

# Pancreatic neuronal melanocortin-4 receptor modulates serum insulin levels independent of leptin receptor

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**Abstract** The leptin-regulated melanocortin (MC) system modulates energy homeostasis and hypothalamic MC neuronal circuits regulate insulin secretion. We therefore hypothesized that MC system components were present in the pancreas. In order to determine the veracity of the hypothesis, we examined c-Fos, melanocortin-4 receptor (Mc4r), and alpha-melanocyte-stimulating hormone ( $\alpha$ -MSH) expression levels in nondiabetic (intact leptin receptor signaling) and Zucker diabetic fatty (ZDF; leptin receptor deficiency) rats. We infused rats via the third ventricle with the  $\alpha$ -MSH analog Nle4, D-Phe7- $\alpha$ -MSH (NDP-MSH), a Mc4r agonist. Subsequently, both hypothalamic and pancreatic c-Fos and Mc4r mRNAs were upregulated. Likewise, immunohistochemical analysis showed that an increased Mc4r and  $\alpha$ -MSH expression in nerves surrounding the pancreatic vasculature and islets. Increases in c-Fos,  $\alpha$ -MSH, and Mc4r expression were independent of leptin receptor function. Conversely, serum insulin was significantly reduced by NDP-MSH treatment, an effect which was reversed by the Mc4r specific blocker HS014. Finally, proopiomelanocortin (POMC) mRNA, the precursor of  $\alpha$ -MSH, was detected by RT-PCR in pancreatic tissue homogenates. These findings suggest that pancreatic Mc4r and autonomic neurons participate in a

communication pathway between the central MC system and pancreatic islets to regulate insulin secretion.

**Keywords** Mc4r ·  $\alpha$ -MSH · NDP-MSH · Pancreas · Zucker diabetic fatty (ZDF) rats · Proopiomelanocortin

## Introduction

The melanocortin (MC) system regulates diverse array of physiological functions. The most important and best characterized functions of the MC system are maintenance of energy balance and insulin action [1–4]. This system consists of five, seven-transmembrane G protein-coupled melanocortin receptors (designated Mcr1–5), three melanocortin peptides  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte-stimulating hormones ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -MSH), the adrenocorticotrophic hormone (ACTH), and two endogenous antagonists, agouti, and agouti-related protein (AGRP) [1]. The  $\alpha$ -MSH [derived from proopiomelanocortin (POMC)] is a trideca-neuropeptide agonist for Mc4r. Among the five Mcrs, Mc4r is strongly implicated in regulation of energy homeostasis and autonomic nerves outflow functions [1, 5]. Although, the MC signaling cascades are recognized to work downstream of leptin signaling pathway [6], some data support the concept of additive or leptin-independent MC signaling [7, 8].

Several hypothalamic centers with Mc4r expression are critical in regulation of energy expenditure, feed intake [2], and insulin action (reviewed in [3, 9]). In addition to a key role in regulation of energy homeostasis [8], studies in wild-type, leptin deficient ob/ob mice, and in mice with diet-induced obesity showed that intracerebroventricular (icv) treatment with melanocortin peptide agonists regulates insulin action and reduces serum insulin levels

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[10–12]. For instance,  $\alpha$ -MSH enhanced insulin actions on glucose uptake and production [11], whereas synthetic  $\alpha$ -MSH analog MTII inhibited basal insulin secretion in a dose-dependent fashion when administered via an icv cannula [10]. These MTII findings were further solidified by observations of increased plasma insulin concentration in young lean Mc4r null mice before they were physically obese. Likewise, decreased serum insulin levels were shown in female mice with MSH overexpression [13]. Further, the inhibitory effects of the MC system on insulin release were direct and not secondary to a lowering of blood glucose [10].

The mechanism by which the MC system regulates serum insulin secretion is likely involves complex pathways. These may include neuroendocrine pathways as well as direct and indirect projections from POMC and/or Mc4r expressing neurons. Previously, POMC neurons in the hypothalamic arcuate nucleus were found to project directly to the thoracic intermediolateral cell column of the spinal cord where sympathetic pre-ganglionic neurons with localized Mc4r are located [14, 15]. These observations suggest that  $\alpha$ -MSH and/or Mc4r may regulate insulin secretion via post-ganglionic sympathetic fibers that may also express Mc4r and/or  $\alpha$ -MSH and directly project to the pancreas from celiacomesenteric ganglion and plexus (the relay center for the pancreas and other abdominal viscera). In one of the aforementioned studies, pharmacological inhibition of the sympathetic system partially abolished the MTII centrally induced serum insulin reduction [10]. This finding suggests direct involvement of the sympathetic system outflow to the pancreas, but does not rule out involvement of pancreatic autonomic neurons that express Mc4r and/or  $\alpha$ -MSH. Mc4r was, for example, shown in peripheral autonomic nerve endings located within organs, such as penile autonomic neurons that modulate sexual function in both rats and human [16].

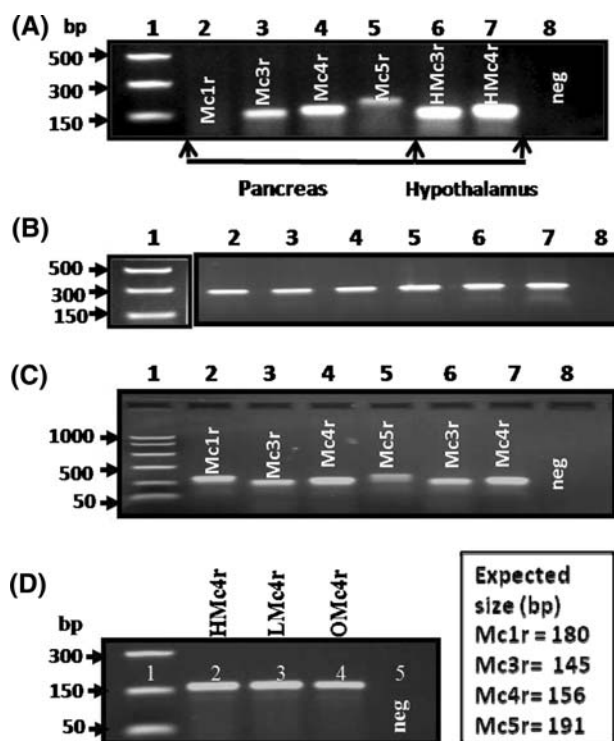
As pancreatic islets are the source of insulin production, and are richly innervated by autonomic nerves [17], the objectives of this study were to: (1) determine the presence and location of melanocortin components (Mc4r and  $\alpha$ -MSH) in the pancreas, (2) assess the acute bidirectional (activation versus inhibition) effects of the  $\alpha$ -MSH analog NDP-MSH, a potent Mc4r agonist, on insulin release, and (3) determine the role of leptin receptor in modulation of NDP-MSH effect on insulin release.

## Results

### Mc4r mRNA transcripts expressed in the pancreas

The objective of this experiment was to determine which Mc4r transcript is expressed in normal rat pancreas. This is

important because  $\alpha$ -MSH analogs, to a different degree, activate all Mc4r isoforms with the exception of Mc2r. Total RNA samples, obtained from normal rat pancreas (lean and/or ZDF), were analyzed separately and as pooled aliquots for presence of Mc4r-1 to -5 mRNA. Representative results in Fig. 1a showed that pancreatic expression of Mc3r, Mc4r, and Mc5r in pooled cDNA aliquots from lean rats. When samples from single rats were tested, a distinct band for each of the above three Mc4r isoforms was detected in six out of eight ZDF and lean rats irrespective of leptin receptor status. Mc1r was, however, not detected in any of the samples tested. S-15 housekeeping ribosomal gene was amplified in parallel from cDNA samples used in Fig. 1a to verify the integrity of cDNA (Fig. 1b). In addition, each primer pair was tested in parallel for the expected product



**Fig. 1** a 2% Agarose gel showing Mc4r isoforms in normal rat pancreas. Lane 1: DNA markers; lanes 2–5: pooled cDNA sample aliquots from lean rats amplified with rat primer pairs for Mc1r, Mc3r, Mc4r, and Mc5r, respectively. Lanes 6 and 7: Mc3r and Mc4r mRNA amplified from rat hypothalamus (HMc4r positive controls), respectively. Lane 8: negative control from pooled RNA (instead of cDNA) plus Mc4r primers. b S-15 housekeeping gene (361 bp) amplified in parallel from samples used in (a) to verify the integrity of cDNA. c Positive control cDNA samples run in parallel for Mc1r (skin), Mc3r (hypothalamus), Mc4r (hypothalamus), and Mc5r (prostate) to verify the expected product size for primer pairs used in (a). d A representative gel for Mc4r in pancreas of lean (LMc4r) and obese ZDF (OMc4r) rats. Lane 1: DNA markers; lane 2: HMc4r; lanes 3 and 4: Mc4r in lean and ZDF rats, respectively; lane 5: negative control as in a. Expected product size for Mc4r isoforms is shown on the right of the gel pictures

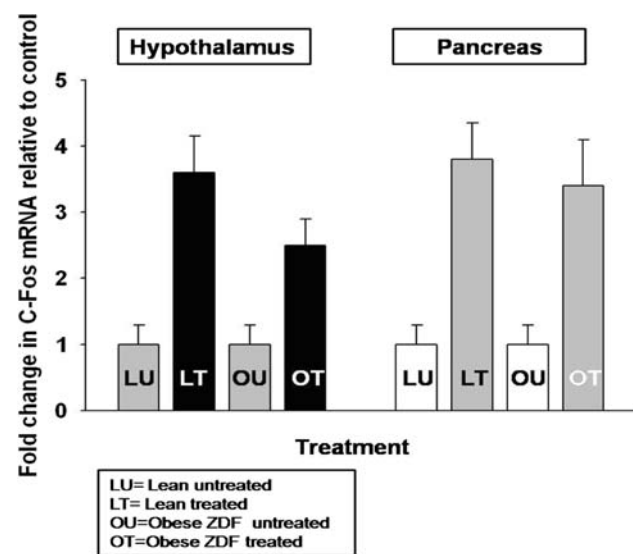
size using positive cDNA transcribed from rat total RNA samples obtained from Zyagen (San Diego, CA, USA). cDNA samples from skin (for Mc1r), rat hypothalamus (for Mc3r and Mc4r), and rat prostate (for Mc5r) were used (Fig. 1c). A representative gel for Mc4r in both lean and obese ZDF rats is shown in Fig. 1d.

#### Effect of acute NDP-MSH infusion on pancreatic and hypothalamic c-Fos and Mc4r mRNA

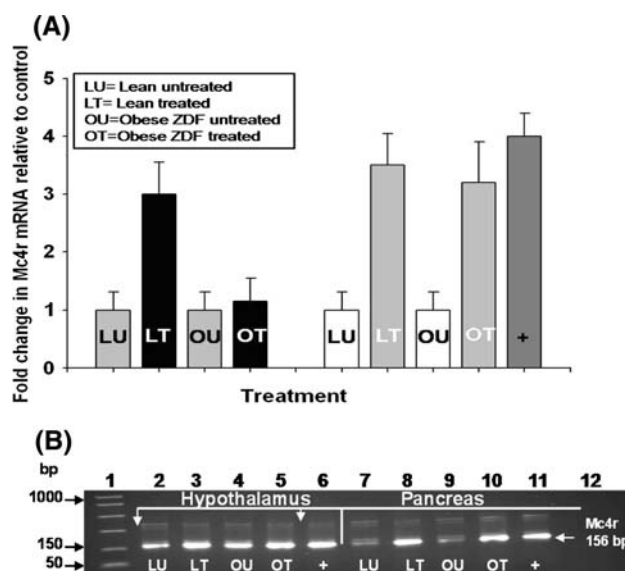
Acute icv infusion (60 min post-treatment) of NDP-MSH in the third ventricle increased c-Fos mRNA (a marker for neuronal activation) between 2 and 3 folds in both the hypothalamus and the pancreas of lean and ZDF-treated rats compared with their respective untreated controls (Fig. 2). Likewise, pancreatic Mc4r mRNA increased between 2 and 3 folds in the pancreas of lean and obese ZDF-treated rats (Fig. 3). Hypothalamic Mc4r mRNA, however, increased more than 2-fold in lean, but not in obese ZDF-treated rats. With the exception of hypothalamic Mc4r in ZDF rats, the increase in gene expression was thus not affected by the leptin receptor status.

#### Effect of NDP-MSH infusion on serum insulin level

Next, we determined the effect of hypothalamic injection of NDP-MSH on serum insulin levels. We compared serum insulin levels in pre- and 60 min post-NDP-MSH treatment. Our results showed that hypothalamic injection of NDP-MSH lowered serum insulin by 81% in lean rats with intact leptin receptor (Fig. 4a). Likewise, 61.4% serum



**Fig. 2** c-Fos mRNA is upregulated more than 3 folds in both hypothalamus and pancreas of lean and over 2–3 folds in obese ZDF rats 60 min post-NDP-MSH treatment. cFos mRNA level in untreated control rats was set at 1.  $n = 4–8$



**Fig. 3** **a** Hypothalamic and pancreatic Mc4r mRNA changes in treated lean and obese ZDF rats compared with their respective untreated controls. Levels of mRNA were determined 60 min post-NDP-MSH treatment. **b** Mc4r mRNA changes shown in (a) were further verified by conventional RT-PCR generated from combined cDNA samples of treatment groups. Upper band in gel represents a ribosomal housekeeping-gene (S-15, 361 bp) co-amplified with Mc4r mRNA (156 bp). Lane 1: DNA markers; lanes 2–5: hypothalamic Mc4r; lane 6: positive hypothalamic cDNA control; lanes 7–10: pancreatic Mc4r; lane 11: positive pancreatic cDNA control; lane 12: Negative reverse transcriptions (RT) control (RNA instead of cDNA).  $n = 4–8$

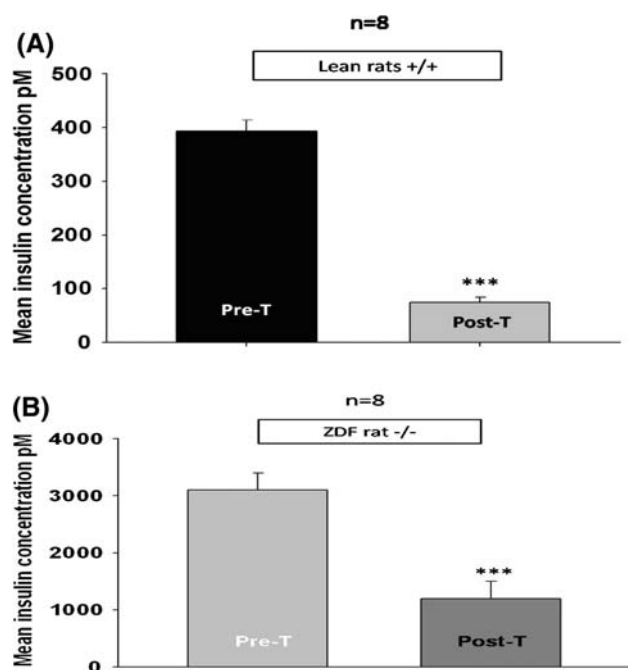
insulin reduction was detected in the hyperinsulinemic ZDF rats with nonfunctional leptin receptor (Fig. 4b).

#### Reversal of suppressive effects of NDP-MSH on insulin serum concentration with Mc4r specific blocker HS014

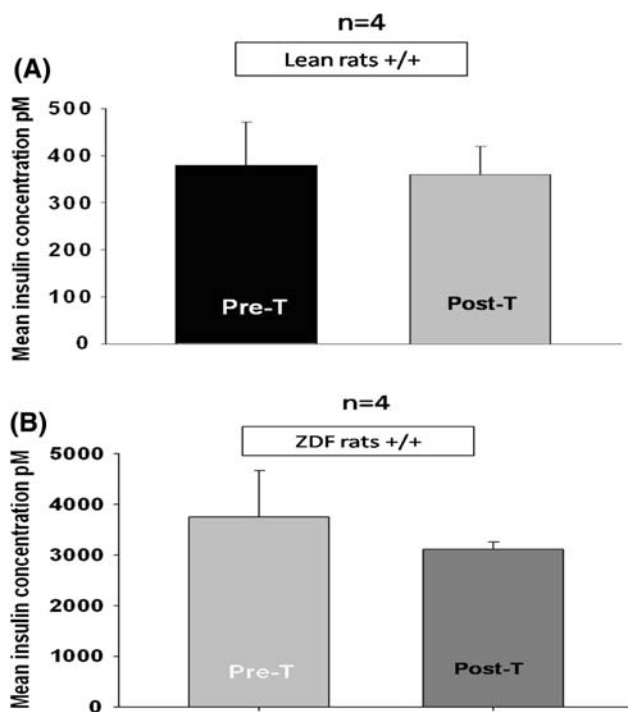
In order to determine the specificity of Mc4r-induced suppression of serum insulin concentration, Mc4r-specific blocker HS014 was used to blunt the NDP-MSH effects. As shown in Fig. 5a and b, pre-treatment with HS014 reversed the suppressive effects of NDP-MSH on basal insulin release by 95% in lean rats (Fig. 5a) and by 84% in hyperinsulinemic ZDF-treated rats (Fig. 5b).

#### Immunohistochemical localization of Mc4r protein and $\alpha$ -MSH peptide in pancreatic neurons and around islets

Immunohistochemical staining with rat Mc4r antibodies showed that localization of Mc4r to pancreatic islets and neurons around vasculature, with increased staining intensity of Mc4r protein in NDP-MSH-treated rats (Fig. 6, 7a, 8a). Dual staining with Mc4r (red color) and neuron-specific beta III tubulin (green color) antibodies showed that Mc4r and beta tubulin III proteins colocalized to nerve



**Fig. 4** Acute icv injection of NDP-MSH potentially suppressed serum insulin 60 minutes post injection (\*\* $P < 0.001$ ) in lean (a) and obese ZDF rats (b) with intact (LepR +/+) and nonfunctional (LepR -/-) leptin receptors, respectively. Pre-T = pretreatment, Post-T = post-treatment.  $n = 8$  per group. Data are expressed as mean  $\pm$  SE



**Fig. 5** Mc4r specific blocker HS014 blunted the NDP-MSH induced reduction in serum insulin ( $P > 0.05$ ) in lean (a) and ZDF rats (b). Pre-T and Post-T = pre- and post-treatment.  $n = 4$  per group. Data are expressed as mean  $\pm$  SE

fibers (Fig. 6, panel 2, further magnified in 2b). No difference in staining intensity of Mc4r was discernable between lean and obese ZDF-treated rats (Fig. 6, panel 5 vs. 6, Fig. 7a) suggesting lack of leptin receptor effect. In addition, there was no staining detected in the exocrine pancreatic serous acini (Figs. 6 and 7a). Consistently, Mc4r was localized in nerve fibers around pancreatic islets (Fig. 6, panels 5 and 6) and vasculature (Fig. 7a).

Like Mc4r protein,  $\alpha$ -MSH peptide was localized in neurons around vasculature and pancreatic islets (Fig. 7b). Pancreatic neurons that express  $\alpha$ -MSH were co-stained with neuron-specific beta III tubulin (Fig. 7c). Statistical analysis of Mc4r (Fig. 8a) and  $\alpha$ -MSH (Fig. 8b) staining intensity in treated and control untreated rats showed that a significant increase of intensity in treated rats irrespective of leptin receptor status ( $P < 0.001$ ).

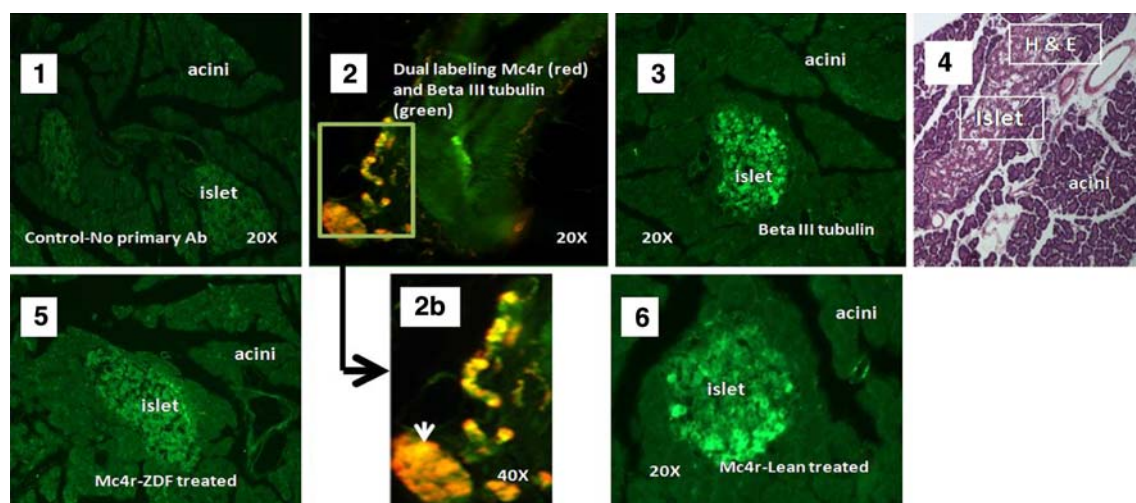
#### Detection of POMC mRNA in pancreatic tissue homogenate

Proopiomelanocortin (POMC) mRNA was detected with RT-PCR in pancreatic cDNA samples obtained from treated and untreated Zucker rats irrespective of leptin receptor status (Fig. 9a–c). cDNA samples from the pituitary gland and hypothalamus were included as positive controls in the RT-PCR analysis (Fig. 9a, b). Analysis of variance of mRNA intensity data in ZDF and lean-treated rats and their respective untreated controls showed that no significant differences as the result of leptin receptor status or treatment (Fig 9c;  $P > 0.05$ ).

#### Discussion

The primary objective of this study was to investigate presence of the MC system components in the pancreas of hyperinsulinemic leptin receptor deficient rats. The findings reported here shed light on possible downstream mechanisms by which the central MC system modulates insulin release under leptin receptor-dependent and -independent conditions. Using protein and RNA indices, we showed that POMC mRNA, the precursor for  $\alpha$ -MSH, and Mc4r protein as well as  $\alpha$ -MSH peptide were present in the pancreas of diabetic and nondiabetic Zucker rats. Both Mc4r and  $\alpha$ -MSH were colocalized in autonomic neurons located around vasculature and pancreatic islets. Further, the study showed that central activation of hypothalamic Mc4r with NDP-MSH triggered downstream activation of pancreatic autonomic neurons as shown by increased c-Fos mRNA level in both the hypothalamus and pancreas. In addition, Mc4r mRNA and Mc4r protein and  $\alpha$ -MSH peptide were increased. These effects were associated with a potent reduction in serum insulin independent of leptin





**Fig. 6** Representative immunohistochemical micrographs showing pancreatic islets marked with Mc4r and/or neuron-specific beta III tubulin antibodies. *Panel 1*: negative control (minus primary antibody); *panel 2* (magnified in 2b): dual labeling Mc4r (red) and beta III tubulin (green); *panel 3*: islet marked with beta III tubulin;

*panel 4*: pancreatic section stained with hematoxylin and eosin for demonstration of normal histology; *panels 5 and 6*: Mc4r protein in ZDF and lean rats 60 min post-treatment, respectively. White arrow points to nerve fibers

receptor function. Collectively, the data suggest a neuronal communication pathway that involves interaction between central and peripheral neuronal pancreatic MC system. The study also added further weight to previous findings that showed that dose-dependent inhibition of serum insulin in ob/ob leptin deficient mice treated centrally with  $\alpha$ -MSH and/or synthetic MTII peptides [10, 11].

The increased expression of c-Fos mRNA in the pancreas of NDP-MSH-treated rats implied that acute stimulation (60 min post-treatment) of hypothalamic Mc4r caused increased activity in both hypothalamic and peripheral autonomic nerve activity in nerve fibers that project to the pancreas. In turn, this effect was associated with upregulation of pancreatic Mc4r and  $\alpha$ -MSH. Importantly, these results were seen in both lean and ZDF-treated rats suggesting a leptin receptor-independent MC effect. This finding is in agreement with data showed that part of MC-mediated energy homeostasis is either independent of or additive to leptin [7].

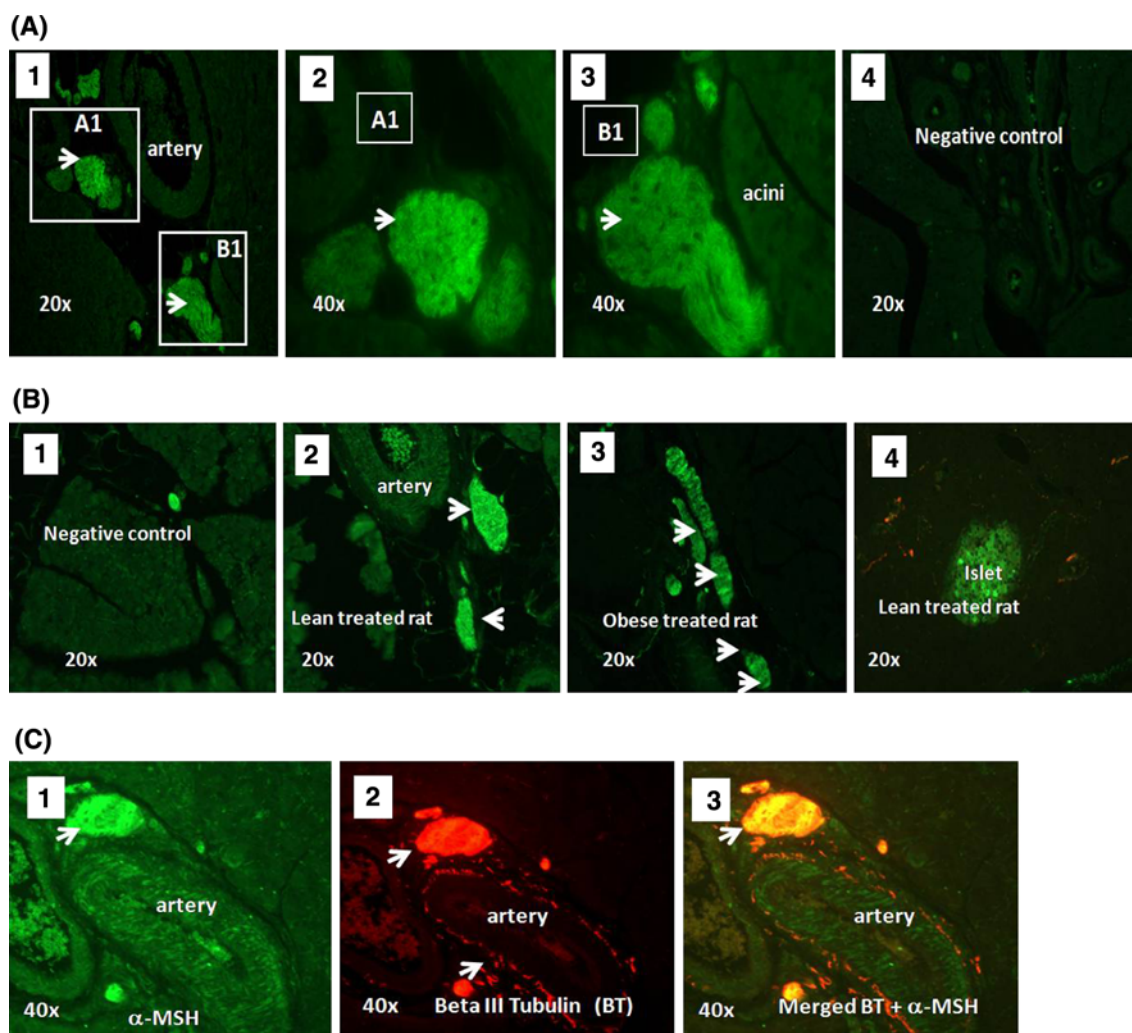
The failure of NDP-MSH treatment to increase hypothalamic Mc4r in ZDF rats with deficient leptin receptor, in contrast with increased expression in lean rats with intact leptin receptor, suggests high occupancy of Mc4r with Mc4r endogenous antagonist AGRP that may have prevented additional activation of Mc4r in ZDF rats. This explanation is supported by a previous study that comparatively analyzed hypothalamic neuropeptides in Zucker lean and ZDF rats, and showed that increased expression of the AGRP in ZDF compared with lean rats [18]. The increased pancreatic Mc4r despite the lack of a corresponding effect on hypothalamic Mc4r in ZDF rats could be explained with previous studies showed that lack of

AGRP in the pancreas [19]. Further, the inhibition of AGRP on  $\alpha$ -MSH action is CNS-specific to hypothalamic Mc3r and Mc4r due to the overlapping distribution of AGRP and POMC expressing cell bodies [20, 21].

The increased in c-Fos expression in the hypothalamus concurs with a previous study showed that a 254% increase in c-Fos-like immunoreactivity in the hypothalamic paraventricular nucleus of Long-Evans rats treated with  $\alpha$ -MSH via an icv route [22].

Hypothalamic icv administration of NDP-MSH potentially suppressed serum insulin secretion independent of leptin receptor. This finding is analogous to previous studies that used a different Mc4r agonist where direct hypothalamic infusion of 1 nM MTII-reduced serum insulin level by 72.8% in hyperinsulinemic leptin deficient ob/ob mice [10]. Conversely, pharmacological inhibition of central MC signaling in rats with ICV administration of the MC receptor antagonist SHU9119 resulted in 259% increase in serum insulin [23]. Inhibition of the sympathetic system by an  $\alpha$ -adrenoreceptor antagonist in one of the aforementioned studies [10] blocked the inhibitory effect of MTII on insulin release suggesting involvement of the sympathetic system. The specific localization of Mc4r and  $\alpha$ -MSH in sympathetic or parasympathetic system was not addressed in our study.

The suppressive effect of the MC system on insulin release in this and previous pharmacological studies is supported by genetic findings where permanent absence of Mc4r signaling is associated with hyperinsulinemia [24]. The level of fasting serum insulin in Mc4r knockout mice was also dependent on Mc4r gene level. Animals heterozygous for Mc4r had intermediate insulin level compared



**Fig. 7** Representative micrographs for Mc4r (a),  $\alpha$ -MSH (b), and merged  $\alpha$ -MSH plus neuron-specific beta III tubulin (c) expressed around pancreatic vasculature and islets in rats treated with NDP-MSH for 10 days. Insert boxes (A1 and B1) are magnified in panels

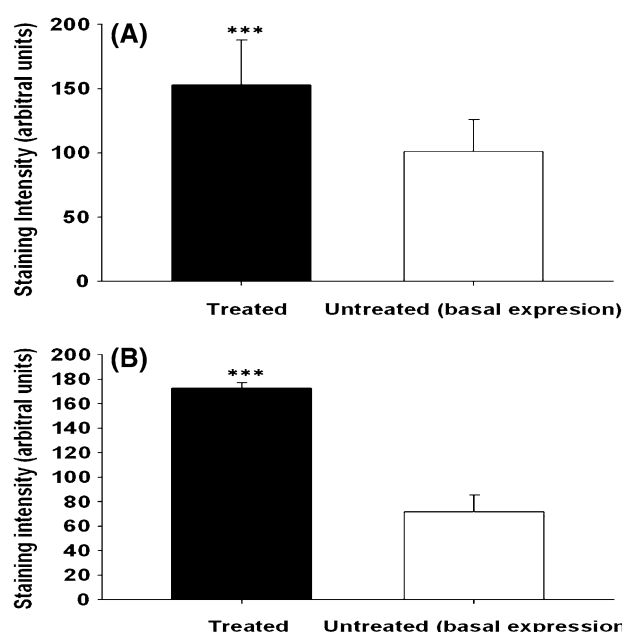
2A and 3A to show the typical wavy appearance of nerve fibers. *White arrows* indicate nerve fibers. Negative control sections were run with omission of the primary antibody

with homozygous null or wild-type Mc4r [24]. In addition, mapping of Mc4r mRNA distribution in rodent brain demonstrated that the expression of Mc4r in several hypothalamic nuclei involved in autonomic and endocrine function, such as the arcuate, paraventricular nucleus, dorsal motor nucleus of the vagus, and the raphe [25]. The mapping and changes in Mc4r and  $\alpha$ -MSH in pancreatic autonomic neurons imply possible involvement of central MC in regulation of peripheral pancreatic neuronal activity. Rich autonomic innervations of islets are known to contribute to stress and physical exercise (sympathetic) and to the cephalic phase of insulin secretion [17].

While NDP-MSH induced the expression of  $\alpha$ -MSH and Mc4r in the absence of a functional leptin receptor, the treated ZDF rats remain hyperinsulinemic. This suggests that both leptin-dependent and independent pathways may

be important in insulin regulation. Alternatively, the central MC involvement in the regulation of insulin secretion under leptin receptor-deficient status may be partially compromised.

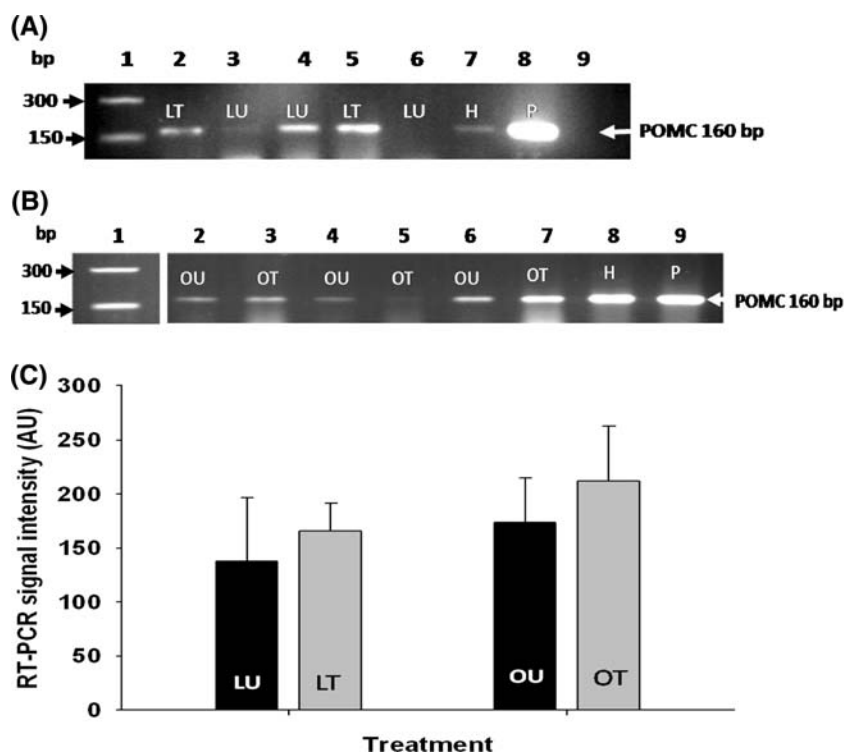
Using a ribonuclease protection assay a previous report showed that lack of Mc4r mRNA, but in the presence of Mc5r in the pancreas of normal rats [26]. The discrepancy between the above report and our findings may be due to differences in the sensitivity and specificity of the techniques used. It is well-established that RT-PCR is a much more sensitive technique than ribonuclease protection assay for quantification of mRNA [27]. The detection of Mc4r mRNA, Mc4r protein, and  $\alpha$ -MSH peptide in the pancreas reported here is in line with previous report showed that wide  $\alpha$ -MSH binding in the pancreas when an enzymatic resistant  $^{125}$ I-labeled NDP-MSH peptide was



**Fig. 8** Graphic depiction of immunohistochemical staining intensity level of Mc4r (a), and  $\alpha$ -MSH (b) in NDP-MSH-treated and untreated rats. Treatment resulted in significant increase in Mc4r and  $\alpha$ -MSH expression irrespective of leptin receptor status ( $P < 0.001$ ).  $n = 4-7$

injected intravenously in rats and mice and later traced to the pancreas [28]. Furthermore, reverse-phase high performance liquid chromatography and radioimmunoassay findings showed that high  $\alpha$ -MSH peptide concentration in pancreatic tissue homogenates obtained from rats [29].

**Fig. 9** POMC mRNA expression in pancreas of lean-treated (LT) and lean untreated (LU) control rats (a). POMC mRNA expression in obese ZDF-treated (OT) and obese ZDF untreated (OU; b). Treatment of NDP-MSH was for 10 days. Control cDNA samples from rat hypothalamus (H) and pituitary gland (P) were included as positive controls. Each band represents an individual rat.  $n = 5$  for treated and 6 for untreated. Graphic representation of POMC mRNA band intensity levels in LT, LU, OT, and OU shown as arbitrary units (AU; c)



As the NDP-MSH peptide used in this study may stimulate the activity of hypothalamic Mc3r [25], activation of central Mc3r by our treatment is possible, since these two receptors colocalize in the same hypothalamic nuclei [9].

Further, the detection of Mc3r in the pancreas in our study and as reported by other [30] requires further investigation for the role, if any, of Mc3r in insulin regulation. However, the over 80–95% reversal effect of the NDP-MSH effect on insulin release with HS014 Mc4r specific blocker in our study suggests a predominating effect for Mc4r action.

This finding tightly agrees with well-characterized role of Mc4r in energy balance and insulin action compared with Mc3r enigmatic functions [1]. In conclusion, the results presented here demonstrate a biological role for peripheral  $\alpha$ -MSH and Mc4r in pancreatic neurons. The presence of active pancreatic Mc4r may also have antiinflammatory benefit as  $\alpha$ -MSH was shown to protect pancreatic islets from cytotoxic injury produced by inflammatory cytokines [31].

## Materials and methods

### Animals

Male obese Zucker Diabetic Fatty (ZDF, fa/fa) rats and their age-matched littermates lean control animals (ZDF

lean, fa/+ or +/+) were obtained from Charles River Laboratories (Charles River, MA, USA) at 7 weeks of age. The fa/fa ZDF rats lack a functional leptin receptor and became hyperphagic and hyperinsulinemic when fed Purina 5008 high fat diet (Purina Mills, Richmond, IN, USA). Lean rats were fed regular rat chow, and were eu-insulinemic. Rats were maintained under standard housing conditions (constant temperature of 22°C, ad libitum food and water, and 12:12 h light/dark cycles) at an AAALAC-accredited lab animal facility at the College of Veterinary Medicine, Auburn University, Alabama.

### Experimental design

Three experiments with a  $2 \times 2$  factorial design were performed. In experiment 1, 12 lean nondiabetic rats (with intact leptin receptor), and 12 obese ZDF rats (with non-functional leptin receptor) were implanted with an icv cannula in the third brain ventricle. Rats were divided into four groups that included lean-treated (8 rats, LT), lean untreated (4 rats, LU), obese ZDF-treated (8 rats, OT), and obese ZDF untreated (4 rats, OU). Treated rats received 0.1 nM NDP-MSH per rat, whereas control rats received sterile 0.9% saline. In experiment 2, 4 lean and 4 obese ZDF rats were injected with 10  $\mu$ l sterile saline containing 4.8 nM HS014 via ICV route (Mc4r specific antagonist, Sigma-Aldrich, St Louis, MO, USA) followed 10 min later with NDP-MSH infusion to blunt the effect of NDP-MSH on serum insulin inhibition.

In experiment 3, 4 lean and 4 ZDF rats received 1 nM NDP-MSH per rat daily for 10 successive days. Similar number of lean and ZDF rats received vehicle. The NDP-MSH dose effect on insulin and the effectiveness of icv cannulation were initially tested using Sprague-Dawley rats. NDP-MSH infusions were performed in 10  $\mu$ l volumes using a microinfusion syringe pump (KD Scientific, Holliston, MA, USA). Serum was obtained from saphenous and/or tail vein before rat treatment, and from the trunk following treatment and euthanasia. Isolated sera were stored at  $-30^{\circ}\text{C}$  for insulin assay.

The pancreas and hypothalamus were harvested and used for RNA isolation and immunohistochemistry (IHC) analyses. Tissues intended for RNA isolation were flash frozen in liquid nitrogen, and those intended for IHC were fixed in 4% paraformaldehyde. All animal procedures were approved by the Institutional Animal Care and Use Committee at Auburn University.

### Surgical placement of ICV cannula

Third ventricle cannulation was accomplished by a modification of a procedure previously established by our group [32]. Briefly, rats were anesthetized by intraperitoneal

injection of ketamine–xylazine (100 mg/kg ketamine and 1 mg/kg xylazine) and placed in a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA, USA). After a surgical plane of anesthesia was attained, the skin over the dorsal surface of the skull was prepared for surgery, and the animal was placed into a stereotaxic frame. A median longitudinal skin incision was made over the dorsal bregmal region of the skull to expose the landmarks of the cranium (bregma and lambda). A 22-gauge stainless steel guide cannula (Plastic One, Roanoke, VA, USA) was implanted into the third ventricle.

Coordinates used were 1.8 mm posterior to bregma, 0.0 mm lateral to the midline, and 8.6 mm ventral to the skull. The guide cannula (10 mm in length) was secured to the skull with four stainless steel screws and acrylic dental cement. A removable 11 mm “dummy” cannula that extended 1 mm beyond the guide cannula was inserted to maintain patency. Rats were placed individually in hanging wire cages and allowed 4–5 days to recover. Cannula placement was confirmed by administration of angiotensin II (American Peptide, Sunnyvale, CA, USA, 50 ng/rat in 6  $\mu$ l volume) to stimulate water consumption. Animals that consumed  $\geq 5$  ml of water within approximately 15–20 min were considered positive.

### Total RNA isolation

Total RNA was isolated using TRIzol reagent (Invitrogen-Life Technologies Inc., Carlsbad, CA, USA), according to the manufacturer’s protocol and as described previously in our laboratory [33]. RNA concentrations were determined at 260 nm wavelength and the ratio of 260/280 was obtained using UV spectrophotometry (DU640, Beckman Coulter Fullerton, CA, USA). The integrity of RNA, indicated by the presence of intact 28S and 18S ribosomal RNA, was verified by 2% denaturing agarose gel electrophoresis. RNA samples were treated with DNase (Ambion-AB Applied Biosystems Inc.) to remove possible genomic DNA contamination according to the manufacturer’s protocol. First strand cDNA synthesis was performed using 2  $\mu$ g total RNA using cDNA first strand synthesis kit (SABiosciences, Frederick, MD, USA).

### PCR and agarose gel electrophoresis

Melanocortin-4 receptor (Mc4r), POMC, and c-Fos mRNA were amplified with validated rat specific primer sets (SABiosciences) according to the manufacturer’s protocol. Quantitative real-time PCR for Mc4r and c-Fos was performed in 25  $\mu$ l reaction mixture containing 12.5  $\mu$ l RT2 Real-Time SYBR/Fluorescein Green PCR master mix (SABiosciences), 1  $\mu$ l first strand cDNA, 1  $\mu$ l RT2 validated primer sets, and 10.5  $\mu$ l PCR-grade water. Reactions



were run in 96-well PCR plates using Bio-Rad PCR cycler (Bio-Rad, MyiQ<sup>TM</sup>, Hercules, CA, USA). Reactions were run in duplicates and the results were normalized to GAPDH housekeeping-gene expression. The amplification protocol was set at 95°C for 15 min, and 40 cycles each at (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s) followed by a melting curve set between 55 and 95°C to ensure detection of a single PCR product. Conventional RT-PCR was used for detection of Mcr isoforms, POMC mRNA, and for verification of Mc4r mRNA real-time PCR data. RT-PCR was performed using Reaction Ready<sup>TM</sup> Hot Start “Sweet” PCR Master Mix (SABiosciences). PCR products were viewed on 2% agarose gel. Staining intensity of the RT-PCR bands was determined by the UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT, USA).

### Immunohistochemistry

Immunohistochemical localization of Mc4r and/or  $\alpha$ -MSH was performed by a modification of an immunohistochemical technique previously described by our laboratory [33]. Briefly, sections from the pancreas were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, and cut at 5- $\mu$ m thickness. Serial sections from each block were stained with hematoxylin and eosin. For IHC staining, sections were deparaffinized in Hemo-D (Scientific Safety Solvents, Keller, TX, USA) and hydrated in distilled water. Antigen retrieval was performed in 10 mM boiling citrate buffer. Sections were incubated in 5% normal goat serum containing 2.5% BSA to reduce nonspecific staining. Mc4r was detected with mouse anti-Mc4r antibody (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:80 in blocker. Likewise,  $\alpha$ -MSH was detected with mouse/rat  $\alpha$ -MSH antibody (Phoenix Pharmaceutical Inc, Burlingame, CA, USA) using 1:400 dilution. Nerves were marked with mouse monoclonal antibody to neuron-specific beta III tubulin (Covance, Inc., Denver, PA, USA) using 1:6000 dilution. The antibody–antigen complexes were visualized with Alexa 488 (red) and Alexa 595 (green)-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) using 1:500 dilution. Sections were examined with a Nikon TE2000E microscope and digital images were made with an attached Retiga EX CCD digital camera (Q Imaging, Burnaby, BC, Canada).

### Insulin concentration

Serum insulin concentration was determined using a rat endocrine LINCOplex kit (Millipore, Billerica, MA, USA) in a 96-well plate format. Assay plates were read using a BioPlex reader according to the manufacturer’s

instructions (Bio-Rad, Hercules, CA, USA). Briefly, pre-treatment and 60 min post-treatment sera were assayed in 10  $\mu$ l volumes in duplicates. A standard curve was generated using duplicate standard insulin samples from the LINCOplex kit with concentration ranging between 0 and 4500 pM. Serum insulin was captured with rat insulin antibody linked to internally dyed beads. A secondary biotinylated antibody was added, and the reaction was detected with streptavidin–phycoerythrin using a dual-laser flow-based reader (Bio-Plex system, Bio-Rad, Carlsbad, CA, USA).

### Statistical analysis

Analysis of real-time PCR data was performed using a modification of the delta delta Ct method ( $\Delta\Delta$  CT) [34, 35].  $\Delta$ CT calculated from real-time PCR data were subjected to analyses of variance using Sigma Stat statistical software (Jandel Scientific, Chicago, IL, USA).  $\Delta$  CT for target (cFos and/or Mc4r) genes in control cDNA samples (from untreated rats) and from NDP-MSH-treated rats was calculated by subtraction of the threshold cycle (CT) of the reference gene (GAPDH) from the CT value of the target (Mc4r/cFos) for normalization of CT values. Expression level (fold changes) were calculated using a formula of  $2^{x-y}$ , where  $x$  is the normalized CT value obtained from NDP-MSH-treated rats for each primer set and  $y$  is the normalized CT value obtained from control untreated rats. RT-PCR intensity data were initially subjected to analysis of variance, and when no differences due to leptin receptor status are found, a T-test was used to analyze difference between treated and untreated rats. Insulin data in pre- and post-treated rats were analyzed using a T-test. Differences between pre-treatment and post-treatment serum insulin levels where  $P < 0.05$  were considered significant. When data were not distributed normally, or heterogeneity of variance was identified, analyses were performed on transformed data or ranked data.

For determination of IHC staining intensity, antibody-stained photographs were captured using a microscope equipped with epifluorescence and NIKON imaging software. Assuming that the intensity of fluorescence was equal to the concentration of receptor or protein expressed, the intensity of the fluorescence on pancreatic nerve fibers was determined in treated and nontreated animals. The area of greatest intensity on each image was determined using the threshold function of Photo Shop CS3. Once the maximum intensity levels were recorded for each image, the data was analyzed using the Statistical Analysis Software program. The significance of the signal intensity was determined using the mixed model for multiple variables test in this program.

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