Melanin-concentrating hormone induces insulin resistance through a mechanism independent of body weight gain

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Abstract

Transgenic hyperexpression of melanin-concentrating hormone (MCH) produces a phenotype of obesity and glucose intolerance. However, it is not known whether under this specific condition, glucose intolerance develops as a direct consequence of hyperexpressed MCH or is secondary to increased adiposity. Here, rats were treated i.c.v. with MCH or with an antisense oligonucleotide to MCH (MCH-ASO). MCH promoted an increase in blood glucose and a decrease in blood insulin levels during a glucose tolerance test. MCH also caused a decrease in the constant of glucose disappearance during an insulin tolerance test. All these effects of MCH were independent of body weight variation and were accompanied by

reduced insulin receptor substrate (IRS)-1 engagement of phosphatidylinositol-3 kinase (PI3-kinase) in white and brown adipose tissues, skeletal muscle and liver and by reduced Akt activation in skeletal muscle. MCH also led to a significant reduction in ERK activation in white adipose tissue. Finally, inhibition of hypothalamic MCH expression promoted a significant increase in ERK activation in brown adipose tissue. We conclude that hypothalamic MCH controls glucose homeostasis through mechanisms that are, at least in part, independent of adiposity.

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Introduction

The hypothalamic neurotransmitter melanin-concentrating hormone (MCH) is expressed predominantly in neurons of the lateral hypothalamus and participates in the control of energy expenditure and food intake (Qu *et al.* 1996, Shimada *et al.* 1998). Transgenic, tissue-specific hyperexpression of this peptide leads to increased feeding accompanied by significantly higher body-weight and adiposity (Ludwig *et al.* 2001). Intracerebroventricular (i.c.v.) treatment with MCH also promotes body weight gain and increased food intake (Della-Zuana *et al.* 2002, Ito *et al.* 2003). Conversely, knockout of the MCH gene produces a lean phenotype, which is accompanied by reduced feeding and, most importantly, by increased resting energy expenditure (Shimada *et al.* 1998).

Although most studies have emphasized the role for MCH in feeding and energy homeostasis, transgenic mice hyperexpressing MCH in the hypothalamus also display an interesting phenotype of insulin resistance (Ludwig *et al.* 2001). According to this study, these transgenic mice present a significantly higher blood glucose level during an intraperitoneal glucose tolerance test (ipGTT), which is accompanied by a markedly increased fed blood insulin level and by severe insulin resistance, as measured by the

insulin tolerance test (ITT). Although the authors state that, in this particular situation, insulin resistance develops secondarily to body weight gain, they do not discard the possibility of a direct effect of MCH in regulating peripheral insulin action and insulin production.

Considering the important epidemiological association between obesity and diabetes mellitus (Kopelman 2000, Flier 2004), the characterization of mechanisms that simultaneously participate in the control of body weight and insulin action may reveal novel candidates for combined therapeutic approaches for both diseases. Therefore, the objective of the present study was to evaluate the effect of short-term i.c.v. treatment with exogenous MCH upon whole-body glucose homeostasis, and upon insulin signal transduction in liver, skeletal muscle, and white and brown adipose tissues of non-genetically manipulated, non-diabetes prone Wistar rats.

Materials and Methods

Chemicals, antibodies and oligonucleotides

Reagents for SDS/PAGE and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA).

Hepes, PMSF, aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, BSA (fraction V) and rat MCH (M-4542), were from Sigma Chemical Co. (St Louis, MO, USA). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), ^{125}I -Protein A and nitrocellulose membranes were from Amersham Corp. (Aylesbury, Bucks, UK). Phosphorothioate-modified oligonucleotides for MCH (sense, 5'-CCC TCA GTC TGG CTG-3' and anti-sense, 5'-ACA GCC AGA CTG AGG-3') were obtained from Life Technologies (GIBCO BRL, Gaithersburg, MD, USA). Sodium amobarbital and insulin were from Eli Lilly (Indianapolis, IN, USA). Polyclonal anti-phosphotyrosine antibodies were raised in rabbits and affinity-purified on phosphotyramine columns (Pang *et al.* 1985). Anti-insulin receptor (IR), anti-insulin receptor substrate (IRS)-1, anti-IRS-2, anti-ERK, anti-pERK (α pERK/Tyr 204, detecting pERK42 and pERK44), anti-Akt1, and anti-phospho [Ser 473]Akt1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p85/phosphatidylinositol-3 kinase (PI3-kinase) antiserum was from UBI (Lake Placid, NY, USA). Insulin (Scott *et al.* 1981), corticosterone (Amersham Corp., Biotrak, Aylesbury, Bucks, UK) and MCH (Phoenix Pharmaceutical Inc., Belmont, CA, USA) were determined by RIA. Glucose was determined by the glucose oxidase method (Trinder 1969).

Intracerebroventricular (i.c.v.) cannulation and i.c.v. injection

Chronic unilateral 26-gauge stainless steel indwelling guide cannulas were stereotactically and aseptically placed into the lateral ventricle (0.2 mm posterior, 1.5 mm lateral and 4.2 mm ventral to bregma) of adult male Wistar rats under sodium amobarbital (15 mg/kg body weight) anesthesia, as previously described (Della-Zuana *et al.* 2002). After a one-week recovery period, all rats were kept in individual cages and i.c.v.-treated with vehicle (TE buffer, 10 mM Tris-HCl, 1.0 mM EDTA), exogenous MCH, MCH sense, or MCH antisense (MCH-ASO) phosphorothioate-modified oligonucleotides. MCH (2.0 μg), MCH sense (4.0 nmoles) and MCH-ASO (4.0 nmoles) were diluted in TE buffer and injected once a day at 1000 h for four days, with a total volume of 2.0 μl per dose. Rats were randomly assigned to four different treatment conditions: TE treatment (control), MCH treatment (MCH), antisense MCH oligonucleotide treatment (MCH-ASO), and MCH sense treatment. Since MCH sense treatment did not modulate hypothalamic MCH concentration, glucose and insulin blood levels and insulin signal transduction, we did not include these data in the results section. Data on the characterization in MCH sense and MCH-ASO have been published previously (Pereira-da-Silva *et al.* 2003). MCH-ASO promoted a reduction in MCH tissue hypothalamic concentration from 0.42 ± 0.07 pmol/mg (control) to 0.34 ± 0.04 pmol/mg (MCH-ASO) ($n=5$, $P<0.05$). Food intake and body

weight were determined daily at 1000 h. The results of food intake are expressed as mean daily consumption (g) during the experimental period, and the results of body weight are expressed as the variation of body weight (g) from the first to the last experimental day.

Glucose tolerance test (GTT)

Intraperitoneal (ip) GTT was carried out at the end of the experimental period, on day 5, after an overnight fast. After collection of an unchallenged sample (time 0), a solution of 20% glucose (2.0 g/kg body weight) was administered into the peritoneal cavity. Blood samples were collected from the tail at 0, 15, 30, 60 and 120 min for determination of glucose and insulin concentrations.

Insulin tolerance test (ITT)

Intraperitoneal (ip) ITT was performed on experimental day five, after an overnight fast. Insulin (6 μg) was injected intraperitoneally and blood samples were collected from the tail at 0, 5, 10, 15, 20, 25 and 30 min for serum glucose determination. The constant rate for glucose disappearance (K_{it}) was calculated using the formula $0.693/t_{1/2}$. Glucose $t_{1/2}$ was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase (Bonora *et al.* 1987).

Tissue extraction, immunoblotting and immunoprecipitation

The abdominal cavity of anesthetized rats was opened and the rats received an infusion of insulin (0.2 ml, 10^{-6} M) or saline (0.2 ml) through the vena cava. After different intervals (2 min for IR, IRS-1, IRS-2 and PI3-kinase and 5 min for Akt and ERK), fragments ($3.0 \times 3.0 \times 3.0$ mm) of brown adipose tissue (BAT), white adipose tissue (WAT) (epididymal fat), liver and skeletal muscle (gastrocnemius muscle) were excised and immediately homogenized in solubilization buffer at 4 °C (1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM PMSF and 0.1 mg aprotinin/ml) with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA) operated at maximum speed for 30 s. Insoluble material was removed by centrifugation for 20 min at 9000 g in a 70.Ti rotor (Beckman) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method. Aliquots of the resulting supernatants containing 5.0 mg total protein were used for immunoprecipitation with antibodies against IR, IRS-1 and IRS-2 at 4 °C overnight, followed by SDS/PAGE, transfer to nitrocellulose membranes and blotting with antiphosphotyrosine, anti-IR, anti-IRS-1, anti-IRS-2 or anti-p85/PI3-kinase. In direct immunoblot experiments, 0.2 mg protein extracts obtained from each tissue were separated

Table 1 Metabolic and hormonal characteristics of rats treated with MCH antisense oligonucleotide (ASO) and exogenous MCH, compared with control. Results are means $\pm$ S.E.M., $n=6$

	Control	ASO	MCH
Food ingestion (g/24 h)	18.3 $\pm$ 1.4	17.0 $\pm$ 1.1	21.2 $\pm$ 2.2
Body weight variation (g/5 days)	+9.3 $\pm$ 1.8	-2.2 $\pm$ 0.5*	+11.3 $\pm$ 1.9
Plasma insulin (ng/ml)	1.86 $\pm$ 0.43	1.63 $\pm$ 0.38	1.28 $\pm$ 0.40
Serum glucose (mg/dl)	80.8 $\pm$ 2.1	83.2 $\pm$ 2.9	90.7 $\pm$ 2.2*
Serum corticosterone (ng/ml)	98.5 $\pm$ 8.9	92.4 $\pm$ 7.7	111.4 $\pm$ 12.3

* $P<0.05$ compared with control.

by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-IR, -IRS-1, -IRS-2, -phospho-Akt or -phospho-ERK antibodies as described (Torsoni *et al.* 2003). To assure proper loading of proteins onto the gels, the nitrocellulose membranes used to evaluate phospho-Akt or phospho-ERK were always reprobated with antibodies against Akt and ERK respectively.

Data presentation and statistical analysis

All numerical results are expressed as the mean $\pm$ S.E.M. of the indicated number of experiments. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software (ScionCorp, Frederick, MD, USA). Data were analyzed by the two-tailed unpaired Student's *t*-test or by repeat-measures analysis of variance (one-way or two-way ANOVA) followed by *post hoc* analysis of significance (Bonferroni test) when appropriate, comparing experimental and control groups. The level of significance was set at $P<0.05$.

Results

Metabolic characterization of experimental animals

Treatment of rats with MCH or MCH-ASO for four days did not modify mean daily food intake, basal plasma insulin levels and basal serum corticosterone levels (Table 1). Treatment with MCH promoted a significant increase in basal glucose levels, while treatment with MCH-ASO led to a significant reduction in 5-day body mass variation (Table 1). Intracerebroventricular MCH led to no variation in body weight (Table 1). During a GTT, the blood levels of glucose were constantly higher in MCH-treated rats (Fig. 1A), which resulted in a significantly higher area under the glucose curve (Fig. 1B). The levels of plasma insulin during the GTT were also affected by MCH treatment (Fig. 1C), which produced a significant reduction in the area under the insulin curve (Fig. 1D). Finally, during ITT, the constant of glucose decay was significantly impaired by the treatment with MCH (Fig. 1E). The

inhibition of MCH expression with MCH-ASO did not modify glucose and insulin levels during the GTT and did not interfere with glucose mobilization during the ITT (Fig. 1).

Insulin signal transduction in peripheral tissues

The treatments with MCH and MCH-ASO did not affect the expression of IR, IRS-1 and IRS-2 in skeletal muscle, liver, WAT and BAT of rats (Fig. 2). Conversely, the treatment with MCH led to significant inhibition of insulin-induced association of IRS-1 with p85PI3-kinase in skeletal muscle (Fig. 3), in liver (Fig. 4), in WAT (Fig. 5) and in BAT (Fig. 6). This was followed by a significant inhibition of insulin-induced [Ser⁴⁷³] phosphorylation of Akt in skeletal muscle (Fig. 3) and by a significant inhibition of insulin-induced [Tyr²⁰⁴] phosphorylation of ERK in WAT (Fig. 5). Finally, the treatment of rats with MCH-ASO promoted a significant increase in insulin-induced [Tyr²⁰⁴] phosphorylation of ERK in BAT (Fig. 6).

Discussion

In recent years, important advances have been made in the characterization of the mechanisms that link obesity to type 2 diabetes. Leptin and insulin, by acting upon specific neurons of the arcuate nucleus, deliver anorexigenic and adipostatic signals to the hypothalamus. Central resistance to these hormones' actions is thought to play an important role in the pathogenesis of obesity by disrupting a complex network of neurotransmitters that coordinate the balance between food intake and energy expenditure (Schwartz *et al.* 2000, Flier 2004). MCH is one of such hypothalamic neurotransmitters that are modulated in response to leptin and insulin (Schwartz *et al.* 2000, Segal-Lieberman *et al.* 2003). The action of these hormones upon hypothalamic circuitries is initiated by the coordinated inhibition or stimulation of neuropeptide Y (NPY)/agouti-related peptide (AGRP)ergic and pro-opiomelanocortin (POMC)ergic (α -melanocyte-stimulating hormone (α -MSH) and cocaine

Figure 1 Serum glucose levels (A), and area under the glucose curve (B), and plasma insulin levels (C), and area under the insulin curve (D) during the ipGTT. (E) Constant of glucose decay (K_{itt}) during the ipITT. Intracerebroventricular cannulated rats were treated with saline (C), MCH or MCH-ASO (ASO) according to protocols described under Materials and Methods. For all experiments $n=6$; * $P<0.05$ compared with control (C).

and amphetamine-related transcript (CART) producing) neurons of the arcuate nucleus respectively. These first-order neurons are connected with downstream second-order neurons located at the periventricular and lateral hypothalamic nuclei. Reduction in NPY/AGRP and increase in α -MSH/CART at the nerve terminals promote increases of anorexigenic and pro-thermogenic neurotransmitters such as corticotropin-releasing hormone and thyrotropin-releasing hormone at the periventricular nucleus and reduction of orexigenic and energy sparing neurotransmitters such as orexin and MCH at the lateral

hypothalamic nucleus (Segal-Lieberman *et al.* 2003, Flier 2004). Since transgenic mice hyperexpressing MCH are obese and insulin resistant, a role for MCH as a central controller of insulin secretion and/or action has been proposed (Ludwig *et al.* 2001).

In the present study, we demonstrate that short-term i.c.v. treatment of rats with MCH does not modify food intake and body weight but does modulate the blood levels of glucose and insulin, leading to glucose intolerance, reduced glucose-stimulated insulin secretion and insulin resistance. All these effects were accompanied by the

Figure 2 Immunoblot (IB) analysis of (A) IR, (B) IRS-1 and (C) IRS-2 expression in skeletal muscle (SM), liver (LV), white adipose tissue (WAT) and brown adipose tissue (BAT) of i.c.v. cannulated rats treated with saline (C), MCH-ASO (ASO) or MCH according to protocols described under Materials and Methods. In all experiments, $n=5$.

modulation of insulin-induced association of IRS-1 with PI3-kinase in skeletal muscle, liver, and white and brown adipose tissues and, especially, by reduced insulin-induced molecular activation of Akt in skeletal muscle. In insulin-sensitive tissues such as skeletal muscle and adipose tissue, Akt connects insulin signal transduction through the

IRSs/PI3-kinase pathway for the control of GLUT-4 translocation from intracellular pools to the cell membrane (Saltiel & Kahn 2001). Therefore, Akt plays a pivotal role in the linkage between the insulin signal and the control of glucose uptake (Saltiel & Kahn 2001). Skeletal muscle is responsible for most of the glucose clearance controlled by insulin (Shulman 2000). Thus, we believe that the inhibitory effect of short-term i.c.v. MCH treatment upon insulin action is mostly dependent on molecular resistance to insulin action through Akt in skeletal muscle. Despite the finding that i.c.v. MCH promoted an apparent impairment of the insulin-induced association of IRS-1 with PI3-kinase in the other tissues tested, this was not translated into the regulation of a more distal event such as the activation of Akt. The reason for these divergent outcomes in different tissues is unknown; however, in a number of animal models of insulin resistance tissue-specific modulation of insulin action has been reported. In a recent study, Rojas *et al.* (2003) have shown that in two different models of insulin resistance, epinephrine- and dexamethasone-treated rats, the expression and functional activation of elements of the insulin signaling pathway occur divergently when comparing liver and skeletal

Figure 3 Evaluation of insulin signal transduction in skeletal muscle of rats treated i.c.v. with saline (C), MCH-ASO (ASO) or MCH according to protocols described under Materials and Methods. Samples of skeletal muscle protein extracts obtained from rats acutely treated iv with saline (-) or insulin (+) were directly separated by SDS-PAGE or employed in immunoprecipitation (IP) assays with anti-IR, -IRS-1 and -IRS-2 antibodies rendering immunoprecipitates that were thereafter separated by SDS-PAGE. After electrophoresis the proteins were transferred to nitrocellulose membranes and blotted (IB) with anti-phosphotyrosine (PY), -p85PI3-kinase (p85PI3K), -p[Ser⁴⁷³]Akt (pAkt) or -p[Tyr²⁰⁴]ERK (pERK) antibodies. Only proteins that suffered significant modulation by any of the treatment protocols have their data graphically represented (in B and C). Open bars, control; darker shaded bars, MCH-ASO; lighter shaded bars, MCH. For all experiments, $n=5$; * $P<0.05$ compared with control (C).

Figure 4 Evaluation of insulin signal transduction in liver of rats treated i.c.v. with saline (C), MCH-ASO (ASO) or MCH according to protocols described under Materials and Methods. Samples of liver protein extracts obtained from rats acutely treated iv with saline (–) or insulin (+) were directly separated by SDS-PAGE or employed in immunoprecipitation (IP) assays with anti-IR, -IRS-1 and -IRS-2 antibodies rendering immunoprecipitates that were thereafter separated by SDS-PAGE. After electrophoresis the proteins were transferred to nitrocellulose membranes and blotted (IB) with anti-phosphotyrosine (PY), -p85PI3-kinase (p85PI3K), -p[Ser⁴⁷³]Akt (pAkt) or -p[Tyr²⁰⁴]ERK (pERK) antibodies. Only proteins that underwent significant modulation by any of the treatment protocols have their data graphically represented (in B). Open bars, control; darker shaded bars, MCH-ASO; lighter shaded bars, MCH. For all experiments, *n*=5; **P*<0.05 compared with control (C).

Figure 5 Evaluation of insulin signal transduction in white adipose tissue of rats treated i.c.v. with saline (C), MCH-ASO (ASO) or MCH according to protocols described under Materials and Methods. Samples of white adipose tissue protein extracts obtained from rats acutely treated iv with saline (–) or insulin (+) were directly separated by SDS-PAGE or employed in immunoprecipitation (IP) assays with anti-IR, -IRS-1 and -IRS-2 antibodies rendering immunoprecipitates that were thereafter separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blotted (IB) with anti-phosphotyrosine (PY), -p85PI3-kinase (p85PI3K), -p[Ser⁴⁷³]Akt (pAkt) or -p[Tyr²⁰⁴]ERK (pERK) antibodies. Only proteins that underwent significant modulation by any of the treatment protocols have their data graphically represented (in B and C). Open bars, control; darker shaded bars, MCH-ASO; lighter shaded bars, MCH. For all experiments, *n*=5; **P*<0.05 compared with control (C).

Figure 6 Evaluation of insulin signal transduction in brown adipose tissue of rats treated i.c.v. with saline (C), MCH-ASO (ASO) or MCH according to protocols described under Materials and Methods. Samples of brown adipose tissue protein extracts obtained from rats acutely treated iv with saline (–) or insulin (+) were directly separated by SDS-PAGE or employed in immunoprecipitation (IP) assays with anti-IR, -IRS-1 and -IRS-2 antibodies rendering immunoprecipitates that were thereafter separated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and blotted (IB) with anti-phosphotyrosine (PY), -p85PI3-kinase (p85PI3K), -p[Ser⁴⁷³]Akt (pAkt) or -p[Tyr²⁰⁴]ERK (pERK) antibodies. Only proteins that underwent significant modulation by any of the treatment protocols have their data graphically represented (in B and C). Open bars, control; darker shaded bars, MCH-ASO; lighter shaded bars, MCH. For all experiments, $n=5$; * $P<0.05$ compared with control (C).

muscle. In another study, Gasparetti *et al.* (2003) have shown that the exposure of rats to a cold environment modulates insulin action and this is associated with a tissue-specific modulation of insulin signal transduction. In a very elegant study, Crozier *et al.* (2003) provided evidence for a tissue-specific regulation of protein synthesis induced by insulin. Finally, in one of the first studies of insulin signal transduction in animal models of insulin resistance, Saad *et al.* (1992) reported a similar tissue specificity concerning the activation of elements of the insulin signaling cascade.

In addition to looking at the activation of the metabolic-controlling PI3-kinase/Akt pathway, we also evaluated the consequences of MCH and MCH-ASO i.c.v. treatment upon the growth- and mitogenic-controlling ERK pathway; we found that the inhibition of hypothalamic MCH expression by MCH-ASO promoted a significant increase in insulin-induced ERK activation in BAT, while i.c.v. MCH promoted a reduction in insulin-induced activation of ERK in WAT. MCH is known to act as an energy-sparing neurotransmitter (Pereira-da-Silva *et al.* 2003, Segal-Lieberman *et al.* 2003) and in a recent study we have shown that inhibition of MCH expression leads to the increase of uncoupling protein-1 (UCP-1) expres-

sion in BAT which is known to increase energy expenditure rates (Pereira-da-Silva *et al.* 2003). Considering that adrenergic stimulation of UCP-1 expression in BAT depends on ERK activation (Valladares *et al.* 2000), we suggest that the short-term treatment of rats with MCH-ASO promotes increased sympathetic signaling to BAT, which engages ERK. Conversely, the ability of MCH to reduce insulin-induced activation of ERK in WAT may be a distal consequence of impaired insulin action in this tissue. In fact, in another experimental model known to maintain increased MCH expression in the hypothalamus, i.e. cold-exposure, impairment of the insulin signal transduction through ERK is also observed (Gasparetti *et al.* 2003).

Finally, in addition to its effects upon insulin signal transduction, short-term i.c.v. MCH treatment promoted an interesting inhibition of glucose-stimulated insulin secretion. A detailed look at the glucose-induced insulin curve suggests that MCH promotes a blunting of the first phase, which is followed by a late increase in insulin secretion. Interestingly, the loss of first phase insulin secretion is a hallmark of insulin-resistance and type 2 diabetes (Steppel & Horton 2004). Although we have not evaluated the mechanism involved in this phenomenon

we believe it was mediated by neural inputs. In two recent studies we observed that during cold-exposure there is an increased expression of MCH in the hypothalamus of rats (Pereira-da-Silva *et al.* 2003). This is accompanied by a reduction in glucose-stimulated insulin secretion which is mediated by sympathetic but not by parasympathetic inputs (De Souza *et al.* 2003).

The present study does not discriminate as to whether the effects of short-term MCH treatment are due to regulation of neural connections from the hypothalamus to the peripheral organs or due to indirect mechanisms such as the control of peripheral hormonal levels. The fact that the treatment protocol was not acute would favor the possibility of an indirect effect. However, since the blood levels of corticosterone were not affected by the treatment and insulin levels were reduced, we believe that the case for the participation of neural mechanisms is strengthened. Moreover, since the period of treatment was very short, and no significant modulation of body weight was detected in MCH-treated rats, we can state that the effects of hypothalamic MCH upon insulin physiological activity and molecular signaling are independent of body mass gain. The present study provides important information to support a role for neural mechanisms in the induction of insulin resistance and development of diabetes. Moreover, MCH is ratified as a potential target for therapeutic actions in obesity and diabetes.

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