Aus der Medizinischen Klinik I

der Universität zu Lübeck

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Hypothalamic insulin signaling regulates white adipose tissue lipolysis and *de novo* lipogenesis *in vivo*

Inauguraldissertation

zur

Erlangung der Doktorwürde

der Universität zu Lübeck

- Aus der Sektion Medizin -

vorgelegt von

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Lübeck 2012

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Tag der mündlichen Prüfung: 23. 05. 2012

Zum Druck genehmigt. Lübeck, den 23. 05. 2012

-Promotionskommission der Sektion Medizin-

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Non-standard Abbreviations

6–OHDA: 6-hydroxydopamine

ABHD5: α/β hydrolase domain containing protein 5

Acc: acetyl–CoA carboxylase

aCSF: artificial cerebrospinal fluid

Akt: Proteinkinase B

Agrp: agouti-related peptide

AL: *ad libitum*

Ampk: AMP–activated protein kinase

ARC: arcuate nucleus of the hypothalamus

Atgl: adipose tissue triglyceride lipase

Atpcl: ATP–citrate lyase

BW: bodyweight

cAMP: cyclic-AMP

CGI-58: comparative gene identification-58

CR: calorically restricted

DM2: diabetes mellitus type 2

DNL: *de novo* lipogenesis index

Erk: extracellular-signal regulated kinase

FAS: fatty acid synthase

GIR: glucose infusion rates

GP: glucose production

Gsk: glycogen synthase kinase

Hsl: hormone sensitive lipase

IACUC: Institutional Animal Care and Use Committee

ICV: intracerebroventricular (3^{rd} ventricle)

IGF: insulin–like growth factor

MBH: mediobasal hypothalamus

NE: norepinephrine

NEFA: non–esterified fatty acid

NIRKO: neuronal insulin receptor knock-out

NPY: neuropeptide Y

PDE3B: phosphodiesterase 3B

PI3K: phosphoinositide 3 kinase

PKA: protein kinase A

Pomc: proopiomelanocortin

PVN: paraventricular nucleus

Ra: rate of appearance

SD: Sprague Dawley

SNS: sympathetic nervous system

TG: triglyceride

VMH: venteromedial hypothalamus

WAT: white adipose tissue

I. Introduction and significance

Access to high caloric food has become temptingly easy over the last decades, while our lifestyle has become more sedentary, resulting in an unprecedented epidemic of obesity (WHO, 2011). The obese state promotes insulin resistance and markedly increases the risk for type 2 diabetes (DM2). White adipose tissue (WAT) plays a critical role in energy homeostasis both as an endocrine organ and as a storage organ of energy rich triglycerides. Obesity is commonly associated with dysfunctional WAT (Gaidhu et al., 2010; Gordon, 1960; Mittendorfer et al., 2009) which is the source of excess non-esterified fatty acids (NEFA) and inflammatory mediators that cause and worsen insulin resistance that sets the stage for DM2 (Boden, 2006). In mammals surplus nutrients are converted mainly into TGs that can be most efficiently stored in WAT. In the fed state nutrients are absorbed in the gut and converted into TGs in the liver. The liver then secretes these TGs as very low density lipoproteins, that will either be utilized by the muscle or, when supply exceeds demand, are stored in WAT (Pond, 1998). Conversely, during fasting or energy demanding states, such as exercise and infection, WAT breaks down its stored TGs during lipolysis, which releases glycerol and NEFAs into the bloodstream to provide energy substrates for processes such as β-oxidation and gluconeogenesis (Hers and Hue, 1983; Lafontan and Langin, 2009). The continuous transition between fasting and feeding requires WAT to dynamically switch from a fatty acid storing to a fatty acid releasing mode according to the metabolic needs. This metabolic flexibility is critical for energy homeostasis. Thus, WAT dysfunction is characterized by the inability of WAT to store lipids or restrain lipolysis in the fed state. WAT dysfunction is observed in lipodystrophic (Garg, 2004), obese and DM2 patients (Groop et al., 1989; Groop et al., 1991; Roust and Jensen, 1993) and results in elevated circulating NEFAs. Excessive lipolysis in WAT causes accumulation of ectopic lipids and promotes a pro-inflammatory state (Boden et al., 2005; Itani et al., 2002; Kosteli et al., 2010), which can cause or worsen insulin resistance in muscle and liver (Boden et al., 2002; Ferrannini et al., 1983; Roden et al., 1996).

WAT is also capable of synthesizing fatty acids during *de novo* lipogenesis. Although, quantitatively WAT *de novo* lipogenesis adds little to the whole body lipid pool (Large et al., 2004), it may serve important metabolic functions that we are just beginning to explore. A recent study has shown that the fatty acid palmitoleate, which seems to be mainly

released by WAT, bears systemic insulin sensitizing properties in mice (Cao et al., 2008). Data on palmitoleate in humans are mixed. While high circulating palmitoleate is associated with improved cholesterol profiles in men, it is also linked to increased TG levels and insulin resistance (Mozaffarian et al., 2010a). However, these studies did not differentiate the source of palmitoleate, which is important since increased hepatic lipid production is associated with insulin resistance (D'Adamo et al., 2010; Gastaldelli et al., 2007) rendering liver derived palmitoleate a possible confounder. Indeed, palmitoleate from exogenous sources (non-liver-derived), such as dairy products, is associated with lower insulin resistance and incidence of DM2 in humans (Mozaffarian et al., 2010b). In rodents, whose lipogenic capacity seems to exceed that of humans (Swierczynski et al., 2000), palmitoleate is implicated in improving glucose uptake *in vitro* and *in vivo* (Cao et al., 2008; Tsuchiya et al., 2010) and to also reduce hepatosteatosis by blocking *de novo* lipogenesis in the liver (Cao et al., 2008). Yet, the exact molecular mechanism of how palmitoleate exerts its insulin sensitizing effects, remains to be elucidated. Furthermore, there is emerging evidence that in human obesity WAT *de novo* lipogenesis is reduced (Diraison et al., 2002; Mayas et al., 2010; Roberts et al., 2009), which may result in lower palmitoleate secretion from WAT contributing to insulin resistance, although this has not been stringently proven. Therefore, failure of WAT *de novo* lipogenesis may represent an additional feature of WAT dysfunction. Apart from lipid production, storage and release, WAT is a highly active endocrine organ that secretes adipokines such as adiponectin and leptin, that control energy homeostasis by regulating appetite and partitioning of glucose and lipids (Kershaw and Flier, 2004).

Regulation of lipid metabolism by leptin

Lipid partitioning is regulated by several circulating factors, such as hormones and cytokines. Two major hormones that control lipid metabolism are insulin, secreted by the endocrine pancreas, and leptin, produced primarily by adipocytes. The two hormones circulate in levels proportional to body fat and are considered the main endocrine adiposity signals in mammals (Schwartz et al., 2000) that communicate current energy availability to the brain. Within the brain, and in particular the hypothalamus, leptin and insulin signaling are integrated with other signals such as neurotransmitters and nutrients. The hypothalamus, in turn, orchestrates nutrient partitioning and appetite (Schwartz and Porte, 2005; Schwartz et al., 2000). The prevailing paradigm of brain insulin's and leptin's role in

the regulation of energy homeostasis is that they act synergistically. Both hormones suppress food intake via signaling in the hypothalamus (Air et al., 2002b; Campfield et al., 1995; Chavez et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Weigle et al., 1995; Woods et al., 1979), although brain insulin's anorectic effects have recently been challenged (Jessen et al., 2010). However, clinically their effects are quite different: Insulin has several anabolic properties and diabetic patients started on insulin tend to gain weight (Franssila-Kallunki and Groop, 1992), while leptin administration reduces adiposity in leptin deficient rodents and humans (Farooqi et al., 2002; Pelleymounter et al., 1995). Leptin action acutely regulates WAT metabolism by inducing lipolysis and inhibiting lipogenesis, which contribute in the long-term to the ability of leptin to reduce adiposity (Shimabukuro et al., 1997; Wang et al., 1999). These effects of leptin (as most of its metabolic effects) are mediated primarily via signaling in the brain. Re-constitution of neuronal leptin receptors completely reverses the lipotoxic, dysmetabolic phenotype of leptin receptor deficient db/db mice (de Luca et al., 2005). Conversely, deletion of the peripheral leptin receptor in mice, but not the brain, results in no obvious dysmetabolic phenotype or alterations in adiposity (Guo et al., 2007). Furthermore, the anti-adiposity effects of systemic leptin, which in part occur independent of its anorexic effects, can be reproduced by infusing only small amounts of leptin intracerebroventricular (ICV) (Gallardo et al., 2007). Conversely, reducing neuronal leptin receptor levels by 50% in mice results in increased fat mass, while food intake seems not to be affected (McMinn et al., 2005). The Buettner lab has previously shown that leptin acutely infused into the mediobasal hypothalamus (MBH) suppresses *de novo* lipogenesis by decreasing protein expression and activation state of key lipogenic enzymes such as fatty acid synthase (FAS) and ATP citrate lyase (Atpcl) (Buettner et al., 2008). Furthermore, MBH leptin suppresses fatty acid uptake into visceral WAT, while it stimulates lipolysis by increasing the activation state of hormone sensitive lipase (Hsl) via phosphorylation of the serine residues 563 and 660 (Buettner et al., 2008). Since these Hsl phosphorylation sites are targets of protein kinase A (PKA) (Anthonsen et al., 1998), an enzyme that is induced via the sympathetic nervous system (SNS), and both pharmacological and surgical denervation of epidydimal fat pads block the effects of central leptin (Buettner et al., 2008), it is likely that MBH leptin stimulates lipolysis by increasing SNS outflow to WAT.

Regulation of lipid metabolism by insulin

While leptin suppresses *de novo* lipogenesis and induces lipolysis in WAT, the acute effects of systemic insulin on WAT metabolism oppose those of leptin. Insulin is considered the major anti-lipolytic (Lafontan and Langin, 2009) and pro-lipogenic regulator (Assimacopoulos-Jeannet et al., 1995) in WAT (Degerman et al., 2003) and these effects are thought to be exclusively mediated via insulin receptors expressed on adipocytes. The importance of intact insulin signaling in maintaining WAT function is highlighted by the fact that humans with insulin receptor mutations exhibit lipodystrophy or even lipoatrophy, a severe reduction in WAT mass with increased circulating fatty acids (Donohue and Uchida, 1954; Hegele, 2003). The classical explanation for this phenotype is that the lipodystrophy results from the loss of peripheral insulin receptor signaling in adipocytes, which leads to unrestrained lipolysis and reduced *de novo* lipogenesis. However, mice that lack the insulin receptor exclusively in fat tissue develop only a mild reduction in adipose tissue mass (Bluher et al., 2002), indicating that the loss of the adipocyte insulin receptor cannot fully explain the lipodystrophic phenotype of patients with insulin receptor defects. Furthermore, the inducible deletion of the insulin receptor throughout the periphery but not the brain only moderately reduces adiposity, while inducible deletion of the insulin receptor in the whole body, that is brain and periphery, leads to severe lipodystrophy within four weeks (Koch et al., 2008; Seibler et al., 2007). Furthermore, chronic ICV insulin infusion can increase fat mass in mice without affecting food intake (Koch et al., 2008).

II. Aims

Collectively, the above mentioned findings indicate that a) the anti-lipolytic and pro-lipogenic effects of insulin are *not* exclusively mediated via the insulin receptor expressed on adipocytes and b) that brain insulin signaling plays a pivotal role in preserving fat mass and retaining NEFAs in WAT opposing the effects of brain leptin (Buettner et al., 2008). The effects of insulin on WAT metabolism are normally counterbalanced by cyclic–AMP (cAMP) signaling, which represents the major pro-lipolytic pathway in adipocytes. cAMP signaling is chiefly regulated by the SNS. However, it is presently unknown whether insulin regulates WAT lipolysis by inhibiting SNS outflow through brain effects, and if so, in which anatomical brain structure. Therefore, the aim of this doctoral thesis was to assess whether insulin acts in the brain to suppress lipolysis and induce *de novo* lipogenesis and to test if intact brain insulin signaling is necessary to maintain adipose tissue functionality and metabolic flexibility. Large parts of this thesis are based on data published in peer-reviewed journals (Buettner et al., 2008; O'Hare et al., 2011; Scherer and Buettner, 2009; Scherer and Buettner, 2011; Scherer et al., 2011).

III. Material and methods

Animal care and husbandry

All animal studies were performed in accordance with the Mount Sinai School of Medicine Institutional Animal Care and Use Committee (IACUC) guidelines under supervision of the Center for Comparative Medicine and Surgery. All the described procedures and experiments are covered under protocols LA09-00174 (Title: Regulation of adipose tissue metabolism by central insulin and leptin; Principal Investigator: Dr. Christoph Buettner; Start: October 20th 2009) and 07-0798 (Title: The Role of STAT3 Signaling in Leptin's Pleiotropic Actions; Principal Investigator: Dr. Christoph Buettner; Start: July 23rd 2007). See **Appendix**.

Rats

Rat experiments were performed in regular chow (RC; Rodent Diet 5001, LabDiet, St. Louis; MO) fed, male Sprague Dawley (SD) rats (Charles River Breeding Laboratories, Wilmington, MA) housed in a temperature and light controlled facility in separate cages. Nutritional information for all diets is provided in **Table 1**.

Mice

Neuronal insulin receptor knock-out (NIRKO) mice were generated as described elsewhere (Bruning et al., 2000; Fisher et al., 2005). Briefly, mice homozygous for the floxed insulin receptor allele ($IR^{lox/lox}$) were crossed with transgenic mice that express the Cre recombinase under control of the rat nestin promoter to generate ($IR^{lox/lox}$: nestin-$Cre^{+/-}$) NIRKO mice. Genotypes were determined by PCR of tail DNA as per protocol described below. Mice were housed on a 12 hr light–dark cycle and fed a standard rodent diet. Mice in clamp experiments were fed Mouse diet 9F, whereas mice in the refeeding experiment were fed PicoLab Rodent Diet 20 (both PMI Nutrition International, St. Louis, MO). See **Table 1.**

Table 1: *Macronutrient content of diets used in rat and mouse studies.*

% kcal	Laboratory Rodent Diet 5001 (RC, rat)	Mouse Diet 9F (RC, clamp)	PicoLab® Rodent Diet 20 (refeeding)
Protein	28,507	23,066	24,651
Carbohydrate	57,996	55,364	62,144
Fat	13,496	21,570	13,205
Physiological fuel value (kcal/gm)	**3,36**	**3,75**	**3,41**

General surgical procedures

Rat stereotaxic cannulae implantation

Stereotactic coordinates were selected according to the Paxinos Rat Brain Atlas (Paxinos and Watson, 1998). Rats were placed in a stereotactic frame (ASI instruments, Warren, MI) under ketamine–xylazine anesthesia (60 mg/kg ketamine; 6 mg/kg xylazine, intraperitoneal) and implanted with a 22–gauge single guide cannula to target the 3rd ventricle (intracerebroventricular, ICV) or a 26–gauge dual guide cannula system for targeting the MBH (both PlasticsOne, Roanoke, VA). The coordinates used for the ICV cannula were 2.5 mm posterior from the bregma, on the midline and 9 mm below the cortical surface. In order to target the MBH the coordinates used were 3.3 mm posterior from the bregma, 0.4 mm bilateral from midline and 9.6 mm below the surface of the skull. Guide cannulae were blocked using dummy cannulae (PlasticsOne, Roanoke, VA) until the day of the experiment. Food dye was routinely infused immediately before removing the brain to confirm correct anatomical placement of the cannulae.

Rat catheter surgery

Approximately 4-7 days prior to the experiment, rats were subjected to aseptic vascular catheter implantation under ketamine-xylazine anesthesia (described above). Catheters were implanted into the right jugular vein and the left carotid artery and secured to their respective vessel with braided 5-0 silk sutures. Catheters were tunneled subcutaneously

and exteriorized at the back of the neck. Any animal that had lost more than 10% of their pre–surgical body weight was excluded from the study.

Mouse catheter surgery

Approximately 7-10 days prior to the experiment, the animals underwent aseptic surgery under isoflurane anesthesia to have vascular catheters implanted into the left femoral artery and right jugular vein. Braided 6-0 silk sutures were used to secure the catheters in the vessels. Catheters were tunneled subcutaneously and exteriorized at the center of the animal's back. Animals were allowed to recover for one week before being studied. Any animal that had lost more than 10% of their pre–surgical body weight was excluded from the study.

Rat hypothalamic signaling studies

Insulin signaling and validation of the insulin receptor antagonist S961

Overnight fasted male SD rats were anaesthetized using ketamine-xylazine and then implanted with cannulae targeting either the 3rd ventricle (ICV) or the MBH (as described above**)**. ICV signaling was performed in 8-week old rats, whereas the MBH signaling experiments were done in 6-weeks old rats. In order to adjust for their smaller body size we changed the coordinates to 2.6 mm posterior of the bregma in the 6-week old rats. Following placement of the ICV cannula anaesthetized rats were given a 10 µl bolus of either vehicle (artificial cerebrospinal fluid (aCSF); Harvard Apparatus, Holliston, MA) or insulin (500 mU; Humulin R Lilly, Indianapolis, IN). The insulin receptor antagonist S961 (a gift from Novo Nordisk; Maaloev, Denmark (Schaffer et al., 2008)) was validated by giving 15 min apart either twice ICV aCSF (2 x 10µl), aCSF (10µl) and then insulin (500 mU in 10 µl aCSF) or 1.75 nmoles S961 (in 10µl aCSF) and then 500 mU with another 1.75 nmoles S961 (all in 10 µl aCSF). In the MBH insulin signaling experiments anaesthetized rats received either aCSF (vehicle) or a 100 mU bolus per side (1 µl per side) into the MBH after cannulae implantation.

All rats were killed by decapitation 15 min after the last bolus and either the MBH or the ARC and VMH were dissected and snap frozen in liquid nitrogen. Correct placement was verified by food dye infusion at the end of the study. ARC and VMH punch biopsies were dissected using N.I.H. style neuro punches (Fine Science Tools, Foster City, CA).

Rat pancreatic euglycemic clamp studies

Prior to the clamp studies rats were stereotaxically fit with indwelling cannulae targeting the 3rd ventricle (ICV) or the MBH. After a one–week recovery period carotid and jugular catheters were implanted for blood sampling and infusion, respectively (see above for more detailed description of the surgical procedures). Rats were allowed to recover for an additional 4 days and required to return to within 10% of their pre–surgical body weight.

Pancreatic clamp procedure

Rat clamp experiments were performed in conscious, non–restrained ad libitum fed male SD rats. During the 360 min study protocol, rats were kept in individual plastic cages with bedding. At the start ICV and MBH infusion cannulae projecting 1 mm from the bevel of the guide cannula were inserted. An ICV (5 µl/hr) or MBH (0.18 µl/hr per side) infusion with either vehicle or human insulin (ICV 30 µU; MBH 2 µU; Humulin R Lilly, Indianapolis, IN) was started and maintained for the entire 6 hrs of the study. aCSF (Harvard Apparatus, Holliston, MA) was used as MBH or ICV vehicle. After 2 hrs of continuous brain infusion a 120 min tracer equilibration period was started (TP = 0 min) consisting of a 20 µCi bolus of [3-^{3}H]-glucose (Radiochemical concentration > 97%; PerkinElmer, Waltham, MA) and a 13.3 µmol bolus of [^{2}H-5]-glycerol (98 atom percent excess) purchased from Isotec (Miamisburg, OH) followed by a 0.5 µCi/min and 0.334 µmol/min infusion of [3-^{3}H]-glucose and [^{2}H-5]-glycerol, respectively. For the last 30 min of the tracer equilibration period, arterial blood samples were collected every 10 min in order to determine baseline glucose production (GP) and rate of appearance (Ra) of glycerol using tracer dilution methodology (see below). At t = 120 min the insulin clamp was started with a primed–continuous infusion of human insulin (17.7 mU · kg^{-1} or 53.2 mU · kg^{-1} bolus followed by a 1 mU or 3 mU · kg^{-1} · min^{-1} infusion; Humulin R, Lilly) while also infusing somatostatin (3 µg · kg^{-1} · min^{-1}). The tracer infusions were continued at the aforementioned rates. **Fig. 1** depicts a detailed schematic of the clamp study protocol. Euglycemia (~110 – 130 mg/dl) was maintained during the clamp by measuring blood glucose every 10 min starting at t = 120 min and infusing 25% glucose as needed. Arterial blood samples were obtained every 10 min for the last hour of the experiment (TP = 180 to 240 min) to calculate GP, the rate of glucose disposal and the Ra glycerol under clamped condition.

Figure 1

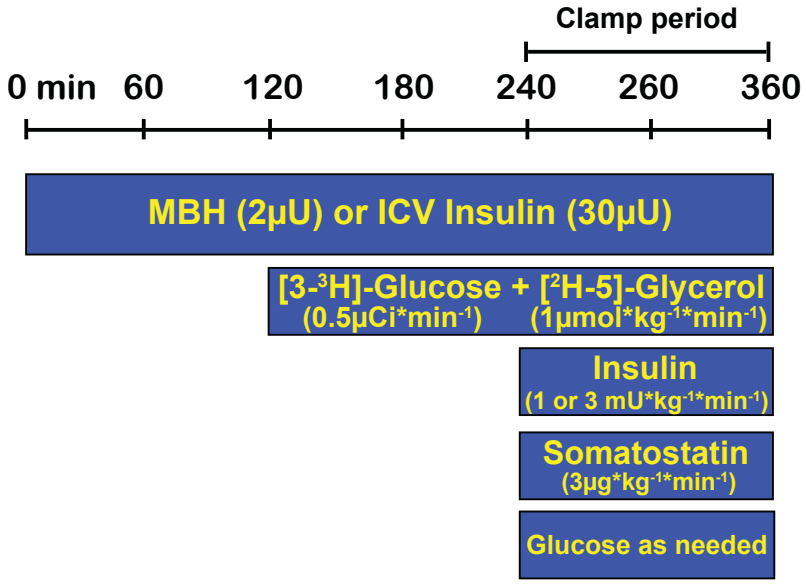

Figure 1 | *Experimental protocol of euglycemic pancreatic clamp studies in SD rats.*

Glucose tracer analyses

Glucose fluxes were analyzed as described elsewhere (Liu et al., 1998; Obici et al., 2002; Pocai et al., 2005). Briefly, to measure plasma [3-^{3}H]-glucose radioactivity samples were de-proteinated using barium hydroxide and zinc sulfate. After centrifugation the supernatant was dried overnight to eliminate tritiated water. Glucose was then re-dissolved in water and counted using Ultima Gold in a MicroBeta TriLux (both PerkinElmer, Waltham, MA) liquid scintillation counter. Under pre-clamp steady state conditions the endogenous glucose production equals the glucose turnover rate, which was determined from the ratio of the [3-^{3}H]-glucose tracer infusion rate and the specific activity of plasma glucose. During the pancreatic clamp period endogenous glucose production was calculated by subtracting the glucose infusion rate from the glucose turnover rate, which in a steady state equals the rate of glucose disposal.

Ra Glycerol analyses

Glycerol flux was analyzed as described elsewhere (Kang et al., 2007). In short, the plasma glycerol Ra in μmol · kg^{-1} · min^{-1} was used as an index for systemic lipolysis, as calculated by the equation

$$R_a = \left(\frac{ENR_{\text{inf}}}{ENR_{pl}} - 1 \right) \cdot R$$

where ENR_{inf} is the fractional isotopic enrichment of the infused glycerol in atom percent excess and ENR_{pl} that in the arterial plasma sample. R is the rate of the isotope infusion in μmol · kg^{-1} · min^{-1}. The ^2H-labeling of plasma glycerol was determined as follows: 20 μl of plasma was de–proteinized with 200 μl of methanol by centrifugation for 10 min at 16,100 g. The fluid fraction was then evaporated to dryness and reacted with 50 μl of bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane for 20 min at 75 °C. Isotope enrichment was determined by gas chromatography – mass spectrometry.

Local denervation experiment

Pharmacological and surgical denervation was performed as previously described (Buettner et al., 2008). In short, for surgical denervation the vascular strand innervating the epigonadal fat pad was identified after laparotomy. The fascia containing the nerve bundle was separated from the vessels and dissected followed by local application of phenol. Pharmacological sympathectomy was performed by injecting 6-hydroxydopamine into the epigonadal fat pad as described elsewhere (Giordano et al., 2006). Rats with intact adipose tissue innervation were infused for 4 hrs with either MBH insulin (total dose: 1.44 μU; Humulin R Lilly, Indianapolis, IN) or vehicle (aCSF, Harvard Apparatus, Holliston, MA). Denervated rats were infused with MBH vehicle. After the infusion rats were anaesthetized with isoflurane, killed and their epigonadal fat pads harvested.

Insulin antagonist infusion experiment

Eight–week old male SD rats were equipped with MBH cannulae and both arterial and venous catheters. Rats were infused for 4 hrs with either aCSF (vehicle) or 0.1 pmoles of the insulin receptor antagonist S961 (Novo Nordisk; Maaloev, Denmark) into the MBH, while also employing a [^2H-5]-glycerol tracer infusion (1 μmol · kg^{-1} · min^{-1}).

Systemic pharmacological sympathectomy experiment

Eight–week old male SD rats were simultaneously implanted with MBH cannulae and jugular catheters during ketamin–xylazine anesthesia. After a 3-day recovery period rats were given two IV bolus injections of 6-OHDA (50 mg/kg BW freshly prepared in 0.9% saline containing 0.1% ascorbic acid) or vehicle (0.9% saline containing 0.1% ascorbic acid) 3 days apart. Vehicle injected animals were pair–fed to 6-OHDA injected rats to match their bodyweights. Three days following the last IV injection rats were either infused with MBH vehicle (aCSF) or S961 (0.1 pmoles) using the protocol depicted in **Fig. 15C**. After 2 hrs a [^{2}H-5]-glycerol tracer infusion was started and maintained (30 μmol · kg^{-1} · min^{-1} for 4 min bolus followed by 3 μmol · kg^{-1} · min^{-1}) for 2 hrs. Blood samples for Ra glycerol analysis were taken at time point (TP) 240 min by tail vein sampling. Only rats recovered within 10% of their pre–surgical BW were used.

Caloric restriction in aged SD rats

At 15 weeks of age, rats were assigned to be fed either ad libitum (AL) or to be calorically restricted (CR) with 55% of the calories consumed by the AL group. The chow contained 64% carbohydrates, 30% proteins, and 6% fats with a physiological fuel value of 3.30 kcal/g chow. Vitamin supplementation was added to the CR rats. Epididymal WAT was harvested and analyzed via Western blot in 4–month–old (young AL) or 18–month–old (aged CR and aged AL) SD rats after 6 hrs of fasting.

Mouse experiments

Mouse pancreatic euglycemic clamp studies

Mouse clamp studies were performed in 16 hr fasted 4–month–old male conscious, non–restrained NIRKO and littermate lox-lox control mice that were equipped with femoral artery and right jugular vein catheters. The protocol consisted of a 100 min tracer equilibration period followed by 110 min clamp period as depicted in **Fig. 2**. A 10 μmol bolus of [^{2}H-5]-glycerol (98 atom percent excess) was given at t = -100 min followed by a continuous infusion at 4 μmol · kg^{-1} · min^{-1} over 210 min. The insulin clamp was initiated at t = 0 min with a continuous infusion of human insulin (4 mU · kg^{-1} · min^{-1} infusion; Humulin R, Lilly) lasting 110 min. Euglycemia was maintained by measuring glucose every 10 min and infusing 50% dextrose as necessary. Plasma samples were collected

before and at the end of the clamp to determine Ra glycerol during baseline and clamped period, respectively. To minimize blood loss, spun–down erythrocytes, which were re–suspended in saline, were re–infused. **Fig. 2** depicts a schematic outline of the clamp study protocol.

Figure 2

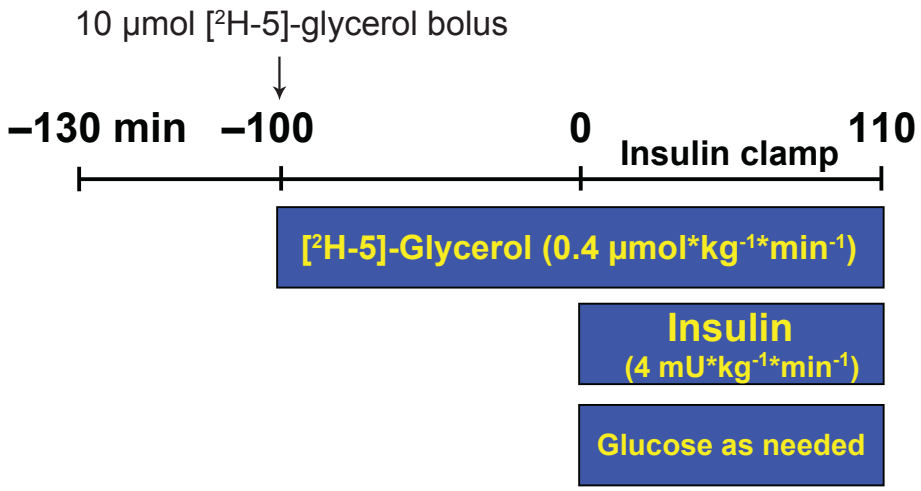

Figure 2 *| Experimental protocol of mouse euglycemic pancreatic clamp studies.*

Mouse fasting–refeeding experiments

Ten-week old Nirko and littermate control mice were fasted for 24 hrs starting at 3 pm the day before the experiment, so the re-feeding period coincided with the onset of the feeding cycle (**Protocol depicted in Fig. 19A**). An initial blood sample was taken from the tail vein at TP 0 min. After 90 min mice were granted *ad libitum* access to regular chow food for 2 hrs. The final blood sample (TP 210 min) was sampled by tail.

Mouse 16 hr fasting challenge

Three–month–old male NIRKO mice and lox-lox littermate controls were subjected to an overnight fast for 16 hrs. Mice were then anaesthetized with isoflurane and blood was collected prior to sacrificing them.

Analytic procedures

Prior to tissue analyses animals were anaesthetized, killed and their tissues snap frozen in liquid nitrogen. Blood was collected in EDTA tubes and glucose measured by Freestyle Freedom glucose analyzer (Abbot, Abott Park, IL). Plasma free–glycerol and triglycerides

were measured with a colorimetric assay from Sigma–Aldrich (St. Louis, MO), plasma NEFAs with a kit from Wako Chemicals (Richmond, VA) and β–hydroxybutyrate levels with an assay form Stanbio Laboratory (Boerne, TX). Rat plasma insulin was measured by radioimmunoassay at the hormone assay core of Albert Einstein Medical College (New York, NY) or with an insulin ELISA kit by Mercodia (Uppsala, Sweden). Rat plasma adiponectin was analyzed by radioimmunoassay at the hormone assay core of Albert Einstein Medical College (New York, NY). Rat plasma glucagon and leptin levels as well as mouse plasma insulin and glucagon were measured with species–specific Luminex MultiAnalyte Profiling (Millipore, Billerica, MA).

WAT norepinephrine content

A 100 mg piece of epididymal WAT was homogenized in a 0.2 M perchloric acid solution with 1 mg/ml ascorbic acid. After centrifugation at 7,500 g for 10 min, catecholamines were extracted from the homogenate with an alumina extraction kit provided by ESA Biosciences (Sunnyvale, CA). The HPLC was equipped with electrochemical detection and ESA HR-80 analytical column and Cataphase II mobile phase.

Fatty acid analysis

Fatty acid analysis was performed as previously described (Scheja et al., 2008). In short, fatty acid methyl ester standards were obtained from Restek, (Bad Homburg, Germany), Cayman (IBL, Hamburg, Germany) and Supelco (Sigma, Munich, Germany). Total lipids from adipose tissue were extracted essentially as described by Folch et al. (Folch et al., 1951). Briefly, 250 µl of butylhydroxytoluene (0.1 mol/l in methanol) and 6 ml of chloroform/methanol (2:1) were added to 50 mg of tissue. After homogenization using an Ultraturrax homogenizer the samples were heated to 50°C for 30 min and centrifuged (1,800 g, 5 min). Fatty acid methyl esters were prepared based on the method of Lepage and Roy except that toluene was used instead of benzene (Lepage and Roy, 1986). Briefly, 100 µl of tissue extract, 2 ml methanol/toluene (4:1), 50 µl heptadecanoic acid (200 µg/ml in methanol/toluene, 4:1) and 200 µl acetylchloride were heated in a capped tube for 1 hour at 100°C. After cooling to room temperature 5 ml of 6% sodium carbonate was added. The mixture was centrifuged (1,800 g 5 min) and 150-200 µl of the upper layer were transferred to auto sampler vials. Gas chromatography analyses were performed using an HP 5890 gas chromatograph (Hewlett Packard) equipped with flame ionization detectors

(Stationary phase: DB-225 30 m x 0.25 mm id., film thickness 0.25µm; Agilent, Böblingen, Germany). Peak identification and quantification were performed by comparing retention times and peak areas, respectively, to standard chromatograms. All calculations are based on fatty acid methyl esters values.

FAS enzyme activity assay

FAS activity in perirenal fat was measured spectrophotometrically using a modified protocol of Nepokroeff *et al.* (Nepokroeff et al., 1975). Briefly, perirenal fat samples (200 mg) were homogenized in ice–cold buffer containing 0.25 M sucrose, 1 mM DTT, 1 mM EDTA and a protease inhibitor cocktail (Roche, Nutley, NJ). The homogenate was centrifuged at 20,000 g at 4°C for 10 min to collect fat–free infranatant, which was then centrifuged at 100,000 g at 4°C for one more hour. Infranatant was mixed 1:1 with 500 mM potassium phosphate buffer containing 0.5 mM DTT (pH 7.4) and incubated at 37°C for 30 min for maximal activation of each sample. In a 96-well plate, 200 µl of potassium phosphate buffer (500 mM, pH 7; containing 0.1 M EDTA and 1 mM β–mercaptoethanol) was added to 5 µl of freshly prepared NADPH solution (10 mM in 100 mM potassium phophate buffer, pH 7) in each well and incubated at 37°C for 10 min. After incubation 100 µl of the activated sample was added and the reaction was started by pipetting the substrate solution to each well (10 µl of 5 mM malonyl-CoA mixed in a ratio of 4:3 with 5 mM acetyl-CoA dissolved in 100 mM potassium phosphate buffer, pH 7). Absorbance was measured every minute at 340 nm for 20 minutes, and the gradient for absorbance at 340nm was calculated. Activity of the FAS enzyme (mU/mg protein) was defined as 1 nmole NADPH consumed · min^{-1}· mg^{-1}. An extinction coefficient of 6220 M^{-1}· cm^{-1} was used in the activity calculation.

Cytosolic and lipid–droplet fraction and TG hydrolase activity

Assay was performed with epididymal fat pads as previously described (Schweiger et al., 2006). Cytosolic and lipid–droplet fraction from rat adipose tissue were obtained using a modified protocol of Schweiger *et al.* (Schweiger et al., 2008). Briefly, rat adipose tissue was disrupted in 300 µl of buffer A (0.25 M sucrose, 1 mM EDTA, 1mM DTT, pH 7, 20 µg/ml leupeptine, 2 µg/ml antipain, 1 µg/ml pepstatin) using the schütthomgen plus (Schüttlabortechnik GmbH, Göttingen, Germany) and centrifuged at 1000 g, 4°C for 15 min to remove nuclei and cell debris. Thereafter, the supernatant was transferred to SW 41

tubes, overlaid with buffer B (50 mM potassium phosphate, pH 7.4, 100 mM KCl, 1 mM EDTA, 20 µg/ml leupeptine, 2 µg/ml antipain, 1 µg/ml pepstatin) and centrifuged in a SW 41 rotor (Beckman, Fullerton, CA) for 2 h at 100,000 g, 4°C. The lipid–droplets were collected as a white band from the top of the tube. The lower 300 µl, corresponding to the cytosolic fraction, were obtained using a hot needle. For the determination of TG hydrolase activity, 12 µg of cytosolic protein (in 100 µl Buffer A) were incubated with 100 µl substrate in a water bath for one hour at 37°C. After incubation, the reaction was terminated by adding 3.25 ml of methanol/chloroform/heptane (10/9/7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid (pH 10.5). After centrifugation (800 g, 15 min), the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting. TG substrate was prepared by emulsifying 1,67 mM triolein (8,000 cpm of [9,10-3H]triolein/nmol) (Perkin Elmer) and 45 µM phosphatidylcholine/phosphatidylinositol (3/1) (Sigma) in 100 mM potassium phosphate buffer (pH 7.0) by sonication (Virsonic 475, Virtis) and adjusted to 5% (w/v) fatty acid free bovine serum albumin (BSA, Sigma).

RNA extraction and quantitative real–time PCR.

Total RNA was obtained from frozen tissue (85–100 mg) using the RNeasy Tissue Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. After treatment with DNase I (Invitrogen, Carlsbad, CA), purified total RNA was used as a template for first–strand cDNA synthesis with Superscript III (Invitrogen, Carlsbad, CA). Quantitative real–time PCR was run with SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Forward and reverse customized primer pairs (Invitrogen, Carlsbad, CA) are listed in **Table 2**. Data was analyzed using the delta-delta-C_t method ($2^{-\Delta\Delta Ct}$) as described in (Livak and Schmittgen, 2001).

Table 2: *Sequences of forward and reverse customized QPCR primers.*

Pck1	5'- CTT CTC TGC CAA GGT CAT CC - 3' 5'- GAG CCA GCC AAC AGT TGT CA - 3'
G6pc	5'- CTA CCT TGC GGC TCA CTT TC - 3' 5'- ATC CAA GTG CGA AAC CAA AC- 3'
Gapdh	5'- ACA CAG CCG CAT CTT CTT GT - 3' 5'- CTT GCC GTG GGT AGA GTC AT- 3'

Western blot analyses

WAT was homogenized in 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.5% NP-40 and complete protease inhibitor cocktail (Roche, Nutley, NJ) and centrifuged at 13,000g for 20 min at -3°C. The supernatant was then collected while carefully avoiding the lipid layer on top and protein concentration was measured with a BCA protein quantification kit (Thermo Scientific, Waltham, MA). Protein extracts were separated on 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA) and blotted onto Immobilon FL PVDF (Millipore, Billerica, MA). Membranes were blocked at room temperature for 1 h in Odyssey LI-COR Blocking Buffer (LI-COR, Lincoln, NE) 1:1 diluted in TBS and incubated in primary antibodies in 1:1 Blocking Buffer/TBS-T overnight at 4°C. Primary antibodies against Acc, phospho–Ampk α, Ampk α, phospho–Akt (Ser 308 and Ser 473), Akt (Proteinkinase B) total, Atgl, Atpcl, phospho–Atpcl, phospho–Erk, Erk total, phospho–Gsk 3 α/β, phospho–Hsl (Ser563, Ser565 and Ser 660), phospho–insulin receptor β, phospho–PKA substrate (all Cell Signaling Technology, Beverly MA), FAS and Enos (BD Bioscience, San Jose, CA), α–tubulin, β–actin, Gapdh, Hsl total (all Abcam, Cambridge, MA), insulin receptor β (Santa Cruz Biotechnology, Santa Cruz, CA) and Perilipin (Souza et al., 2002), a gift from Dr Andrew Greenberg, were used. After three consecutive 5 min washes in TBS-T (0.1%), blots were incubated with Dylight 680-conjugated goat anti–rabbit IgG and Dylight 800-conjugated goat anti–mouse IgG (both Thermo Scientific, Waltham, MA) for 1 hr at room temperature in blocking buffer containing 0.1% TBS-T and 0.1% SDS. After three washes in TBS-T and a final wash in TBS, the blots were scanned with the LI-COR Odyssey (LI-COR, Lincoln, NE) and quantified with Odyssey 3.0 software based on direct fluorescence measurement.

DNA extraction and genomic PCR

DNA was isolated by incubating tail biopsies in tail buffer (100 mM Tris pH 8, 200 mM NaCl, 5 mM EDTA pH 8, 0.2 % SDS) and Proteinase K (Quiagen, Venlo, Netherlands) at 56°C overnight in a water bath. DNA was precipitated with isopropanol. To determine the presence of the *Cre* transgene and the *floxed insulin receptor* (*IR*) allele, DNA was amplified with PCR using the primer sequences in **Table 3** and visualized with ethidium bromide using agarose (1%) gel electrophoresis (See **Fig. 3**).

Table 3: *Sequences of forward and reverse primers for genomic PCR*

Cre	5'- ACC TGA AGA TGT TCG CGA TTA TCT- 3' 5'- AC CGT CAG TAC GTG AGA TAT CTT - 3'
IR	5'- TGC ACC CCA TGT CTG GGA CCC- 3' 5'- GCC TCC TGA ATA GCT GAG ACC- 3'

Figure 3

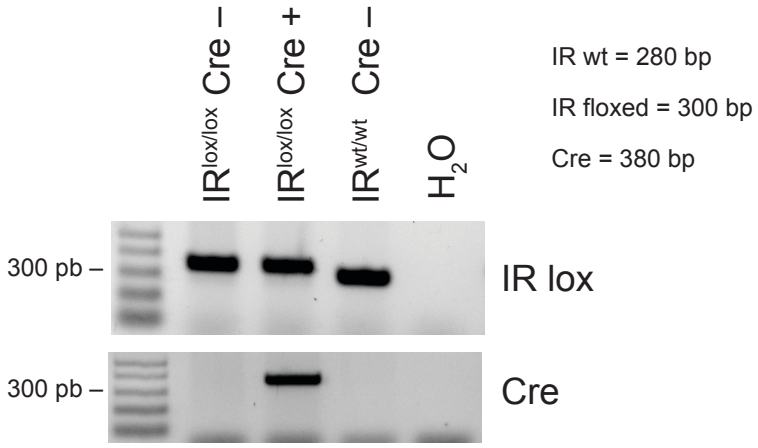

Figure 3 *| Representative results of PCR-based genotyping of Cre and IR, in tails of control (IR$^{lox/lox}$: nestin-Cre$^{-/-}$) and NIRKO (IR$^{lox/lox}$: nestin-Cre$^{+/-}$) mice. Genomic DNA of tails was amplified for 35 cycles using the primers displayed in* ***Table 3****. Tail DNA of C57BL6 mouse was used as a control.*

Statistics

All data are represented as mean ± s.e.m. Comparisons among groups were made using one–way ANOVA followed by unpaired two–tailed Student's t–tests if not otherwise indicated. Differences were considered statistically significant at $P < 0.05$. For **Figs. 10A, C, D and 21B** Pearson correlation and a two–tailed t–test was performed in Graphpad Prism 5.0b for Mac (GraphPad Software, San Diego California USA)

IV. Results

Brain insulin suppresses lipolytic flux

In order to test whether brain and specifically hypothalamic insulin signaling is able to regulate WAT metabolism, insulin levels were increased locally by infusing insulin directly into the 3rd ventricle (ICV) or the MBH of 10-week-old male SD rats using stereotactic cannulae. To delineate insulin signaling in specific hypothalamic nuclei within the MBH, acute insulin signaling studies in ICV (3rd ventricle) and MBH insulin infused rats were performed at doses that have been commonly used by others (Carvalheira et al., 2003; Rahmouni et al., 2004; Roman et al., 2005). Punch biopsies of the arcuate nucleus (ARC) and the venteromedial hypothalamus (VMH) were harvested and analyzed by Western blot. Brain insulin infusion markedly increased insulin receptor phosphorylation and activated classic downstream targets of insulin signaling such as Akt, glycogen synthase kinase 3 (Gsk 3) and extracellular-signal regulated kinase (ERK) 1/2. The activation of the insulin-signaling cascade in this experimental setup was more pronounced in the ARC compared to the VMH (**Fig. 4 A and B**). This is likely due to differences in the anatomical location of these two nuclei. The ARC directly adjoins to the 3rd ventricle, where insulin is delivered via the ICV cannula, whereas the VHM is located more lateral. The MBH cannulae using the aforementioned coordinates also predominantly target the ARC (Paxinos and Watson, 1998), however in **Fig. 4B** the differences between ARC and VMH insulin signaling seem to be less pronounced.

Figure 4

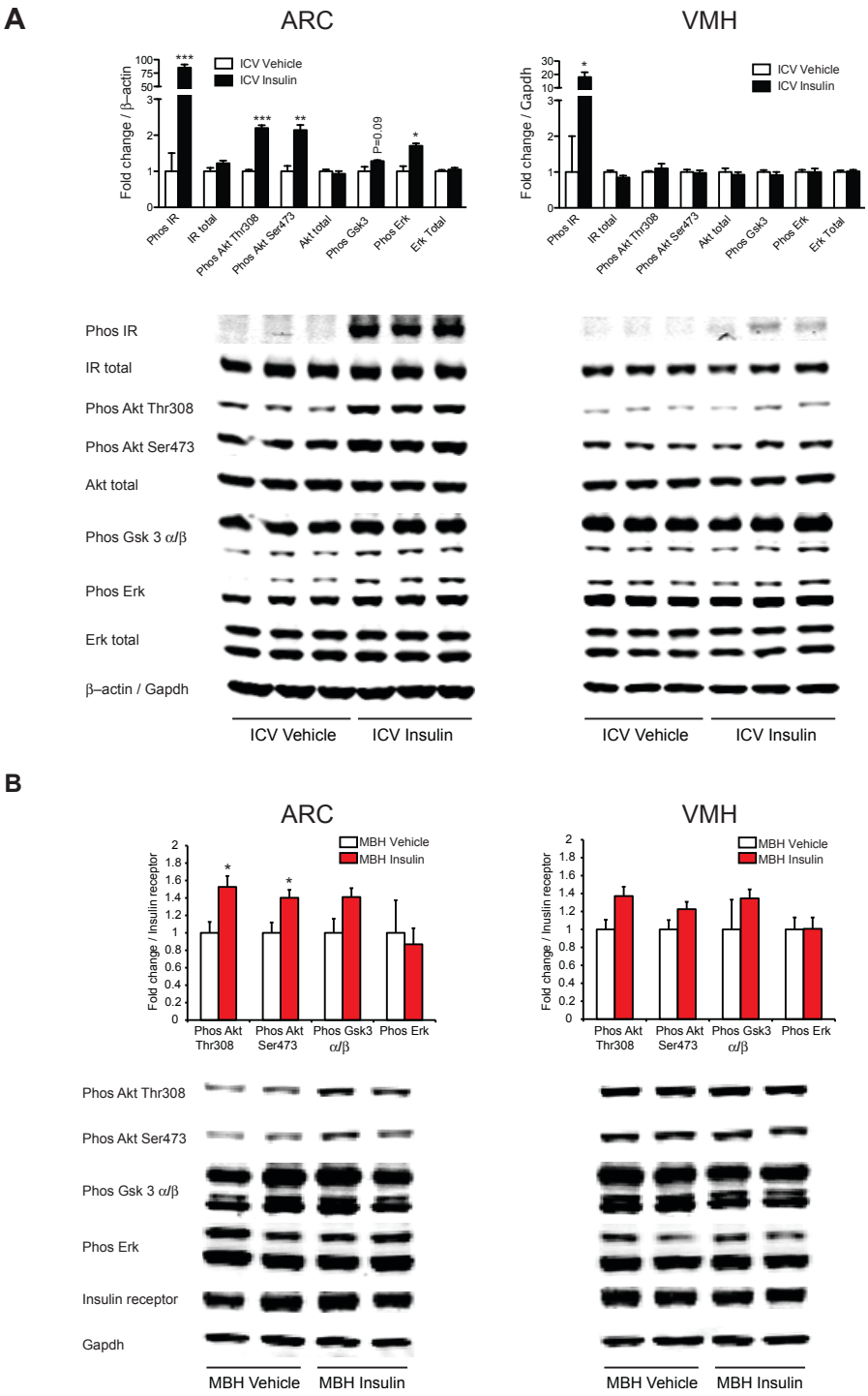

Figure 4 | Insulin infusion either into the 3rd ventricle (ICV) or directly into the MBH activates insulin signaling predominantly in the ARC compared to the VMH. (A, B) *Rats were killed 15 min after administration of insulin or vehicle. Punch biopsies of the ARC and VMH were harvested for Western blot analyses. (A) Top, quantification of Western blot analyses expressed as relative*

*change from vehicle treated group for ARC and VMH insulin signaling. Bottom, Western blot analyses of total protein as well as phosphorylation of insulin receptor, Akt, Erk and Gsk3 (n = 3 per group). (**B**) Representative Western blot analyses and quantifications of ARC (left) and VMH (right) punch biopsies harvested from 6–week old male SD rats 15 min after an MBH insulin bolus (n ≥ 3 per group). All error bars represent s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 versus vehicle treated group.*

Next, insulin was continuously infused ICV or intraparenchymally into the MBH for the duration of 6 hrs. To study the role of brain insulin in regulating lipolysis *in vivo* a stable isotope glycerol tracer, [^{2}H-5]–glycerol, was infused at the same time. To reduce the likelihood for pharmacological effects of the insulin doses administered, the doses used were more than 15,000–fold lower than those commonly utilized for ICV insulin infusion experiments (Air et al., 2002a; Brief and Davis, 1984; Rahmouni et al., 2004). Since the intracerebral infusion of hormones can alter pancreatic insulin secretion and circulating glucose levels, we subjected the animals to pancreatic clamps (protocol depicted in **Fig. 1**) to maintain euglycemia during the brain infusion of insulin or artificial cerebrospinal fluid (vehicle) (**Fig. 5A**). To compare the effects of isolated brain insulin signaling with the effects of systemic insulin (which includes the direct effects of insulin on adipocytes) plasma insulin levels were kept either at baseline (1 mU · kg^{-1} · min^{-1}) or hyperinsulinemia (3 mU · kg^{-1} · min^{-1}) was induced to mimic the fed state (**Fig. 5B**). Since glucose metabolism and lipolysis are regulated by insulin, both were assessed simultaneously by employing tracer dilution techniques to determine glucose and glycerol fluxes utilizing [3-^{3}H]-glucose and [^{2}H-5]-glycerol, respectively.

Figure 5

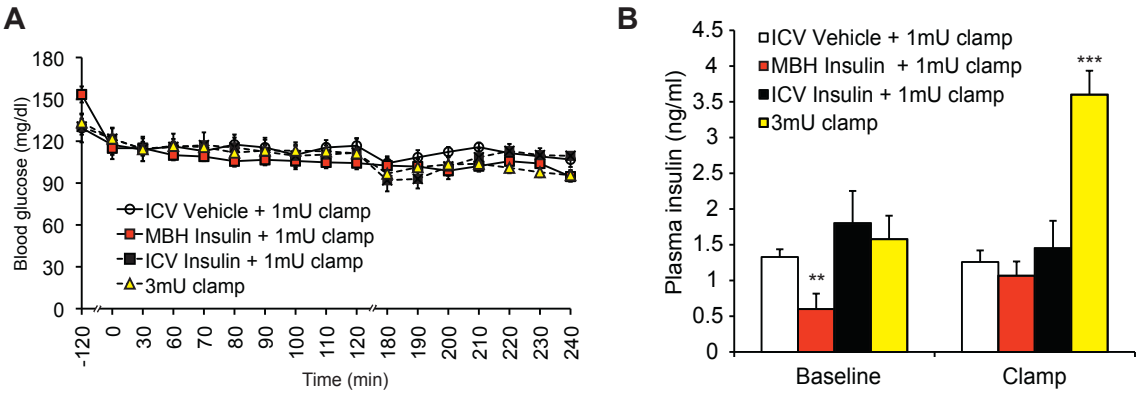

Figure 5 | Plasma glucose and insulin levels during baseline and the euglycemic pancreatic clamp. (A) *Plasma glucose levels (n ≥ 6 per group).* ***(B)*** *Plasma insulin levels during baseline (i.e. time point -120 to 120 min) and the clamp (i.e. 120 to 240 min; n ≥ 6 per group). All error bars represent s.e.m.; ** P < 0.01, *** P < 0.001 versus vehicle treated group*

Both ICV and MBH insulin administration markedly suppressed the Ra glycerol under basal and clamped conditions indicating that brain insulin, and more specifically MBH insulin signaling, suppresses lipolysis (**Figs. 6A and B**). Systemic glucose and insulin levels were not different between ICV vehicle, ICV insulin and MBH insulin infused rats during a 1 mU · kg^{-1} · min^{-1} basal pancreatic clamp, while insulin levels were raised about three fold in the 3 mU · kg^{-1} · min^{-1} hyperinsulinemic clamp group (**Figs. 5A and B**). Thus, brain insulin infusion inhibited lipolysis independent of increases in peripheral insulin levels. Hyperinsulinemia induced by a 3 mU · kg^{-1} · min^{-1} clamp decreased the Ra glycerol by about 65% compared to a 1 mU · kg^{-1} · min^{-1} clamp in vehicle infused animals (**Fig. 6B**). Thus, at the doses administered, brain insulin infusion inhibited lipolysis to a similar extent as that achieved with peripheral hyperinsulinemia.

Figure 6

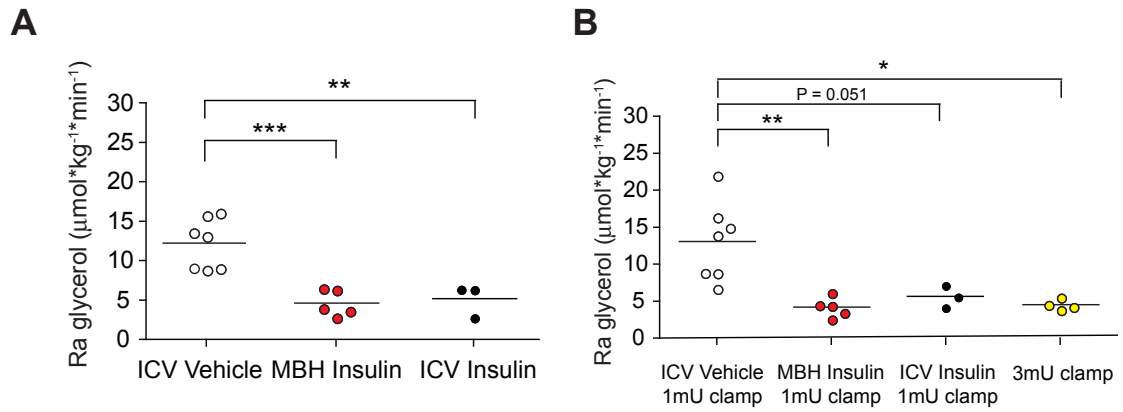

Figure 6 | Brain insulin signaling suppresses lipolysis in vivo. (A, B) *Ra glycerol during baseline* ***(A)*** *and euglycemic pancreatic clamp* ***(B)*** *(n ≥ 3 per group). * P < 0.05, ** P < 0.01, *** P < 0.001 as indicated.*

In a separate series of studies MBH insulin infusions were repeated and compared to MBH vehicle infused animals. Again, MBH insulin markedly suppressed Ra glycerol compared to MBH vehicle infused rats independent of circulating glucose and insulin levels (**Figs. 7A – C**).

Figure 7

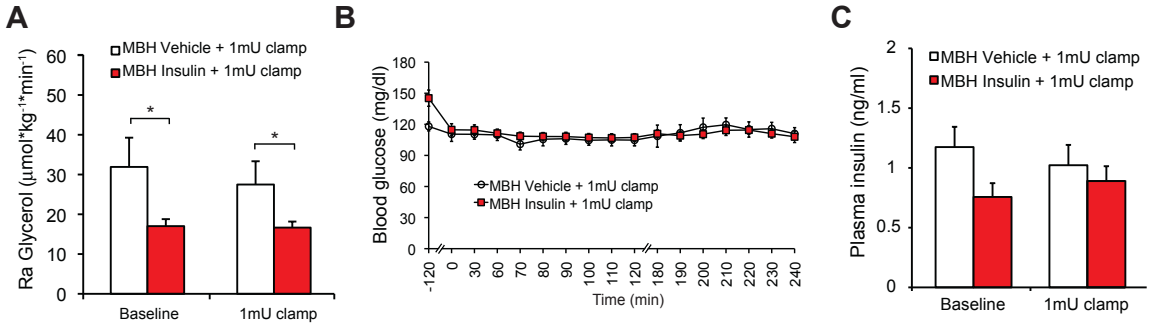

Figure 7 | MBH insulin suppresses lipolytic flux as assessed by the Ra of a stable glycerol tracer. ***(A)*** *Ra glycerol during baseline and 1 mU · kg^{-1} · min^{-1} clamp. MBH insulin and MBH vehicle infused rats were compared (n ≥ 4 per group).* ***(B)*** *Plasma glucose levels were equal in both groups (n ≥ 6 per group).* ***(C)*** *Plasma insulin levels during baseline and the clamp (n ≥ 6 per group). All error bars represent s.e.m.; * P < 0.05 as indicated.*

These dynamic changes in the rates of whole body lipolysis were reflected by changes in static measurements of plasma parameters of fatty acid metabolism, albeit to a lesser

degree. NEFA levels trended to increase during the basal period in vehicle infused animals, which is due to the transitioning of the animal into the fasted state and the flushing of catheters with saline and heparin, a lipoprotein lipase activator, commonly used to prevent clotting of the vascular access. This rise in plasma NEFA levels was prevented by both MBH and ICV insulin administration (**Fig. 8A**), which was also reflected in a significantly lower AUC (**Fig. 8B**). Of note, the effects of centrally administered insulin closely mimicked the effects of systemic insulin infusion in humans, where heparin-induced increase in plasma NEFAs is completely blunted by insulin treatment (Chaudhuri et al., 2007). Plasma glycerol and triglyceride levels trended in the same direction, although this failed to reach statistical significance (**Figs. 8C and D**). As expected, due to the administration of somatostatin, glucagon levels decreased during the clamp, but neither glucagon nor the levels of the adipokines leptin and adiponectin were different between groups and can therefore not explain the differences in lipolytic flux (**Figs. 8E–G**).

Figure 8

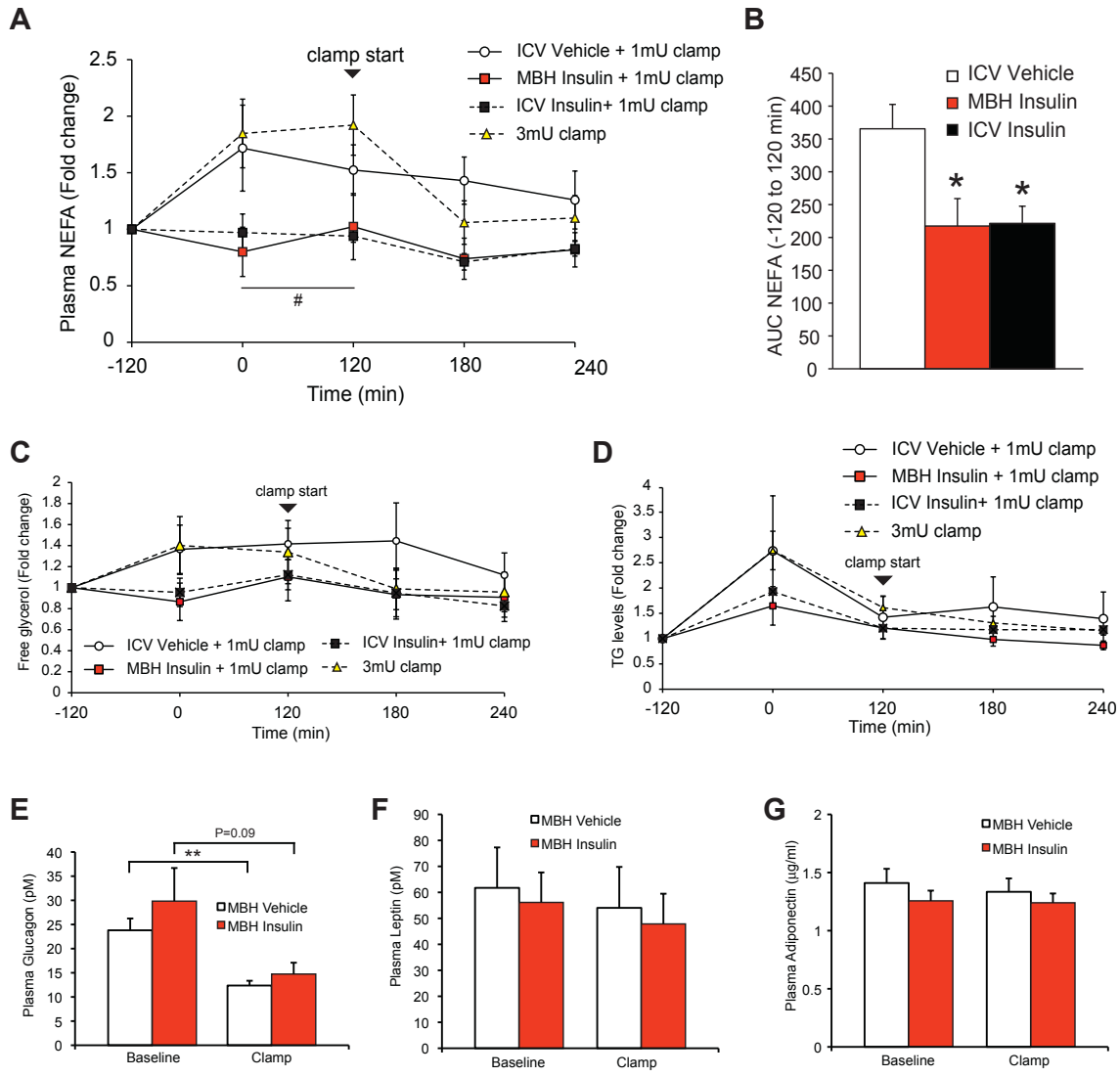

Figure 8 | Brain insulin infusion suppresses whole body lipolysis. *(**A**) Change of plasma NEFA levels compared to baseline during the 6 h infusion protocol. Arrowhead marks the start of the clamp at time point 120 min (n ≥ 4 per group). (**B**) AUC of **Fig. 8A** comparing vehicle to ICV and MBH insulin infused rats prior to the start of the clamp study (timepoint -120 to 120 min, n ≥ 4 per group). (**C, D**) Change of plasma glycerol and triglyceride (TG) levels from baseline. Arrowhead indicates the start of the clamp (n ≥ 4 per group). (**E, F, G**) Plasma glucagon, leptin and adiponectin levels of MBH vehicle and insulin infused rats at baseline and during the clamp (n ≥ 6). All error bars are s.e.m.; * P < 0.05, ** P < 0.01, versus ICV vehicle + 1 mU · kg^{-1} · min^{-1} clamp group if not otherwise indicated. # P < 0.05 versus 3 mU· kg^{-1} · min^{-1} clamp group.*

Lipolytic flux correlates with hepatic glucose production (GP)

Lipolytic flux can regulate hepatic GP potentially through two mechanisms (Mittelman and Bergman, 2000; Rebrin et al., 1996): 1) by providing the liver with the gluconeogenic precursor glycerol and 2) by enabling the synthesis of high energy NADH substrates required during gluconeogenesis through β–oxidation of NEFAs (Hers and Hue, 1983). To uncover a potential interdependence, glucose fluxes were assessed simultaneously in these studies. During the clamp MBH insulin infused rats required higher glucose infusion rates (GIR) compared to vehicle infused rats (**Figs. 9A**) and suppressed hepatic GP to a similar extent as rats treated with systemic hyperinsulinemia (**Figs. 9C and D**), which is consistent with prior reports (Pocai et al., 2005). Notably, despite the ability of MBH insulin to suppress hepatic GP during the clamped period, circulating glucose levels (**Fig. 5A and 7B**) and hepatic GP (**Fig. 9B**) did not change during the basal period where MBH insulin was infused, but no IV somatostatin. Glucose disposal into peripheral tissues, as assessed through the use of [3-^{3}H]–glucose tracer dilution technique, was not altered by central insulin infusion during basal clamps (**Figs. 9E**). As expected, systemic hyperinsulinemia increased glucose utilization in peripheral tissues like muscle and WAT, which is mainly a function of the direct effects of insulin on both tissues (**Fig. 9E**).

Figure 9

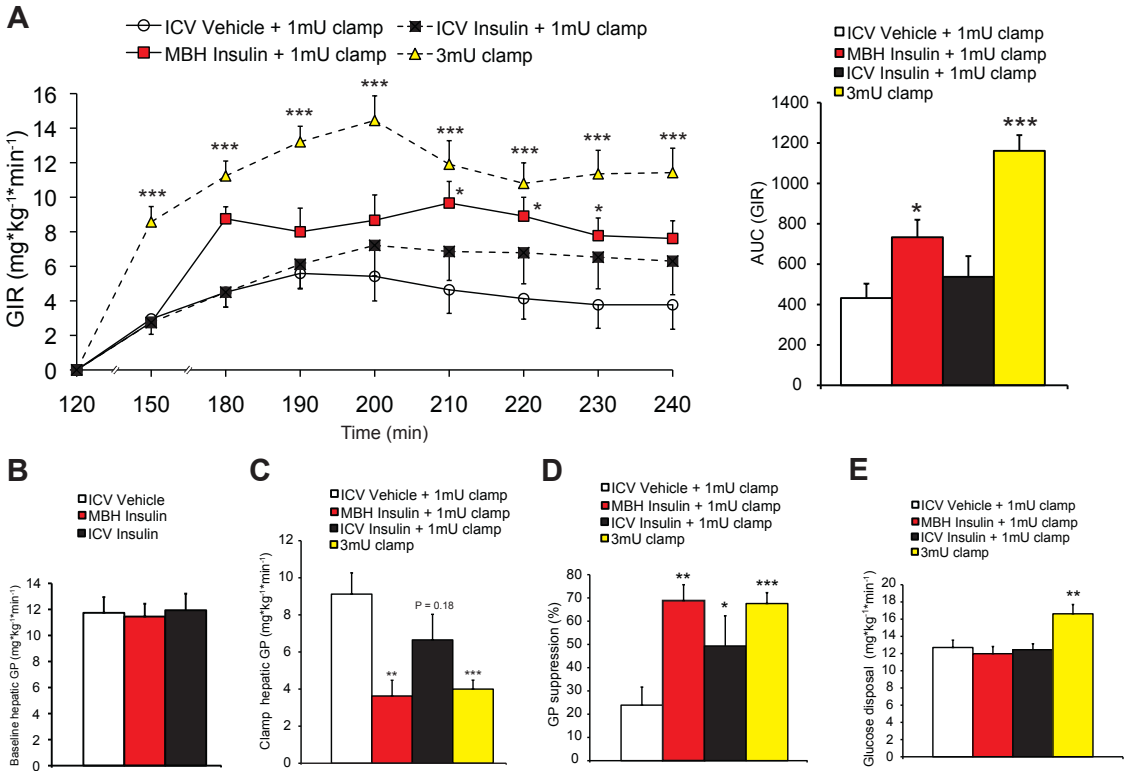

Figure 9 | MBH Insulin suppresses hepatic GP. (A) *GIR required to maintain euglycemia. Right, AUC of line graph on the right (n ≥ 6 per group).* ***(B)*** *Baseline hepatic GP (n ≥ 6 per group).* ***(C)*** *Clamp hepatic GP (n ≥ 6 per group).* ***(D)*** *Percent suppression of GP during the clamp from baseline (n ≥ 6 per group).* ***(E)*** *Rate of glucose disposal during the clamp (n ≥ 6 per group). All error bars are s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 versus ICV vehicle + 1 mU · kg^{-1} · min^{-1} clamp group*

Of note, the Ra glycerol tightly correlated with hepatic GP during the 1mU clamps (**Fig. 10A**), while neither of these parameters correlated with circulating insulin levels (**Figs. 10C and D**). Since glucose levels were controlled through euglycemic clamps it can be ruled out that hepatic GP drives WAT lipolysis in these studies. However, the tight correlation between lipolytic flux and hepatic GP during the MBH insulin infusion as well as the fact that MBH insulin did not change hepatic gluconeogenic gene expression of *Pck1* and *G6pc* (**Fig. 10B**) suggests that one of the mechanisms through which hypothalamic insulin regulates hepatic GP is by controlling lipolytic flux from WAT.

Figure 10

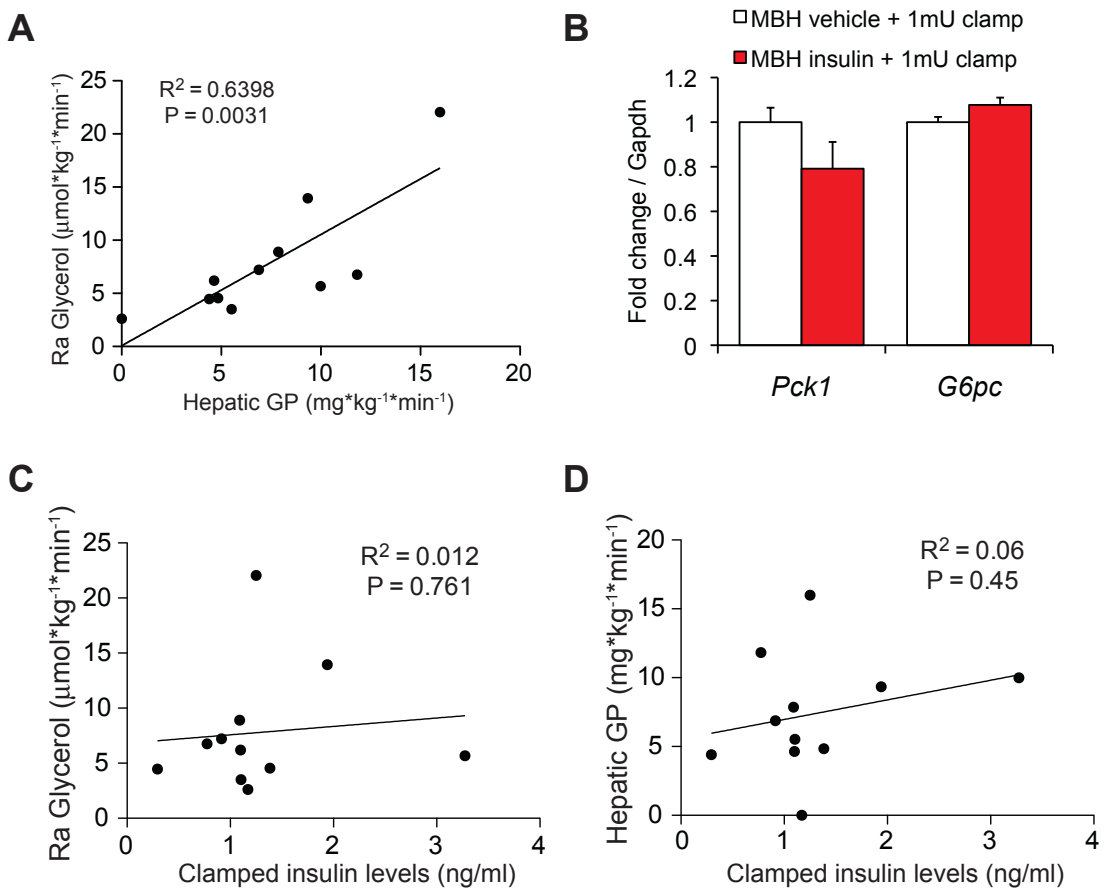

Figure 10 | Lipolytic flux as assessed by the Ra glycerol correlates with hepatic GP. (A) *Correlation between Ra glycerol and hepatic GP during the 1mU clamp (n = 11).* ***(B)*** *Fold change in hepatic mRNA levels of Pck1 and G6pc, which encode for the gluconeogenic enzymes phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase respectively, are shown. Liver tissue was harvested at the end of the clamp studies after MBH insulin or vehicle treatment (n ≥ 6).* ***(C, D)*** *Ra glycerol* ***(C)*** *and hepatic GP* ***(D)*** *did not correlate with circulating insulin levels during the clamp (n = 11). All error bars are s.e.m.*

MBH insulin decreases systemic lipolysis by decreasing Hsl and Atgl activation in WAT by suppressing sympathetic outflow

In order to delineate the molecular mechanisms through which hypothalamic insulin regulates WAT lipolysis, expression and the activation state of lipolytic proteins such as Hsl (encoded by *Lipe*) were analyzed. Hsl hydrolyzes diacylglycerols to monoacylglycerols and serves as a marker for SNS outflow to WAT (Bartness et al., 2009; Buettner et al., 2008). Consistent with the glycerol flux data central insulin suppressed the

activation state of Hsl (**Fig. 11A**). PKA activates Hsl through phosphorylation of Ser 563 and 660 (Anthonsen et al., 1998). Central insulin decreased phosphorylation at both these sites, while phosphorylation of Ser 565, which inactivates Hsl (Anthonsen et al., 1998), trended to be increased (**Fig. 11A**). Hsl is phosphorylated at Ser 565 by AMP–activated protein kinase (Ampk) (Anthonsen et al., 1998; Garton and Yeaman, 1990), which has been shown to inhibit adipocyte lipolysis (Anthony et al., 2009). Indeed, phospho–Ampk α (Thr 172) levels, an indicator of Ampk activation, was increased in WAT from MBH insulin infused animals (**Fig. 11B**). To assess whether the phosphorylation state of the aforementioned lipolytic proteins is consistent with changes in lipolytic activity, triglyceride hydrolase activity in lipid–droplet–depleted adipose tissue homogenates harvested from the same MBH insulin and vehicle infused rats were analyzed. The activation of Hsl by PKA leads to its translocation from the cytosol to the surface of the lipid droplet (Egan et al., 1992; Granneman et al., 2007). As total Hsl levels in total cell extracts were not different between MBH insulin and vehicle infused rats (**Fig. 11D**), activation of Hsl should lead to its cytosolic depletion. Since MBH insulin infusion suppressed Hsl phosphorylation, one would predict higher cytosolic Hsl activity compared to vehicle infused controls after lipid droplet removal, which indeed was the case (**Fig. 11E**). Thus, MBH insulin suppresses Hsl activity in WAT by inhibiting its phosphorylation and thereby likely its translocation to lipid droplets. Adipose tissue triglyceride lipase (Atgl, encoded by *Pnpla2*) hydrolyzes triacylglycerols to diacylglycerols and, together with Hsl, accounts for over 90% of the WAT acyl–hydrolase activity (Zimmermann et al., 2004). MBH insulin infusion did not alter Atgl protein expression in WAT (**Fig. 11A**). However, Atgl activity is not solely determined by total protein levels, but also regulated through cAMP dependent and independent posttranslational modification. Atgl activity is increased upon β–adrenergic stimulation via a posttranslational mechanism (Haemmerle et al., 2006). This activation critically depends on the phosphorylation of the lipid droplet associated protein perilipin, which involves the dissociation of α/β hydrolase domain containing protein 5 (ABHD5; also known as *comparative gene identification-58* (CGI-58)) from perilipin and its subsequent activation of Atgl (Granneman et al., 2007; Miyoshi et al., 2007; Zimmermann et al., 2009). Phospho–perilipin as assessed by a PKA substrate motif antibody trended ($P = 0.053$) to be suppressed in WAT after MBH insulin infusion (**Fig. 11C**). These results suggest that in addition to suppressing Hsl activity, MBH insulin

reduces Atgl activation by decreasing perilipin phosphorylation and thereby favoring CGI-58 retention. Thus, brain insulin regulates Hsl activity and likely Atgl activation via PKA.

Figure 11

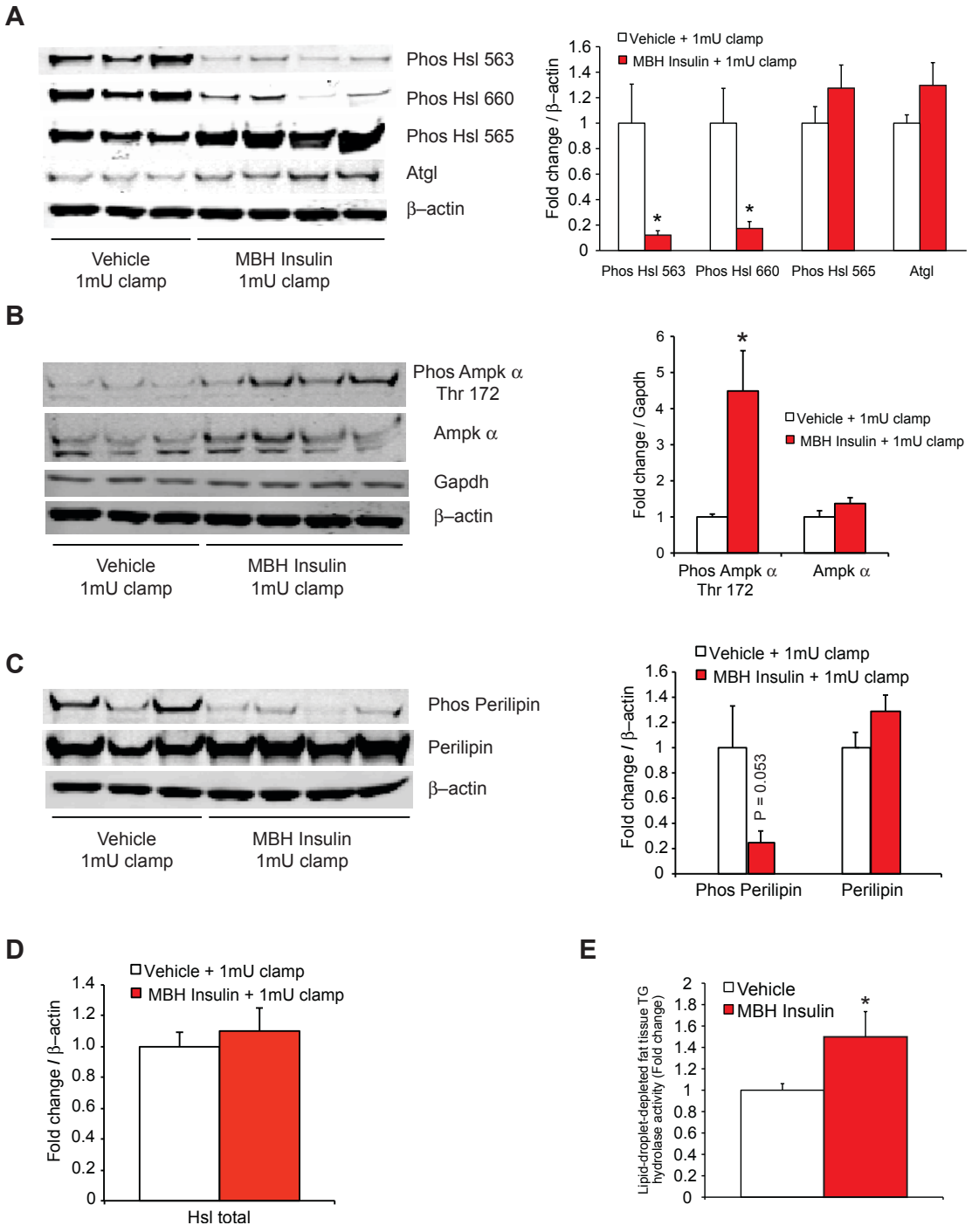

Figure 11 | MBH insulin suppresses Hsl activation. (A-D) *Western blot analyses of epididymal fat pads harvested at the end of the clamp studies as described in* ***Fig. 1****. **(A)** Left, representative Western blot of key proteins of lipolysis. Right, quantification of the Western blot analyses (n ≥ 5*

per group). ***(B, C)*** *Left, representative Western blot analyses of total protein and phosphorylation of Ampk* ***(B)*** *and Perilipin* ***(C)****. Right, quantification of Western blot analyses (n ≥ 3 per group).* ***(D)*** *Quantification of Western blot analyses of total Hsl protein (n ≥ 4 per group).* ***(E)*** *Lipid-droplet-depleted cytosolic triglyceride hydrolase activity (n ≥ 8 per group, one-tailed t-test was applied). All error bars are s.e.m.; * P < 0.05 versus vehicle group.*

Insulin antagonizes cAMP signaling through activation of phosphodiesterase 3B (PDE3B) in an Akt dependent manner (Degerman et al., 2003). It has been shown that hypothalamic leptin signaling can increase insulin signal transduction as assessed by Akt phosphorylation in peripheral organs like the liver (German et al., 2009). Both leptin and insulin regulate autonomic outflow within the MBH (Buettner and Camacho, 2008), which could influence insulin signaling through crosstalk from cAMP signaling, for example. Although circulating insulin levels were controlled during the clamp studies, it is still conceivable that hypothalamic insulin could enhance insulin signaling in WAT. Thus, it has to be ruled out that MBH insulin alters Akt activation in WAT. I found that, MBH insulin did not change Akt activation in WAT as assessed by Akt phosphorylation at Ser473 (**Fig. 12**), indicating that the effects of brain insulin on WAT metabolism occur independent of changes in peripheral insulin signaling.

Figure 12

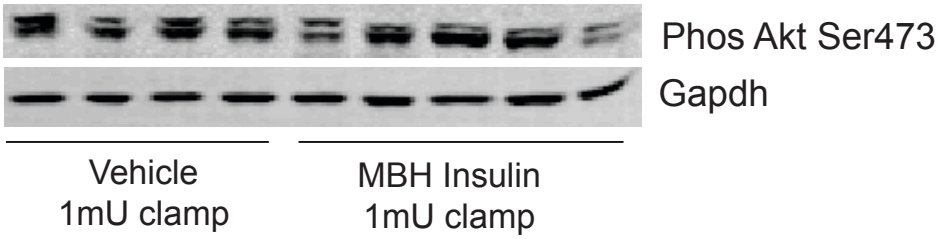

Figure 12 | MBH insulin does not change WAT Akt signaling. *Western blot of Akt phosphorylation at Ser473 in epididymal fat pads after a 6 hr MBH insulin infusion (n ≥ 4).*

Brain insulin induces lipogenesis in WAT

Lipogenesis in WAT has recently been shown to regulate whole body insulin sensitivity by generating palmitoleate that improves insulin sensitivity (Cao et al., 2008). Furthermore, the Buettner lab has found in prior studies that lipogenesis in WAT is upregulated in metabolically beneficial states. For example, lipogenesis is elevated in young versus aged rats (**Fig. 13A**) or calorically restricted (CR) aged rats versus ad libitum (AL) fed aged rats

(**Fig. 13A**). Since hyperinsulinemia induces lipogenesis in WAT (Assimacopoulos-Jeannet et al., 1995), I tested whether brain insulin signaling is sufficient to increases lipogenesis in WAT in the absence of peripheral hyperinsulinemia and assessed lipogenic protein expression in the same tissue lysates that had been used for the Hsl Western blot analysis (**Fig. 11A**). MBH insulin increased the expression of the key lipogenic proteins FAS (encoded by *Fasn*) and Acetyl–CoA carboxylase (Acc, encoded by *Acaca*) (**Fig. 13C**). Adipose tissue FAS enzyme activity of MBH insulin infused rats was increased by 60% just below statistical significance (P = 0.086) (**Fig. 13B**). Protein expression of Atpcl and its phosphorylation, a marker of the activated state, trended to be induced (**Fig. 13C**). These findings implicate that *in vivo* a) WAT lipogenesis and lipolysis are inversely regulated by hypothalamic insulin and that b) MBH insulin seems to oppose the acute actions of MBH leptin. The Buettner lab has previously shown that MBH leptin induces lipolysis and inhibits lipogenesis by increasing sympathetic outflow to WAT (Buettner et al., 2008).

Figure 13

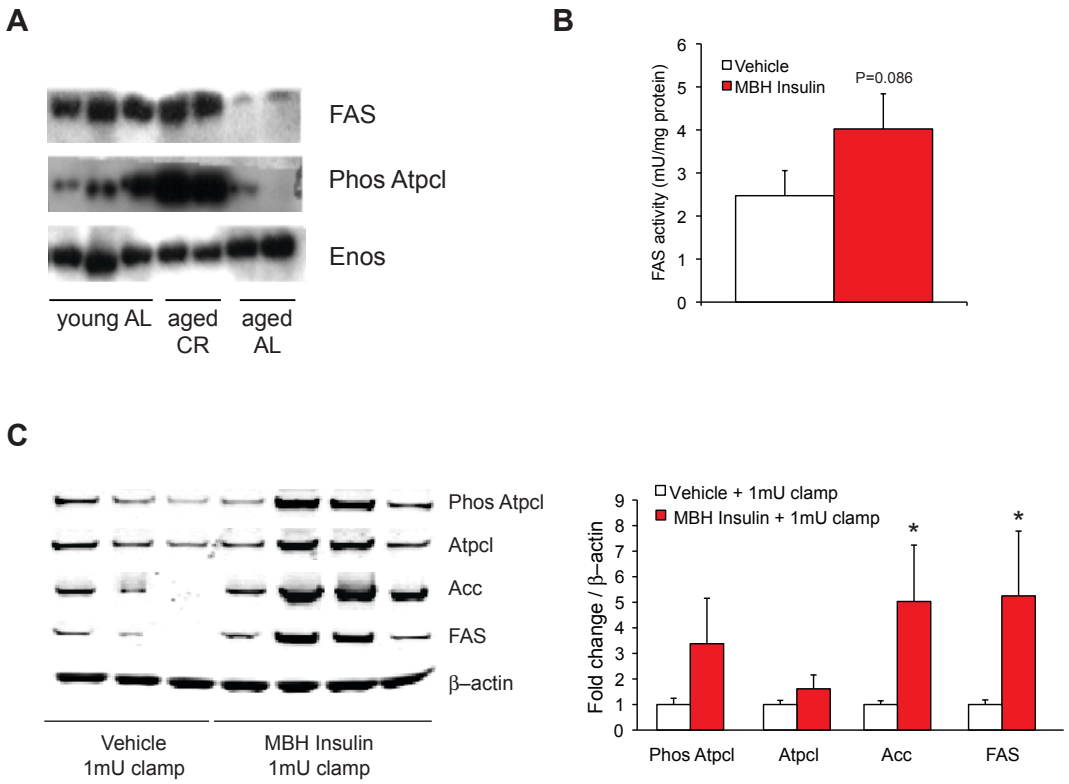

Figure 13 | MBH insulin increases lipogenic protein expression and activity. *(A) Western blot analyses of epidydimal fat pads of either 4–month–old (young) or 18–month–old (aged) rats after*

*they have been subjected to a 6 hr fast. Young rats had ad libitum (AL) access to food, whereas aged rats were either calorically restricted (CR) or AL fed. Aging impairs protein expression of lipogenic enzymes compared to young rats. Caloric restriction reverses the aging induced suppression of lipogenic protein expression. (**B**) FAS activity measured in perirenal fat depots (n = 4 per group, one-tailed t-test was applied) (**C**) Left, representative Western blot analyses of key lipogenic proteins in epididymal fat pads from clamped animals. Right, quantification of the Western blot analyses (n ≥ 5 per group). All error bars are s.e.m.; * P < 0.05 versus vehicle group.*

MBH insulin dampens SNS activity

To delineate whether the effects of MBH insulin on WAT metabolism are mediated via the autonomic nervous system, surgical denervation as well as pharmacological sympathectomy, of rat epigonadal fat pads using 6–hydroxydopamine (6–OHDA), were performed. Surgical denervation and pharmacological sympathectomy suppressed basal WAT Hsl activation, a marker for sympathetic outflow to WAT (Bartness et al., 2009; Buettner et al., 2008). This effect was replicated with MBH insulin infusion (**Fig. 14**). Although this experiment is not an ultimate proof of a linear pathway, these data suggest that hypothalamic insulin regulates Hsl activation via suppression of SNS outflow to WAT.

Figure 14

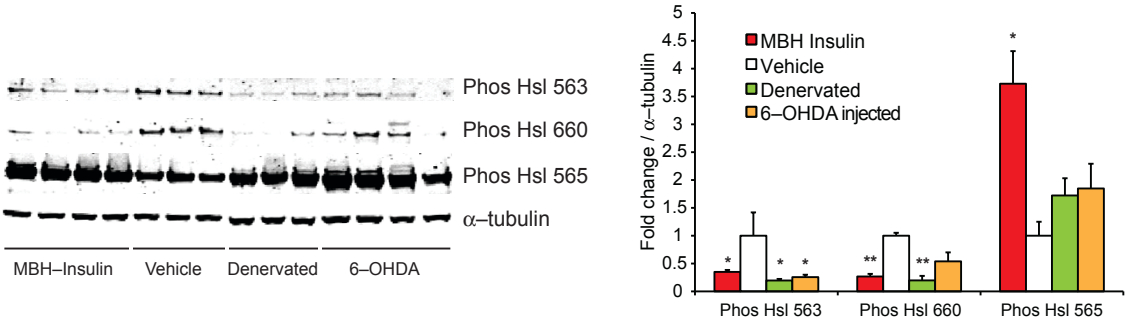

***Figure 14** | MBH insulin suppresses Hsl activation to a similar degree as surgical denervation or selective pharmacological sympathectomy of the epididymal fat pad, indicating that insulin suppresses lipolysis through a reduction of SNS outflow to WAT. WAT Hsl protein expression was assessed by Western blot analyses. Epigonadal fat pads with intact sympathetic innervation from rats that were either infused with MBH insulin or vehicle were compared with surgically/pharmacologically denervated fat pads of vehicle infused rats. All error bars are s.e.m.; * P < 0.05, ** P < 0.01 versus vehicle group.*

Acute inhibition of brain insulin signaling unrestrains lipolysis, but depends on sympathetic innervation of WAT.

I next asked if the acute induction of brain insulin resistance is sufficient to alter lipolysis. S961 is a recently identified peptide inhibitor that blocks insulin binding to the insulin receptor, while not inhibiting insulin or insulin–like growth factor (IGF) 1 binding to the IGF receptor (Schaffer et al., 2008) or IGF 1 binding to the insulin receptor. To first validate the compound, S961 was injected ICV in insulin signaling experiments. S961 successfully blocked insulin induced insulin receptor phosphorylation in the MBH (**Figs. 15A),** which also obliterated downstream insulin signaling **(Fig. 15B)**. To assess if the inhibition of endogenous insulin signaling in the MBH is sufficient to unrestrain lipolysis, either the insulin receptor antagonist S961 or vehicle were infused into male SD rats for 4 hrs, while simultaneously infusing [^{2}H-5]-glycerol tracer systemically (**Fig. 15C**). During the infusion study systemic insulin and glucose levels were similar between groups (**Figs. 15E and F**). Indeed, inhibiting endogenous insulin signaling in the MBH doubled the Ra glycerol (**Fig. 15D**) suggesting that hypothalamic insulin resistance can contribute to the unrestrained lipolysis seen in the insulin resistant state. Since increased glucose production is a major component of widespread SNS activity (Gellhorn, 1954) and glucose levels throughout the entire infusion experiment were not different in S961 versus vehicle infused rats, it is unlikely that S961 is an unspecific activator of the SNS.

Figure 15

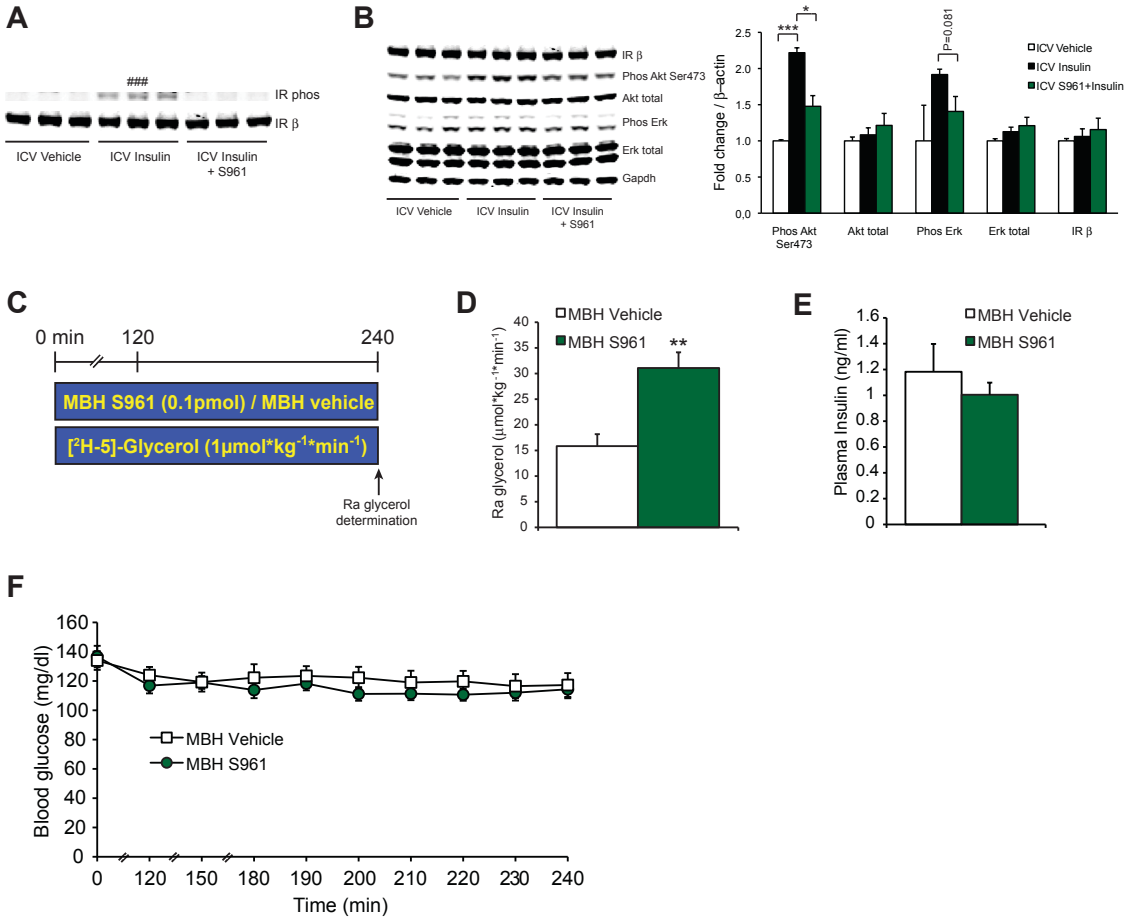

Figure 15 | Acute inhibition of endogenous MBH insulin signaling is sufficient to unrestrain lipolysis. ***(A)*** *Validation of the insulin receptor antagonist in vivo. S961 was co–infused with insulin in equimolar amounts into the 3rd ventricle of male SD rats. S961 blocked insulin induced MBH insulin receptor phosphorylation (n = 3 per group)* ***(B)*** *Left, Western blot analyses of protein extracts derived from the MBH of rats infused with either vehicle, insulin or insulin plus S961 harvested 15 min after an insulin bolus. Akt and Erk phosphorylation were assessed. Right, quantifications of Western blot analyses (n = 3 per group).* ***(C)*** *Schematic study outline. Male SD rats were infused with glycerol tracer and received either vehicle or S961 for 4 hrs into the MBH.* ***(D)*** *Ra glycerol of MBH vehicle and S961 infused rats as per protocol depicted in* ***Fig. 15C*** *(n ≥ 4 per group)* ***(E)*** *Plasma insulin levels at time point 240 min. (n ≥ 4 per group).* ***(F)*** *Plasma glucose levels measured during the 4 hr infusion of the insulin receptor antagonist S961. All error bars are s.e.m.; * P < 0.05, ** P < 0.01 versus vehicle group unless otherwise indicated; ### P < 0.001 versus ICV vehicle and ICV insulin + S961 group*

The denervation studies shown in **Fig. 14** suggested that MBH insulin reduces WAT lipolysis via the autonomic nervous system, since MBH insulin suppressed Hsl phosphorylation comparable to chemical sympathectomy. I further tested this concept by asking if the unrestrained lipolysis induced by the acute inhibition of insulin signaling in the MBH depended on intact sympathetic innervation. To this end 10-week-old male SD rats were systemically sympathectomized by injecting 6–OHDA. After a short recovery period, the animals were subjected to the same infusion studies depicted in **Fig. 15C** in order to test if MBH S961 would still be able to increase Ra glycerol. As expected systemic sympathectomy obliterated the pro–lipolytic effects of the insulin antagonist (**Fig 16A)**. Importantly, WAT norepinephrine (NE) content was markedly reduced verifying the sympathectomy (**Fig. 16B**). Ra glycerol trended to be lower in the denervated animals compared to sham controls, suggesting that sympathectomy per se lowers pro-lipolytic input to WAT. Thus, these results provide further support for the concept that MBH insulin signaling controls WAT lipolysis by dampening SNS activity.

Figure 16

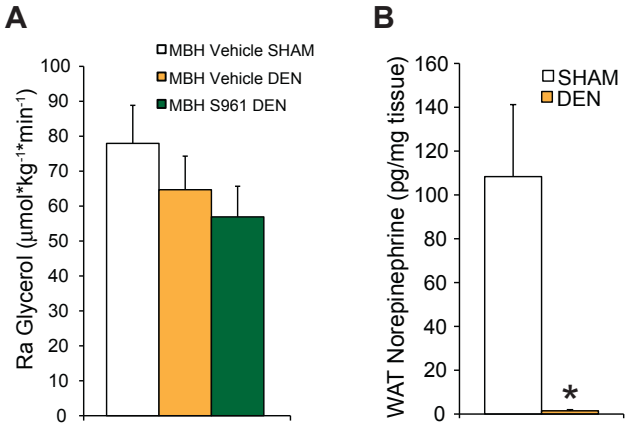

Figure 16 | The acute inhibition of endogenous MBH insulin signaling depends on intact sympathetic innervation in order to unrestrain lipolysis. *(A) Ra glycerol of sham and pharmacologically sympathectomized rats that were either infused with MBH S961 or vehicle. A similar protocol as depicted in* ***Fig. 15C*** *was used (n ≥ 6 per group).* ***(B)*** *WAT NE levels in sham versus 6–OHDA denervated rats (n = 4 per group). All error bars are s.e.m.; * P < 0.05 versus MBH Vehicle SHAM group.*

Genetic deletion of the neuronal insulin receptor increases lipolysis in mice

To test if neuronal insulin signaling mediates the role of brain insulin in regulating lipolysis, glycerol fluxes in neuronal insulin receptor knock–out (NIRKO) and littermate lox-lox control mice (**Fig. 17A**) were studied during hyperinsulinemic euglycemic clamp studies (**Figs. 17C and D,** protocol depicted in **Fig. 17B**). NIRKO mice have been reported to be susceptible to diet–induced obesity, although their body weight is not altered when they are young and fed a standard chow diet. Furthermore, they develop mild insulin resistance when they get older than 6 months (Bruning et al., 2000). In this study four-month-old NIRKO mice required equal GIR during a hyperinsulinemic clamp indicating no or only mild impairment of glucose homeostasis in young NIRKO mice (**Fig. 17E**). In the fasted state NIRKO mice exhibited a two–fold higher Ra glycerol compared to lox-lox littermate controls (**Fig. 17F**) although insulin levels were not different (**Tab. 4 and Fig 17C**), demonstrating that neuronal insulin signaling controls basal lipolytic rate. Induction of hyperinsulinemia during a 4 $mU \cdot kg^{-1} \cdot min^{-1}$ euglycemic clamp raised insulin levels equally in both groups (**Fig. 17C**) and while glycerol fluxes were suppressed in both NIRKO and control mice (presumably through the direct actions of insulin on adipocytes), the Ra glycerol remained higher in the NIRKO mice (**Fig. 17F**), while glucose and glucagon levels were not different between groups (**Figs. 17D and Tab. 4**).

Figure 17

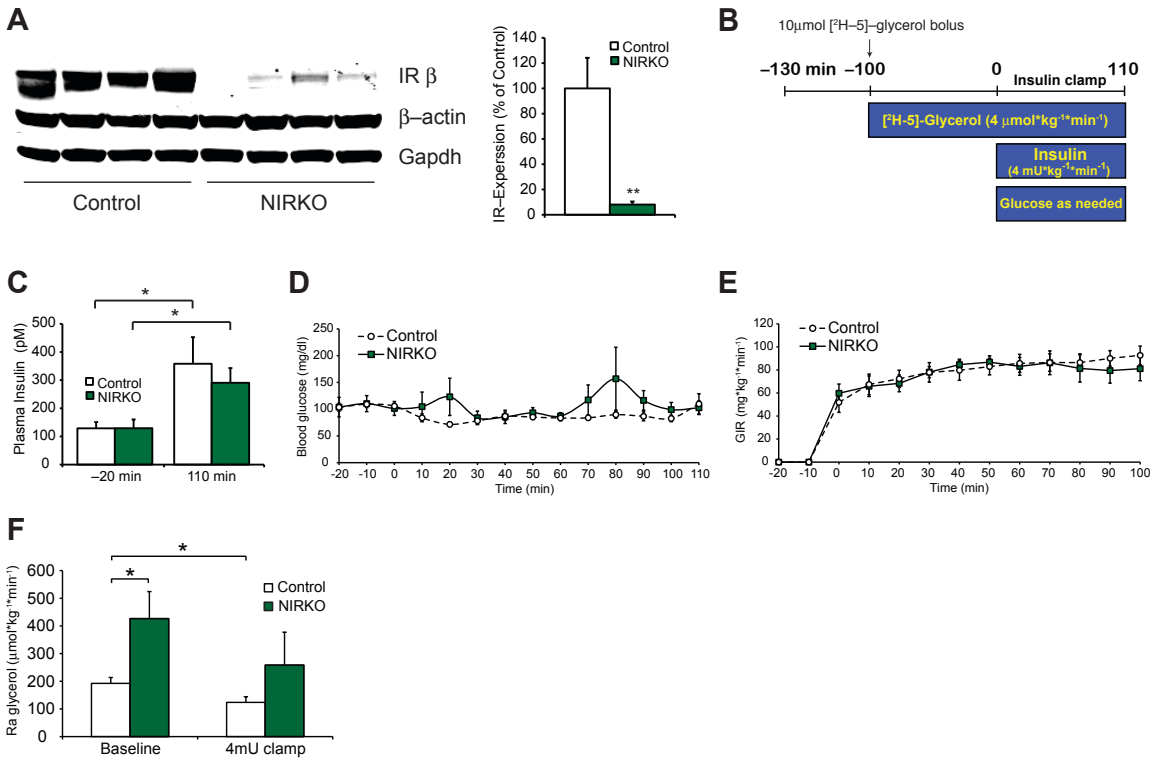

Figure 17 | Genetic disruption of neuronal insulin signaling increases whole body lipolytic flux. ***(A)*** *Insulin receptor (IR) protein expression was reduced by > 90% in brain extracts of NIRKO mice as assessed by Western blot (n = 4 per group).* ***(B)*** *Schematic representation of the clamp studies in NIRKO mice. Following a 16 hr fast, NIRKO and littermate control mice were subjected to a 110 min 4 mU · kg^{-1} · min^{-1} hyperinsulinemic euglycemic clamp study.* ***(C)*** *Plasma insulin levels were not different between groups and increased three–fold during the pancreatic clamp. (n = 5 per group).* ***(D)*** *Plasma glucose levels of NIRKO and lox-lox littermate control mice during baseline and the hyperinsulinemic clamp (n = 5 control and n = 6 NIRKO).* ***(E)*** *Glucose infusion rate (GIR) required to maintain euglycemia during the 4 mU · kg^{-1}· min^{-1} hyperinsulinemic clamp (n = 5 control and n = 6 NIRKO).* ***(F)*** *Baseline and clamp glycerol fluxes as assessed by [^{2}H-5]-glycerol tracer infusion are increased in the NIRKO mice (n ≥ 5 per group). All error bars represent s.e.m.; * P < 0.05, ** P < 0.01 versus lox-lox control mice or as indicated.*

Table 4 | Baseline characteristics of NIRKO mice after an overnight 16 hr fast. *(n ≥ 3 per group) All values represented as mean ± s.e.m. * P < 0.05 versus lox-lox control animals.*

	Control	NIRKO
Age (weeks)	16.7 ± 0.9	16.0 ± 0.7
Bodyweight (g)	23.6 ± 1.2	20.4 ± 0.9 *
Blood glucose (mg/dl)	104 ± 18	104 ± 17
Plasma NEFA (mM)	0.72 ± 0.09	0.65 ± 0.05
Plasma triglycerides (µg/µl)	0.78 ± 0.06	0.59 ± 0.1
Plasma free glycerol (µg/µl)	0.030 ± 0.002	0.027 ± 0.003
Plasma insulin (pM)	129 ± 22	129 ± 31
Plasma glucagon (pM)	61 ± 14	70 ± 10

These data indicate that lipolytic flux is exquisitely sensitive to brain insulin action, more so than hepatic glucose fluxes. Changes in levels of plasma NEFA and glycerol did not show significant differences (**Fig. 18A**). Since unrestrained lipolysis is expected to increase ketogenic substrate flux to the liver, I further assessed whether NIRKO mice display increased ketogenesis. Indeed, NIRKO mice challenged with a 16 hr fast had elevated β–hydroxybutyrate levels compared to control animals (**Fig. 18B**), supporting the concept that brain insulin signaling can regulate both lipolysis and ketogenesis. Plasma NEFA and free glycerol levels were not different between groups following the 16 hr fast (**Figs. 18C and D)**.

Figure 18

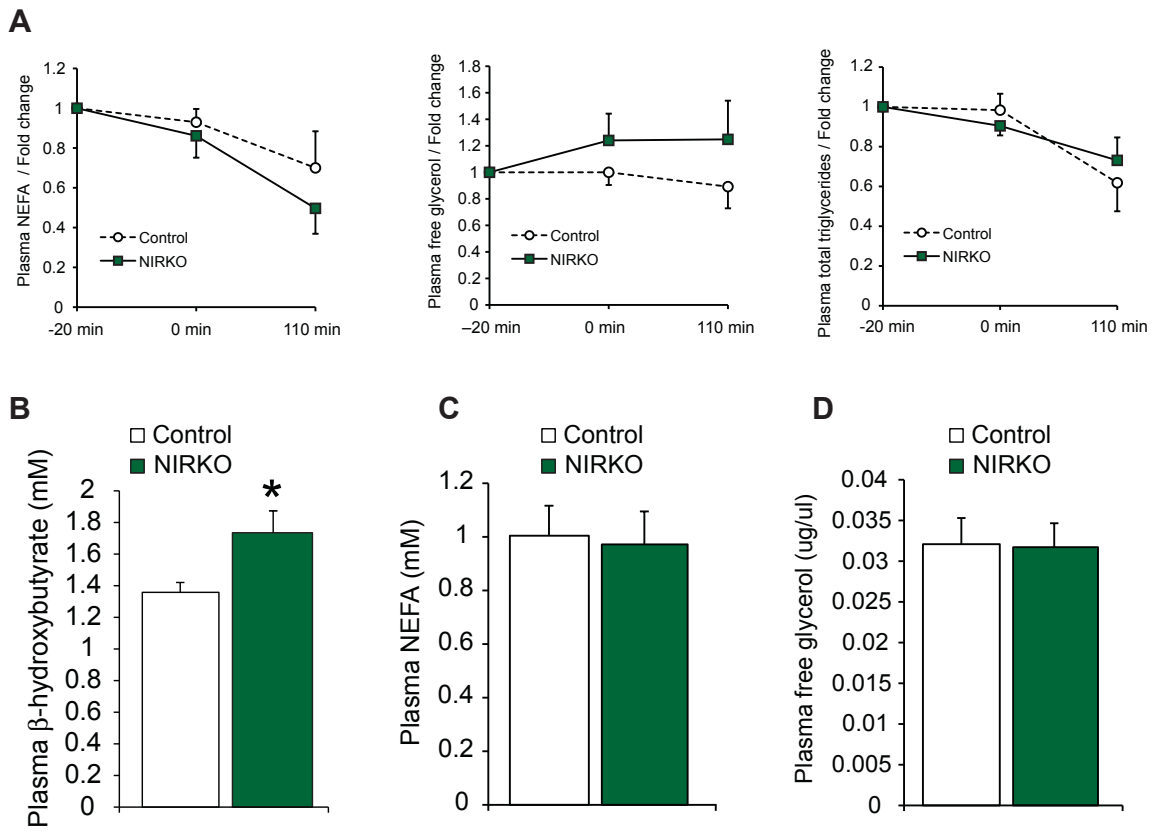

Figure 18 | Brain insulin signaling affects ketogenesis. (A) *Change of plasma NEFA, glycerol and triglyceride levels during baseline and the 4 mU· kg^{-1} · min^{-1} hyperinsulinemic euglycemic clamp (n ≥ 5 per group).* ***(B)*** *Plasma β–hydroxybutyrate levels are elevated in the NIRKO mice following a 16 hr fasting challenge (n = 9 per group).* ***(C, D)*** *Plasma NEFA and free glycerol levels measured from plasma collected after a 16 hr fasting period (n = 9 per group). All error bars represent s.e.m.; * P < 0.05 versus lox-lox control mice.*

Loss of neuronal insulin receptor signaling impairs the metabolic switch from fasting to re-feeding.

The transition from fasting to re–feeding is a physiological challenge that requires adipose tissue to readily switch from NEFA release to NEFA retention. To assess the role of neuronal insulin receptor signaling in this metabolic adaptation, NIRKO mice and control littermates were fasted overnight and then re–fed during the onset of their feeding cycle (**Protocol depicted in Fig. 19A**). This allows assessing the suppression of NEFA release after re–feeding. While fasting NEFAs were not different between NIRKO and control mice, the suppression of NEFAs following a 2 hr re-feeding period in NIRKO mice was

significantly lower (**Fig. 19C**), despite higher peripheral insulin levels after feeding (**Fig. 19B**). Insulin levels of control mice were unchanged after feeding, which is consistent with prior reports that showed that lean insulin-sensitive mice do not exhibit a change in insulin levels after oral glucose administration (Andrikopoulos et al., 2008). Body weight and food intake during re-feeding were not different between groups (**Figs. 19D and E**). These data indicate that the neuronal insulin receptor regulates lipolysis in WAT during the fasting to feeding transition.

Figure 19

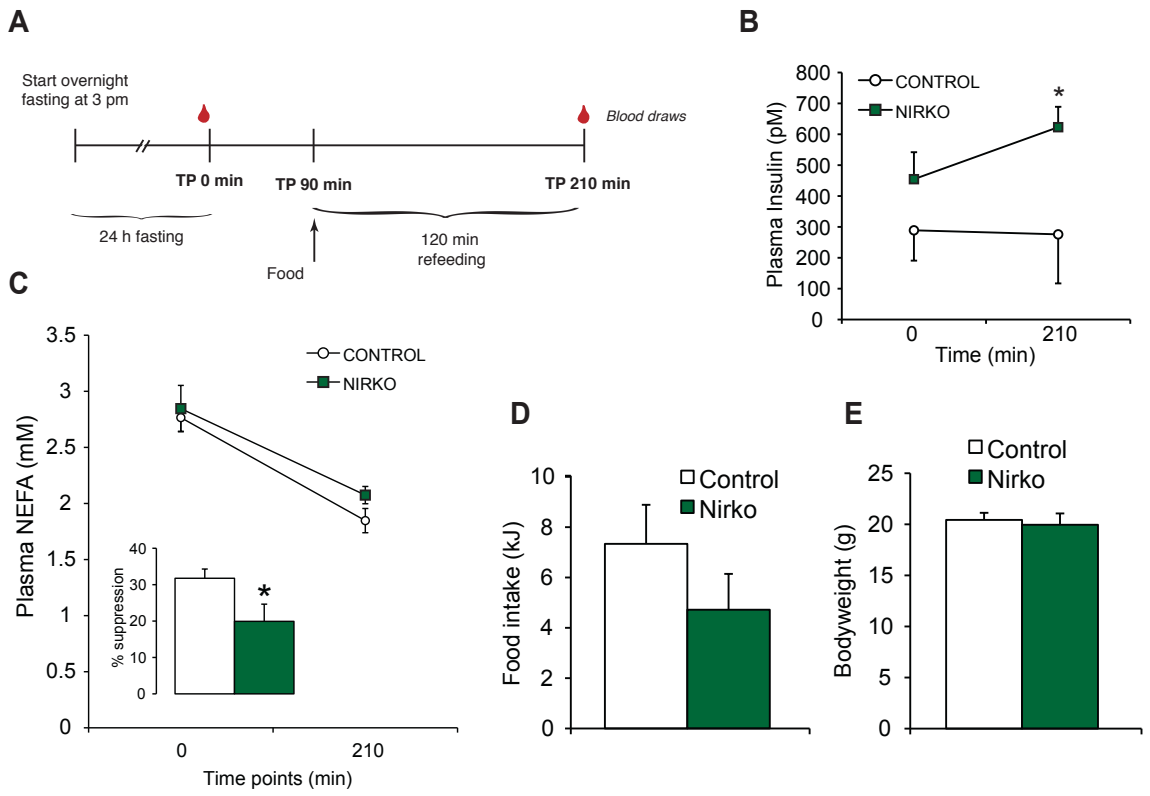

Figure 19 | Genetic blockade of neuronal insulin signaling impairs the switch from fasting to re–feeding. ***(A)*** *Schematic of the fasting re–feeding protocol* ***(B)*** *Plasma insulin before and after re–feeding (n ≥ 8 per group)* ***(C)*** *Plasma NEFA levels before and after re–feeding. Insert depicts % suppression of plasma NEFA levels after food intake (n ≥ 7 per group)* ***(D)*** *Food intake during re-feeding (n ≥ 9 per group)* ***(E)*** *Bodyweights after fasting (n ≥ 9 per group). All error bars are s.e.m.; * P < 0.05 versus control mice.*

Neuronal insulin receptor signaling regulates *de novo* lipogenesis in WAT

Consistent with the findings from the rat studies where MBH insulin increased lipogenic protein expression, loss of brain insulin signaling in the NIRKO mice decreased Acc and FAS protein expression as well as activated Phospho–Atpcl in WAT under clamped and overnight fasted conditions (**Figs. 20A** and **B**), suggesting that the lifelong absence of brain insulin signaling disrupts WAT lipogenesis.

Figure 20

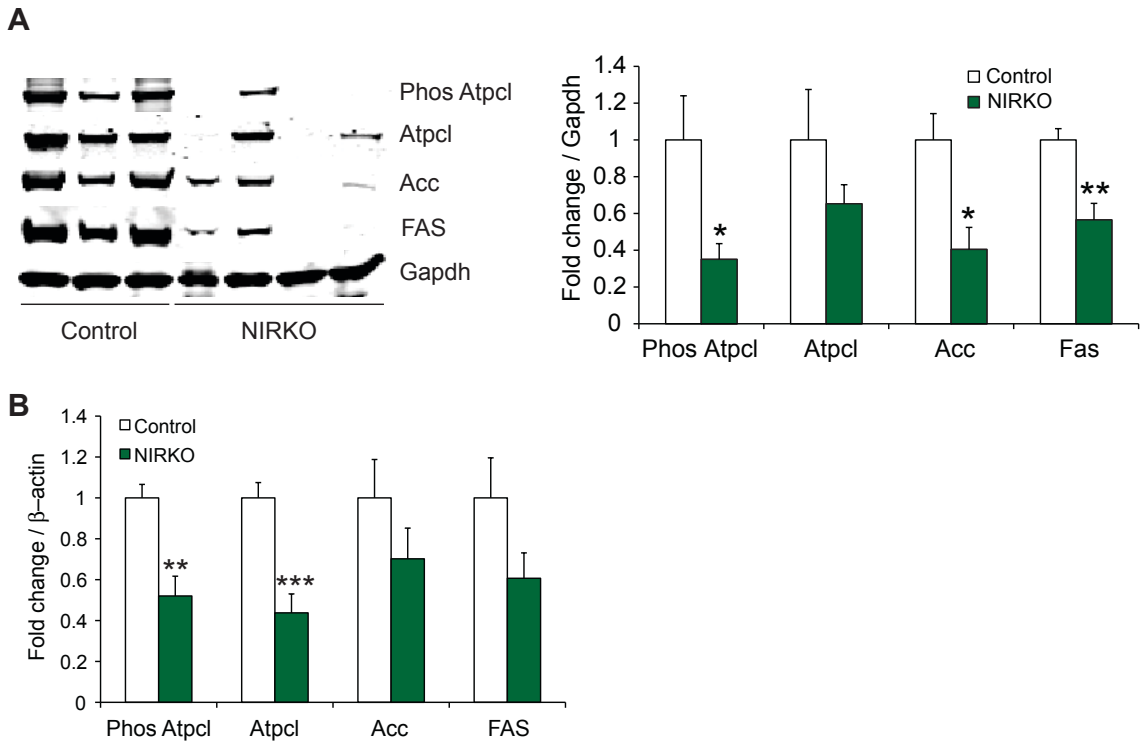

Figure 20 | Loss of neuronal insulin receptor signaling impairs de novo lipogenesis in WAT. ***(A)*** *Left, representative Western blot analyses of lipogenic protein expression and the activation state of Atpcl in epididymal fat pads obtained at the end of the clamp study. Right, quantification of Western blot data compared to littermate control mice (n ≥ 5 per group).* ***(B)*** *Western blot quantifications of epididymal fat pads of NIRKO and littermate control mice harvested after an overnight fast (n = 8 per group). All error bars are s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 versus control mice.*

To test whether the decreased WAT lipogenic enzyme expression in the NIRKO mice translated into alterations in WAT fatty acid composition, fatty acid lipid profiles were

generated from epididymal fat pads of fasted control and NIRKO mice. Several fatty acid species (14:0, 14:1n5, 16:0, 16:1n7) associated with *de novo* lipogenesis were markedly suppressed in the epididymal fat depots of the NIRKO mice (**Fig. 21A**). I also found a lower *de novo* lipogenesis index (DNL) (**Fig. 21C)**, which is commonly used as a marker for lipogenesis (Chong et al., 2008; Hudgins et al., 1996). However, oleate (18:1n9), the most abundant fatty acid in WAT, was not significantly changed, suggesting that triglyceride content per se was not different between groups. Notably, the insulin sensitizing lipokine palmitoleate (16:1n7), which is produced during *de novo* lipogenesis in WAT (Cao et al., 2008), was decreased in the NIRKO mice (**Fig. 21A**, red arrowhead). Furthermore, palmitoleate levels in WAT correlated closely with the expression of the lipogenic enzyme Acc and the activation state of Atpcl in the fasted state, further supporting the notion that brain insulin regulates palmitoleate synthesis through regulation of WAT lipogenesis (**Fig. 21B**). These findings assign a critical role to neuronal insulin signaling in maintaining WAT function. Hypothalamic insulin resistance may therefore contribute to the decreased lipogenic protein expression in WAT observed in obesity. This in turn could lead to decreased production of the insulin sensitizing fatty acid palmitoleate, further worsening systemic insulin resistance.

Figure 21

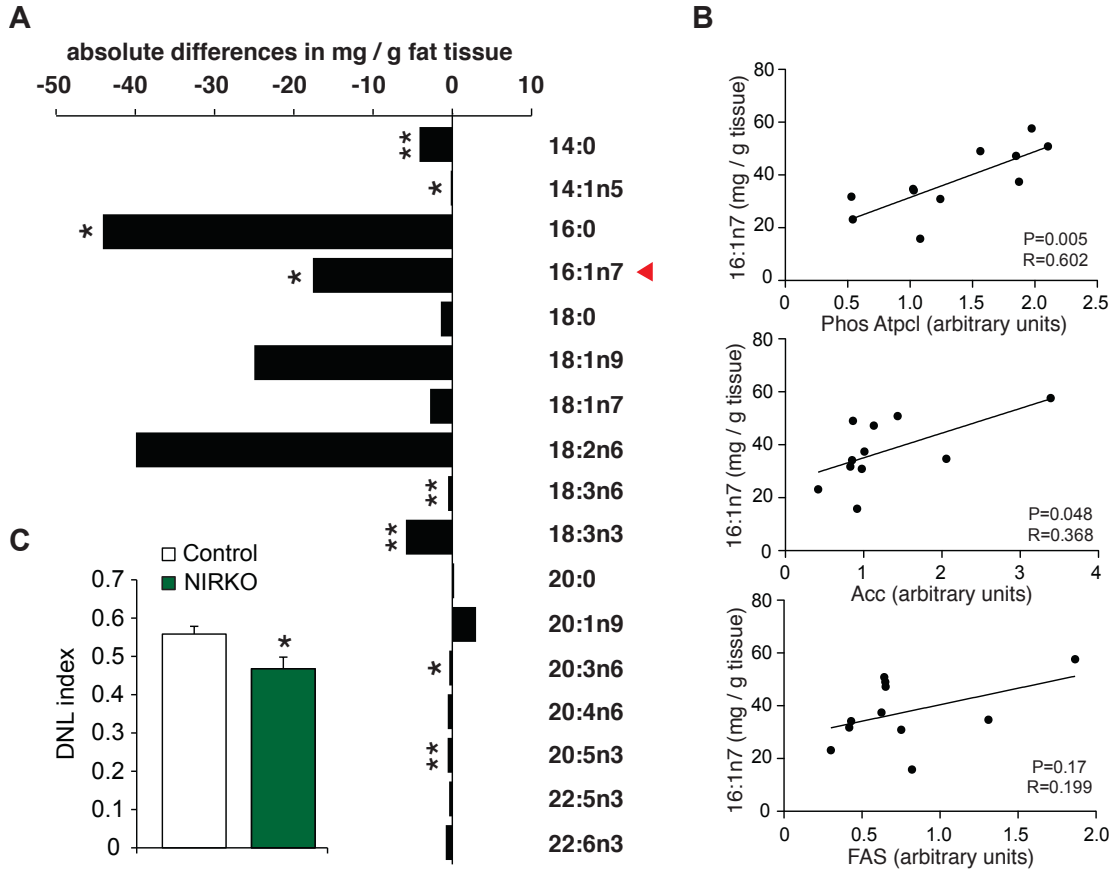

Figure 21 | (A) *Differences of WAT fatty acid species of overnight fasted NIRKO and control mice. (n = 6 per group). Red arrowhead marks palmitoleate.* ***(B)*** *Correlation between the expression of lipogenic proteins and palmitoleate levels (n = 11).* ***(C)*** *De novo lipogenesis (DNL) index calculated using the ratio of palmitic (16:0) to linoleic acid (18:2n6) (n ≥ 5 per group).; All error bars are s.e.m.; * P < 0.05, ** P < 0.01, *** P < 0.001 versus control mice.*

V. Discussion

WAT has emerged as a critically important organ for whole body glucose and lipid homeostasis. WAT is an important metabolic sink, clearing and storing circulating lipids, thereby protecting other organs from ectopic lipid accumulation. In addition, WAT is an important source of adipokines like leptin and adiponectin, and inflammatory mediators like tumor necrosis factor alpha and interleukin 6 that circulate and can impair insulin signaling in distant organs including the hypothalamus (Zhang et al., 2008). During fasting, WAT is the main source of NEFAs that provide energy substrates to muscle and liver. Unrestrained lipolysis during the absorptive state induces lipotoxicity and a low–grade inflammation that is commonly associated with obesity and diabetes (Boden, 2006). Lastly, *de novo* lipogenesis in WAT produces insulin sensitizing fatty acid species like palmitoleate (Cao et al., 2008).

This thesis establishes that neuronal and in particular hypothalamic insulin action is a critical regulator of WAT metabolism. The presented studies demonstrate that brain insulin action restrains lipolysis by reducing sympathetic outflow to WAT and regulates *de novo* lipogenesis in adipose tissue opposing the effects of brain leptin (Buettner et al., 2008). Impaired brain and hypothalamic insulin signaling increases lipolytic flux and decreases *de novo* lipogenesis hampering the production of the insulin sensitizing fatty acid palmitoleate. These conclusions are based on the findings from two independent rodent models: 1) SD rats, where brain or MBH insulin signaling was either increased or inhibited and 2) mice with a lifelong disruption of neuronal insulin signaling. The molecular mechanism through which MBH insulin regulates WAT metabolism within the adipocyte comprises of an increase in lipogenic protein expression and activity and a decrease in the activation state of lipolytic enzymes like Hsl (**see proposed model in Fig. 22**). Whether other anti-lipolytic regulators such as, adenosine, prostaglandin E_2, neuropeptide Y (NPY) or lactate participate in the regulation of WAT metabolism by brain insulin remains to be determined (Ahmed et al., 2010; Jaworski et al., 2009; Lafontan and Langin, 2009). Furthermore, the role of the parasympathetic nervous system in the central regulation of WAT by insulin and leptin is untested at present, however parasympathetic innervation of WAT remains controversial (Giordano et al., 2006; Kreier et al., 2002).

Lipolytic flux from WAT drives hepatic GP

The finding that intact brain insulin signaling is essential for maintaining WAT functionality has implications not only for lipid metabolism, but is also likely to be relevant for glucose homeostasis, since lipolytic flux can contribute to hepatic gluconeogenesis (Mittelman and Bergman, 2000; Rebrin et al., 1996). NEFA levels closely correlate with hepatic GP, independent of systemic insulin or glucose levels (Rebrin et al., 1995). Furthermore, insulin's anti-lipolytic properties are essential for the suppression of hepatic GP (Rebrin et al., 1996), because glycerol acts as a gluconeogenic precursor, while NEFAs provide important energy substrates to the liver to fuel gluconeogenesis (Hers and Hue, 1983). Indeed, the presented data indicate that lipolytic flux is exquisitely sensitive to brain insulin action, more so than hepatic glucose fluxes. MBH insulin infusion in rats did not alter hepatic GP even after 4 hrs, while Ra glycerol flux was already altered. Only after somatostatin infusions were started an effect on hepatic GP became apparent. Several reports show that both brain leptin and insulin have the ability to alter hepatic glucose flux (reviewed in (Buettner and Camacho, 2008)). Brain insulin infusion in rodents suppresses hepatic glucose output by decreasing gluconeogenesis, while glycogenolysis in the liver is not affected (Obici et al., 2002; Pocai et al., 2005). Central leptin infusions, on the other hand, acutelely *induces* gluconeogenesis, while *suppressing* glycogenolysis. Thus, although central leptin alters glucose partitioning in the liver, it does not change net hepatic glucose output (Gutierrez-Juarez et al., 2004). Therefore, the opposing effects of brain insulin and leptin on WAT lipolysis are mirrored in the regulation of hepatic gluconeogenesis through brain signaling by both these hormones. Lipolytic flux closely correlated with hepatic glucose production in rats that received brain insulin infusions (**Fig. 10A**), raising the possibility that hypothalamic insulin action may either 1) regulate both lipolysis and hepatic GP via a suppression of sympathetic outflow to the liver or 2) may indirectly regulate hepatic GP by decreasing the flux of glycerol and NEFA to the liver via suppression of lipolysis in WAT. Therefore, the brain control of hepatic GP may in part occur via the central regulation of WAT lipolysis.

Figure 22

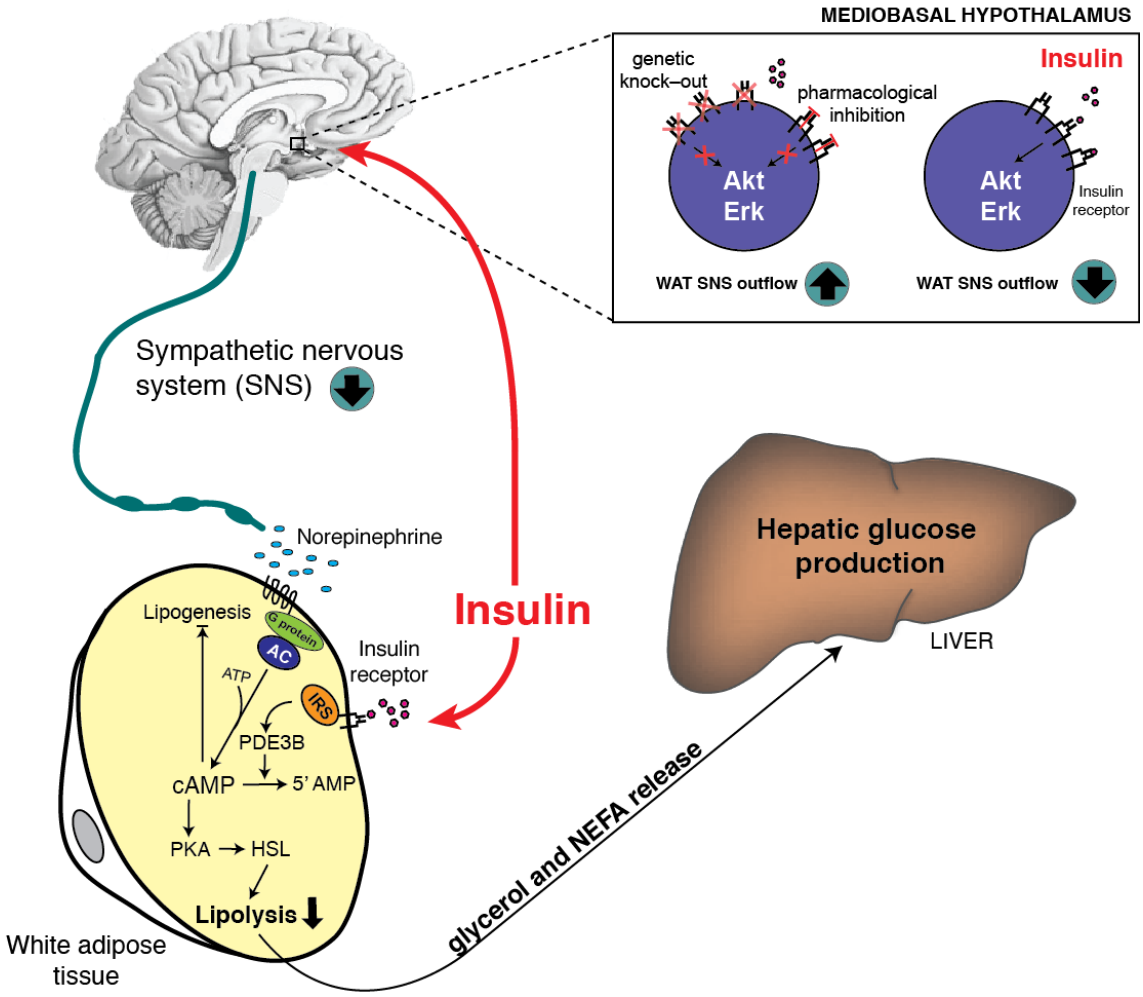

Figure 22 | Proposed model of the role of brain insulin in regulating WAT metabolism. *Insulin inhibits WAT lipolysis through both direct and indirect effects: insulin binding to the insulin receptor expressed on adipocytes results in inactivation of PDE3B leading to the degradation of cAMP (Degerman et al., 1990; Smith et al., 1991). In addition to the direct effects of insulin on adipocytes, hypothalamic insulin signaling suppresses lipolysis and induces lipogenesis indirectly by dampening SNS outflow to WAT. The reduction in lipolysis contributes to a decrease in hepatic GP by limiting the flux of the gluconeogenic precursor glycerol and NEFAs, which provide energy substrates for gluconeogenesis.*

Brain insulin signaling and *de novo* lipogenesis

There is growing evidence that in obesity and diabetes lipogenic capacity of adipose tissue is reduced in rodents as well as humans (Diraison et al., 2002; Moraes et al., 2003; Nadler et al., 2000). The stimulatory effects of brain insulin on WAT lipogenesis plus the finding that the absence of the neuronal insulin receptors impairs the lipogenic capacity of WAT suggest that the dysregulation of WAT lipogenesis in obesity is at least in part a function of brain insulin resistance.

Furthermore, the presented data suggest that brain insulin action has important anabolic function in WAT maintenance, which is independent of, but complements cell-autonomous effects of insulin on adipocytes. In further support of this concept, a recent study showed that the presence of the brain insulin receptor is critically important to prevent lipodystrophy in mice (Koch et al., 2008). The same study showed that chronic ICV insulin infusion in mice leads to increased fat pad mass and hypertrophy of adipocytes, which the authors ascribed to increased lipogenesis. Equally, if not more important in the control of adiposity is likely the regulation of WAT lipolysis through brain insulin signaling. One could draw this conclusion from the finding that denervation of WAT leads to no change in lipogenic protein expression, but completely abrogates Hsl activation leading to increased adipose depot mass (Buettner et al., 2008). The absence of WAT renders humans (Hegele, 2003; Petersen et al., 2002) and mice (Shimomura et al., 1999) severely insulin resistant, due to the ectopic accumulation of lipids in tissues such as muscle and liver.

The role of Agrp and Pomc neurons in regulating lipid metabolism.

The presented studies raise several questions. One is which neuronal subtype within the CNS and the MBH mediates the effects of insulin on the regulation of WAT metabolism. It is tempting to speculate that the effects of brain insulin and leptin are mediated by a single neuronal subpopulation within the hypothalamus. Two likely candidates are proopiomelanocortin (Pomc) and agouti–related peptide (Agrp) expressing neurons, as they play signal roles in controlling energy homeostasis and are both targets of insulin and leptin (Schwartz and Porte, 2005). Deletion of leptin receptors in either Pomc or Agrp neurons leads to moderate obesity, while the combined knock-out of leptin receptors in Agrp and Pomc neurons has an additive effect resulting in an approximately 30% increase in adiposity despite equal food intake among all strains (Balthasar et al., 2004; van de Wall

et al., 2008). These findings suggest that brain leptin signaling in both Pomc and Agrp neurons plays an important role in the regulation of adiposity. Mice that lack both insulin and leptin receptors in Pomc neurons are markedly insulin resistant. However, double knock-out of leptin and insulin receptors on Pomc neurons partially reverses the obesity phenotype of isolated leptin receptor Pomc knock-out mice (Hill et al., 2010). This suggests that the opposing effects of insulin and leptin could be integrated in Pomc neurons, where insulin signaling seems to preserves fat mass, whereas leptin signaling decreases it. Yet, deletion of the insulin receptor in either Pomc or Agrp neurons causes no change in adiposity, indicating that other redundant pathways compensate. Furthermore, only Agrp insulin receptor knock-out mice, but not Pomc, fail to suppress hepatic glucose production during hyperinsulinemia (Konner et al., 2007). Although several neuron specific insulin receptor KO models have been generated (reviewed in (Belgardt et al., 2009)), analyses of lipolytic fluxes or WAT lipogenesis have not been undertaken as of yet. However, judging from the energy balance and glucose homeostasis phenotype of the aforementioned models, it is unlikely that a single neuronal subtype can explain both insulin and leptin's effects on lipid metabolism, rather that it is integrated within a complex network of neurons that is maintained in a delicate balance. In some cases insulin signaling in one neuronal subtype can even antagonize the effects of insulin signaling in another (Lin et al., 2009). Thus, lifelong Cre-lox knock-out models have several limitations in identifying first order neurons that mediate the acute effects of brain insulin and leptin in regulating energy metabolism: First, it is unclear which neuronal populations in the CNS express Pomc and Agrp during development. Thus, during development Cre recombinase potentially targets cells that are different from the classic Agrp/Pomc neurons in adult animals (Padilla et al., 2010). Secondly, even if a specific neuronal knock-out model blunts the ability of brain insulin and/or leptin to regulate WAT metabolism, this does not necessarily imply that the particular neuronal population is the main target, only that the balance within the neuronal network is disturbed. This imbalance renders the hypothalamus insensitive to the acute effects of leptin and/or insulin, but also possibly other effectors such as nutrients.

The neurophysiologic effects of insulin and leptin signaling in Pomc and Agrp neurons

Another question is, how are the insulin and leptin signals integrated within the CNS and what neuronal network (2^{nd} / 3^{rd} order neurons) is involved in conveying the signals to the adipose tissue. Insulin and leptin affect Pomc and Agrp neurons in a distinct manner. While leptin increases the frequency of action potentials in some Pomc expressing neurons (Al-Qassab et al., 2009; Cowley et al., 2001), insulin hyperpolarizes a subset of Pomc neurons (Al-Qassab et al., 2009; Hill et al., 2010; Konner et al., 2007). Both hormones seem to depend on intact phosphoinositide 3 kinase (PI3K) signaling to exert these effects (Al-Qassab et al., 2009) and, surprisingly, both insulin and leptin stimulate PI3K activity in Pomc cells (Xu et al., 2005). Yet, how can two hormones affect neuronal activity in an opposing fashion and at the same time activate the same intracellular signaling cascade? This apparent paradox was solved by the demonstration that the opposing neuronal responses to insulin and leptin are integrated in two distinct subpopulations of Pomc neurons that reside in different areas of the hypothalamus, rather than in the same exact neurons (Williams et al., 2010). However, other reports suggest that insulin is able to hyperpolarize a small number of Pomc neurons, which were pre-stimulated with leptin (Al-Qassab et al., 2009), suggesting that in rare cases, insulin and leptin signals can be integrated in single Pomc neurons. Taken together, leptin and insulin seem to induce opposing electrophysiological responses in subpopulations of Pomc neurons in the hypothalamus (also reviewed in (Belgardt and Bruning, 2010; Belgardt et al., 2009)).

In Agrp neurons insulin and leptin induce different signaling cascades. Insulin increases while leptin suppresses PI3K in a process that requires synaptic transmission from Pomc and other inhibitory presynaptic neurons (Xu et al., 2005). Insulin's electrophysiological effects in Agrp neurons are heterogeneous; only a small subset of Agrp neurons is insulin responsive and insulin has either depolarizing (Al-Qassab et al., 2009; Claret et al., 2007) or hyperpolarizing effects (Konner et al., 2007). Leptin seems to not affect Agrp neuron spike frequency in studies performed in mice (Al-Qassab et al., 2009; Claret et al., 2007), but to hyperpolarize pacemaker neurons in the rat ARC (van den Top et al., 2004). Thus, insulin and leptin affect subsets of Agrp and Pomc neurons in an opposing fashion. The finding that in Agrp neurons insulin activates while leptin suppresses PI3K, as well as the opposing electrical responses evoked in Pomc neurons, could represent potential

mechanisms through which brain insulin and leptin exert opposing effects on WAT metabolism.

The autonomic innervation of WAT

Retrograde transneuronal viral tracer studies in rodents, which are helpful tools in characterizing organ-specific SNS outflow, revealed multiple CNS regions that are involved in the autonomic innervation of WAT. The paraventricular nucleus (PVN) of the hypothalamus, which is known to project directly to spinal sympathetic neurons (Yamashita et al., 1984), stained positive at an early stage after virus injection into WAT (Song et al., 2005; Stanley et al., 2010). At later time points the infection spread to the ARC and lateral hypothalamus (Stanley et al., 2010), suggesting that these brain regions are upstream of the PVN. Interestingly, only Pomc expressing, but not NPY expressing neurons in the ARC co-localized with viral infection (Stanley et al., 2010). Pomc expressing neurons release α-melanocyte stimulating hormone, a cleavage product of Pomc, which activates melanocortin receptors. Central melanocortin agonists increase lipolysis and SNS outflow to WAT in siberian hamsters (Brito et al., 2007) and melanocortin 4 receptor mRNA expression highly co-localizes with pseudorabies virus infected neurons (Song et al., 2005), making Pomc neurons a likely integration site of MBH insulin and leptin signals to WAT. Given the distinct localization of the insulin and leptin reactive Pomc subpopulations within the MBH, it may be that these neuronal subsets project to different 2^{nd} order neurons that then activate or block SNS outflow to WAT integrating the opposing effects of insulin and leptin on a topographic basis. There is evidence for this in rats, since neurons of the anterior half of the ARC (insulin responsive neurons) were found to project to autonomic areas such as the dorsal vagal complex (Zheng et al., 2005), whereas neurons of the caudal portion (leptin responsive Pomc neurons) primarily connect to the PVN (Baker and Herkenham, 1995; Williams et al., 2010).

Future directions

Pomc and Agrp neurons, while important in the regulation of energy homeostasis, are only two of many neuronal subtypes that reside within the MBH. As leptin and insulin receptors are expressed widely within the CNS, other neuronal populations besides Agrp and Pomc are likely to participate in the regulation of WAT metabolism through brain leptin and

insulin and await further characterization. Furthermore, the electrophysiology and the intracellular signaling events that are triggered by insulin and leptin, require additional study. This knowledge could prove critical in understanding the molecular events that lead to the diverging effects of brain insulin and leptin signaling in regulating WAT metabolism.

Adiposity is regulated by many factors and the direct regulation of WAT lipolysis and lipogenesis represents only two among these. The metabolic phenotyping of currently available mouse models of neuron specific insulin and leptin receptor deletions has been mostly limited to the study of body composition, glucose fluxes and serum lipid profiles, parameters that are only indirectly affected through alterations in lipolytic flux from WAT. A more comprehensive assessment of lipid fluxes during fasting to re-feeding transitions or hyperinsulinemic clamps as well as in vivo determinations of WAT *de novo* lipogenesis in these mouse models will further our understanding of the regulation of WAT metabolism through the CNS.

It is important to point out that although long-term leptin treatment improves energy homeostasis in leptin deficient rodents and humans, the acute effects of leptin in non-leptin deficient rodents are conceivably detrimental if for example increased lipolysis is not counterbalanced by increased fatty acid utilization resulting in lipotoxicity. Under physiologic circumstances plasma levels of insulin and leptin change in parallel - both hormones are increased in the fed state and low during fasting although these changes during the fasting to re-feeding transition are much more pronounced in the case of insulin and subtle with leptin (Frederich et al., 1995). In the fed state NEFA release from WAT should be restrained while nutrient storage in WAT should increase, which are both a function of brain insulin signaling. To the contrary, leptin increases lipolysis and lowers *de novo* lipogenesis in WAT, both signs of WAT dysfunction. The fact that leptin administration in leptin deficient animals is not detrimental is likely due to the coordinated regulation of a number of metabolic effects such as an induction in β-oxidation. Thus, the balance between brain insulin and leptin action are a critical determinant of metabolic flexibility. The obese state is characterized through both leptin and insulin resistance. One can speculate that in the obese state, which is characterized by hyperleptinemia, the effects of leptin on WAT metabolism are preserved and not decreased as a consequence of leptin resistance. If this would turn out to be true, than leptin should drive WAT lipolysis and hamper *de novo* lipogenesis in obesity and DM2 and thus contribute to WAT dysfunction.

Finally, conditions in which brain insulin signaling is compromised, such as high fat feeding (Ono et al., 2008), chronic inflammatory conditions (Zhang et al., 2008) and obesity (Posey et al., 2009; Zhang et al., 2008), are likely to dysregulate the control of lipolysis and *de novo* lipogenesis in WAT due to impaired insulin action in the hypothalamus. Thus, hypothalamic insulin resistance unrestrains lipolysis and reduces the lipogenic capacity in WAT, which in turn induces lipotoxicity resulting in peripheral insulin resistance and thereby perpetuating a vicious cycle.

In summary, these studies provide evidence that the opposing effects of leptin and insulin on WAT metabolism are at least in part mediated via the brain. Impaired leptin and/or insulin signaling in the brain disrupt the brain control of lipolysis and *de novo* lipogenesis in WAT. This is mirrored by mouse models of either brain insulin and leptin receptor deficiency, which reproduce key components of WAT dysfunction in the obese and diabetic state. WAT is a driver of hepatic gluconeogenesis through its control of lipolytic flux, yet WAT is also an important source of insulin sensitizing fatty acid species. Thus, the control of WAT metabolism through brain insulin and leptin is likely to play an important role in lipid and glucose homeostasis.

VI. Summary / Kurzfassung in deutscher Sprache

White adipose tissue (WAT) dysfunction in obesity is a key player in the development of type 2 diabetes. In an insulin sensitive organism, WAT is able to readily switch from a fatty acid storing to a fatty acid releasing mode according to metabolic needs. This metabolic flexibility is critically important for metabolic homeostasis. To the contrary, WAT dysfunction, characterized by the inability of WAT to store lipids or unrestrained fatty acid release results in lipotoxicity, while impaired *de novo* lipogenesis in WAT decreases the synthesis of insulin sensitizing fatty acid species like palmitoleate. Insulin is considered the principal anti-lipolytic and pro-lipogenic hormone, which is thought to exert its effects exclusively via the insulin receptors expressed on adipocytes. Humans with insulin receptor mutations exhibit severe lipodystrophy with increased circulating fatty acids. Similarly, deletion of the murine insulin receptors in both the brain and periphery results in severe reduction of WAT mass, while deletion of insulin receptors in peripheral tissues including WAT but not the brain only mildly alters adiposity. This suggests that brain insulin signaling plays a pivotal role in WAT function. To test this male Sprague Dawley rats received a 6 hr infusion of insulin either intracerebroventricular (ICV) or directly into the mediobasal hypothalamus (MBH), while circulating glucose and insulin levels were controlled by a euglycemic clamp. *In vivo* lipolysis was assessed by [^{2}H-5]-glycerol tracer dilution technique. MBH or ICV insulin infusion markedly suppressed glycerol flux compared to vehicle infused controls, indicating a suppression of lipolysis, while increasing lipogenic protein expression and fatty acid synthase activity in epidydimal fat pads. Consistent with the lipolytic flux hormone sensitive lipase activation, as assessed by western blots of activating and inactivating serine phosphorylation sites, was significantly lower in the MBH insulin infused rats. The suppression of WAT lipolysis by hypothalamic insulin is likely due to a restraint of sympathetic nervous system outflow as surgical denervation or pharmacological sympathectomy lead to a similar suppression of hormone sensitive lipase activation in epidydimal fat pads. Consistent with these results, mice chronically lacking the neuronal insulin receptor exhibit unrestrained lipolysis and decreased *de novo* lipogenesis in WAT. These data demonstrate that insulin signaling in the MBH restrains WAT lipolysis and induces *de novo* lipogenesis, and that any alteration within this brain-WAT circuitry can contribute to WAT dysfunction and increased fatty acid release, which in turn can induce insulin resistance and lipotoxicity.

Ausführliche Zusammenfassung in deutscher Sprache

Stand der Forschung: Gesundes, funktionales Fettgewebe ist essentiell für einen normalen Glukose- und Fettstoffwechsel. Die Aufgabe des Fettgewebes ist es einerseits durch den Prozess der Lipolyse aus den gespeicherten Triglyzeriden freie Fettsäuren und Glyzerin als Energieträger, z.B. während des Fastens, in die Zirkulation freizusetzen und andererseits postprandial Lipide zu speichern (Degerman et al., 2003). In adipösen Menschen sowie Patienten mit Diabetes mellitus Typ 2 (DM2) ist diese dynamische Regulation gestört und der netto Fettsäureflux erhöht (Mittendorfer et al., 2009). Freie Fettsäuren sind dann vermehrt verfügbar und werden teils ektopisch in anderen Organen wie z.B. den Muskeln oder der Leber abgelagert (Boden et al., 2005; Itani et al., 2002; Kosteli et al., 2010), wo diese toxische Effekte haben können. Seit kurzem ist bekannt, dass durch *de novo* Lipogenese im Fettgewebe Fettsäuren, wie z.B. Palmitoleat, gebildet werden, die systemisch insulinsensitivierende Wirkung haben (Cao et al., 2008). Es gibt Hinweise, dass in adipösen Patienten die Kapazität zur *de novo* Lipogenese im Fett gestört ist (Diraison et al., 2002; Roberts et al., 2009). Diese Beobachtungen unterstreichen zusätzlich die zentrale Rolle des Fettgewebes in der Pathogenese des DM2. Allerdings ist der Hintergrund für die Funktionsstörung des Fettgewebes in der Adipositas und dem DM2 ungeklärt.

Menschen mit Mutationen des Insulinrezeptors entwickeln eine schwere Lipodystrophie mit erhöhten zirkulierenden freien Fettsäuren (Hegele, 2003). Dies zeigt, dass das Insulinsignal ein essentieller Faktor für funktionales Fettgewebe ist. Insulin ist eines der wichtigsten anti-lipolytischen Hormone. Nach jetzigem Stand der Forschung vermittelt Insulin seine anti-lipolytische Wirkung ausschließlich direkt über den auf Adipozyten exprimierten Insulinrezeptor (Degerman et al., 2003). Gegenspieler des Insulins ist der Sympathikus, der im Fettgewebe über die Produktion von zyklischem AMP pro-lipolytische Wirkung hat. Es ist jedoch unklar, ob Insulin auch durch Bindung und Aktivierung neuronaler Insulinrezeptoren die Sympathikusaktivität zum Fettgewebe verringert und somit die Lipolyse auch indirekt blockieren kann. Die Hypothese, dass Insulin durch Aktivierung von Insulinrezeptoren im Gehirn Lipolyse steuern kann, wird von Studien unterstützt, die zeigten, dass neuronale Insulinrezeptoren wichtige Funktionen in der Erhaltung der Fettgewebsmasse haben. Während eine induzierte generalisiert genetische Ablation von Insulinrezeptoren in Gehirn und Peripherie bei Mäusen zu

schwerer Lipodystrophie führt, kommt es bei einer Deletion ausschließlich peripherer Insulinrezeptoren unter Erhaltung der Insulinrezeptoren des Gehirns nur zu geringen Veränderungen der Fettgewebsmasse (Koch et al., 2008).

Ziele und wissenschaftliche Fragestellung: Die Hypothese war daher, dass zirkulierendes Insulin Fettgewebsfunktionen, wie Lipolyse und *de novo* Lipogenese, auch indirekt via Insulinrezeptoren im Gehirn regulieren kann. Das Ziel dieser Doktorarbeit war es, diesen neuen physiologischen Regelkreislauf zu prüfen, zu charakterisieren und folgende wissenschaftliche Fragen zu beantworten: Kann Insulin, unabhängig seiner Wirkungen auf periphere Fettgewebsinsulinrezeptoren, Fettgewebsstoffwechsel durch Bindung an hypothalamische Insulinrezeptoren und Modulierung der Sympathikusaktivität steuern und inwieweit trägt hypothalamische Insulinresistenz in Diabetikern zur Erhöhung des Fettsäurefluxes bei?

Methodik: Männliche Sprague Dawley Ratten, die stereotaktisch mit Kanülen implantiert wurden, sind über sechs Stunden entweder in den dritten Ventrikel (ICV) oder direkt in den mediobasalen Hypothalamus (MBH) mit Insulin oder artifizieller Cerebrospinalflüssigkeit (Kontrolle) infundiert worden, wobei simultan systemische Insulin- und Glukosespiegel durch euglykämisch-pankreatische Klemmen kontrolliert wurden. Gleichzeitig wurden [3-^{3}H]-Glukose und [^{2}H-5]-Glyzerin Tracer infundiert, um Glukoseflux bzw. Lipolyse zu messen. Die pharmakologischen Studien wurden zusätzlich durch ein genetisches Mausmodell, das keinen Insulinrezeptor im Gehirn exprimiert (NIRKO Mäuse) (Bruning et al., 2000), ergänzt, um die Auswirkungen chronisch hypothalamischer Insulinresistenz auf den Fettgewebsmetabolismus zu testen. NIRKO Mäuse und Kontrollen des gleichen Wurfes, die für 16 bzw. 24 Stunden keinen Zugang zu Futter hatten, wurden entweder hyperinsulinämisch-euglykämisch geklemmt oder für zwei Stunden *ad libitum* gefüttert und dann auf Veränderungen im lipolytische Flux mittels [^{2}H-5]-Glyzerin Tracer bzw. Messung freier Fettsäuren im Plasma untersucht. Fettgewebsproben der Ratten und NIRKO Mäuse wurden im Anschluss mit Western Blot auf Veränderungen in der Proteinexpression lipolytischer und lipogener Enzyme untersucht. Zusätzlich wurden epididymale Fettgewebsdepots der Ratten sowohl chirurgisch denerviert als auch pharmakologisch mittels 6-Hydroxydopamin sympathektomiert, um die Rolle des Sympathikus in der zentrale Regulation des Fettgewebes zu untersuchen.

Ergebnisse: Zentral appliziertes Insulin, sowohl ICV als auch direkt in den MBH, führte zu einer beträchtlichen Reduktion des systemischen Glyzerinfluxes im Vergleich zu Kontrollen, unabhängig von zirkulierenden Insulin- und Glukosespiegeln, welche durch euglykämisch-pankreatische Klemmen kontrolliert wurden. Dabei waren intrahypothalamische Insulininfusionen potenter. Diese konnten sogar eine vergleichbare Verringerung der Lipolyse wie systemische Hyperinsulinämie erzielen. Andererseits hatte die Applikation eines Insulinrezeptorantagonisten (S961) (Schaffer et al., 2008) direkt in den MBH, was zur Blockade des endogenen Insulinsignals im MBH führte, eine Steigerung des Lipolysefluxes zur Folge.

Lipolytischer Flux vom Fettgewebe kann über verschiedene Mechanismen auch hepatische Glukoseproduktion regulieren (Mittelman and Bergman, 2000; Rebrin et al., 1996). Zum einen dient das freigesetzte Glyzerin als Ausgangsstoff für die hepatische Glukoneogenese, zum anderen versorgen die durch Lipolyse freigesetzten Fettsäuren die Leber mit Energie in Form von, durch β–Oxidation gewonnenes, NADH, um die Glukoneogenese aufrechtzuerhalten (Hers and Hue, 1983). Zentrales Insulin supprimierte hepatische Glukoseproduktion, erhöhte allerdings im Gegensatz zu systemischem Insulin nicht die Glukoseaufnahme in die Peripherie, was mit Ergebnissen anderer Studien übereinstimmt (Pocai et al., 2005). Interessanterweise korrelierten Glyzerinflux (Lipolyse) und hepatische Glukoseproduktion in den Tieren, die zentral Insulin appliziert bekamen, während keiner dieser Parameter mit zirkulierendem Insulin korrelierte. Diese Daten deuten darauf hin, dass möglicherweise hypothalmisches Insulinsignaling hepatische Glukoseproduktion über die Kontrolle von Lipolyse im Fett steuern kann.

Western Blots der epidydimalen Fettgewebsdepots der Ratten, die entweder zentrales Insulin oder Kontrolle erhielten, ergaben, dass zentrales Insulin den Aktivierungszustand von hormonsensitiver Lipase verringert. Proteinkinase A aktiviert die hormonsensitive Lipase durch Phosphorylierung an Serin 563 und 660 (Anthonsen et al., 1998). Zentrales Insulin reduzierte die Serin-Phosphorylierung an diesen beiden Stellen, während die Phosphorylierung an Serin 565, welches das Enzym inaktiviert (Anthonsen et al., 1998), erhöht war. Diese Ergebnisse zeigen zusätzlich, dass Insulin im Gehirn anti-lipolytisch wirkt. Gleichzeitig wurde die Expression von Schlüsselproteinen der *de novo* Lipogenese im Fett, wie z.B. Fettsäure-Synthase, Acetyl-CoA-Carboxylase und ATP-Citrat-Lyase, durch zentrales Insulin induziert und die Fettsäure-Synthase-Aktivität im perirenalen

Fettgewebe erhöht. Sowohl chirurgische Denervierung als auch pharmakologische Sympathektomie des epidydimalen Fettgewebes führten zu einer vergleichbaren Reduktion der hormonsensitiven Lipase-Aktivierung wie zentrales Insulin. Blockierte man dann den Insulinrezeptor im MBH in systemisch sympathektomierten Ratten, verlor der Insulinrezeptorantagonist S961 seine pro-lipolytische Wirkung. Zusammengefasst deuten diese Studien darauf hin, dass hypothalamisches Insulin Lipolyse im Fettgewebe über eine Suppression der Sympathikusaktivität im Fett reguliert.

Korrespondierend zu den vorher präsentierten Ergebnissen entwickelten NIRKO Mäuse nach 16-stündigem Nahrungsentzug ungehemmten lipolytischen Flux im Vergleich zu Kontrollen des gleichen Wurfes. Systemische, durch euglykämisch-pankreatische Klemmen induzierte, Hyperinsulinämie konnte die Lipolyseraten der NIRKO Mäuse nicht komplett normalisieren. In einem unabhängigen Experiment wurden NIRKO und Kontroll-Mäuse nach 24-stündigem Nahrungsentzug für zwei Stunden *ad libitum* gefüttert, wobei NIRKO Mäuse im Vergleich zu Kontrolltieren ihre freien Fettsäuren im Plasma schwächer supprimierten. Beide Experimente zeigen, dass die systemische Kontrolle der Lipolyse maßgeblich von intakten neuronalen Insulinrezeptoren abhängt. Analog zu den Ratten-Studien, in denen zentrales Insulin *de novo* Lipogenese im Fett induzierte, war die Expression pro-lipogenetischer Enzyme im Fett der NIRKO Mäuse erwartungsgemäß reduziert. Fettsäureprofile der NIRKO Mäuse, erstellt von Gewebsproben des epidydimalen Fettes, ergaben einen verringerten *de novo* Lipogeneseindex sowie eine signifikante Reduktion der insulinsensitivierenden Fettsäure Palmitoleat. Fettgewebs-Palmitoleatspiegel korrelierten zudem mit der Proteinexpression pro-lipogenetischer Enzyme, was darauf hindeutet, dass zentrales Insulin durch Kontrolle der *de novo* Lipogenese die Palmitoleatproduktion steuern kann. Diese Daten unterstreichen wiederum die essentielle Rolle von zentralem Insulin in der Kontrolle des Fettgewebsstoffwechsels.

Zusammengefasst zeigen die Daten dieser Doktorarbeit, dass Insulin durch Aktivierung neuronaler Insulinrezeptoren im MBH und der dadurch ausgelösten Dämpfung des Sympathikussignals zum Fettgewebe, Lipolyse reduziert und *de novo* Lipogenese im Fettgewebe induziert. Veränderungen in diesem Gehirn-Fettgewebe Regelkreislaufes kann eine Fettgewebsdysfunktion zur Folge haben und zu pathologisch erhöhter Fettsäuresekretion führen, was wiederum Insulinresistenz und Lipotoxizität induzieren kann.

VII. References

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VIII. Appendix

IACUC approval for animal studies:

Protocol: LA09-00174

Title: Regulation of adipose tissue metabolism by central insulin and leptin

MOUNT SINAI
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October 22, 2009

PRINCIPAL INVESTIGATOR: Christoph Buettner, M.D.

DEPARTMENT: Endocrinology

BOX: 1055

Please be advised that the application referenced below has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) for the period: 10/20/09 – 10/19/12. A progress report for this project is due 10/19/10.

AGENCY: National Institute of Diabetes and Digestive and Kidney Diseases /NIH / DHHS

TITLE: Regulation of Adipose Tissue Metabolism by Central Insulin and Leptin

SPECIES APPROVED: Rats / Mice

GCO Ref #08-0619
InfoEd Ref #LA09-00174

GM/jg

Protocol: 07-0798

Title: The Role of STAT3 Signaling in Leptin's Pleiotropic Actions

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AGENCY: NIH / DHHS

The application identified below was submitted to your Agency for consideration. Please be advised that this application was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) on 7/23/07.

PRINCIPAL INVESTIGATOR: Christoph Buettner, M.D. / Ph.D.

TITLE: The Role of STAT3 Signaling in Leptin's Pleiotropic Actions

SPECIES APPROVED: Mouse

DEPARTMENT: Medicine

BOX: 1055

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A3111-01.

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IX. Acknowledgements

I am extremely grateful to my mentor, Prof. Dr. Christoph Buettner, for supporting this thesis project. He always had an open ear for my problems, motivated me constantly, inspired me and really taught me how to be an efficient scientist. I would also like to thank my thesis advisor Prof. Dr. Hendrik Lehnert for his help in making this doctoral thesis possible.

I am particularly thankful for all the support and encouragement I received from my family: my mum, Carmen, my dad, Josef, and my brother, Stefan! Guys, you are simply the best! A big shout out also goes to my girlfriend Lisa, who was very patient, supportive and understanding for my moods throughout this entire project.

I am extremely thankful for the help from my colleagues at the Buettner Lab and all my collaborators on this project: James O'Hare, Liz Zielinski, Bob Cheng and Kai Su, who helped to perform the numerous rat clamp studies, Claudia Lindtner for the real-time PCR and for being the best colleague one could possibly imagine, Prashant Vempati and Leslie Roteta, who helped with genotyping, Simon Fisher and Kelly Diggs-Andrews, who performed the NIRKO clamp studies, Ludger Scheja, who conducted the WAT lipid profiles, and Martina Schweiger and Rudi Zechner for performing the TG hydrolase assay and their excellent technical advice. I would also like to thank Drs Ronald Kahn and Jens Bruening for making available the NIRKO mice, Dr Michelle Puchowicz at Case Western University MMPC (supported by U24 DK76169) for the mass spectrometry analyses, and the Yale Center of Clinical investigations for the NE measurements (supported by CTSA Grant UL1 RR024139 from the National Center for Research Resources). I further would like to thank Abott for providing glucose strips, Dr Andrew Greenberg for the Perilipin antibody and Dr Lauge Schaeffer from Novo Nordisk for the insulin receptor antagonist S961. Finally, I would like to thank Drs Nir Barzilai and Radhika Muzumdar for the epididymal fat samples from calorically restricted, aged rats and Dr Gary Schwartz for his advice and very helpful discussions. This thesis project was supported by NIH Grants DK074873, DK083568 and DK082724 awarded to Christoph Buettner. In addition I was supported by travel grants from the European Foundation for the Study of Diabetes and the provincial government of Vorarlberg, Austria.

X. Curriculum vitae

Thomas Josef Scherer

geb. 5. Oktober 1981

BILDUNGSWEG

Seit Okt. 2011	Assistenzarzt, Innere Medizin III, Abteilung für Endokrinologie und Stoffwechsel, Medizinische Universität Wien, Österreich
Jun. 2008 - Aug. 2011	Research fellow, Buettner Lab, Endocrinology Division, Mount Sinai School of Medicine, New York, USA *(Dissertation wurde während dieser Zeit verfasst)*
Mär. 2008 - Jun. 2008	Turnusarzt Allgemeinmedizinische Lehrpraxis Dr. Helena Franc, Wien, Österreich
Nov. 2007 - Jan. 2008	Forschungspraktikum Buettner Lab, Endocrinology Division, Mount Sinai School of Medicine, New York, USA
Okt. 2001 - Jul. 2007	Diplomstudium Humanmedizin N202 an der Medizinischen Universität Wien, Österreich

PROFESSIONELLE MITGLIEDSCHAFTEN

Endocrine Society (seit 2010)

European Association for the Study of Diabetes (seit 2008)

FORTBILDUNGEN

Metabolic Syndrome Course: "An organ systems approach to experimental targeting of the Metabolic Syndrome." Vanderbilt University School of Medicine, Nashville, Tennessee, USA

AUSZEICHNUNGEN

2011	Keystone Symposia Scholarship Winner
2010	The Endocrine Society Outstanding Abstract Award
2008	Albert Renold Travel Fellowship (European Foundation for the Study of Diabetes)
2001	Leistungsstipendium der Medizinischen Universität Wien für das Studienjahr 2001/2002

XI. Publikationsliste

Yin and Yang of Hypothalamic Insulin and Leptin Signaling in Regulating White Adipose Tissue Metabolism. Scherer T and Buettner C. Rev Endocr Metab Disord. 2011 Jun 29 doi: 10.1007/s11154-011-9190-4

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Abstracts und Präsentationen:

Short-term Overfeeding Disrupts Brain Insulin Control of Liver and Adipose Tissue Function. T Scherer, E Zielinski, J O'Hare, C Lindtner and C Buettner. Vortrag und Posterpräsentation Keystone Symposia on Lipid Biology and Lipotoxicity, Killarney, Ireland 2011

The role of brain insulin signaling in regulating adipose tissue metabolism. T Scherer, J O'Hare, K Diggs-Andrews, S Dighe, C Lindtner, T Milsom, B Cheng, K Su, M Puchowicz, S J Fisher, S F Previs & C Buettner. Vortrag und Posterpräsentation D-Cure meeting 2010, New York Academy of Medicine

Hypothalamic Insulin Signaling Regulates Lipolysis in Adipose Tissue. T Scherer, J O'Hare, KD Andrews, B Cheng, C Lindtner, S Dighe, T Milsom, K Su, M Puchowicz, SJ Fisher, SF Previs and C Buettner. Posterpräsentation Endocrine Society Meeting 2010, San Diego

Diet-Induced Hepatic Insulin Resistance Is Mediated by Endocannabinoids Via CB1 Receptor-Mediated Dephosphorylation of Akt-2 by PHLPP1. J Liu, L Zhou, K Xiong, J O'Hare, **T Scherer**, C Buettner, G Kunos. Co-Autor Poster Endocrine Society Meeting 2010, San Diego

Brain insulin signaling increases VLDL production in vivo. T Scherer, J O'Hare, K Su, L Scheja and C Buettner. Posterpräsentation Keystone Symposia "Adipose tissue biology" 2010, Keystone, CO

P20 NIH Mini-Center Grant Progress Report for Pilot Feasibility Project: A role of hypothalamic insulin resistance in the pathogenesis of alcoholic liver disease. C Lindtner, **T Scherer**, C Buettner; Vortrag (Progress Report), 2010 Mount Sinai School of Medicine.