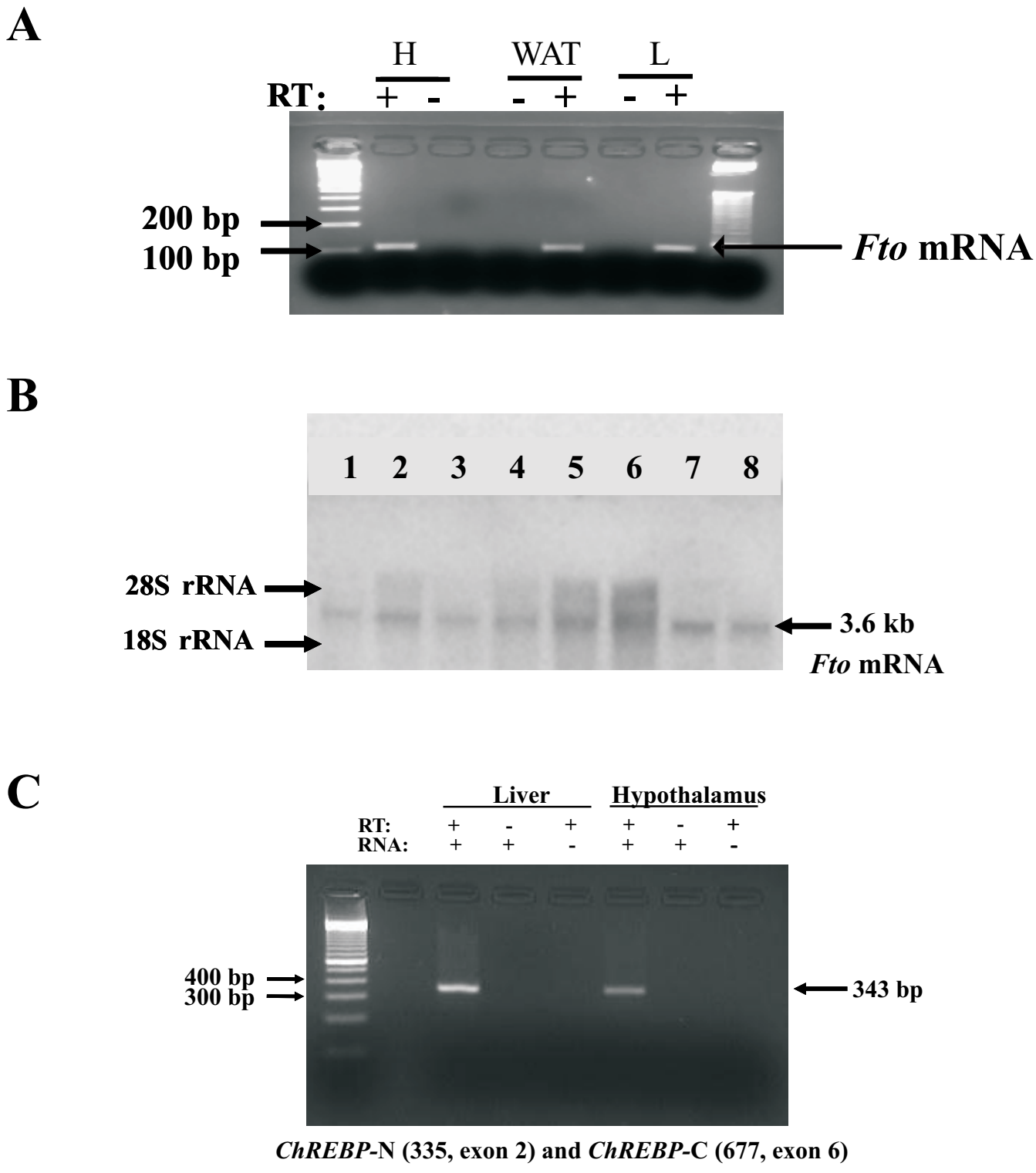


Figure 18: *Fto* expression in the brain of male C57BL/6 mice. (A) *Fto* mRNA expression analyzed by RT-PCR analysis, in the hypothalamus (H), white adipose tissue (WAT) and liver (L). (B) *Fto* mRNA expression, analyzed by Northern blot analysis, in the hypothalamus (1), thalamus (2), prefrontal cortex (3), cortex (4), hippocampus (5), brain stem (6),cerebellum (7), and spinal cord (8). (C) RT-PCR analysis of *ChREBP* mRNA expression in the liver and hypothalamus. Reverse transcriptase (RT).

Hypothesis II: Hypothalamic Fto expression is regulated by nutritional signals, such as glucose

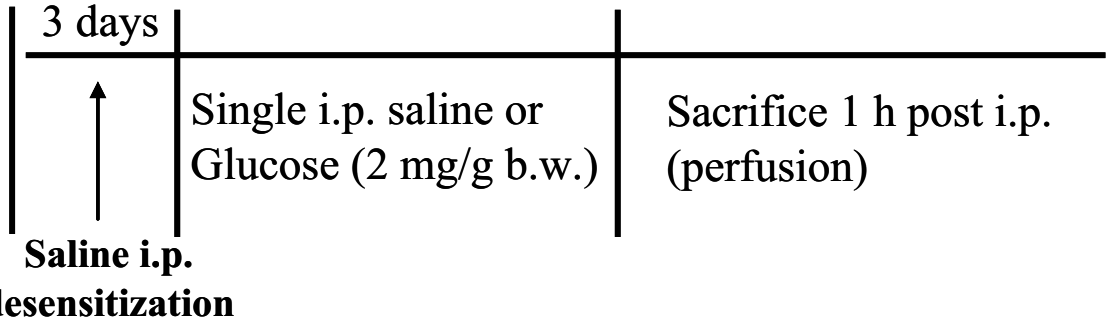
Thesis Study II: Investigate the effect of either 16-h or 30-h fasting on the expression of hypothalamic *Fto* → mRNA ANALYSIS

Animals: Wild-type mice; age 6-8 weeks

Sacrifice (9-11 AM)

Thesis Study III: Investigate the effect of 30-h fasting and intraperitoneal (i.p.) glucose on the expression of hypothalamic *Fto* → mRNA ANALYSIS

Animals: Wild-type mice; age 6-8 weeks



Thesis Study IV: Investigate the effect of 30-h fasting and intracerebroventricular (i.c.v.) glucose on the expression of hypothalamic *Fto* → mRNA ANALYSIS

Animals: Wild-type mice; age 6-8 weeks

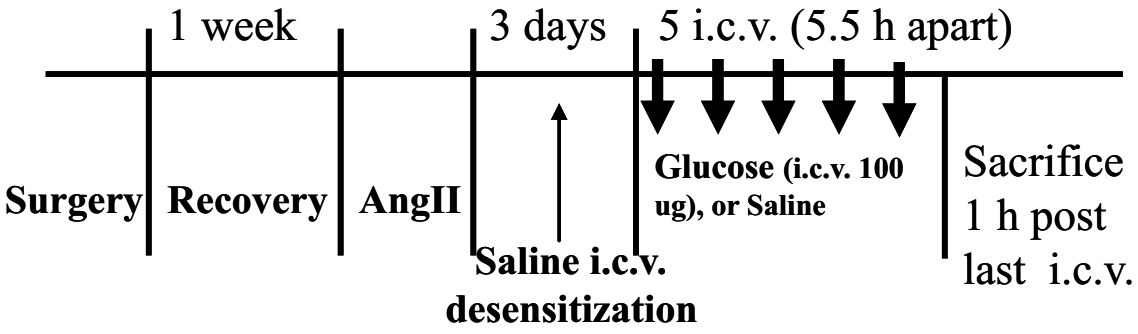


Figure 19: Experimental outline in pursuit of Studies II, III and IV aimed at addressing the Thesis Hypothesis II.

Table 4. Effect of single intracerebroventricular (i.c.v.) glucose on body weight and blood glucose concentration in mice fasted overnight (16 hours) and sacrificed 30 min post-i.c.v.

	Fasted-aCSF	Fasted-Glucose	*P**
Body weight (g)	21.7 ± 1.9	22.1 ± 1.0	0.608
Blood glucose (mg/dL)	119 ± 13	105 ± 18	0.065

Values are means ± SEM (N = 9-10/group).
*: P values by Student's t-test or Wilcoxon test.

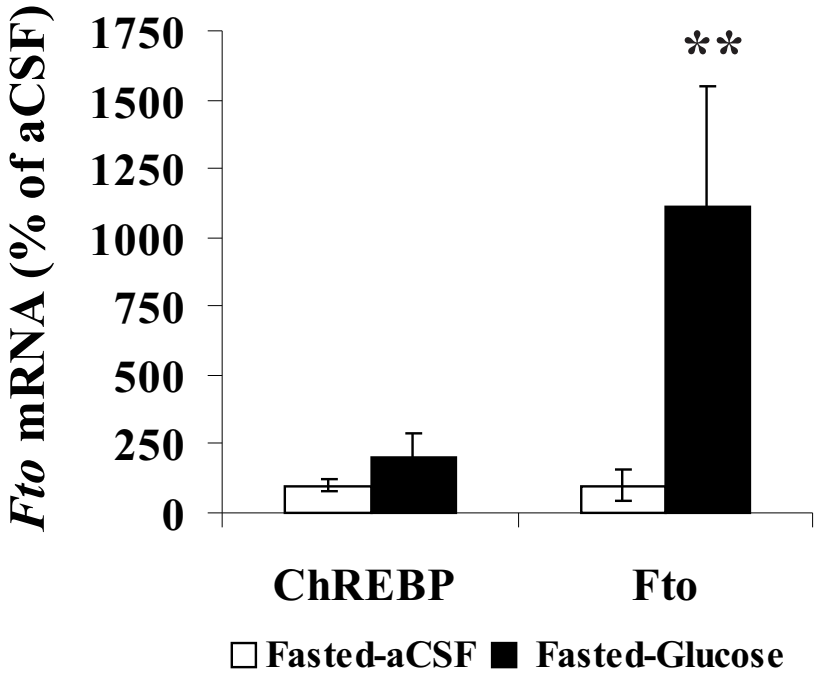


Figure 20: Glucose increases hypothalamic *Fto* expression in lean mice fasted overnight. The effect of single intracerebroventricular (i.c.v.) glucose injection on hypothalamic *ChREBP* and *Fto* mRNA expression was investigated in male C57BL/6 mice fasted for overnight (16 hours) and sacrificed 30 min post-i.c.v. Data are means ± S.E.M. (N = 7-10 mice/group). **: $P < 0.01$, Student's t-test.

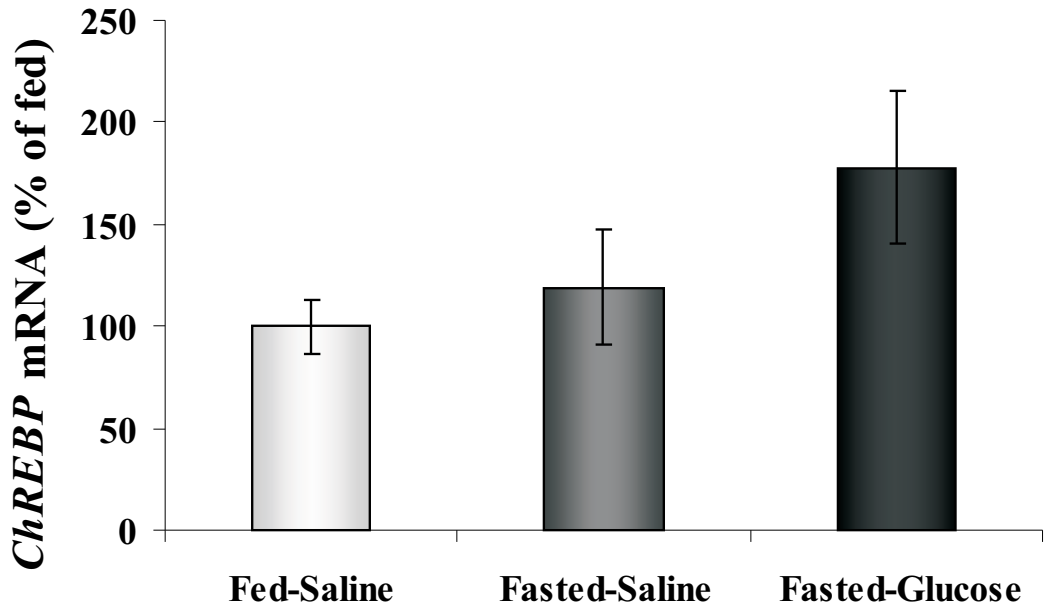


Figure 21: Changes in nutritional state by fasting or central administration of glucose do not alter hypothalamic *ChREBP* expression in lean mice. The effect of multiple (5) intracerebroventricular (i.c.v.) glucose injections on hypothalamic *ChREBP* mRNA expression in male C57BL/6 mice fasted for 30 hours. Data are means ± S.E.M. (N = 9-10 mice/group). There was no significant difference by one-way ANOVA.

Table 5. Effect of 30-h fasting on body weight, concentrations of blood glucose, serum insulin and leptin in mice

	Fed	Fasted	*P**
Body weight (g)	24.9 ± 0.5	21.3 ± 0.6	< 0.0001
Blood glucose (mg/dL)	118 ± 11	63 ± 3	< 0.0005
Serum insulin (ng/mL)	0.71 ± 0.12	0.18 ± 0.04	< 0.001
Serum leptin (ng/mL)	2.56 ± 0.21	ND	N/A

Values are means ± SEM (N = 9-10/group).
*: P values by Student's t-test or Wilcoxon test.
ND: Levels were below the detection limit.
N/A: Not applicable

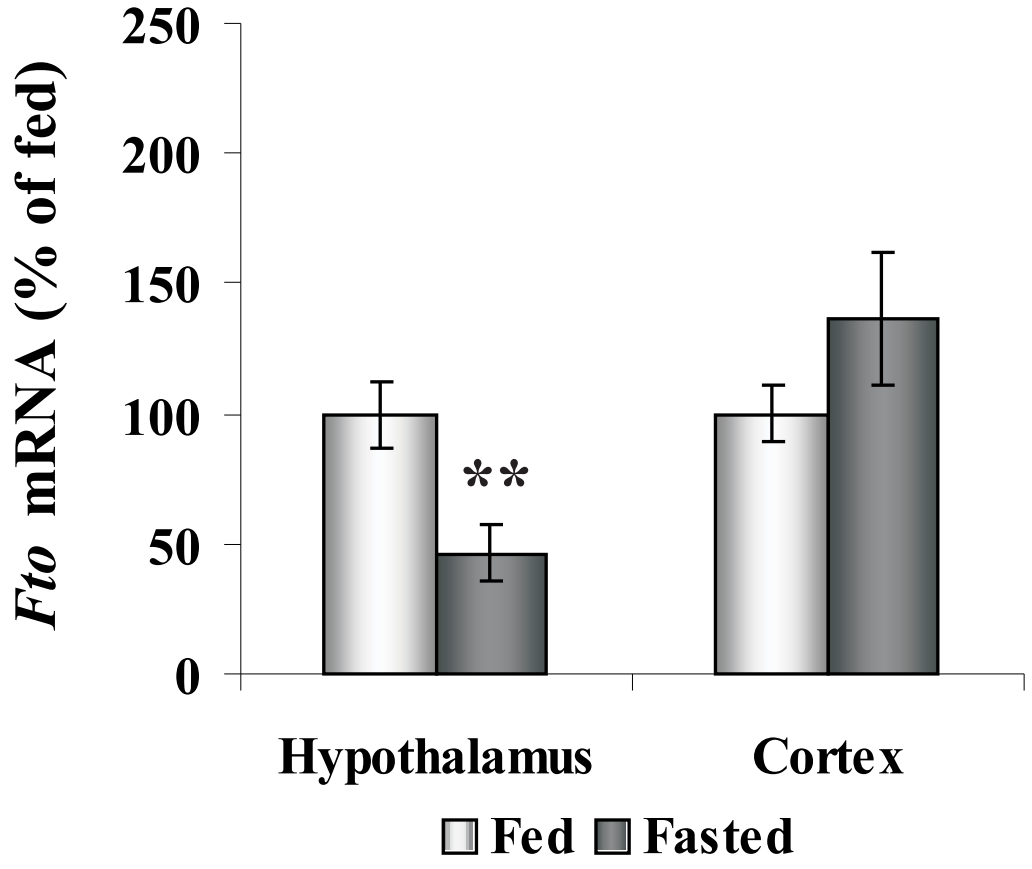


Figure 22: Fasting reduces *Fto* expression in the hypothalamus but not in cerebral cortex. The effect of fasting (30 h) on *Fto* mRNA expression was tested in male C57BL/6 mice. Data are means ± S.E.M. (N = 9-10 mice/group). **: $P < 0.01$, Student's t-test.

Table 6. Effect of single intraperitoneal (i.p.) injection of glucose on body weight, WAT weight, concentrations of blood glucose, serum insulin and leptin in mice fasted for 30 hours

	Fed-Saline	Fasted-Saline	Fasted-Glucose
Body weight (g)	26.0 ± 0.4^{A}	19.7 ± 0.4^{B}	19.8 ± 0.4^{B}
WAT weight (g)	0.30 ± 0.02^{A}	0.07 ± 0.03^{B}	0.08 ± 0.02^{B}
Blood glucose (mg/dL)	166 ± 12^{A}	106 ± 18^{B}	152 ± 10^{AB}
Serum insulin (ng/mL)	0.81 ± 0.07^{A}	0.17 ± 0.06^{B}	0.41 ± 0.05^{B}
Serum leptin (ng/mL)	3.41 ± 0.63	ND	ND

Values are means ± SEM (n = 7-8/group).
ND: Levels were below the detection limit.
Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey-Kramer test).

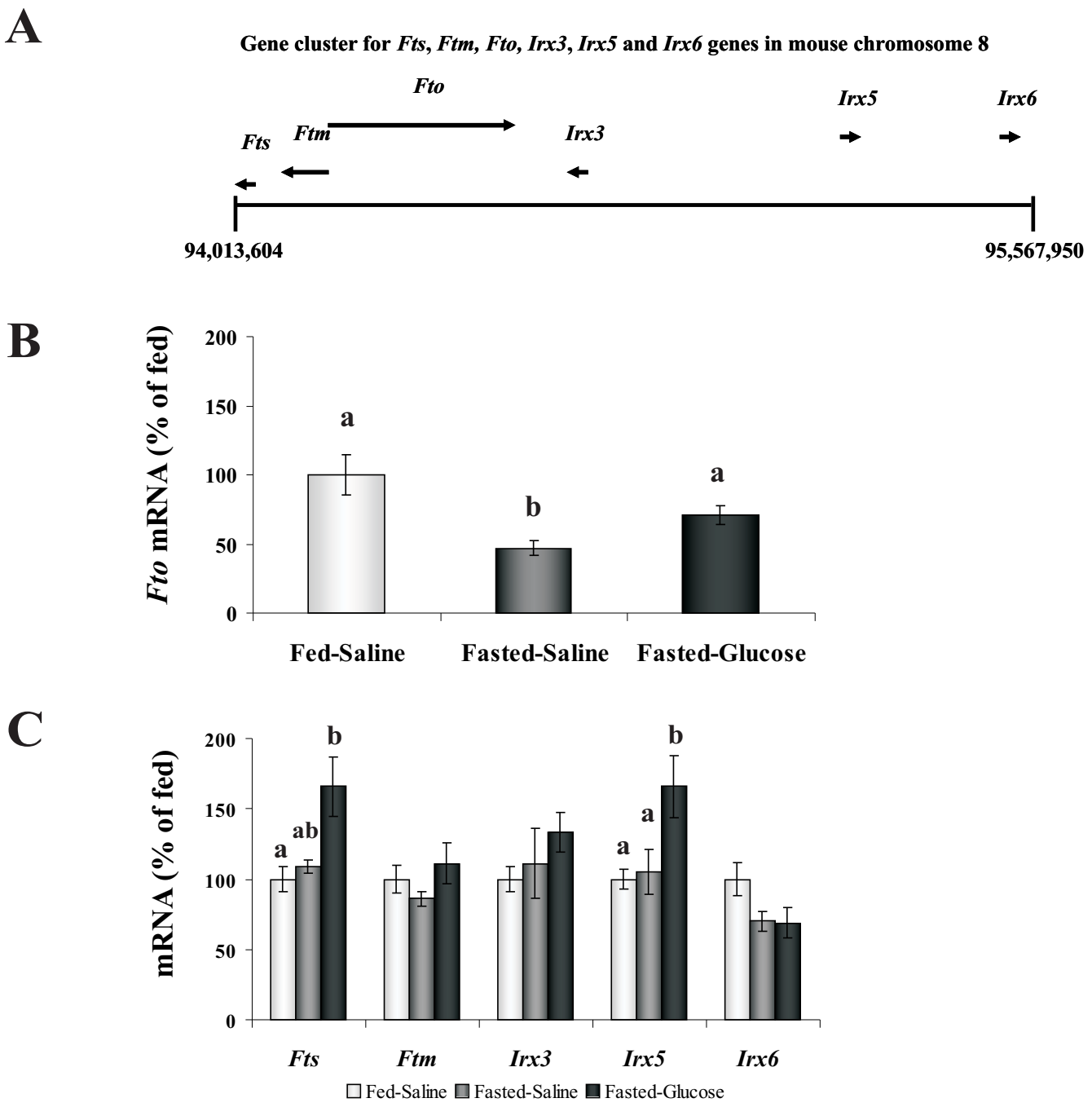


Figure 23: Fasting reduces *Fto* expression and glucose treatment increases *Fto* and *Irx5* expression in the mouse hypothalamus. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose injection on hypothalamic expression of Fto and its neighboring genes, *Fts, Ftm, Iroquois homeobox 3 (Irx3), Irx5 and Irx6* was examined in male C57BL/6 mice. (A) Drawing of gene cluster for *Fts, Ftm, Fto*, *Irx3, Irx5* and *Irx6* in the mouse chromosome 8 (not to scale). (B) Fasting reduces and i.p. glucose increases hypothalamic *Fto* mRNA expression. (C) A 30-h fast does not alter the mRNA expression of hypothalamic *Fts, Ftm, Irx3*, and *Irx6*. Glucose treatment increases *Irx5* mRNA, but not *Fts, Ftm,Irx3*, and *Irx6* mRNA levels. Data are means ± S.E.M. (N = 9-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test or Wilcoxon test with Bonferroni correction). There was no significant difference between the groups in *Irx6* mRNA levels ($P = 0.07$ by one-way ANOVA).

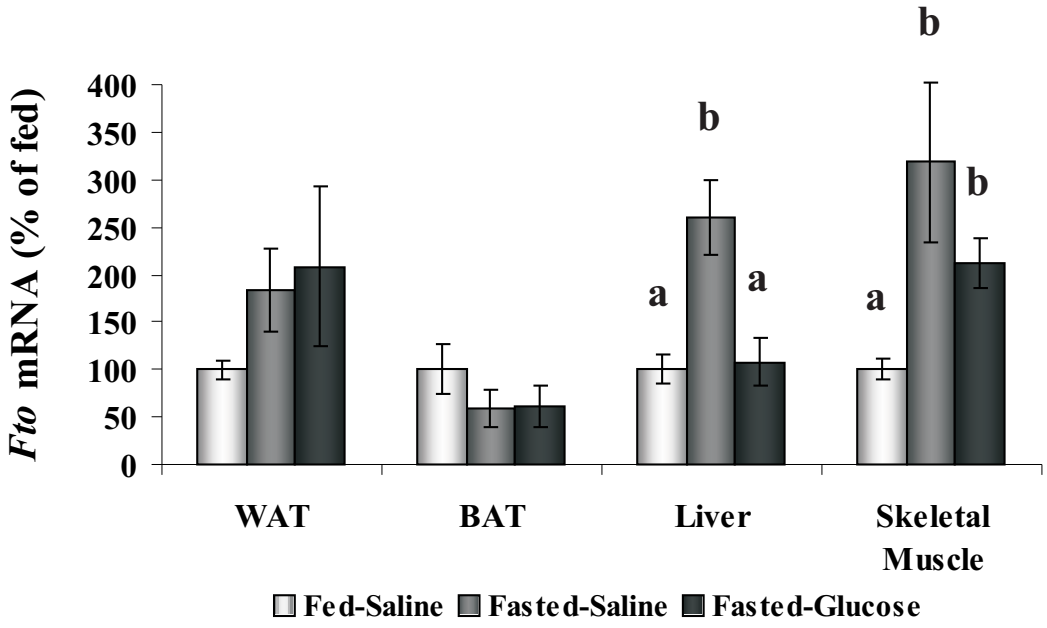


Figure 24: Fasting increases *Fto* expression in the liver and skeletal muscle and glucose treatment reduces Fto expression in the liver of lean mice. The effect of fasting (30 h) and intraperitoneal (i.p.) glucose injection on *Fto* mRNA expression in peripheral tissues was tested in male C57BL/6 mice. WAT: White adipose tissue; BAT: brown adipose tissue. Data are means ± S.E.M. (N = 9-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test or Wilcoxon test with Bonferroni correction). There were no significant differences between the groups in WAT ($P = 0.31$) and BAT ($P = 0.22$) *Fto* mRNA levels (one-way ANOVA).

Table 7. Effect of multiple (5) i.c.v. injections of glucose on body weight, concentrations of blood glucose, serum insulin and leptin in mice fasted for 30 hours.

	Fasted-Saline	Fasted-Glucose	*P**
Body weight (g)	21.3 ± 0.6	21.6 ± 0.6	0.6864
Blood glucose (mg/dL)	63 ± 3	88 ± 4	< 0.0001
Serum insulin (ng/mL)	0.18 ± 0.04	0.16 ± 0.03	0.6205
Serum leptin (ng/mL)	ND	ND	N/A

Values are means ± SEM (N = 9-10/group).
*: P values by Student's t-test or Wilcoxon test.
ND: Levels were below the detection limit.
N/A: Not applicable

Table 8. Effect of multiple (9) i.c.v. injections of glucose on body weight, concentrations of blood glucose, serum insulin and leptin in mice fasted for 48 hours

	Fed-Saline	Fasted-Saline	Fasted-Glucose
Body weight (g)	25.6 ± 0.4 ^{A}	19.2 ± 0.3 ^{B}	18.6 ± 0.4 ^{B}
Blood glucose (mg/dL)	118 ± 11 ^{A}	74 ± 5 ^{B}	72 ± 3 ^{B}

Values are means ± SEM (N = 9-10/group).
Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey-Kramer test).

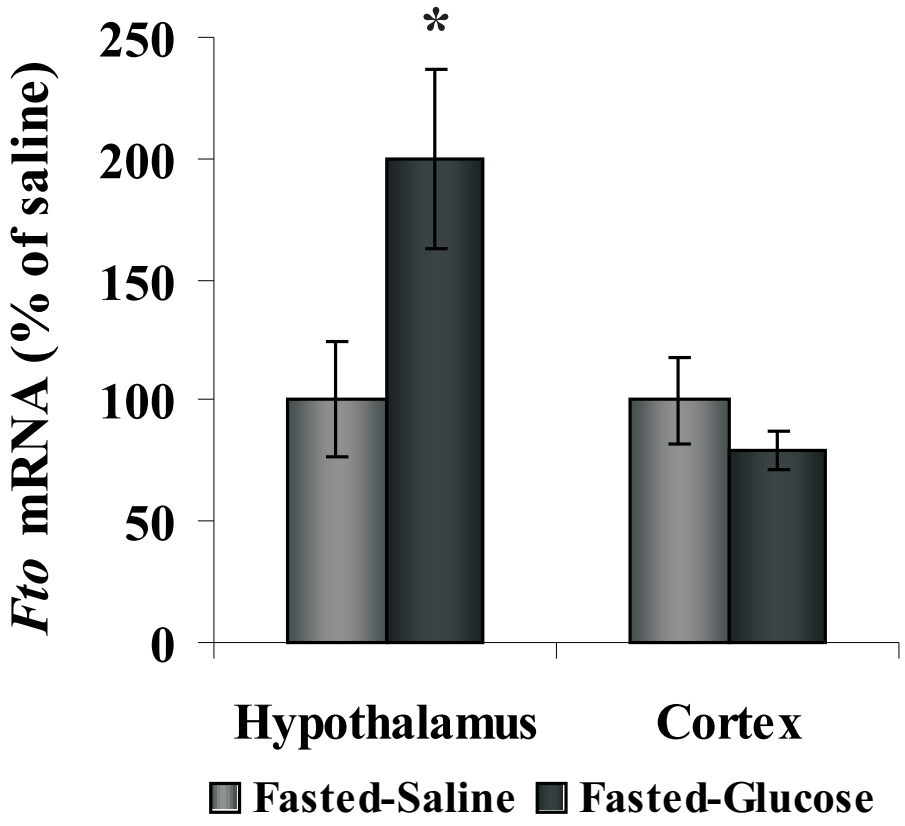


Figure 25: Glucose increases *Fto* expression in the hypothalamus but not in cerebral cortex. The effect of multiple (5) intracerebroventricular (i.c.v.) glucose injections on *Fto* mRNA expression was tested in male C57BL/6 mice fasted for 30 hours. Data are means ± S.E.M. (N = 9-10 mice/group). *: $P < 0.05$, by Student's t-test.

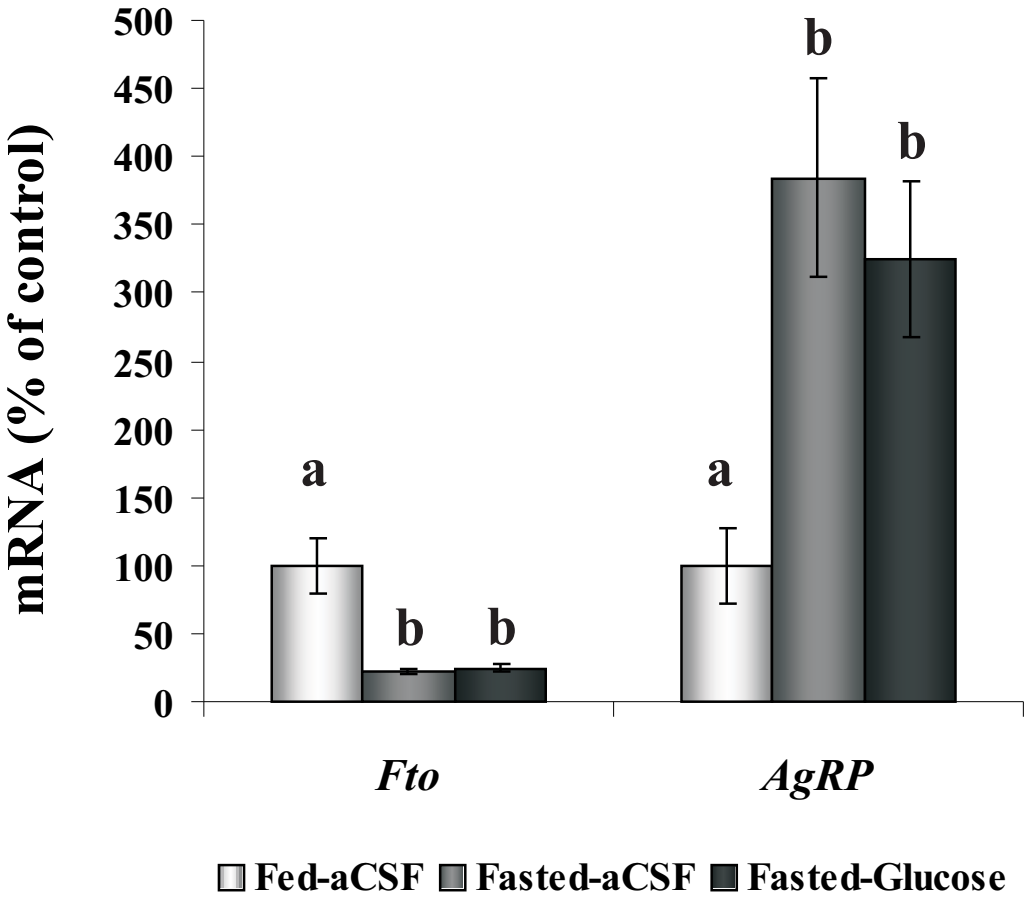


Figure 26: Opposite expression pattern of hypothalamic *Fto* and *AgRP* in response to prolonged fasting. The effect of fasting (48 h) and multiple (9) intracerebroventricular (i.c.v.) glucose injections on hypothalamic *Fto* and *AgRP* mRNA expression was investigated in male C57BL/6 mice. Data are means ± S.E.M. (N = 9-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test or Wilcoxon test with Bonferroni correction).

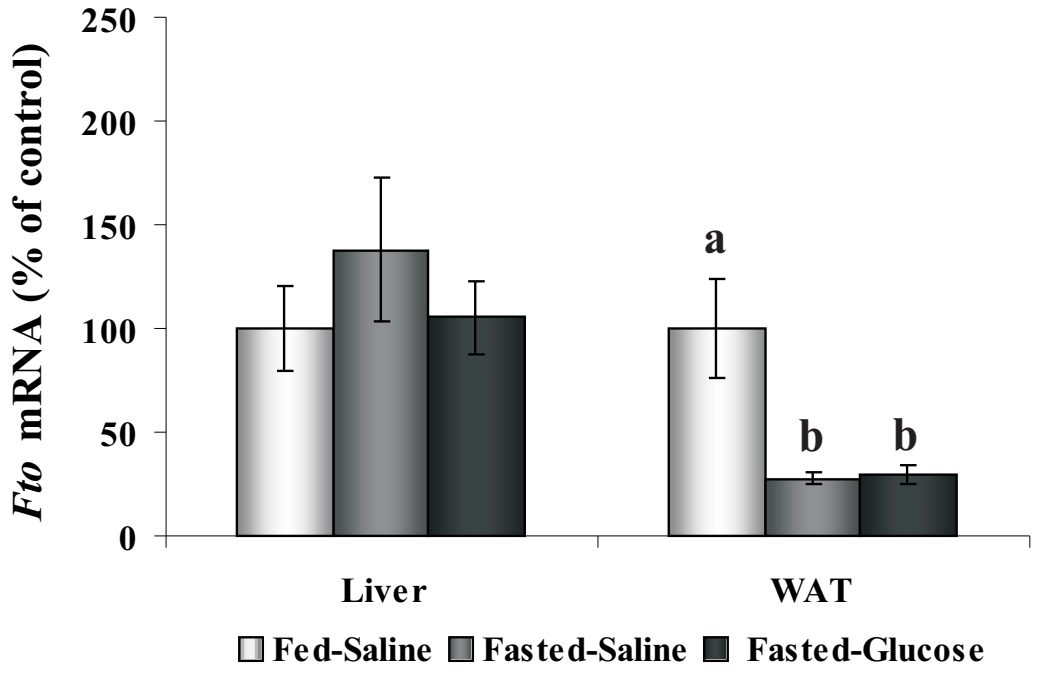


Figure 27: Prolonged fasting reduces *Fto* expression in the white adipose tissue (WAT) but not in the liver of lean mice. The effect of fasting (48 h) and multiple (9) intracerebroventricular (i.c.v.) glucose injections on *Fto* mRNA expression in peripheral tissues was tested in male C57BL/6 mice. Data are means ± S.E.M. (N = 9-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test or Wilcoxon test with Bonferroni correction).

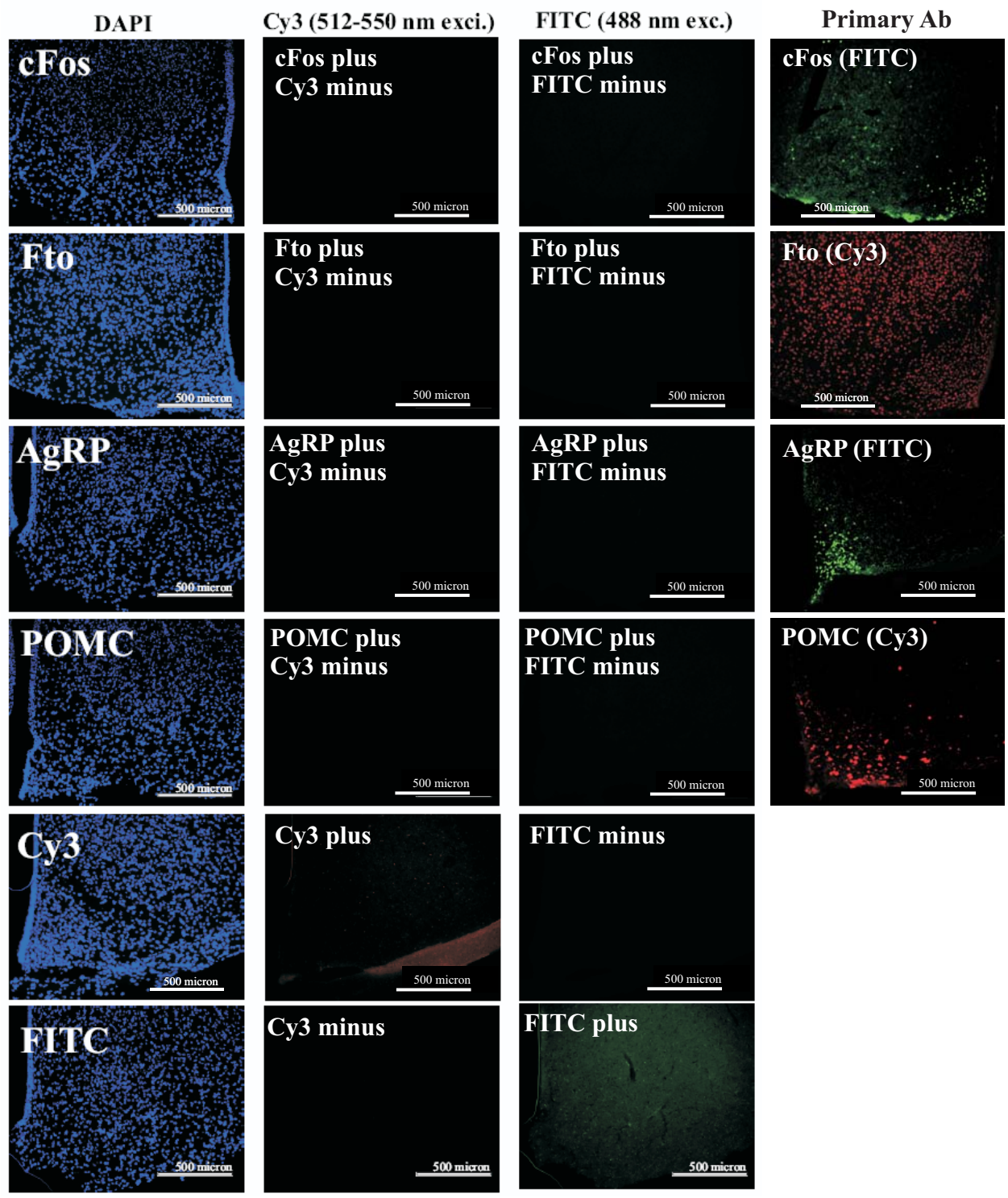


Figure 28: Negative controls for immunohistochemical analysis in hypothalamic regions of C57BL/6 male mice. Magnification: 10X. DAPI stains nuclei blue. Cy3 emits red fluorescence and FITC emits green fluorescence.

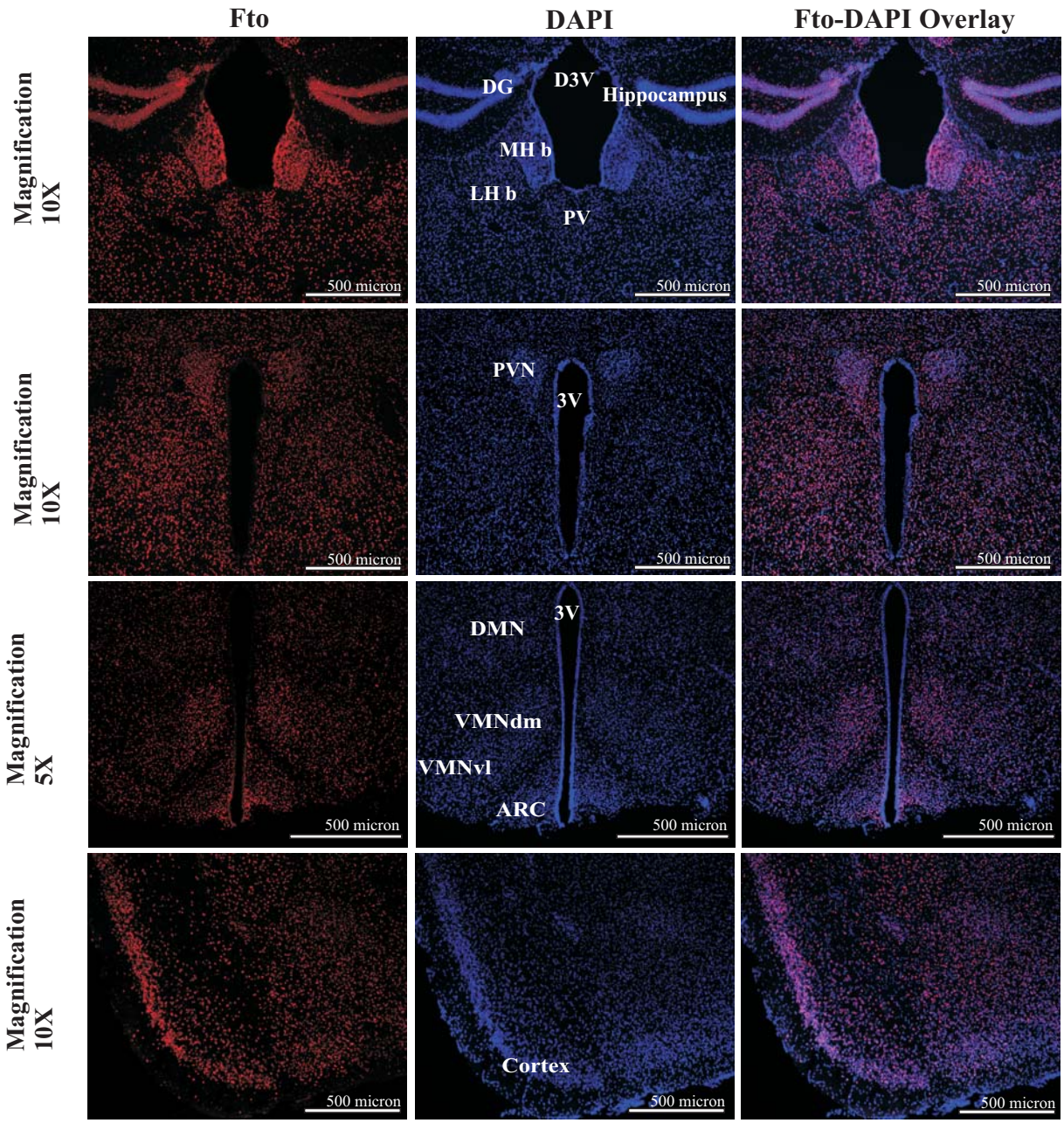


Figure 29: Fto expression pattern in the brain of male C57BL/6 mice. Fto-immunopositive cells were found in hippocampus, dentate gyrus (DG), paraventricular thalamic nucleus (PV), lateral habenular nucleus (Lhb), medial habenular nucleus (Mhb), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), VMN dorsomedial (VMNdm), VMN ventrolateral (VMNvl), arcuate nucleus (ARC) and cortex. Third ventricle (3V), dorsal 3V (D3V).

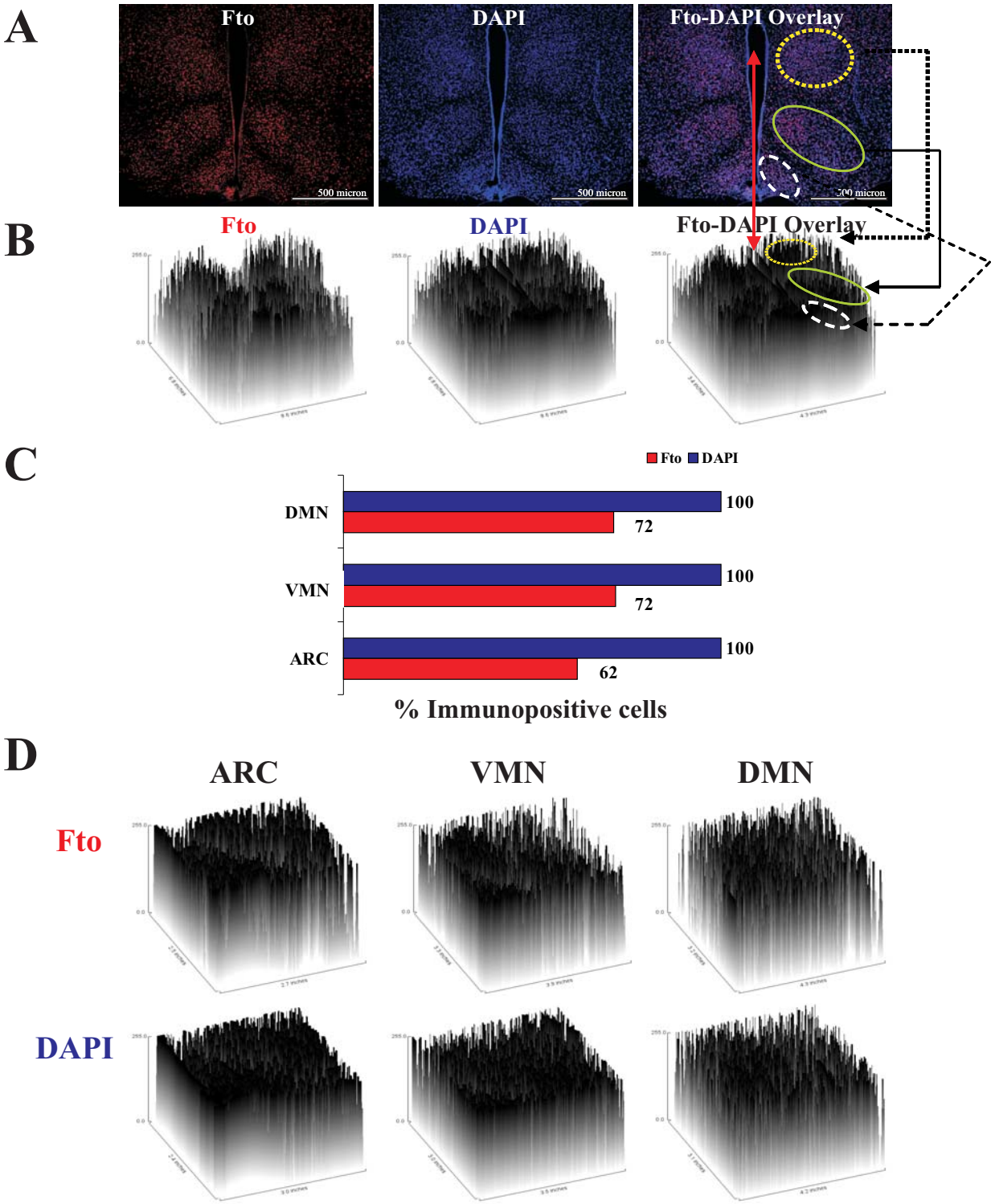


Figure 30: The abundantly expressed nuclear protein Fto is not present in every cell of the hypothalamus. The distribution pattern of Fto-immunopositive (neuronal) nuclei (Cy3) in the hypothalamus was investigated in male C57BL/6 mice. (A) Representative photomicrographs of Fto-immunopositive cells and DAPI-stained nuclei in the hypothalamus, showing the distribution of Fto, DAPI and Fto-DAPI co-localization. (B) Surface plot analysis of Fto-immunopositive cells, DAPI and Fto-DAPI distribution in the entire hypothalamus corresponding to sections in panel A. (C) Percent of DAPI-stained nuclei co-expressing Fto in the arcuate nucleus (ARC), ventromedial nucleus (VMN) and dorsomedial nucleus (DMN). (D) Representative Surface plot analysis of Fto and DAPI, depicting cell staining, distribution and intensity in a 3-dimentional image.

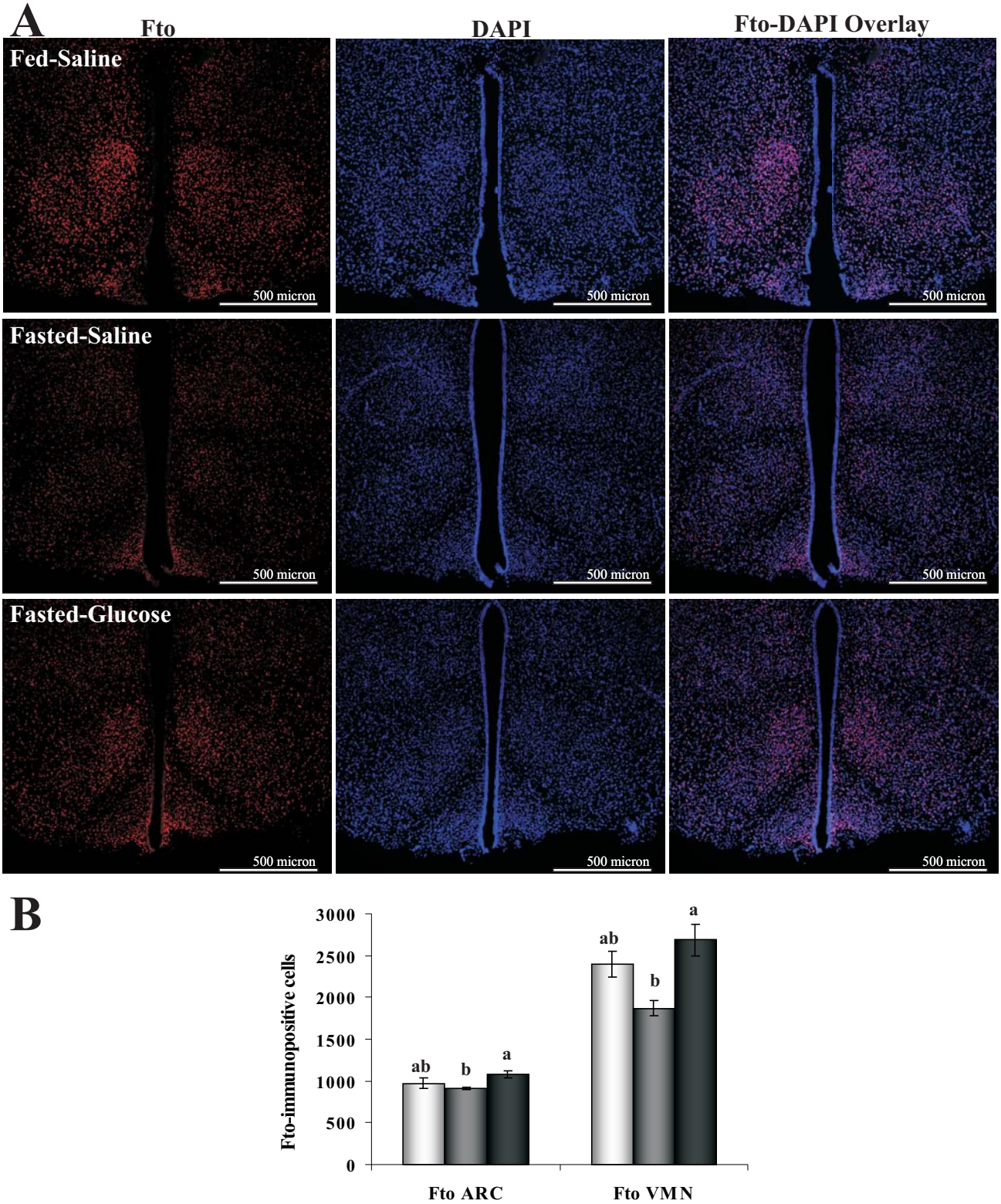


Figure 31: Hypothalamic Fto expression is increased by glucose in lean mice. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive neurons in the arcuate nucleus (ARC) and ventromedial nucleus (VMN) was tested in male C57BL/6 mice. (A) Representative photomicrographs showing distribution of Fto-immunopositive neuronal nuclei. Fto (Cy3). Magnification: 5X. (B) Number of Fto-immunopositive nuclei in ARC and VMN. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

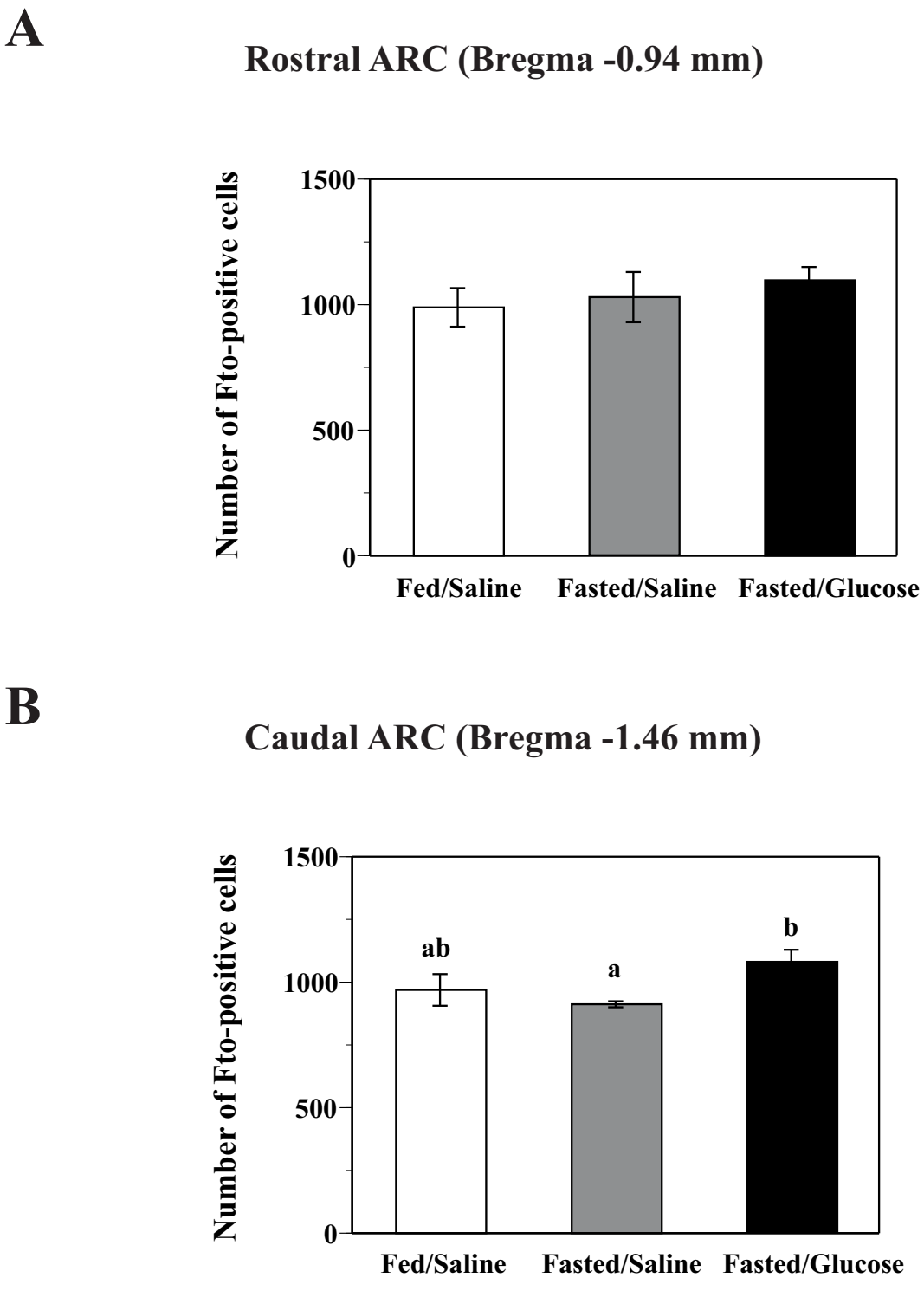


Figure 32: Glucose-induced increase in Fto expression in the caudal, but not rostral, ARC. Effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive cells was examined in different anterior-posterior levels of the arcuate nucleus (ARC) in male C57BL/6 mice. Number of Fto-immunopositive (neuronal) nuclei in rostral (A) and caudal (B) ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

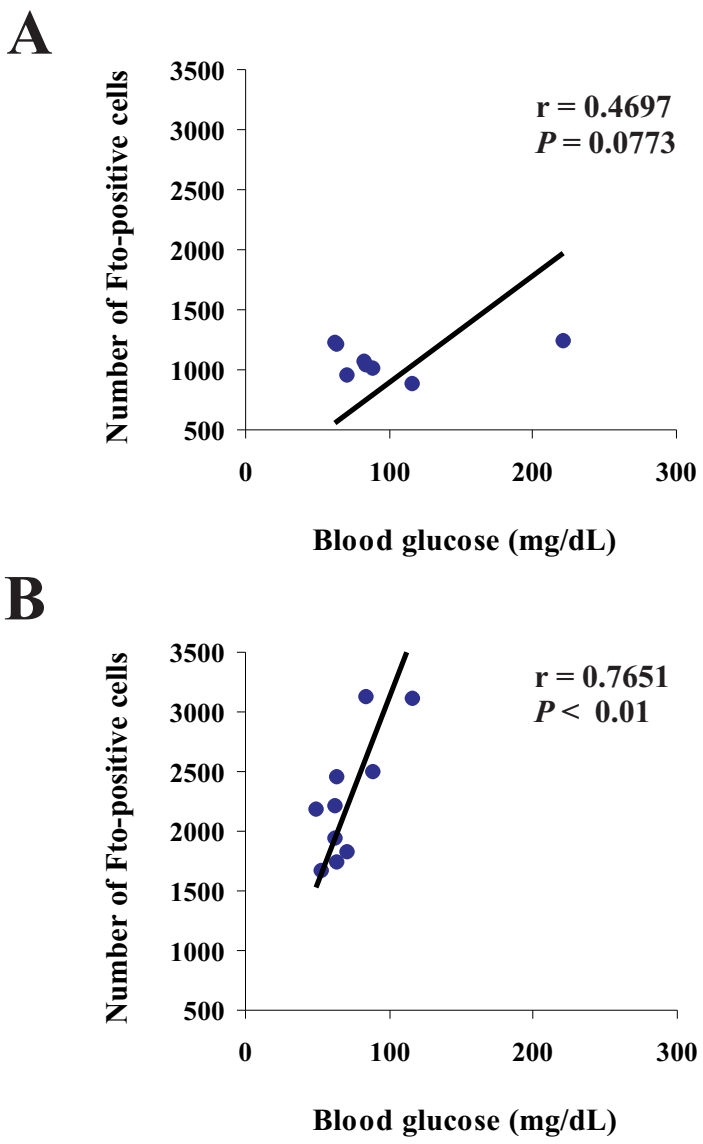


Figure 33: Under 30-h fasted conditions the levels of circulating glucose positively correlate with the number of Fto-immunopositive cells in the VMN of lean mice. Correlation analysis between blood glucose and Fto-immunopositive cell number in the (A) ARC and (B) VMN in male C57BL/6 mice fasted for 30 h and treated intraperitoneally with either saline or glucose.

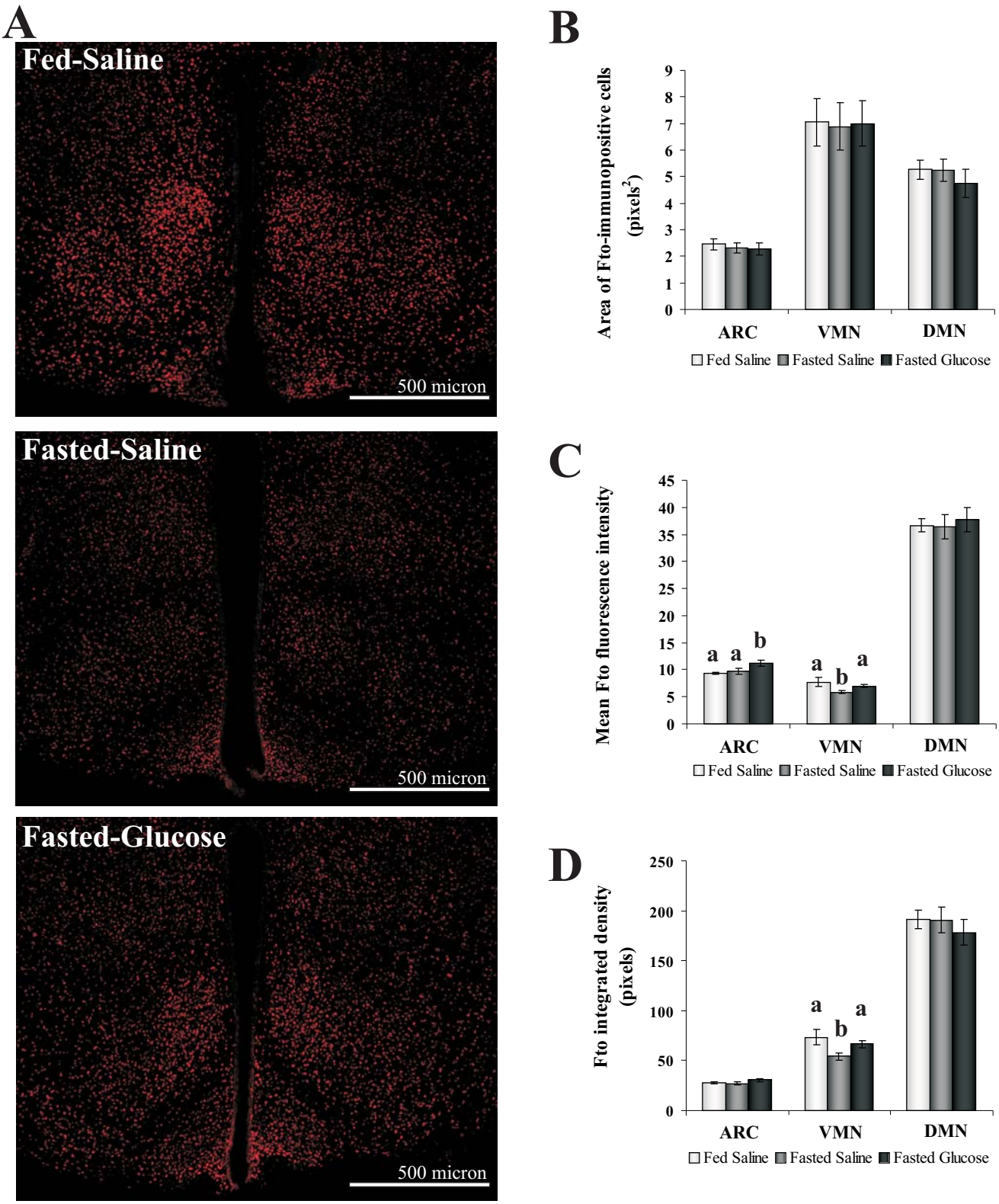
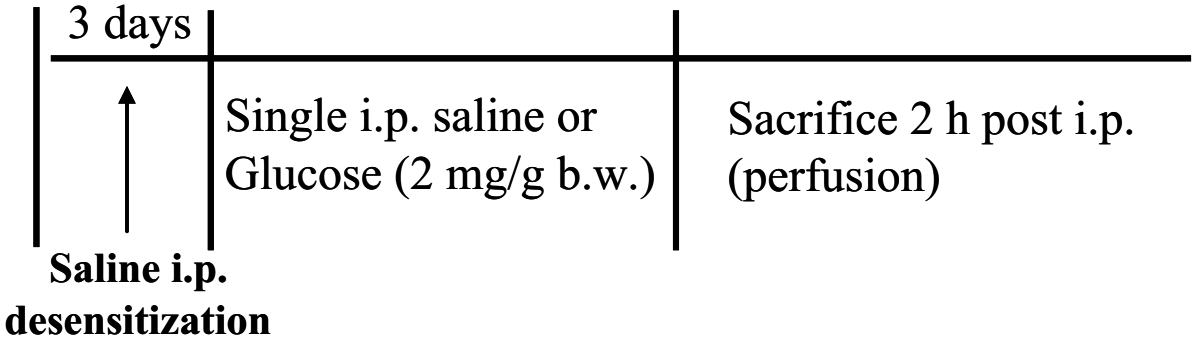


Figure 34: Fasting reduced Fto expression in the VMN and glucose treatment increases Fto expression in the VMN and ARC in lean mice. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive neurons in the arcuate nucleus (ARC) and ventromedial nucleus (VMN) was tested in male C57BL/6 mice. (A) Representative photomicrographs of Fto-immunopositive cells, showing distribution of Fto-immunopositive neuronal nuclei in the hypothalamus. Fto (Cy3). Magnification: 5X. (B) Total area of Fto-immunopositive nuclei. (C) Mean gray value for Fto fluorescence. (D) Integrated density of Fto expressing regions. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

Hypothesis III: Fto function is part of hypothalamic nutritional sensing mechanisms related to the central melanocortin system

Thesis Study V: Investigate the effect of intraperitoneal (i.p.) glucose on Fto expression pattern in the brain under fasted conditions (30 h) → PROTEIN ANALYSIS (immunohistochemistry)

Animals: wild-type mice; age 6-8 weeks



Immunohistochemical analysis of Fto, POMC and AgRP expression in the brain

Figure 35: Experimental outline in pursuit of Study V aimed at addressing the Thesis Hypothesis III.

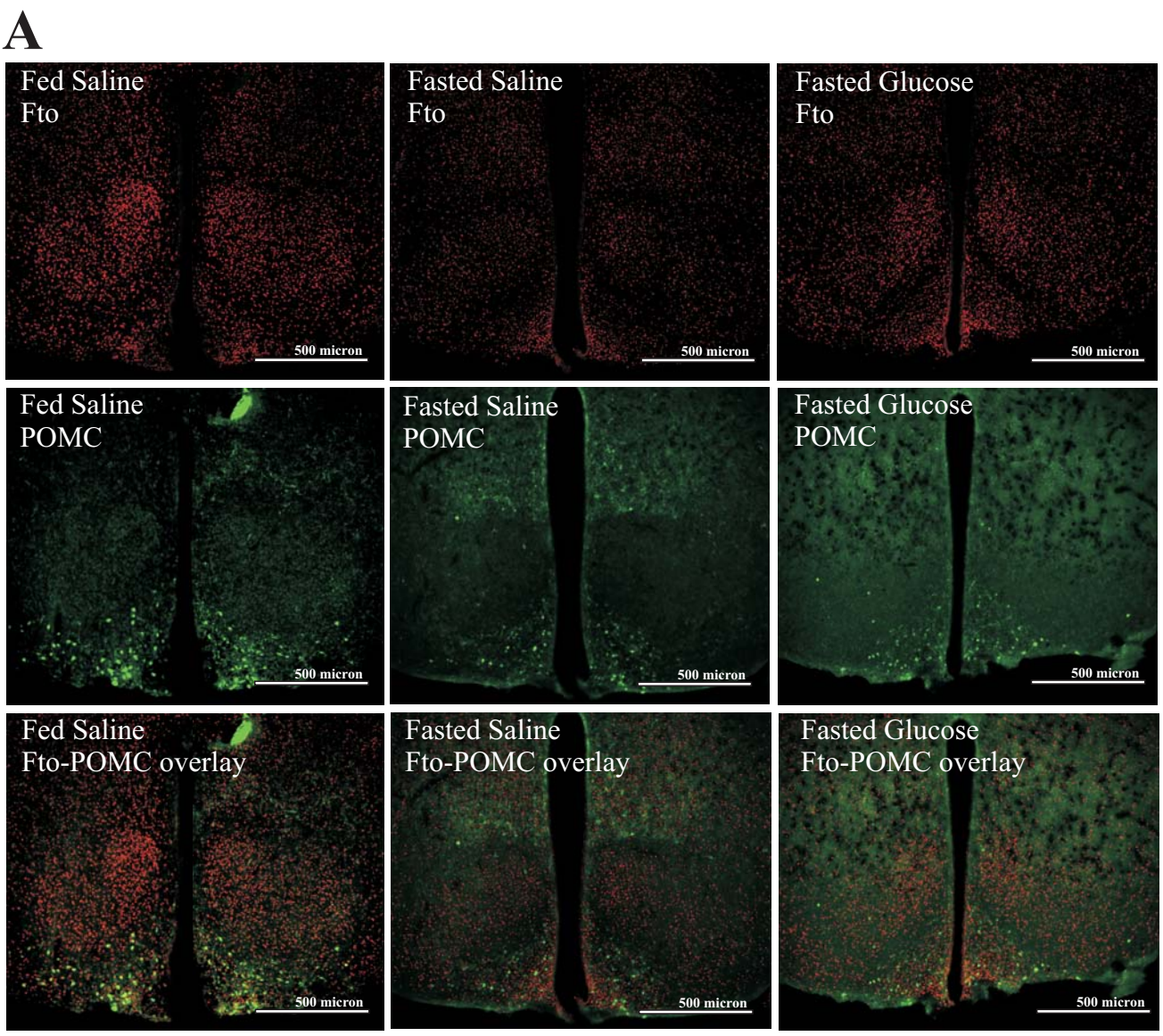


Figure 36: Fto expression exhibits the same pattern as POMC in response to fasting and glucose. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto- and POMC-immunopositive neurons in the arcuate nucleus (ARC) was tested in male C57BL/6 mice. (A) Representative photomicrographs of Fto- and POMC-immunopositive cells in the hypothalamus. Distribution of Fto-immunopositive nuclei (Cy3) and POMC neurons (FITC) were visualized. (B) Number of POMC-immunopositive nuclei in the ARC. Magnification: 5X. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

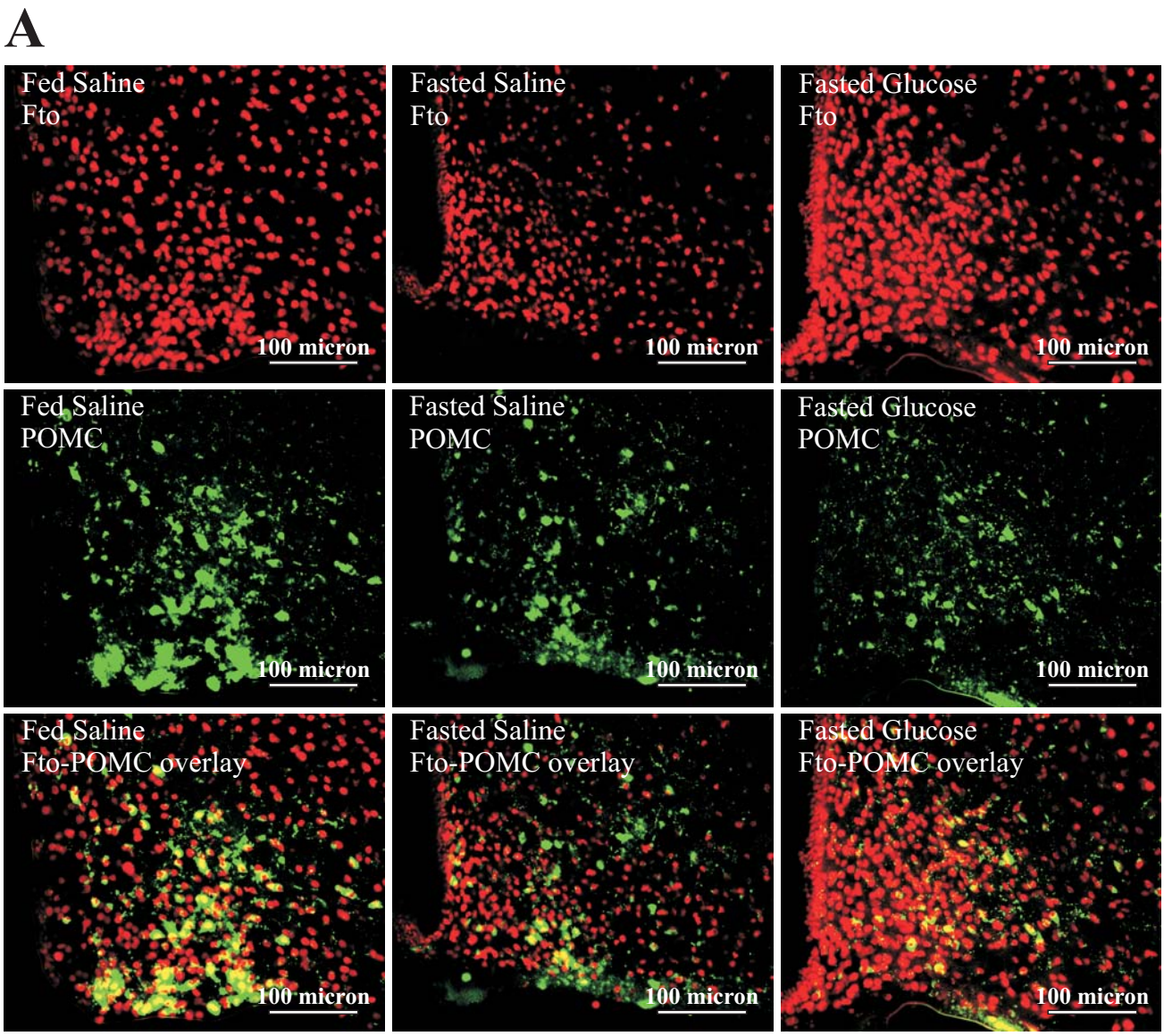


Figure 37: Glucose treatment reduces the extent of co-localization between Fto and POMC neurons. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto- and POMC-immunopositive neurons in the arcuate nucleus (ARC) was tested in male C57BL/6 mice. (A) Distribution of Fto-immunopositive nuclei (Cy3), and POMC neurons (FITC). Magnification: 20X. (B) Number of POMC-Fto co-expressing cells. (C) Percentage of POMC-positive cells co-expressed with Fto-immunopositive nuclei in the ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

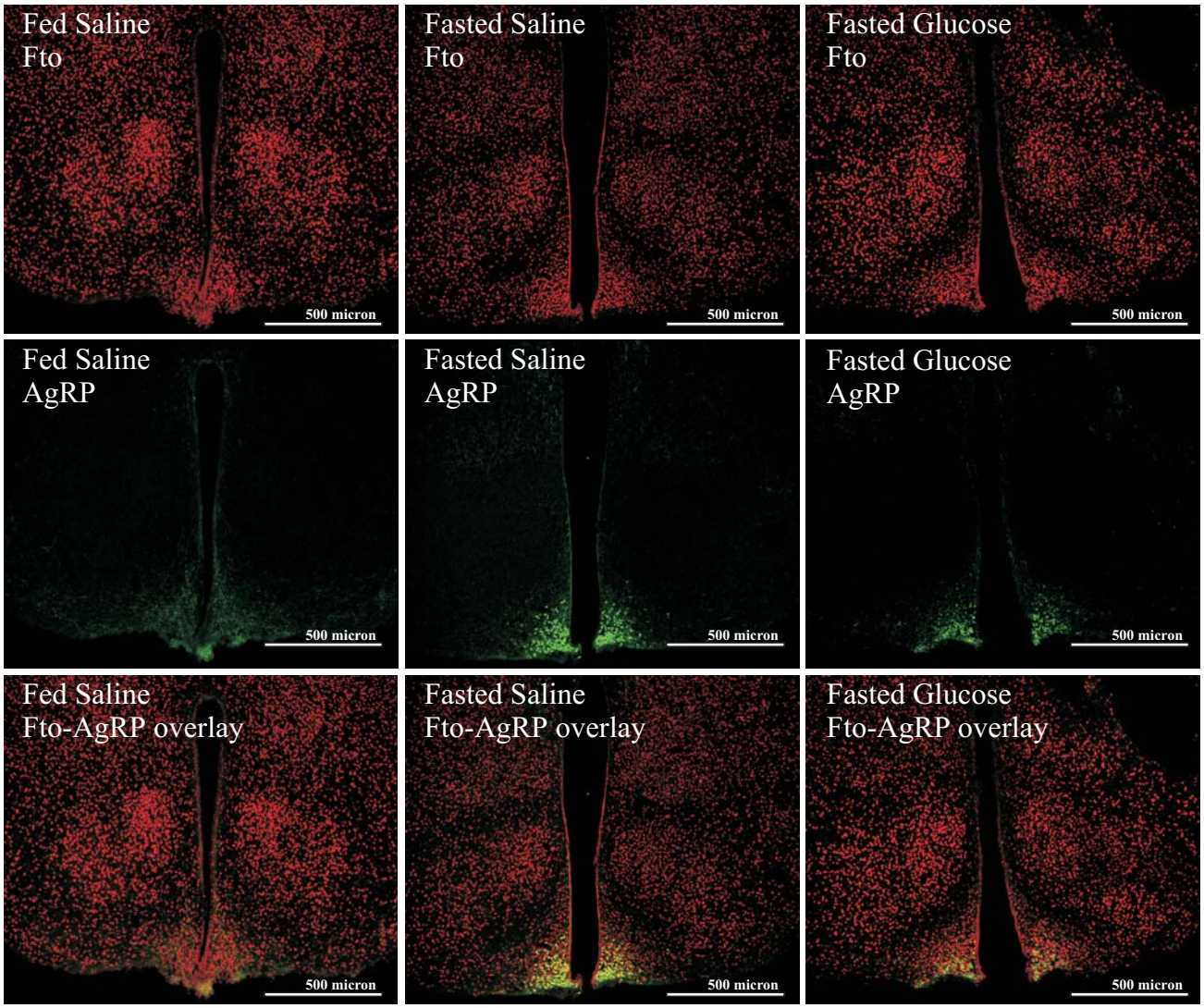


Figure 38: Fto exhibits an opposite expression pattern as compared with AgRP in response to fasting and glucose treatment. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto- and AgRP-immunopositive neurons in the arcuate nucleus (ARC) was tested in male C57BL/6 mice. (A) Representative photomicrographs of Fto- and AgRP-immunopositive cells in the hypothalamus. Distribution of Fto-immunopositive nuclei (Cy3) and AgRP neurons (FITC) were visualized. Magnification: 5X. (B) Number of AgRP-immunopositive nuclei in the ARC. (C) Percentage of AgRP neurons co-expressing Fto in the ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

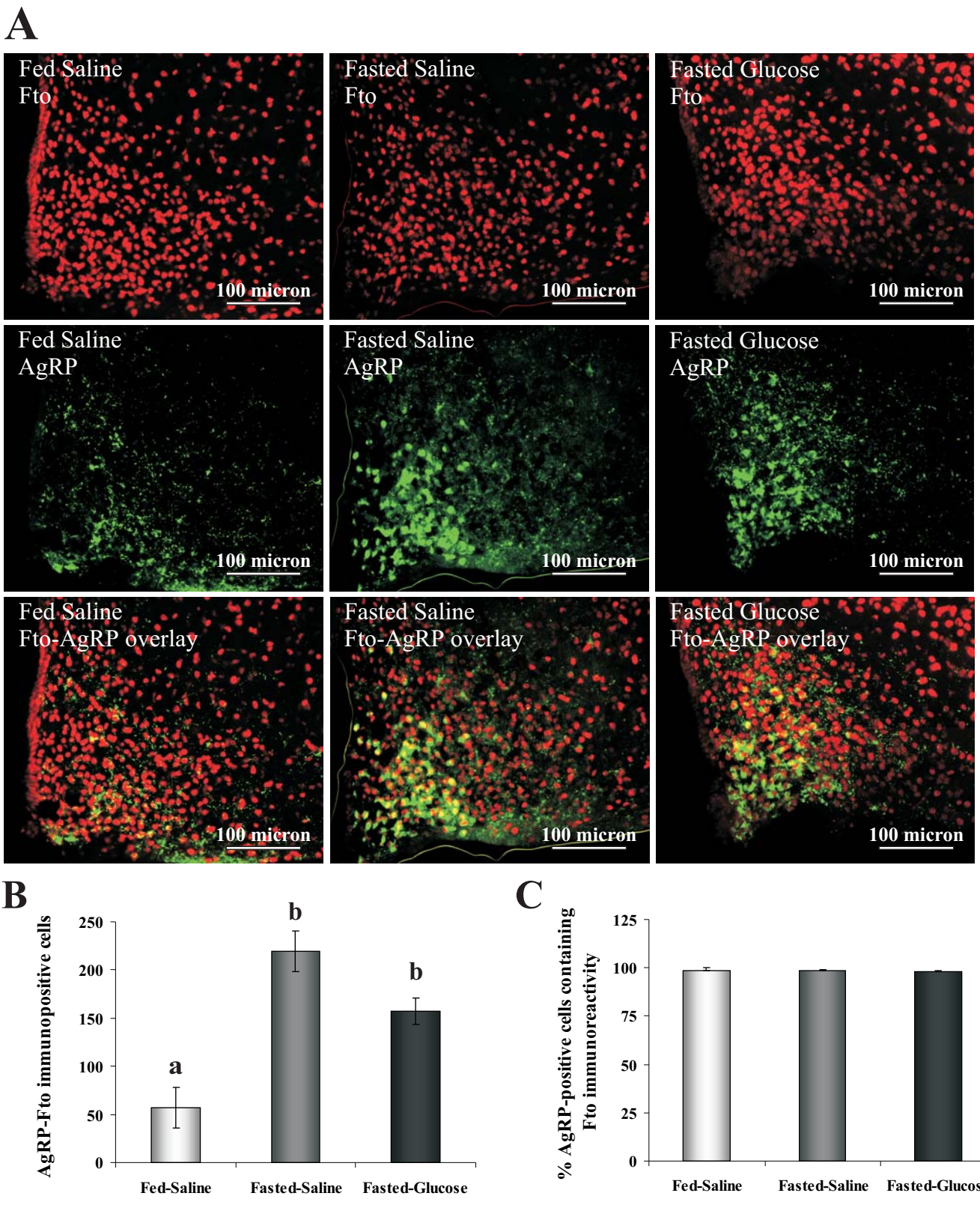


Figure 39: Fto is co-localized with AgRP neurons during changes in nutritional states. The effect of fasting (30 h) and single intraperitoneal (i.p.) glucose treatment on Fto- and AgRP-immunopositive neurons in the arcuate nucleus (ARC) was tested in male C57BL/6 mice. (A) Representative photomicrographs of Fto- and AgRP-immunopositive cells in the hypothalamus. Distribution of Fto-immunopositive nuclei (Cy3) and AgRP neurons (FITC) were visualized. Magnification: 20X. (B) Number of AgRP-Fto co-expressing cells. (C) Percentage of AgRP neurons co-expressing Fto in the ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

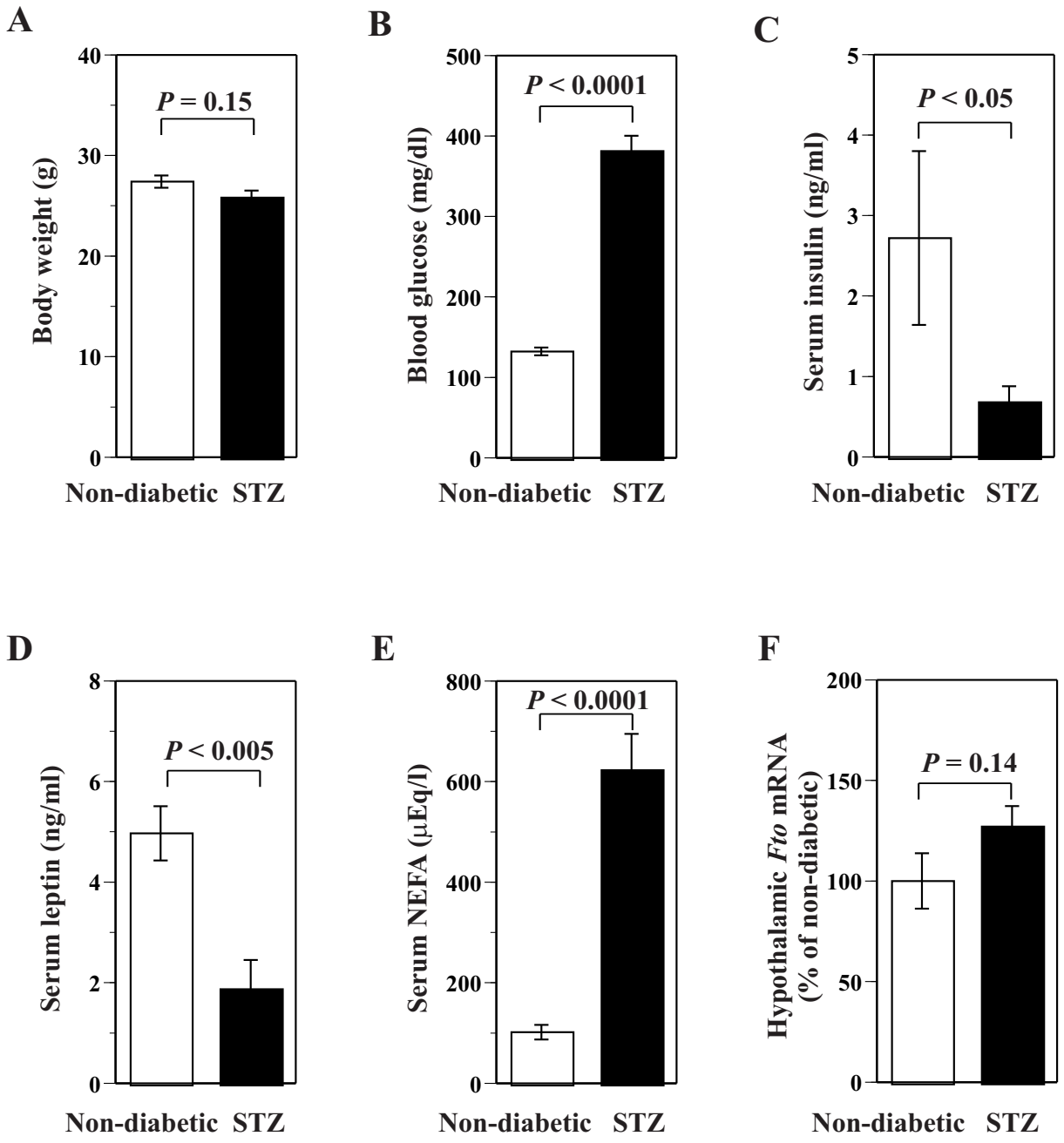


Figure 40: Diminished levels of circulating insulin do not alter hypothalamic *Fto* mRNA expression. The effect of streptozotocin (STZ)-induced diabetes on body weight (A), blood glucose (B), serum insulin (C), serum leptin (D), serum NEFA (E), and hypothalamic *Fto* mRNA levels (F). Data are means ± S.E.M. (*N* = 5-7 animals/group). Statistical analysis was performed by either Student's *t*-test or Wilcoxon test.

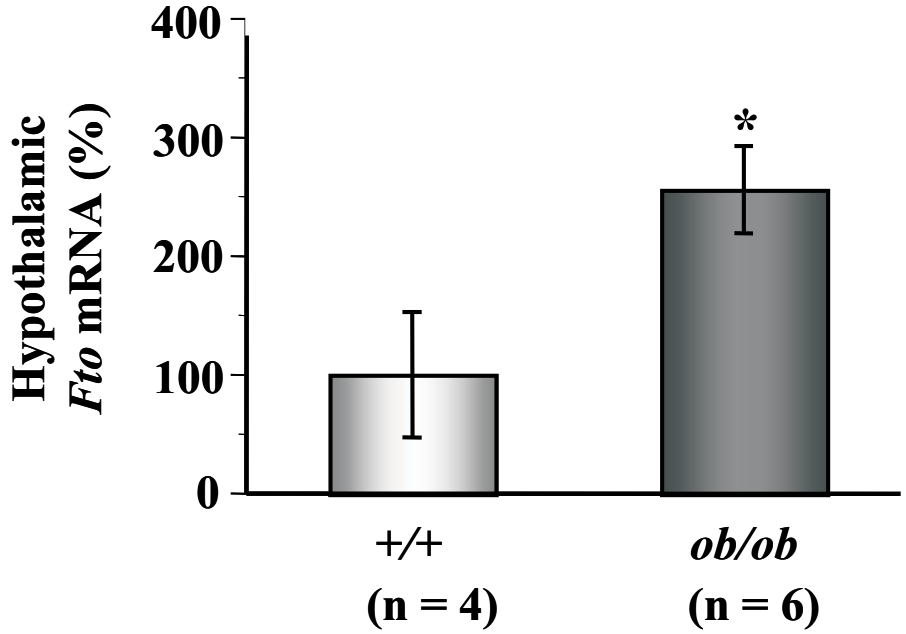
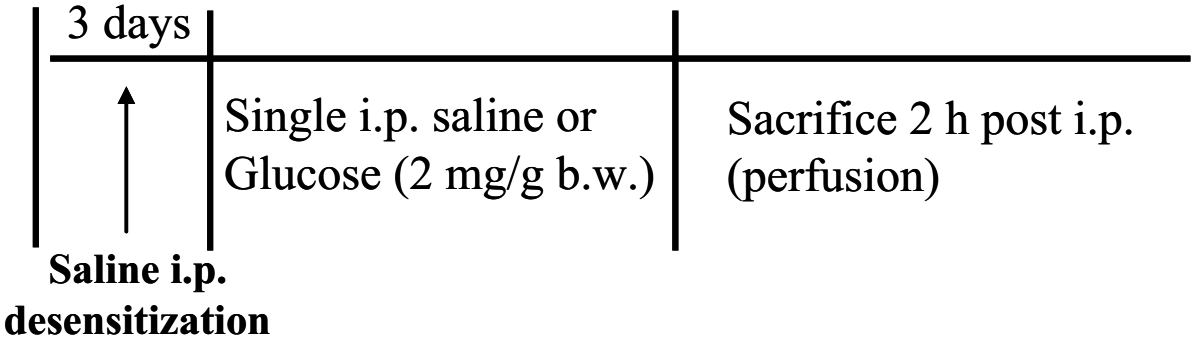


Figure 41: Obesity increases the expression of hypothalamic *Fto* mRNA. The effect of obesity on hypothalamic *Fto* mRNA was tested in the lepin deficient *ob/ob mice*. Student`s *t*-test, * $P < 0.05$. Data are means ± S.E.M.

Hypothesis IV: Nutritional regulation of hypothalamic Fto expression is impaired in obesity

Thesis Study VI: Investigate the effect of intraperitoneal (i.p.) glucose on Fto expression pattern in the brain under prolonged fasted conditions (48 h) in obese animals → PROTEIN ANALYSIS (immunohistochemistry)

Animals: Wild-type and *ob/ob* mice; age 6-8 weeks



Immunohistochemical analysis of Fto, POMC, AgRP and cFos expression in the brain

Figure 42: Experimental outline in pursuit of Study VI aimed at addressing the Thesis Hypothesis IV.

Table 9. The effect of 48-h fasting and glucose treatment (single i.p.) in wild-type C57BL/6J male mice

	Fed-Saline	Fasted-Saline	Fasted-Glucose
Body weight (g)	24.9 ± 0.4^{A}	18.4 ± 0.3^{B}	18.7 ± 0.2^{B}
Body weight change (g)	-0.17 ± 0.09^{A}	-6.10 ± 0.17^{B}	-6.39 ± 0.24^{B}
Blood glucose (mg/dl)	183.8 ± 12.9^{A}	80.9 ± 4.9^{B}	128.0 ± 11.3^{C}

Values are means ± SEM. (N = 10/group)
Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey-Kramer test).

Table 10. The effect of 48-h fasting and glucose treatment (single i.p.) in *ob/ob* male mice

	Fed-Saline	Fasted-Saline	Fasted-Glucose
Body weight (g)	51.4 ± 0.7^{A}	42.3 ± 0.6^{B}	42.3 ± 1.1^{B}
Body weight change (g)	-0.18 ± 0.23^{A}	-6.09 ± 0.15^{B}	-5.62 ± 0.24^{B}
Blood glucose (mg/dl)	417.7 ± 30.4^{A}	89.6 ± 9.5^{B}	267.1 ± 39.5^{C}

Values are means ± SEM. (N = 10/group)
Groups that do not share a common letter are significantly different ($P < 0.05$, Tukey-Kramer test).

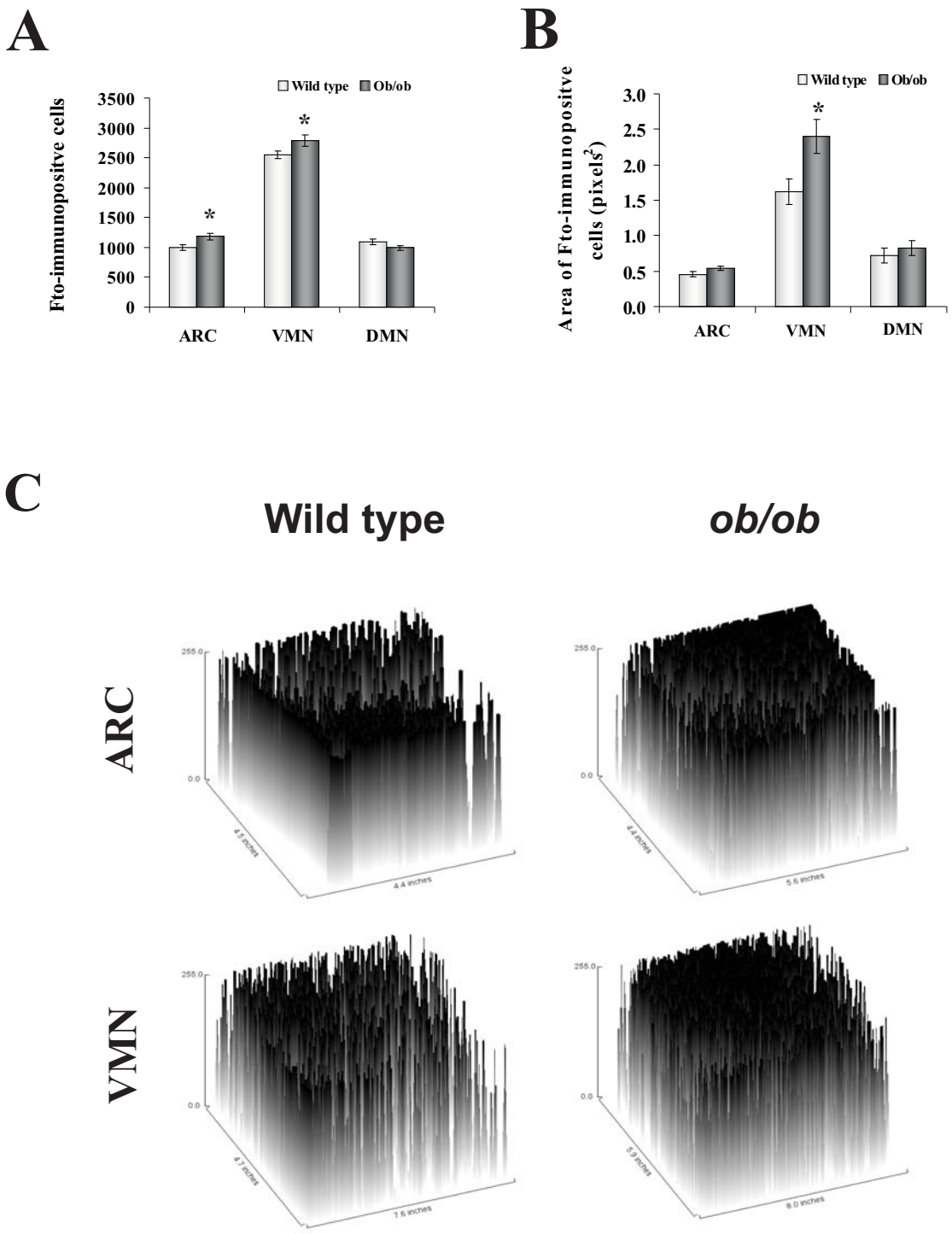


Figure 43: Up-regulation of Fto expression in the hypothalamus of obese mice. The effect of obesity on hypothalamic Fto-immunopositive cells was tested in male C57BL/6J wild-type and *ob/ob* mice fed *ad libitum*. (A) Number of Fto-immunopositive cells in ARC, VMN and DMN. (B) Total area of Fto-immunopositive regions in ARC, VMN and DMN. (C) Representative Surface plot analysis showing both distribution and density of Fto-immunopositive cells in the ARC and VMN of wild-type and *ob/ob* mice. Data are means ± S.E.M. (N = 7-10 mice/group). *: $P < 0.05$, Student's t-test.

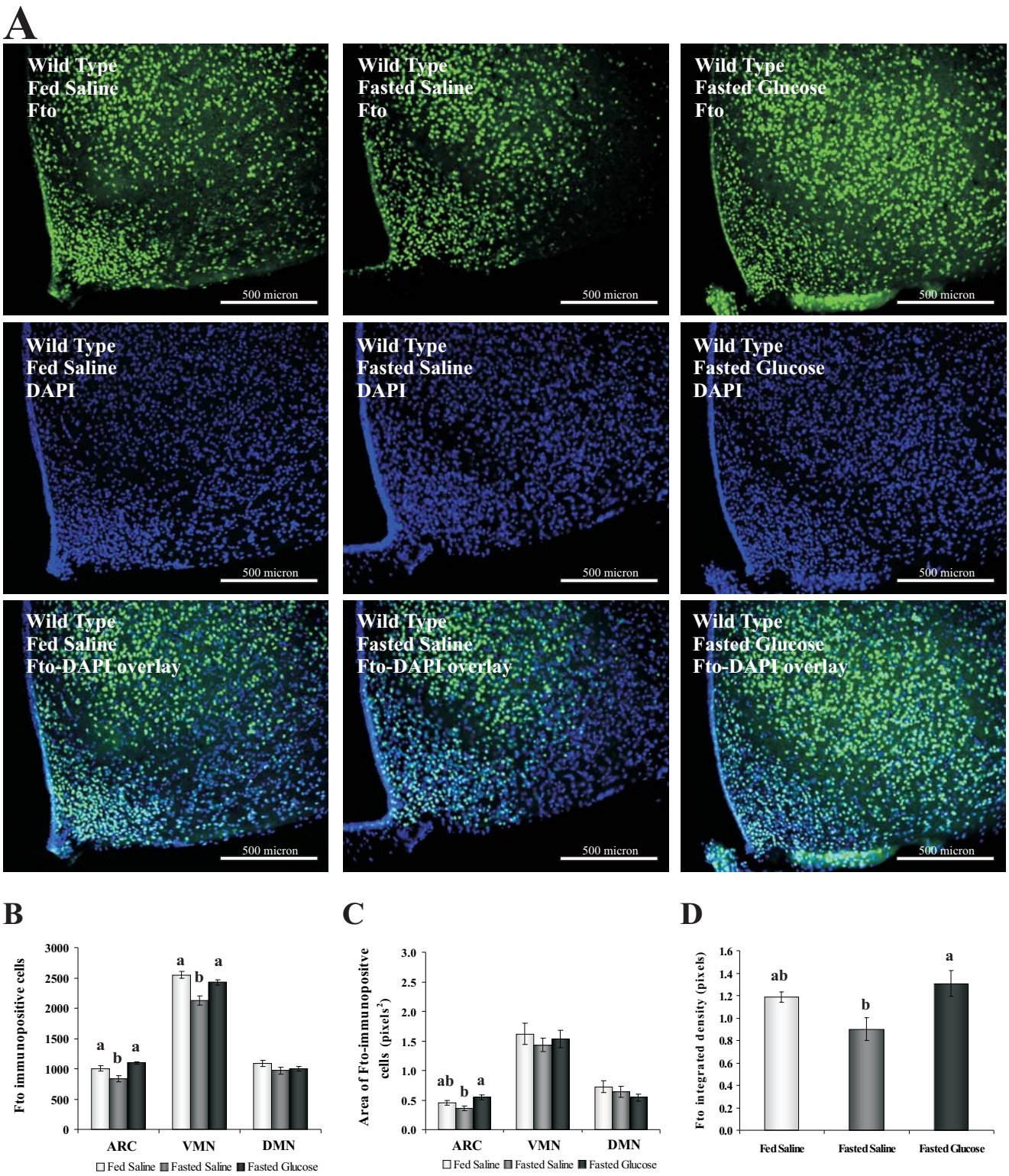


Figure 44: Fasting reduces and glucose treatment increases Fto expression and distribution in the hypothalamus of lean mice. The effect of fasting (48 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive cells in the arcuate nucleus (ARC), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN) was tested in male C57BL/6J mice. (A) Representative photomicrographs of Fto-immunopositive cells in the hypothalamus. Distribution of nuclei (DAPI) and Fto-immunopositive nuclei (FITC) were visualized. (B) Number of Fto-immunopositive nuclei in ARC, VMN and DMN. (C) Total area of Fto-immunopositive regions. (D) Integrated density of Fto-immunopositive cells in ARC. Magnification: 10X. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different within each brain region ($P < 0.05$, Tukey-Kramer test).

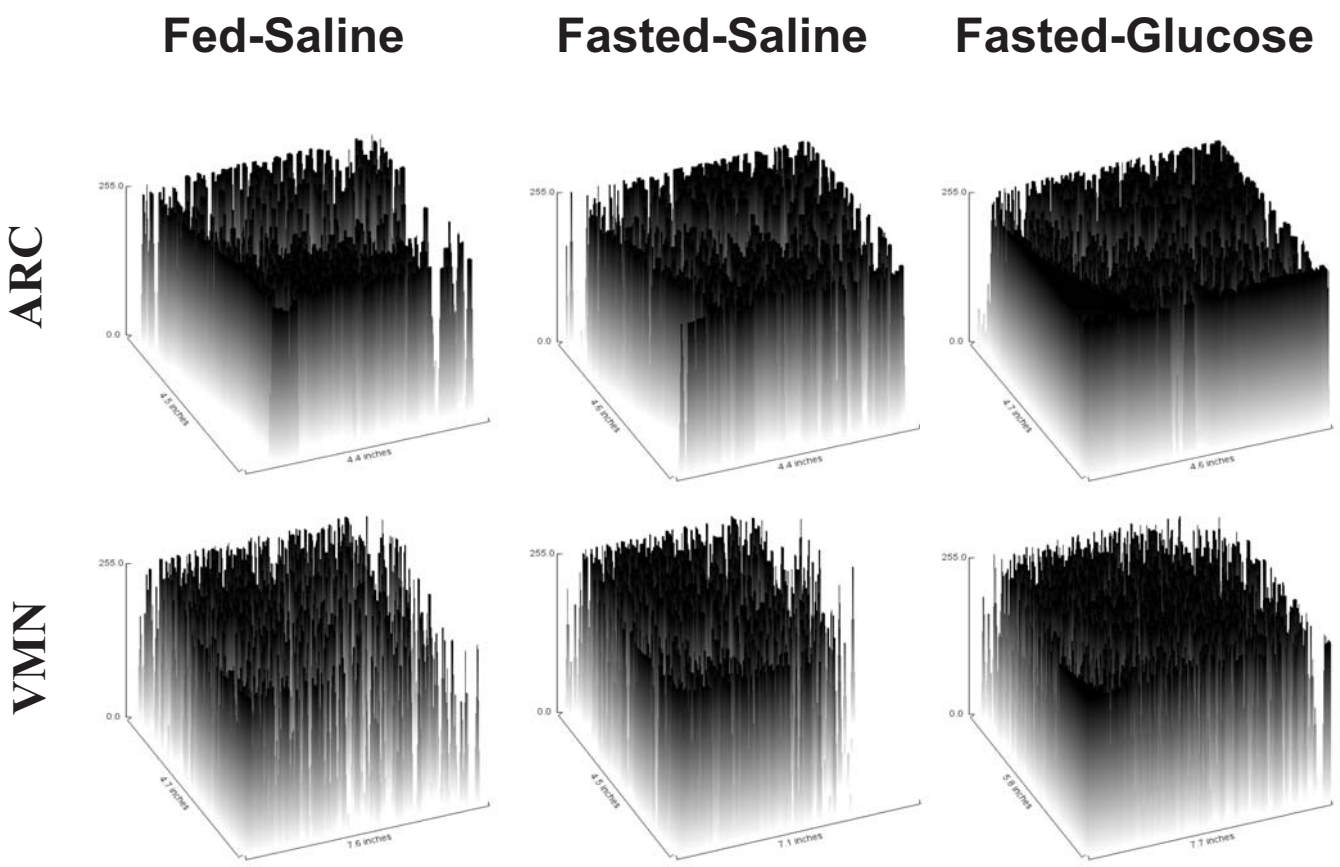


Figure 45: Fto expression pattern changes and Fto-expressing cell density decreases by fasting and increases by glucose in lean mice. The effect of fasting (48 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive cells was tested in male C57BL/6J mice. Representative Surface plot analysis showing distribution and density of Fto-immunopositive cells in the arcuate nucleus (ARC) and ventromedial nucleus (VMN).

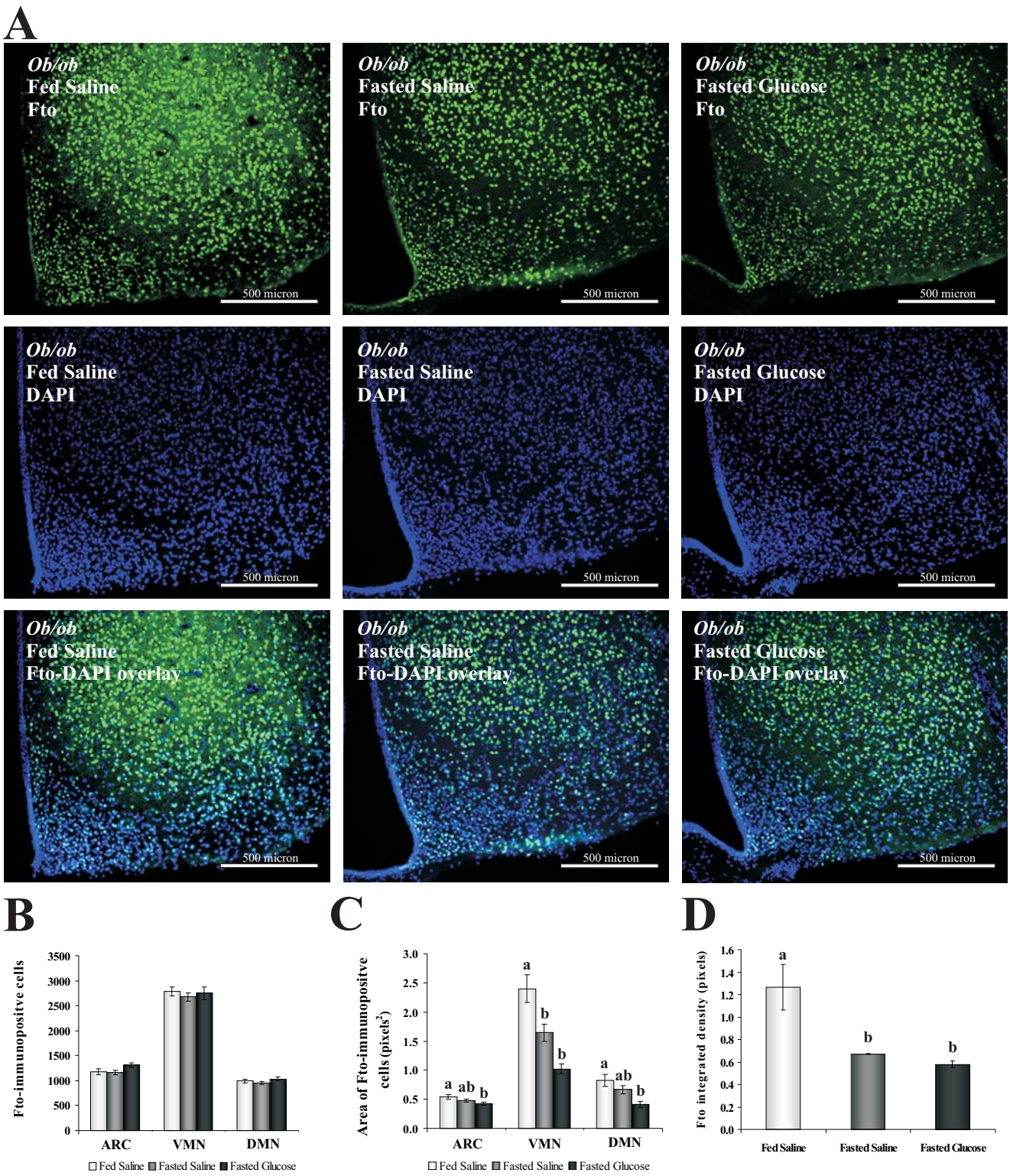


Figure 46: In the presence of obesity the number of Fto-immunopositive cells is not altered in response to changes in nutritional state. The effect of fasting (48 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive cells in the arcuate nucleus (ARC), ventromedial nucleus (VMN), and dorsomedial nucleus (DMN) was tested in male *ob/ob* mice. (A) Representative photomicrographs of Fto-immunopositive cells in the hypothalamus. Distribution of nuclei (DAPI) and Fto-immunopositive nuclei (FITC) were visualized. (B) Number of Fto-immunopositive nuclei in ARC, VMN and DMN. (C) Total area of Fto-immunopositive regions. (D) Integrated density of Fto-immunopositive cells in ARC. Magnification: 10X. Data are means ± S.E.M (N =7-10 mice/group). Groups with different letters are statistically different within each brain region ($P < 0.05$, Tukey-Kramer test).

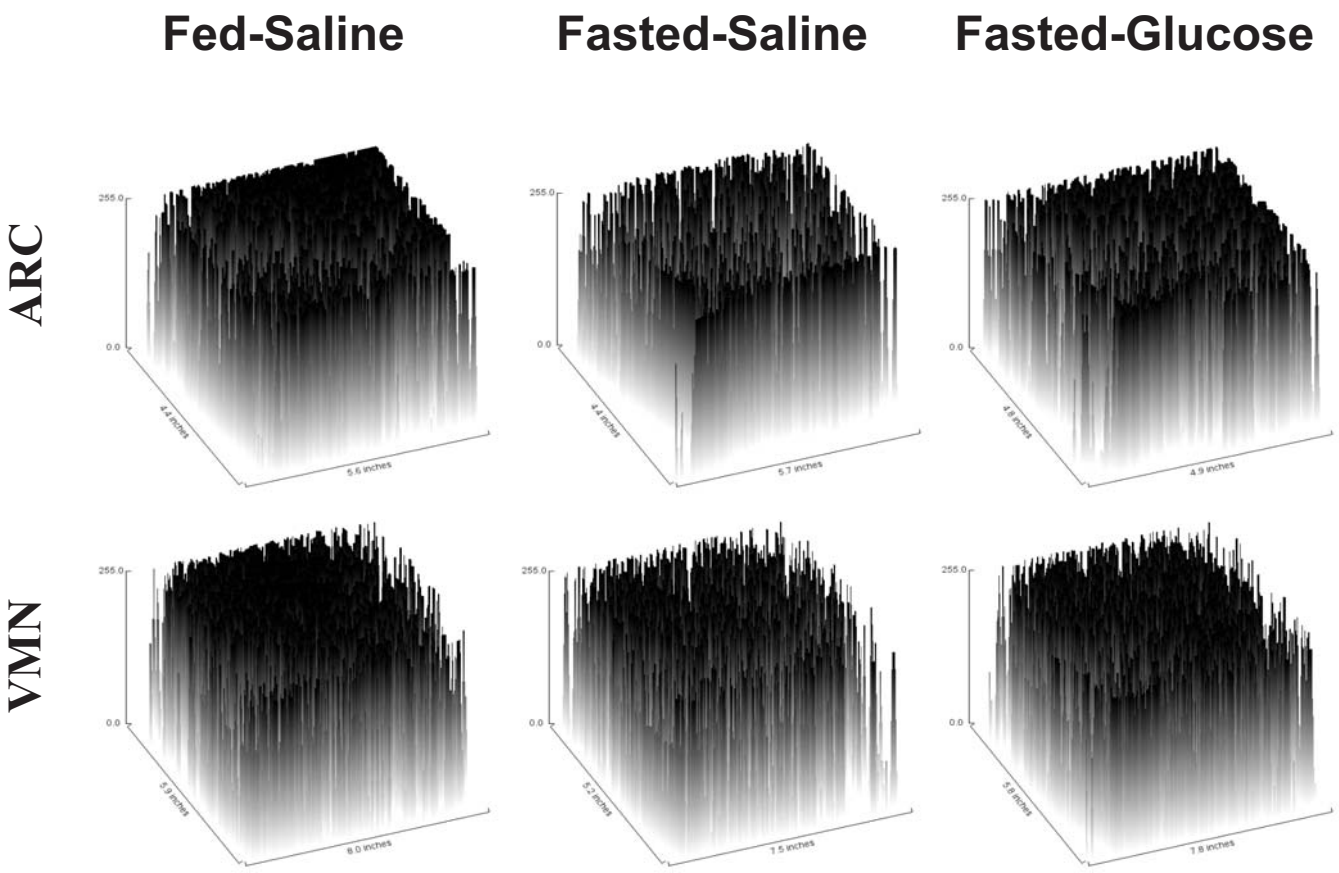


Figure 47: Fto-immunopositive cell density is decreased by fasting in the hypothalamus of obese mice. The effect fasting (48 h) and single intraperitoneal (i.p.) glucose treatment on Fto-immunopositive cells was tested in male *ob/ob* mice. Representative Surface plot analysis showing distribution and density of Fto-immunopositive cells in the arcuate nucleus (ARC) and ventromedial nucleus (VMN).

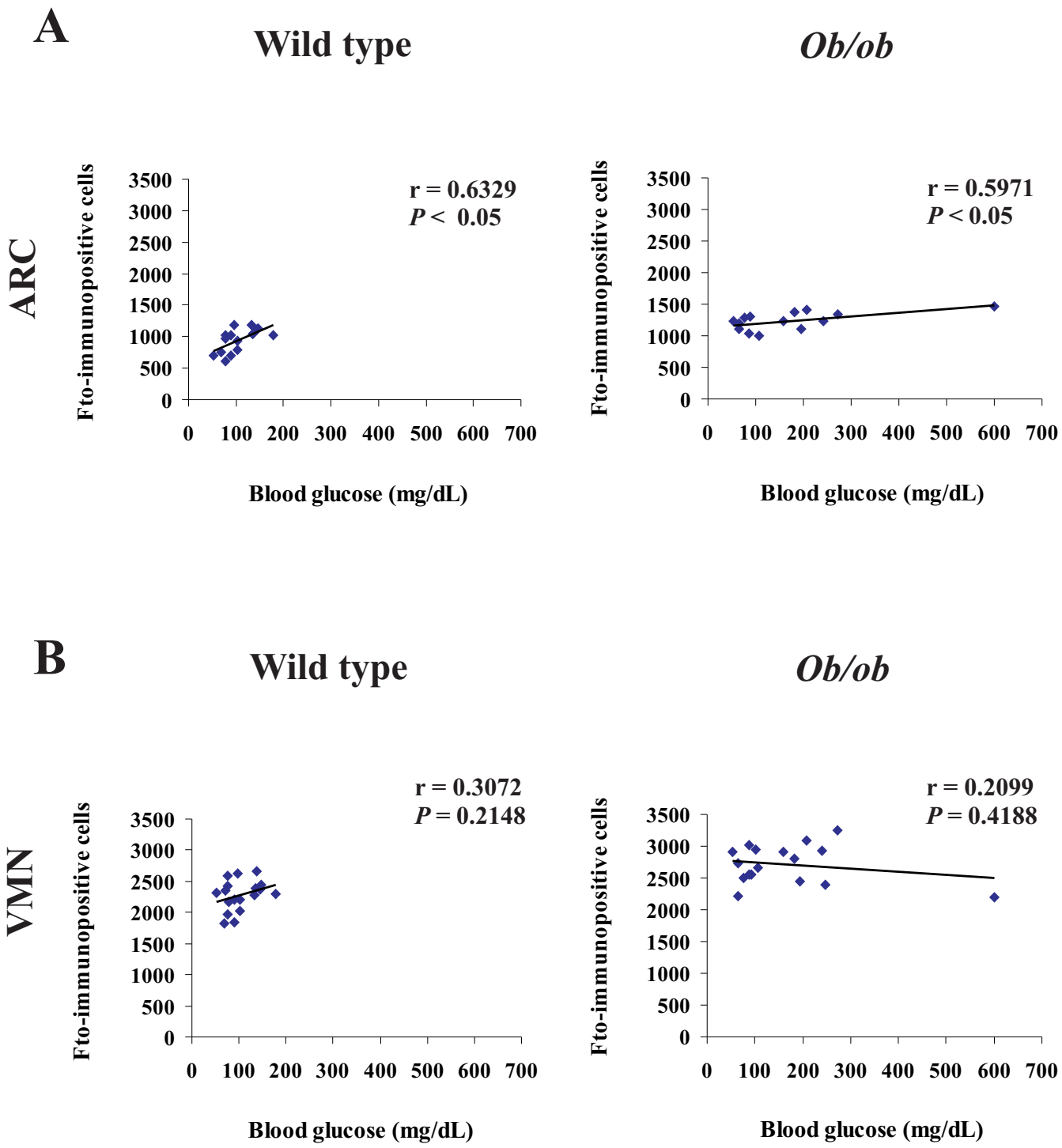


Figure 48: Positive correlation between circulating blood glucose and ARC Fto expression is present in lean mice, but not in obese mice. Correlation analysis between blood glucose and Fto-immunopositive cell number in the ARC (A) and VMN (B) in male wild-type and *ob/ob* mice fasted for 48 h and treated intraperitoneally with either saline or glucose (N =7-10 mice/group).

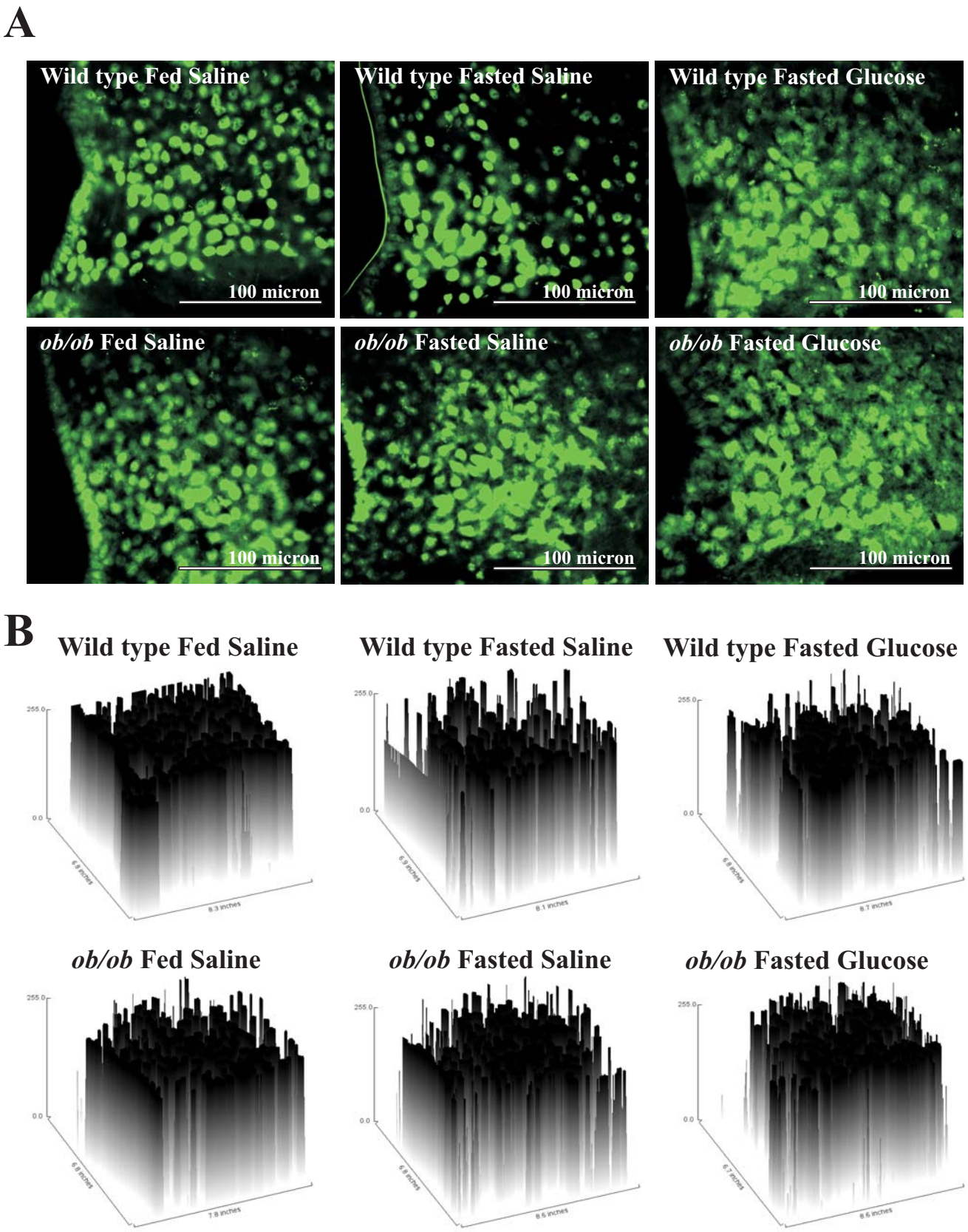


Figure 49: Blunted response in hypothalamic Fto expression to changes in nutritional states. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto-immunopositive neurons in the arcuate nucleus (ARC) was examined in male C57BL/6J wild-type and *ob/ob* mice. (A) Representative photomicrographs of Fto expression pattern in the ARC. Distribution of Fto-immunopositive nuclei (FITC) was visualized. Magnification: 40X. (B) Surface plot analysis of Fto-immunopositive cell distribution and density in the ARC.

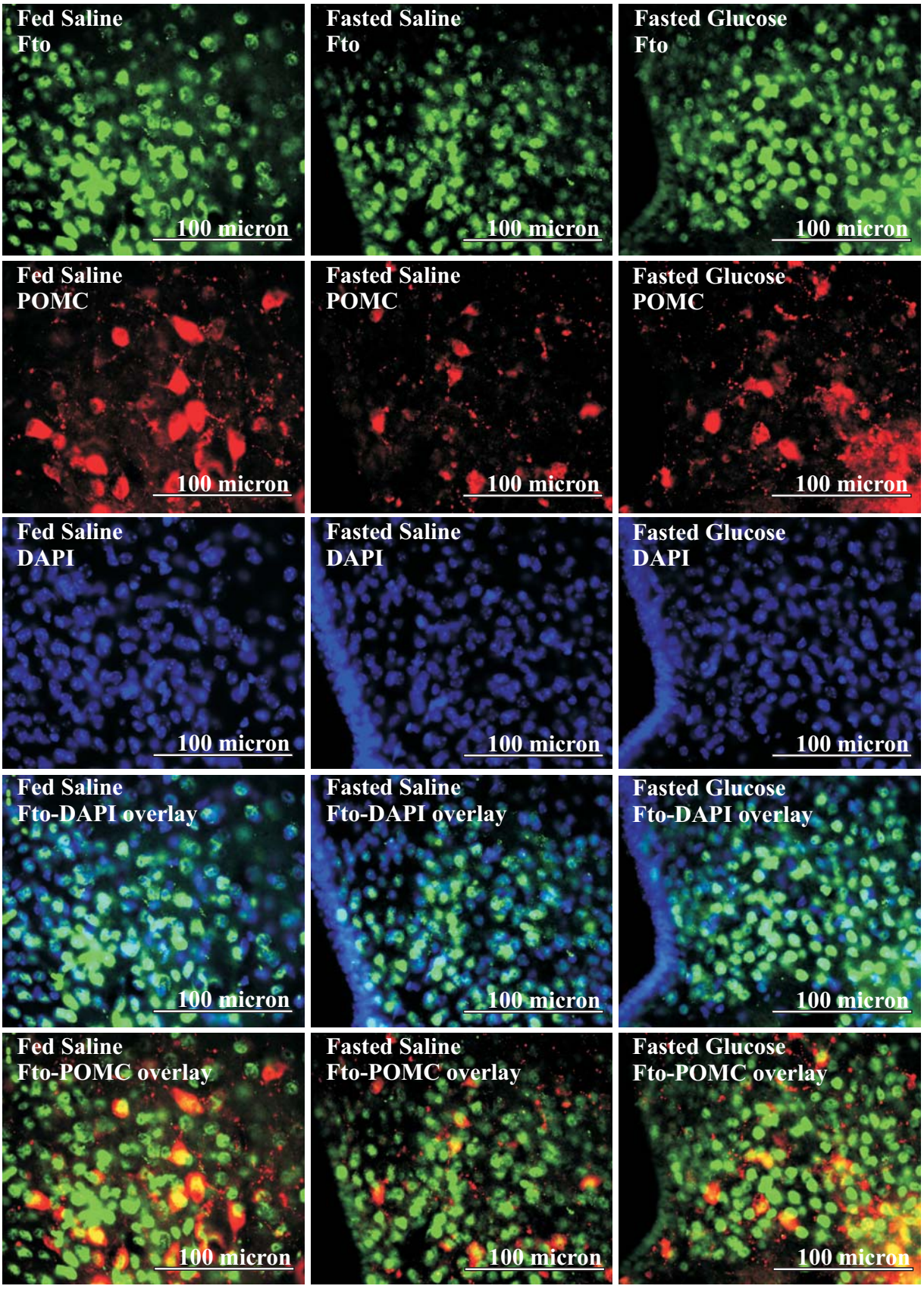


Figure 50: Fasting decreases and glucose treatment increases hypothalamic Fto and POMC expression in lean mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto and POMC immunoreactivities in the arcuate nucleus (ARC) was tested in male C57BL/6 mice. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and POMC neurons (Cy3) were visualized. Magnification: 40X.

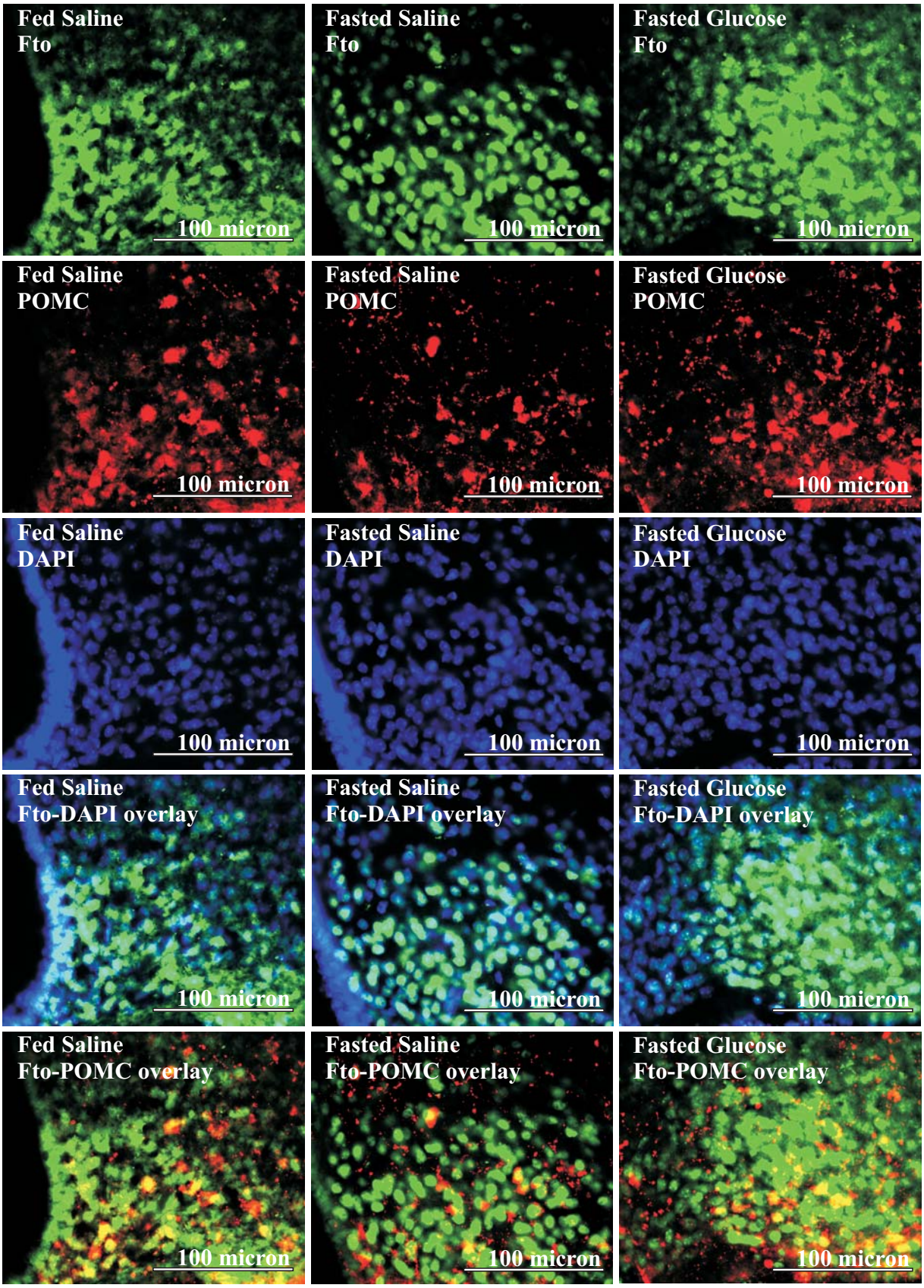
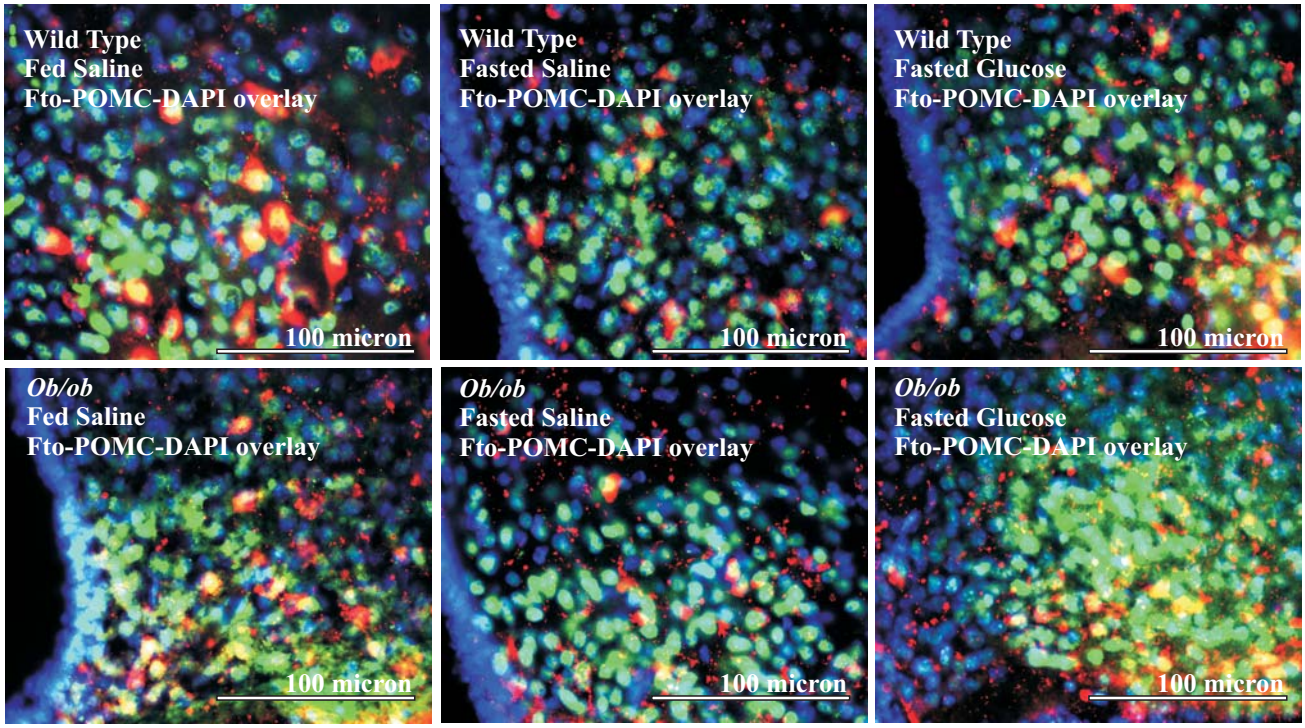


Figure 51: Blunted response in hypothalamic Fto and POMC expression to changes in nutritional state in obese mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto- and POMC-immunopositive neurons in the arcuate nucleus (ARC) was tested in male *ob/ob* mice. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and POMC neurons (Cy3) were visualized. Magnification: 40X.

A



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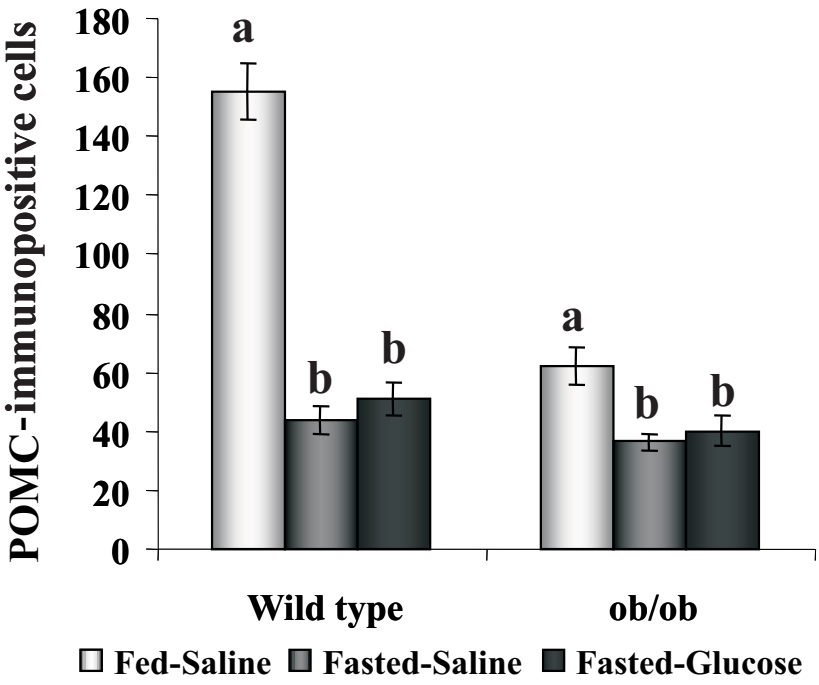


Figure 52: Reduced hypothalamic POMC expression in obesity. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto- and POMC-immunopositive neurons in the arcuate nucleus (ARC) was tested in male wild-type and *ob/ob* mice. (A) Representative photomicrographs of cells co-expressing Fto and POMC. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and POMC neurons (Cy3) were visualized. Nuclei were visualized by DAPI staining in the ARC. Magnification: 40X. (B) Number of POMC-immunopositive nuclei in ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

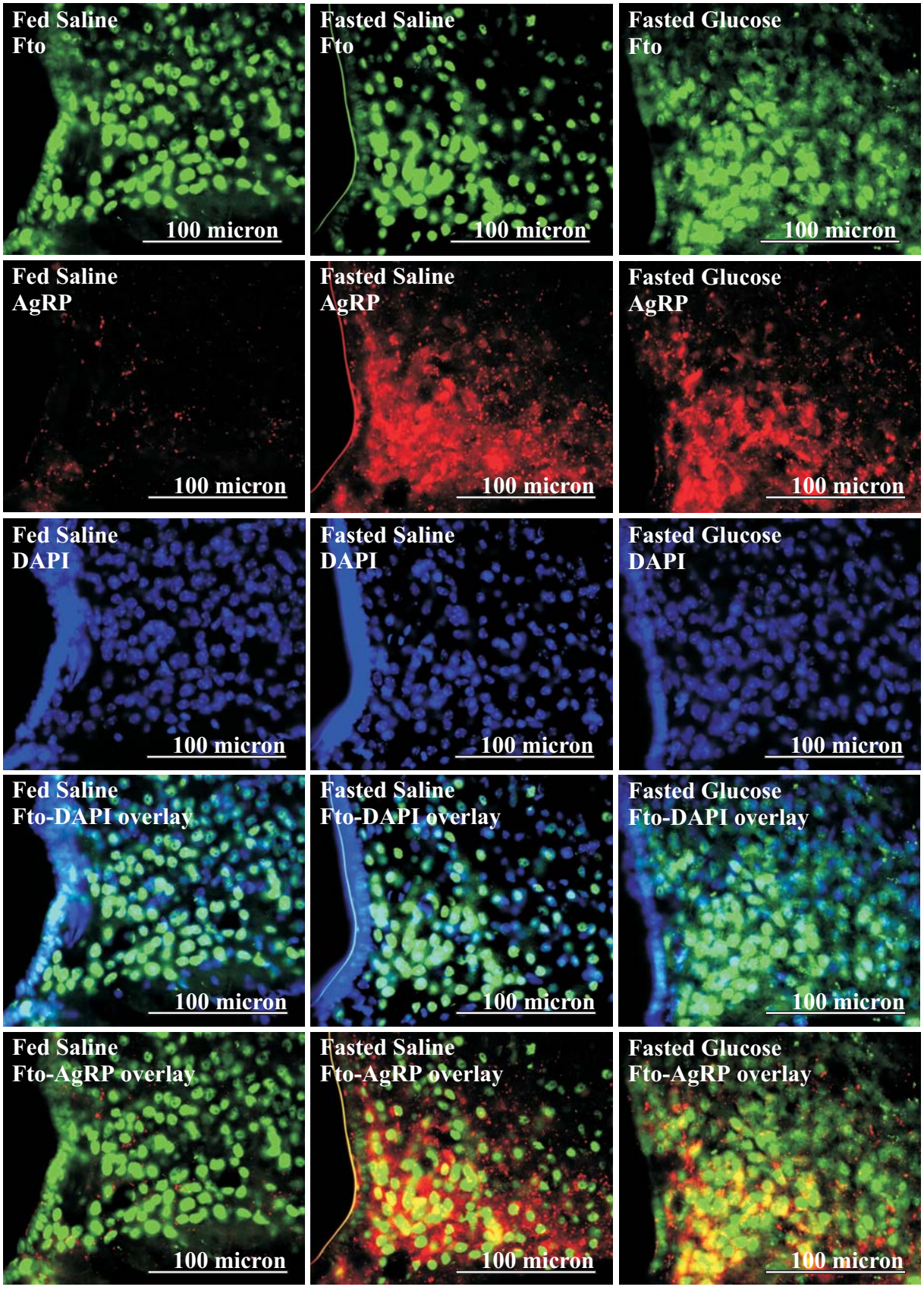


Figure 53: Fto and AgRP exhibit an opposite trend of hypothalamic expression pattern in response to changes in nutritional status in lean mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto- and AgRP-immunopositive neurons in the arcuate nucleus (ARC) was tested in male C57BL/6J mice. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and AgRP neurons (Cy3) were visualized. Magnification: 40X.

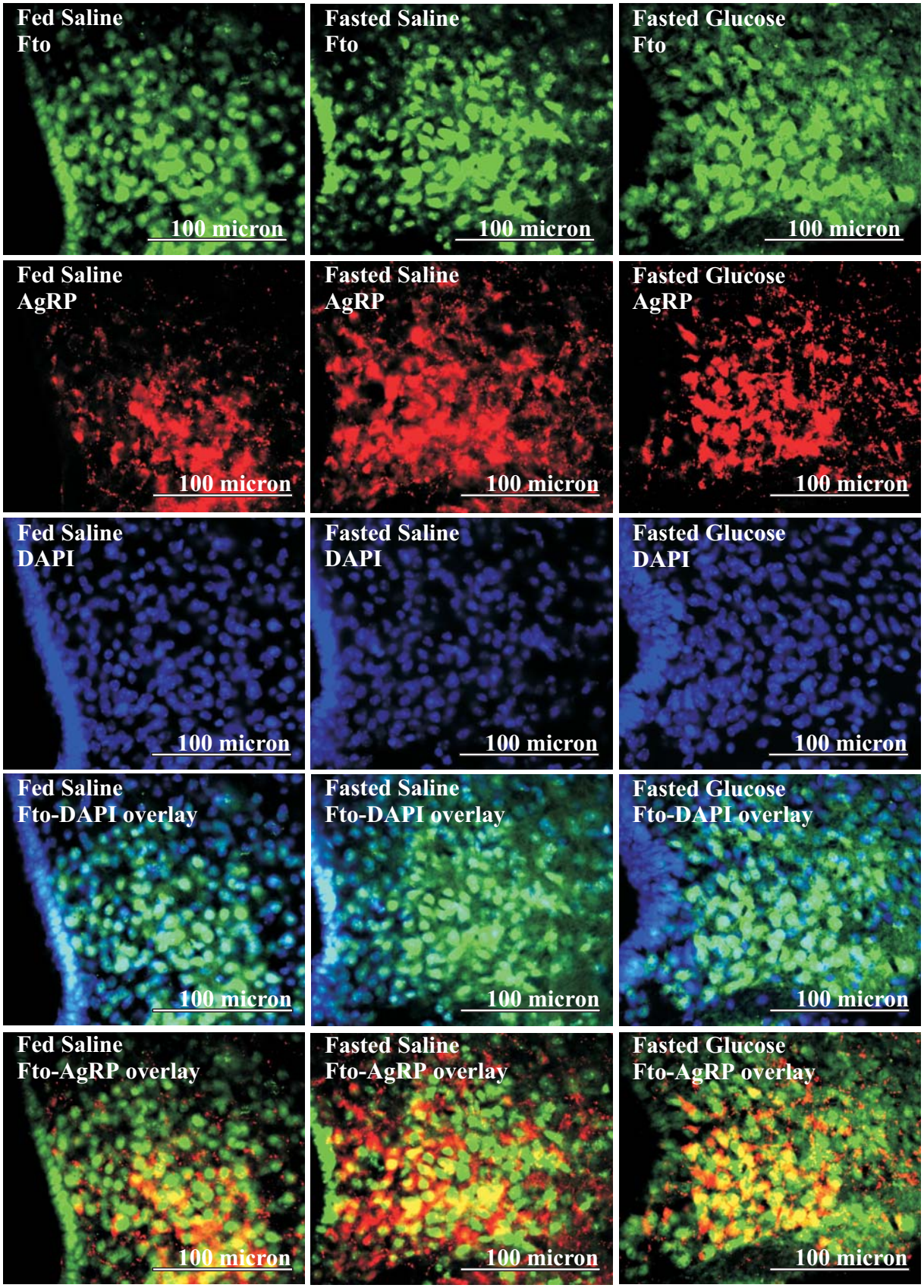


Figure 54: Expression pattern of hypothalamic Fto and AgRP exhibits reduced response to changes in nutritional state in obese mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto- and AgRP-immunopositive neurons in the arcuate nucleus (ARC) was tested in male *ob/ob* mice. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and AgRP neurons (Cy3) were visualized. Magnification: 40X.

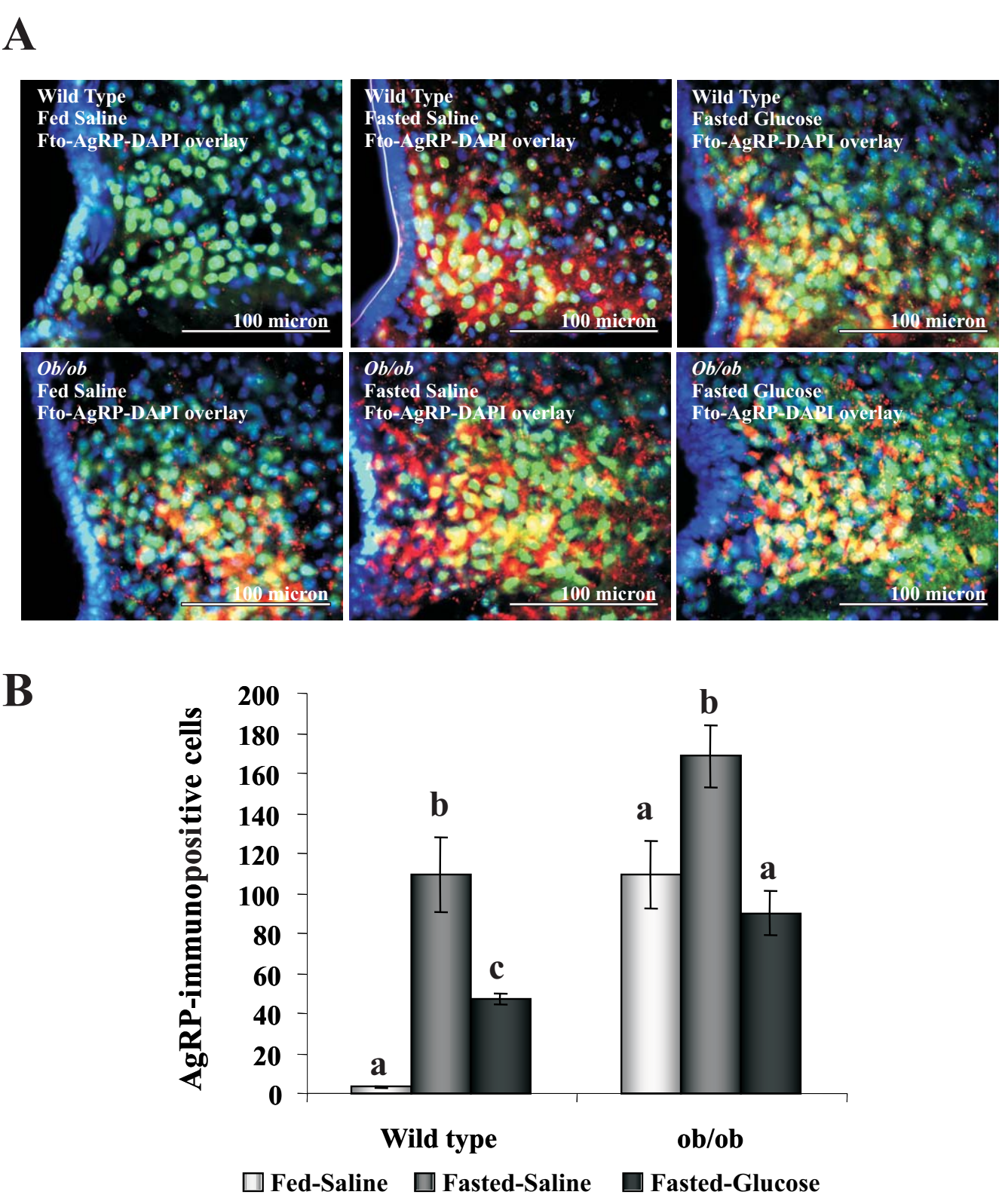


Figure 55: Up-regulation of hypothalamic AgRP expression in obesity. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on Fto- and AgRP-immunopositive neurons in the arcuate nucleus (ARC) was tested in male wild-type and *ob/ob* mice. (A) Representative photomicrographs of cells co-expressing Fto and AgRP. Distribution and co-expression of Fto-immunopositive nuclei (FITC) and AgRP neurons (Cy3) were visualized. Nuclei were visualized by DAPI staining in the ARC. Magnification: 40X. (B) Number of AgRP-immunopositive nuclei in ARC. Data are means ± S.E.M. (N =7-10 mice/group). Groups with different letters are statistically different ($P < 0.05$, Tukey-Kramer test).

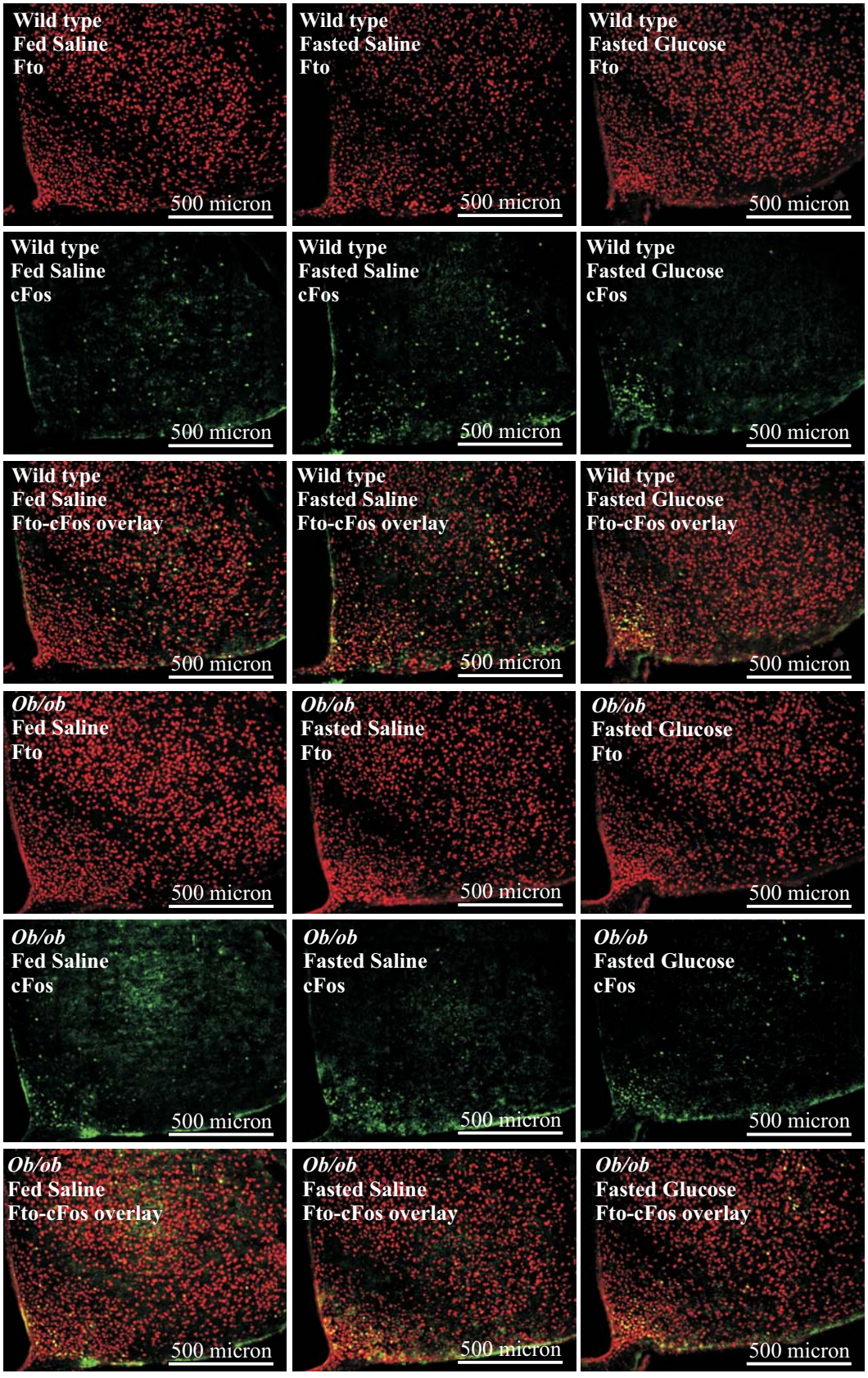


Figure 56: Representative photomicrographs of cFos expression pattern in response to fasting and glucose treatment in lean and obese mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on cFos and Fto immunoreactivities in the arcuate nucleus (ARC) and ventromedial nucleus (VMN) was tested in male wild-type and *ob/ob* mice. Distribution and co-expression of Fto-immunopositive nuclei (Cy3) and cFos-immunopositive nuclei (FITC) in wild-type (A) and *ob/ob* (B) mice were visualized. Magnification: 10X.

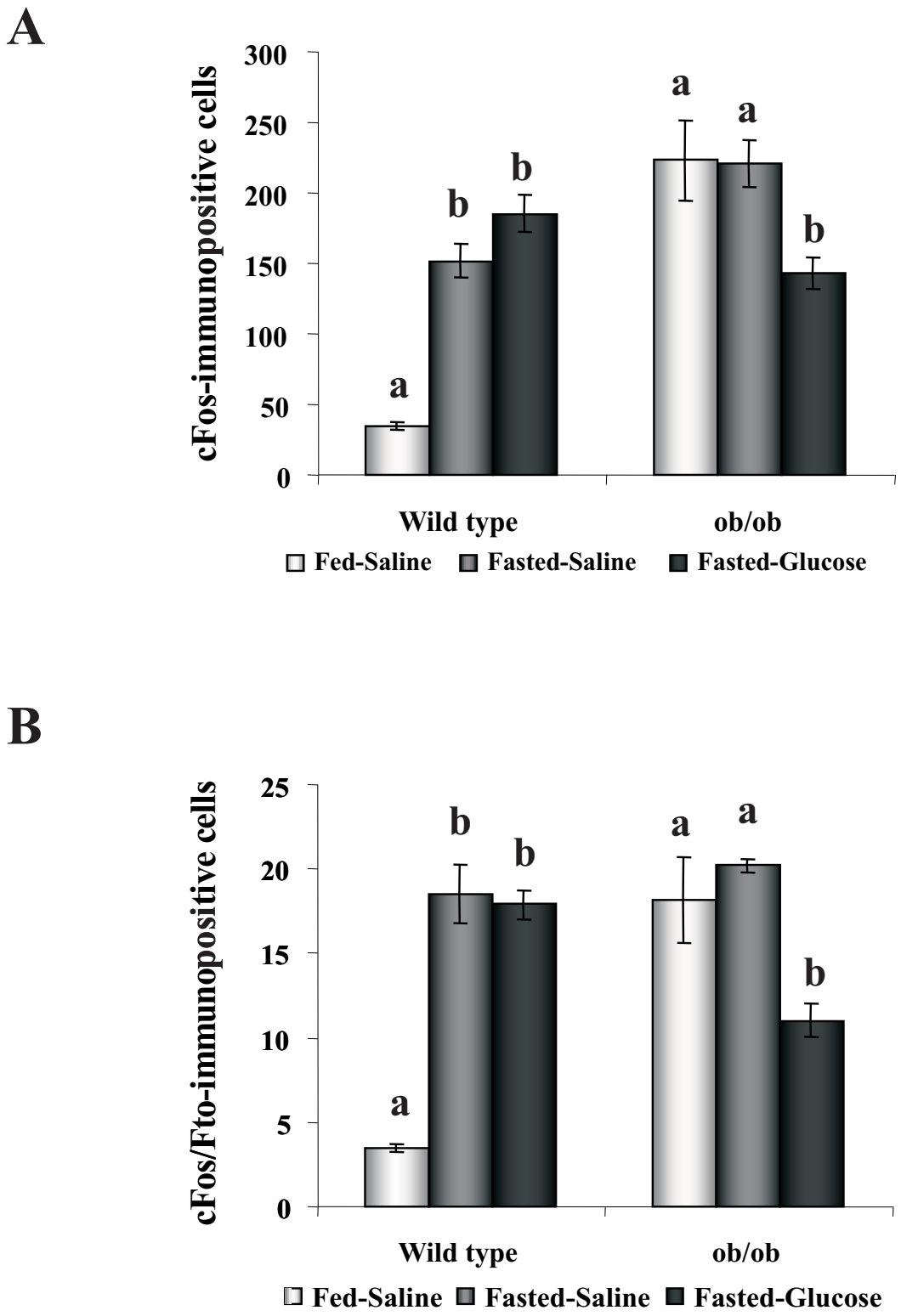
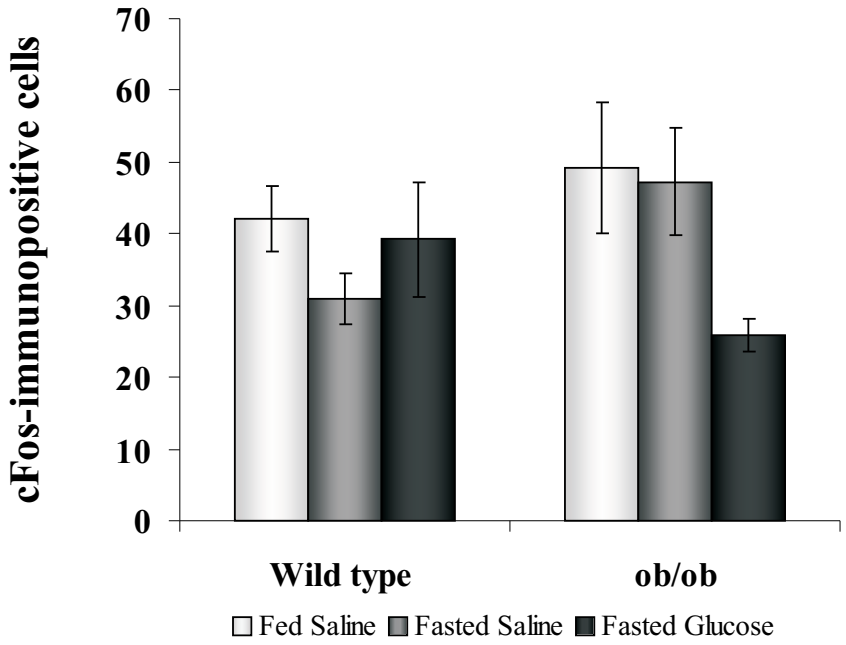


Figure 57: Fasting increases cFos and %cFos/Fto cells in the ARC of lean but not obese mice, while glucose reverses this effect only in obese mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on cFos expression in the hypothalamic ARC (A) and in the Fto-expressing neurons (B) was tested in male C57BL/6J wild-type and *ob/ob* mice. (A) Number of cFos-immunopositive nuclei. (B) Percentage of Fto-immunopositive cells co-expressing cFos in the ARC. Groups with different letters are statistically different within each genotype ($P < 0.05$, Tukey-Kramer test or Wilcoxon test with Bonferroni correction).

A



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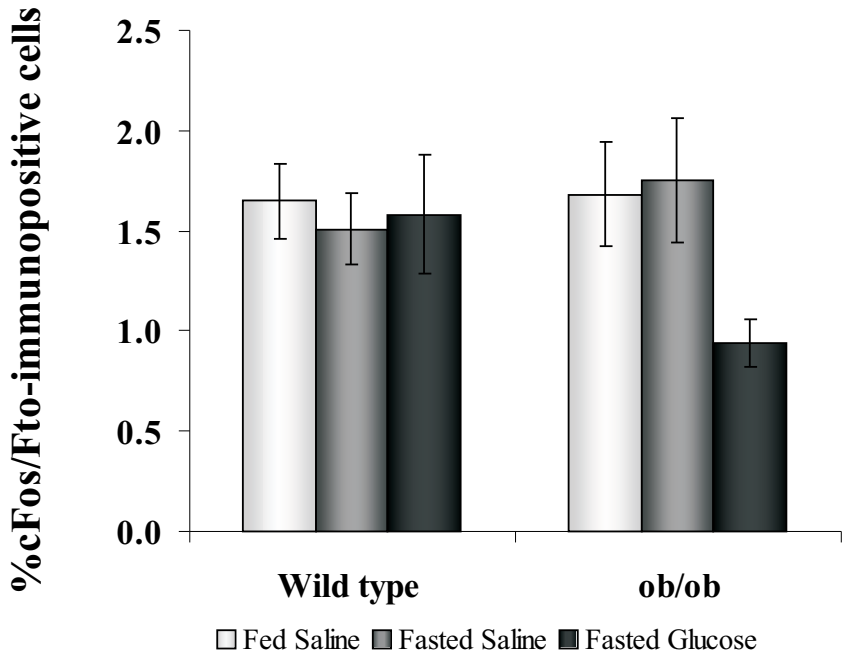

Figure 58: Fasting did not affect cFos and %cFos/Fto cells in the VMN of lean and obese mice. The effect of fasting (48 h) and intraperitoneal (i.p.) glucose treatment on cFos expression in the hypothalamic VMN (A) and in the Fto-expressing neurons (B) was tested in male C57BL/6J wild-type and *ob/ob* mice. (A) Number of cFos-immunopositive nuclei. (B) Percentage of Fto-immunopositive cells co-expressing cFos in the VMN. No statistical difference was detected by one-way ANOVA or Kruskal-Wallis test. Differences between fasted-saline and fasted-glucose are not significant.

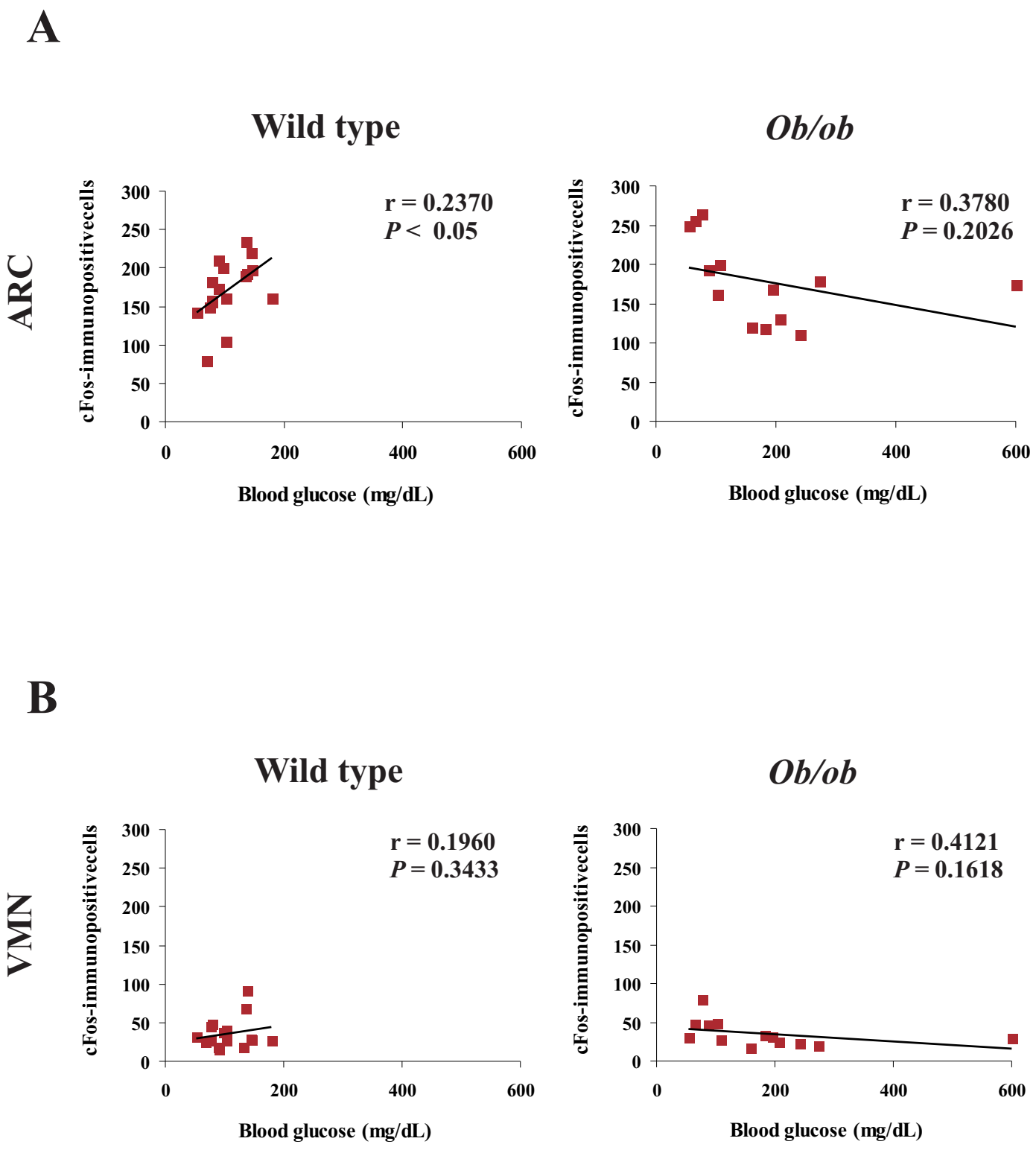

Figure 59: Under fasted conditions, the effect of glucose on the expression of cFos is present only in the ARC of lean mice. Correlation analysis between blood glucose and cFos-immunopositive cell number in the (A) ARC and (B) VMN in male wild-type and *ob/ob* mice fasted for 48 h and treated intraperitoneally with either saline or glucose. Data are means ± S.E.M. (*N* =7-10 mice/group).

GENERAL DISCUSSION

It has been well established that CNS melanocortin signaling plays a critical role in the regulation of energy balance. Impairments in the melanocortin system cause obesity, while enhanced CNS melanocortin signaling reverses obesity. It is well established that the activity of the hypothalamic melanocortin system is altered in response to changes in metabolic signals including nutrients and hormones. The CNS melanocortin system maintains the body's metabolic states within the normal range by monitoring changes in these metabolic signals, as well as, by producing appropriate metabolic adjustments. Thus, impairments in the regulation of CNS melanocortin activity by metabolic signals may cause obesity and other metabolic impairments including fatty liver disease. The *FTO* gene has been recently identified as a possible regulator of metabolism via its action in the CNS melanocortin pathway. These findings led this Ph.D. thesis work toward the hypothesis that hypothalamic melanocortinergic neurons regulate metabolism, including hepatic lipid metabolism by monitoring changes in metabolic signals, such as glucose, via Fto and that impairments in this regulatory system causes fatty liver disease. The present study was designed to partially address this hypothesis. Specifically, the study examined (1) the effect of alterations in CNS melanocortin signaling on hepatic lipogenic gene expression and lipid accumulation, (2) the effect of metabolic states (fasting and glucose treatment) on hypothalamic Fto expression, (3) the effect of obesity on hypothalamic Fto expression and (4) expression of Fto in hypothalamic POMC and AgRP neurons.

In the present study, MTII treatment reduced body weight in *ob/ob* mice and MTII-induced weight loss was associated with reduced expression of hepatic lipogenic genes. Conversely, SHU9119 treatment increased lipid accumulation in the liver through an increase in lipogenic gene expression that may be partly mediated by SREBP-1c- and PPARγ2- dependent pathways. MTII is a lactam Ac-Nle^4-cycle[Asp^5-D-Phe^7-Lys^{10}] α-MSH-(4-10)-NH2 with an agonist activity. Although MTII is a non-specific melanocortin agonist, it shows a higher selectivity for the MC4R and has a 136-fold higher affinity for the MC4R compared with α-MSH (Schioth et al., 1997). SHU9119 is a synthetic peptide with a β-(2-naphthyl)-D-alanine (D-Nal) substituted with the D-Phe7 residue of MTII that exhibits an antagonistic activity (Hruby et al., 1995). Although SHU9119 is a non-specific melanocortin antagonist, it shows a higher selectivity for the MC4R and has a 11-fold higher affinity for the MC4R compared with agouti peptide (Fan et al., 1997). *In vivo* assays demonstrated that the inhibitory effect of 3 nmol MTII was blocked by 6 nmol of SHU9119 in C57BL/6J mice, indicating that the SHU9119 concentration required to produce a competitive inhibitory effect on MC4R is 2-fold the concentration of MTII (Fan et al., 1997). Thus, our findings suggest that alterations in CNS signaling through MC4R affect hepatic lipogenic gene expression. However, the present study cannot rule out the possibility that signaling through MC3R may also play a role in the regulation of hepatic lipid metabolism. Other melanocortin peptides, such as HS024 and HS014, that have higher specificity for MC4R may be useful to further clarify the role of MC4R in the regulation of hepatic lipid metabolism (Kask et al., 1998). Alternatively, MC4R and MC3R knockout mice can be used to assess specific

contributions of these receptors to MTII-induced and SHU9119-induced changes in hepatic lipid metabolism (Butler et al., 2000; A. S. Chen et al., 2000; Huszar et al., 1997).

The present study also suggested that CNS melanocortin signaling regulates the expression of hepatic lipogenic genes, at least partly, through SREBP-1c- and PPARγ2-dependent mechanisms. This conclusion is primarily based on the findings that SHU9119 treatment increased the expression levels of genes encoding lipogenic enzymes and transcription factors. However, changes in mRNA levels do not necessarily reflect the activity of the gene product. To overcome this limitation and ascertain the present findings, it is necessary to measure the activity of lipogenic enzymes and the levels of the active form of transcription factors in future studies.

STZ-treated mice and *ob/ob* mice were used in the present study as a model of type 1 diabetes and obesity/type 2 diabetes/fatty liver disease, respectively. STZ mouse model has been widely used as an animal model of type 1 diabetes. The STZ mouse is characterized by extreme hypoinsulinemia and hyperglycemia. One of advantages of this mouse model is that nutritional manipulations, such as fasting, do not cause changes in serum insulin levels, thus eliminating the effect of changes in insulin levels on other parameters, such as gene expression, from the analysis (Mizuno et al., 1999). In the present study, hypothalamic *Fto* mRNA levels were not different between non-diabetic control and STZ-induced diabetic mice suggesting the possibility that insulin may not play a major role in the regulation of hypothalamic Fto expression. However, further studies are necessary to draw this conclusion. Although STZ-treated mice have the extremely low levels of insulin, they are not complete absent of insulin. Thus, this model cannot completely rule out the possible action of the left-over insulin. To specifically

address the central effects of insulin on Fto expression, neuronal insulin receptor knockout (NIRKO) or knockdown of hypothalamic insulin receptor with RNA interference can be used (Reviewed in: Biddinger & Kahn, 2006).

The *ob/ob* mice have a mutation in the *leptin* gene and have been widely used as a model of the early-onset obesity (Zhang et al., 1994). This mouse model has a significant advantage to examine the regulation of gene expression. It is clear that leptin plays a significant role in the regulation of hypothalamic nutrient-sensitive genes, such as POMC and AgRP, which are involved in the regulation of metabolism (Benoit et al., 2002; Cheung et al., 1997; Mizuno et al., 1998; Mizuno & Mobbs, 1999; Schwartz et al., 1997). Since, *ob/ob* mice do not have functional leptin, this mouse model enables the investigation of leptin-independent regulatory mechanism of hypothalamic gene expression. Thus, the present finding that fasting reduces hypothalamic Fto expression in leptin-deficient *ob/ob* mice is a clear indication that metabolic factors other than leptin also play a role in the regulation of Fto expression. The present study also showed that glucose fails to reverse fasting-reduced reduction in hypothalamic Fto expression. Thus, it is likely that this defect in the hypothalamic glucose sensing is a contributing factor to the leptin-deficiency-induced metabolic impairments. Similar to the *ob/ob* mice, leptin deficiency is associated with obesity in human. However, the incident rate of the mutation in the *leptin* gene is very low in human (Montague et al., 1997; Strobel et al., 1998). It became apparent that the majority of human obesity is associated with leptin resistance rather than the absolute leptin deficiency. Thus, it should be noted that the present findings have to be replicated in other animal models of obesity, specifically those associated with leptin resistance such as diet-induced obese mice.

The present study demonstrated that Fto is expressed in hypothalamic POMC and AgRP neurons, while fasting reduced hypothalamic Fto expression and glucose treatment (both i.p. and i.c.v.) increased Fto expression in fasted mice. These changes in Fto expression were found in the hypothalamus in a site-specific manner. Furthermore, fasting-induced reduction in hypothalamic Fto expression was observed in both lean wild-type and obese *ob/ob* mice, the stimulatory effect of glucose on hypothalamic Fto expression was absent in *ob/ob* mice. These findings are consistent with the hypothesis that hypothalamic Fto expression is regulated by metabolic signals, including glucose and that impairments in this regulation may cause obesity. Additionally, the present study suggests that fasting and glucose regulate Fto expression specifically in the VMN and ARC hypothalamic regions, as well as, the hypothalamic POMC and AgRP neurons. In addition to changes in blood glucose levels, changes in other nutrients and hormones are associated with the state of feeding condition. Therefore, the present study cannot address the possible involvement of these other metabolic factors in the regulation of hypothalamic Fto expression. To further confirm the present findings and understand the role of other metabolic signals in the regulation of Fto expression, further studies are necessary. These studies may involve the assessment of the effect of the blockade of CNS or hypothalamic glucose metabolism, insulin signaling and leptin signaling on changes in hypothalamic Fto expression *in vivo* and *in vitro* using specific inhibitors, RNA interference and brain-specific knockout models.

In summary, although further studies are necessary to fully understand the mechanism by which metabolic signals regulate hypothalamic Fto expression, melanocortin activity and metabolism, the present study suggests that hypothalamic Fto is

regulated, at least partly, by changes in CNS glucose availability and/or glucose action in POMC and AgRP neurons, and that impairments in this regulation may cause metabolic impairments including obesity and fatty liver disease. The present study was not designed to identify specific biological functions of hypothalamic Fto in the regulation of metabolism. The answer to this question awaits future studies (see "**Future experiments**"). Information obtained from the present study and future studies may enhance the design of future obesity prevention efforts and help improve the health of patients with obesity and obesity-associated impairments, such as fatty liver disease.

FUTURE EXPERIMENTS

Study 1: Test how changes in hypothalamic Fto activity can modify hypothalamic POMC and AgRP neuronal activity.

To determine the effect of either increased or decreased hypothalamic Fto activity on glucose sensing of POMC and AgRP neurons, mice will be given i.c.v. injections, respectively, of either adenoviral Fto or Fto-specific siRNA for 8 weeks in the presence of glucose treatments (i.c.v. and i.p.). The effects of Fto on POMC and AgRP neuronal activity will be tested by measuring the mRNA and protein expression of α-MSH and AgRP, respectively. It is expected that if Fto mediates the glucose sensing effects of POMC, then in the presence of glucose, knocking down Fto will result in reduced neuronal activity of POMC. Alternatively, if Fto has a regulatory role in the glucose-sensing mechanisms of AgRP neurons, then knocking down Fto is expected to result in changes (i.e. reduction) in AgRP production in the presence of glucose. It is expected that Fto over-expression may increase the rate of demethylation at the hypothalamic POMC promoter either in *in vivo* (ie. in obese animal models where hypermethylation in the *Pomc* promoter may exist) or *in vitro* (N38 hypothalamic cell line transfected with a constract containing hypermethylated *Pomc* promoter).

Study 2: Determine if Fto has a contributing function on the glucose-sensing mechanism in POMC and AgRP neurons.

Fto will be ablated in either POMC or AgRP neurons using the Cre-loxP strategy. Fto transgenic mice will be generated to have loxP sites flanking exon2 and exon3 of the murine *Fto* gene. In the animal model of Fto-deficiency, Fischer et al. have shown a total ablation of Fto when exons 2 and 3 of the *Fto* coding sequence were deleted (Fischer et al., 2009). Intercrossing $Fto^{flox/flox}$ mice and mice that have Cre recombinase under the control of either Pomc (*TgPomcCre*) or Agrp (*TgAgrpCre*) promoter can selectively ablate the region encoding *Fto* in POMC or AgRP neurons, respectively. The resulting Fto null transgenic mice are expected to have impaired glucose sensing mechanisms in response to either i.c.v. or i.p, glucose treatments.

Study 3: Generate mice with neuronal-specific overexpression of Fto to investigate its effect on whole body metabolism.

To determine if Fto overexpression in the brain is beneficial in whole body metabolism or preventative towards the development of obesity, floxed Fto transgenic mice (study 2) will be crossed with transgenic mice expressing Cre. First, the metabolic phenotype of POMC-Cre x floxed Fto ($POMC^{Fto-/-}$) or AgRP-Cre x floxd Fto ($AgRP^{Fto-/-}$) transgenic animals will be assessed under normal chow and high-fat diet conditions. It is expected that both $POMC^{Fto-/-}$ and $AgRP^{Fto-/-}$ transgenics will be sensitive to the effects of diet induced obesity accompanied with impairements in both central nutrient sensing and melanocortin signalling. Second, a whole brain Fto knockout transgenic line will be used to assess the functional role of Fto in the entire brain and its effects in obese animal

models (*ob/ob*) under normal chow and high-fat diet conditions. Transgenic mice will be generated by having the Fto coding region floxed (see study 2) and crossed with Nestin-Cre transgenic mice to delete Fto specifically in the brain. It is probable that whole brain knockout mice crossed with obese animal models will not reverse or prevent the development of obesity. Lastly, Fto-overexpressing transgenic mice will be tested for their response to diet induced obesity. Transgenic mice will be generated having the human Fto coding region under the regulation of neuron specific enolase (NSE) promoter, targeting Fto expression specifically to neurons. The hypothesis exists that Fto functions to prevent the development of obesity, possibly through a stimulatory or inhibitory effect on POMC and AgRP neurons, respectively. Consequently, Fto-overexpressing mice may be resistant to diet-induced obesity mainly through the function of Fto on the central melanocortin system.

Study 4: To address the hypothesis that glucose excitation in Fto neurons is mediated by the closure of K-ATP channels, two approaches will be used.

First, the effects of the K-ATP blocker glibenclamide on the ability of glucose to reduce hypothalamic AMPK phosphorylation and AMPK activity will be confirmed as previously described (Lam et al., 2007). If it is proven that glucose sensing of Fto neurons is mediated via the K-ATP channel mechanism, then the second approach will be to assess the physiological function of K-ATP channel closure (depolarization) in Fto neurons in the development of obesity and type 2 diabetes. Transgenic mice expressing a mutant Kir6.2 subunit in Fto neurons will be generated such that K-ATP channels become insensitive to changes in ATP. Patch clamp studies will be performed to assess

the membrane potential and basal firing rates of Fto neurons under normal and glucose treated conditions. Neuronal activities will be recorded from the neurons in the ventrolateral VMN and the lateral peri-arcuate area where glucose-excited neurons are abundant. Using the approach as previously published (Parton et al., 2007), loose-patch recordings of Fto neurons from wild-type (expressing Fto-green fluorescent protein) and transgenic mice having mutated K-ATP channels in Fto neurons (Fto-mut-Kir6.2) will be performed. Electrophysiological recordings will be performed in the presence of basal glucose conditions (aCSF solution with 3 mM glucose) and hyperglycemic conditions (5 mM, 10 mM and 25 mM of glucose). It is expected that Fto-mut-Kir6.2 transgenic mice will have impairments in glucose sensing mechanisms. I.p. glucose tolerance tests will be performed in both wild-type and Fto-mut-Kir6.2 transgenic mice. If a functional K-ATP channel mechanism is required for glucose sensing by Fto neurons, then Fto-mut-Kir6.2 will have impaired glucose tolerance. Alternatively, the already-existing Kir6.2 ko can be used to record the activities of neurons in the VMNvl or lateral peri-arcuate area using a patch clamp followed by identification of Fto-positive or –negative cells by a single cell real-time PCR using RNA from the recorded neurons isolated by laser capture.

Study 5: Investigate whether glucose sensing in Fto neurons is impaired in obesity.

Diet-induced obesity is known to result in impaired glucose sensing by hypothalamic neurons (Parton et al., 2007). Electrophysiological recordings will be performed to assess the effects of glucose sensing in Fto neurons (Fto-GFP) of mice fed normal chow diet or high-fat diet for 8 weeks. If hypothalamic Fto functions as a glucose sensor, then it is expected that under high-fat diet conditions, Fto neurons will have reduced firing rate

in response to an increase in glucose. The ability of Fto neurons to respond to glucose will be tested in the presence of obesity using the leptin-deficient animal model (*ob/ob*). The results are expected to be in line with those from the diet-induced obesity, which is impaired glucose sensing in Fto neurons.

THESIS CONCLUSIONS AND SUMMARY

CONCLUSIONS

Increased hepatic *de novo* lipogenesis in response to impaired central melanocortin signaling

To address the hypothesis that reduced central melanocortin signaling stimulates hepatic lipid synthesis and accumulation towards the development of fatty liver, the effect of i.c.v. SHU9119, a melanocortin receptor antagonist, on the expression levels of genes involved in lipid metabolism was investigated. Reduced central melanocortin signaling induces lipid accumulation in the liver through an increase in lipogenic gene expression and *de novo* lipogenesis that may be primarily mediated by SREBP-1c- and PPARγ2- dependent pathways. Additionally, enhanced CNS melanocortin signaling by i.c.v. treatment with MTII reduced the expression of hepatic lipogenic genes in association with reduction in body weight in *ob/ob* mice, a mouse model of fatty liver disease.

These findings are consistent with the hypothesis that central melanocortin signaling regulates hepatic lipid metabolism, and impairments in the central melanocortin pathway lead to the development of hepatic steatosis, while enhanced central melanocortin signaling may be beneficial in reversing abnormal hepatic lipid metabolism in fatty liver disease (Poritsanos et al., 2008).

Regulation of hypothalamic Fto expression by nutritional signals, such as glucose

To address the hypothesis that hypothalamic expression of Fto is regulated by metabolic signals, including glucose, the expression pattern of Fto was assessed in metabolically relevant tissues and brain regions. Fasting increased *Fto* mRNA levels in liver and skeletal muscle, and i.p. glucose treatment reversed this effect in the liver. In the hypothalamus, *Fto* mRNA levels were reduced by fasting and increased by glucose treatment (i.p. and i.c.v.). Furthermore, fasting reduced and glucose treatment increased Fto expression in hypothalamic ARC and VMN, but not in DMN.

These findings are consistent with the hypothesis that Fto expression is regulated by metabolic signals, including glucose (Poritsanos et al, 2010). Reduction in blood glucose levels and/or hypothalamic glucose availability, at least partly mediates the inhibitory effects of fasting on hypothalamic Fto expression.

Fto as part of the CNS melanocortin pathway

To assess the possible involvement of Fto in the regulation of the activity of hypothalamic melanocortin system, Fto expression was examined in hypothalamic POMC and AgRP neurons by double-staining immunohistochemistry. Fto was co-expressed in both POMC and AgRP neurons.

These findings support the hypothesis that Fto functions in hypothalamic nutritional sensing as part of the central melanocortin system.

Impaired hypothalamic Fto expression in response to fasting and glucose in obese mice

To address the hypothesis that obesity is associated with impairments in hypothalamic Fto expression by nutrients, such as glucose, the effect of fasting and glucose treatment on hypothalamic Fto expression was examined in obese *ob/ob* mice. Fasting reduced hypothalamic Fto expression in both lean wild-type and obese *ob/ob* mice. In contrast, glucose-induced increase in hypothalamic Fto expression was abolished in *ob/ob* mice.

The findings support the hypothesis that obesity is associated with an impairement in the sensitivity of hypothalamic Fto-expressing neurons to the changes in CNS glucose availability and/or glucose action. Consequently, the blunted response of hypothalamic Fto-expressing neurons to glucose may cause metabolic impairements.

SUMMARY

An intact hypothalamic glucose sensing mechanism is necessary for stable hepatic glucose and lipid metabolism. Regulatory mechanisms underlying nutritional regulation of hypothalamic Fto expression may be also associated with hepatic lipid and glucose metabolism. Although, this thesis work only showed an association of hypothalamic Fto neurons with the melanocortin peptides, it is important to address in the future whether Fto serves as a hypothalamic molecular sensor of nutrients, such as glucose, for the central melanocortin system and consequently be involved in the regulation of hepatic lipid metabolism. The findings of this Ph.D. thesis work support the idea that hypothalamic Fto plays a beneficial role in protecting against the development of obesity. In this regard, impairments in the ability of FTO to function as a molecular mediator of nutritional sensing in the central melanocortin system may ultimately result in the development of obesity and obesity-associated impairments including hepatic steatosis.

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