ISSN 2472-1972

Effects of Naltrexone on Energy Balance and Hypothalamic Melanocortin Peptides in Male Mice Fed a High-Fat Diet

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The hypothalamic melanocortin system composed of proopiomelanocortin (POMC) and agouti-related protein (AgRP) neurons plays a key role in maintaining energy homeostasis. The POMC-derived peptides, α-MSH and β-EP, have distinct roles in this process. α-MSH inhibits food intake, whereas β-EP, an endogenous opioid, can inhibit POMC neurons and stimulate food intake. A mouse model was used to examine the effects of opioid antagonism with naltrexone (NTX) on *Pomc* and *Agrp* gene expression and POMC peptide processing in the hypothalamus in conjunction with changes in energy balance. There were clear stimulatory effects of NTX on hypothalamic *Pomc* in mice receiving low- and high-fat diets, yet only transient decreases in food intake and body weight gain were noted. The effects on *Pomc* expression were accompanied by an increase in POMC prohormone levels and a decrease in levels of the processed peptides α-MSH and β-EP. Arcuate expression of the POMC processing enzymes *Pcsk1*, *Pcsk2*, and *Cpe* was not altered by NTX, but expression of *Prcp*, an enzyme that inactivates α-MSH, increased after NTX exposure. NTX exposure also stimulated hypothalamic *Agrp* expression, but the effects of NTX on energy balance were not enhanced in *Agrp*-null mice. Despite clear stimulatory effects of NTX on *Pomc* expression in the hypothalamus, only modest transient decreases in food intake and body weight were seen. Effects of NTX on POMC processing, and possibly α-MSH inactivation, as well as stimulatory effects on AgRP neurons could mitigate the effects of NTX on energy balance.

Freeform/Key Words: POMC, AgRP, α-MSH, β-EP, naltrexone

The hypothalamic melanocortin system, comprising proopiomelanocortin (POMC) and agouti-related protein (AgRP)–expressing neurons located in the arcuate nucleus (ARC) [1, 2] and downstream melanocortin receptor expressing neurons, plays a key role in regulating energy balance and metabolism [3–5]. The POMC-derived peptide α-melanocyte stimulating hormone (α-MSH) inhibits food intake and stimulates energy expenditure via activation of MC4-R–expressing neurons, whereas AgRP antagonizes the effect of α-MSH on MC4-R, causing an increase in food intake and a decrease in energy expenditure [6]. Although the primary effects of POMC on energy balance are thought to be mediated by α-MSH, there is evidence that another POMC-derived peptide, β-endorphin (β-EP), an endogenous opioid peptide, can affect feeding behavior. Endogenous opioid peptides, including β-EP, dynorphins, and enkephalins, have distinct effects on feeding behavior mediated by brain μ, κ, and δ opioid receptors, respectively [7–9]. When injected into the brain in rats, β-EP has short-term stimulatory effects on food intake and can also antagonize the

Abbreviations: AgRP, agouti-related protein; AH, anterior hypothalamus; ARC, arcuate nucleus; BW, body weight; KO, knockout; MBH, medial basal hypothalamus; MOR, μ opioid receptor; NC, normal chow diet; NTX, naltrexone; POMC, proopiomelanocortin; PRCP, prolylcarboxypeptidase; WT, wild type; α-MSH, α-melanocyte stimulating hormone; β-EP, β-endorphin.

Received 20 November 2018
Accepted 23 January 2019
First Published Online 28 January 2019

March 2019 | Vol. 3, Iss. 3
doi: 10.1210/js.2018-00379 | Journal of the Endocrine Society | 590–601

inhibitory effects of α-MSH on food intake [10]. The opioid antagonist naltrexone (NTX) has high affinity for the μ opioid receptor (MOR) and can inhibit food intake, especially in rat models [11].

Hypothalamic POMC and AgRP neurons express the MOR [12–14]. Electrophysiological studies show that the MOR on POMC neurons functions as an autoinhibitory receptor in response to the release of β-EP [15], β-EP has also been shown to inhibit AgRP neurons [14, 16]. NTX has well-established stimulatory effects on POMC neurons and it has been postulated that NTX decreases food intake in part by stimulating POMC-derived α-MSH release [17]. We have shown that NTX stimulates hypothalamic POMC mRNA expression and that this is associated with a marked decline in the concentrations of α-MSH and β-EP in the hypothalamus [18]. We have also shown that NTX stimulates POMC peptide release from the hypothalamus *in vitro* [19]. The decrease in peptide content could thus result from increased peptide release, but an effect on POMC processing is also a possibility. In those studies, effects of NTX on POMC were not correlated with changes in food intake or energy balance. NTX has also been shown to affect feeding in humans, but the effects are not very robust [20].

In this study, we used a mouse model to examine the effects of NTX on POMC gene expression and peptide processing in conjunction with changes in food intake and body weight (BW) of mice fed low- and high-fat diets. Processing was assessed by measuring hypothalamic levels of the POMC prohormone and processed peptides and expression of POMC processing enzymes. Levels of prolylcarboxypeptidase (PRCP), an enzyme that inactivates α-MSH, were also measured [21]. We also examined the effects of NTX on hypothalamic *Agrp* expression, given the possibility that stimulation of AgRP neurons by NTX could mitigate the stimulatory effects of POMC on energy balance. Finally, the effects of NTX were examined in *Agrp*-null mice to determine if NTX would be more effective in suppressing food intake and weight gain in the absence of AgRP.

1. Materials and Methods

A. Animals

All animal experiments were approved by the Columbia University Institutional Animal Care and Use Committee. All animals were housed in a pathogen-free barrier facility and all manipulations were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Adult male C57BL/6J mice were purchased from Jackson Laboratories, Farmington, CT and studied at 3 months of age for most of the experiments. *Agrp* knockout (KO) mice were originally obtained from Dr. Lex Van der Ploeg and studied on a C57BL/6J background [22]. Male $Agrp^{-/-}$ and wild-type (WT) littermates were studied at 5 months of age. Before the experiments, the mice were exposed to repeated handling to minimize stress during the experimental protocol.

Naltrexone hydrochloride (catalog no. 16676-29-2; Tocris Bioscience, Minneapolis, MN) was dissolved in normal saline and infused via subcutaneously implanted Alzet osmotic minipumps (Model 1007D; Durect Corp, Cupertino, CA) for 7 days. Control animals were infused with saline. Minipumps were implanted while isoflurane anesthesia was administered to the mice.

In an initial experiment, animals were studied on a normal chow diet with different doses of NTX and no statistically significant inhibition of food intake, and BW gain was observed. Subsequently, NTX infusion studies were performed while simultaneously switching animals from the normal chow diet (NC; 13% kcal from fat; diet no. 5053; Research Diets, New Brunswick, NJ) to a 60% high-fat diet (HFD; diet no. D12492; Research Diets). NTX was infused at a dose of 20/mg/kg/d for 7 days. In one experiment, mice were maintained and studied with NTX or saline on a breeder chow diet (22% kcal from fat; diet no. 5058; Research Diets). Animals were euthanized at the end of the NTX or saline infusion period, between 9 AM and 11 AM.

B. Experimental Protocols

B-1. Experiment 1: Effects of saline or NTX infusion on an HFD vs NC diet

The effects of NTX (20 mg/kg/d) or saline infusion for 7 days were studied in C57BL/6 male mice that were simultaneously switched from the NC to HFD; a control saline group continued receiving the NC. Three groups of animals were studied: Saline NC (n = 12), Saline HFD (n = 14), and NTX HFD (n = 15). At the time of euthanization, half of the animals from each group were used for hypothalamic mRNA isolation and quantitation, and the other half were used for POMC peptide measurements.

B-2. Experiment 2: Effects of saline or NTX infusion in Agrp$^{-/-}$ and WT mice

The effects of NTX or saline infusion for 7 days were studied in *Agrp*$^{-/-}$ and WT mice during the switch to the HFD to see if there was a more robust effect of NTX on feeding in *Agrp*$^{-/-}$ mice compared with the WT controls. Four groups of animals were studied: Saline-WT (n = 8), NTX-WT (n = 9), Saline *Agrp*$^{-/-}$ (n = 8), and NTX-*Agrp*$^{-/-}$ (n = 9). At the time of euthanization, half of the animals from each group were used for hypothalamic mRNA isolation and quantitation and half were used for POMC peptide measurements.

B-3. Experiment 3: Effect of saline or NTX infusion on POMC processing enzymes in the ARC of the hypothalamus and other hypothalamic regions

C57BL/6J mice were maintained on a breeder chow before and during NTX (n = 8) or saline (n = 8) infusion for 7 days. The hypothalamus of each animal was processed for both mRNA and peptide measurements, as detailed in the next paragraph.

C. Tissue Dissection and Isolation of RNA and Peptides

Mice were euthanized by decapitation after a brief exposure to CO_2. Brains were dissected immediately and placed in ice-cold Hanks balanced salt solution (catalog no. 24020117; Gibco). The hypothalamus was dissected using a mouse brain matrix, as previously described [23]. The medial basal hypothalamus (MBH) was dissected from a 2-mm coronal hypothalamic section caudal to the optic chiasm and the anterior hypothalamus (AH) containing the paraventricular nucleus was dissected from a 1-mm coronal section rostral to the MBH section. In experiment 3, the ARC was dissected from the MBH using a dissecting microscope. The remaining MBH is denoted as MBH-ARC. Sections were processed differently for RNA or protein isolation as described below.

D. RNA Isolation and Real-Time PCR

For experiments 1 and 2, RNA isolation was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) in conjunction with the RNase-Free DNase set (Qiagen). For experiment 3, brain regions were initially homogenized in AT buffer (10 mM TRIS-Cl, pH 8.0; 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.15% Triton X-100) containing RNAse inhibitor [24]; half of the homogenate was then acidified with 0.2 N HCl for peptide isolation and half was suspended in Qiazol reagent for RNA isolation. cDNA was synthesized using the Superscript III First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Grand Island, NY) and analyzed using quantitative RT-PCR performed with Lightcycler 480 SYBR Green I Master in a Lightcycler 480 Real-Time PCR system (both from Roche Applied Science, Indianapolis, IN). Samples were normalized to β-actin. The following primer pairs were used in this study: *Agrp*: 5′-GCAAAGGCCATGCTGACTGC-3′ (forward) and 5′-CTTCTTGA GGCCATTCAGAC-3′ (reverse); *Cpe*: 5′-GCAACGCCCAGGGAATAGAT-3′ (forward) and 5′-GTCTCCTCCGTGCAGATTGG-3′ (reverse); *Pcsk1*: 5′-CGTTCAGTT-CAAAAA GACTC-3′ (forward) and 5′-GGCAGAGCTGCAGTCATTCT-3′ (reverse); *Pcsk2*:

5′-CCAGGCCATGGCTGATGGCGTG-3′ (forward) and 5′-CGTAGCTGCCACCGTCCC CAG-3′ (reverse); *Pomc*: 5′-CAGTGCCAGGACCTCAC CACGG-3′ (forward) and 5′-CGGTC-CCAGCGGAAGTGACCC-3′ (reverse); *Prcp*: 5′- GCTTCTGCCCCTATCTGG CACG-3′ (forward) and 5′-GGGCCAAGCAGGCAAAGG CT-3′ (reverse); and *β-Actin*: 5′-CCCTGAA-CCCTAAGGCCAACCGTGAAAA-3′ (forward) and 5′-TCTCCGGAGT CCATCACAATGCC-TGTG-3′ (reverse).

E. Protein Isolation and Peptide Analysis

For experiments 1 and 2, hypothalamic samples were homogenized immediately in 0.1 N HCl, as previously described [18, 25]. α-MSH and β-EP were measured by RIA using antibodies raised in this laboratory, as previously described [18, 25]. The α-MSH antibody cross-reacts fully with desacetyl α-MSH, but there is no cross-reactivity with ACTH, β-EP, or POMC [25, 26]. The β-EP antiserum is directed at $\beta\text{-EP}_{18–25}$; it cross-reacts fully with $\beta\text{-EP}_{1–27}$, and $\beta\text{-EP}_{1–26}$, and 2.6% with POMC on a molar basis; there is no cross-reactivity with α-MSH or ACTH [25, 27].

POMC was assayed using an in-house two-site ELISA with antibodies provided by Dr. Anne White, with the capture monoclonal antibody directed against $\text{ACTH}_{10–18}$ [28, 29] and the detection antibody directed against γ-MSH [30, 31]. There is 100% cross-reactivity with 22K pro-ACTH. There is no cross-reactivity with ACTH, α-MSH, γ-MSH, or β-EP [32]. Affinity-purified human 31K POMC was used for standards.

PRCP levels were measured by sandwich ELISA from MyBioSource (catalog no. 929190; San Diego, CA). Protein content of each brain region was determined by the Bradford method, using BSA as the standard.

Processing of β-EP was analyzed by reverse-phase HPLC, as previously described [19]. HPLC was performed on pooled MBH and AH samples from WT mice in experiment 2 that were treated with either saline or NTX. Samples were evaporated in a Speed Vac Concentrator (RVT 4104, Savant Instruments Inc., Holbrook, NY) and then dissolved in 0.1% trifluoroacetic acid containing 24% acetonitrile, applied to the C18 column, and eluted with an acetonitrile gradient. The column was calibrated with synthetic mouse $\beta\text{-EP}_{1–31}$, $\beta\text{-EP}_{1–27}$, and $\beta\text{-EP}_{1–26}$.

F. Statistics

Data were analyzed by two-tailed Student *t* test or one-way ANOVA using Prism (GraphPad Software, La Jolla, CA). Results are presented as mean ± SEM and statistical significance is defined as $P < 0.05$.

2. Results

A. Experiment 1: Effects of Saline or NTX Infusion on HFD vs NC

As expected, there was a significant increase in food intake and BW of the mice fed the HFD compared with those fed the NC (Fig. 1A and 1B). NTX infusion resulted in a significant transient reduction in food intake and BW gain on the first day of the HFD, but this was not sustained throughout the 7-day period (Fig. 1A and 1B). No significant differences in food intake or BW were seen after NTX vs saline infusion in mice fed the NC (data not shown).

Effects of the HFD and NTX treatment on *Pomc* and *Agrp* mRNA levels in the MBH and on POMC peptide levels in the MBH and AH are shown in Fig. 2. The HFD did not affect *Pomc* expression vs NC. However, NTX stimulated *Pomc* expression in mice receiving the HFD vs Saline HFD ($P = 0.017$) and Saline NC ($P = 0.002$) treatments (Fig. 2A). In contrast, *Agrp* expression decreased in animals after 7 days of the HFD vs NC ($P = 0.006$). However, as with *Pomc,* NTX also stimulated expression of *Agrp* in mice receiving the HFD vs Saline HFD ($P = 0.014$) treatments (Fig. 2B).

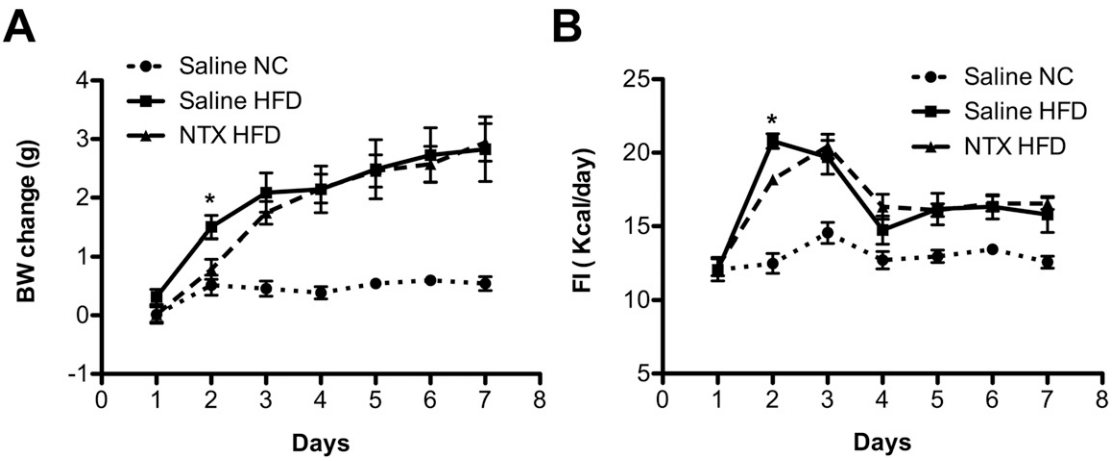

Figure 1. Effects of NTX or saline infusion for 7 d on (A) mean cumulative BW change (± SEM) and (B) daily food intake (kcal/d) after animals were switched to a Saline HFD and NTX HFD as compared with animals that remained on a Saline NC. *$P < 0.05$. FI, food intake.

Levels of the POMC prohormone increased by 31% in MBH of the HFD vs Saline NC groups ($P = 0.007$); however, α-MSH and β-EP levels did not change. POMC levels increased by an additional 25% in the MBH after NTX treatment; this was accompanied by a nearly 50% decline in α-MSH and β-EP levels (Fig. 2C and 2D). POMC levels did not change in the AH after the HFD switch or NTX infusion; however, similar to the MBH, α-MSH and β -EP levels declined significantly after NTX infusion (Fig. 2F and 2G). There was a marked decline in the α-MSH-to-POMC ratio and β-EP-to-POMC ratio in both brain regions after NTX treatment (Fig. 2E and 2H). A decline in the α-MSH-to-POMC ratio was also noted in the MBH in mice fed the HFD vs NC.

B. Experiment 2: Effects of Saline or NTX Infusion in AgRP$^{-/-}$ and WT Mice

The effects of NTX infusion for 7 days on food intake and BW change in *Agrp* knockout and WT mice after being switched to an HFD are shown in Fig. 3. NTX treatment resulted in a transient decrease in food intake and BW gain that was comparable in the WT and *Agrp*$^{-/-}$ mice (Fig. 3A–3D). Thus, the effects of NTX on energy balance were not more robust in the absence of *Agrp*. *Pomc* mRNA levels increased in the MBH of the WT and *Agrp* KO mice when treated with NTX (Fig. 4A). POMC prohormone levels increased (Fig. 4C) and α-MSH and β-EP levels decreased to a similar extent in the MBH of the WT and *Agrp* KO mice treated with NTX (Fig. 4D and 4E). POMC prohormone levels did not change in the AH, but α-MSH and β-EP levels decreased to the same extent in WT and *Agrp* KO mice treated with NTX (Fig. 4I and 4J).

Moreover, the α-MSH-to-POMC and β-EP-to-POMC ratios also decreased to the same extent in MBH (Fig. 4F and 4G) and AH of NTX-treated WT and *Agrp* KO mice (Fig. 4K and 4L). Consistent with previous results, *Agrp* expression also increased in the WT animals receiving NTX treatment (Fig. 4B).

C. Experiment 3: Effects of Saline or NTX Infusion on POMC Processing Enzymes and PRCP in the ARC of the Hypothalamus and Other Hypothalamic Regions

POMC prohormone levels increased in the ARC after NTX vs saline infusion. The increase was accompanied by a simultaneous decrease in the levels of the processed peptides β-EP and α-MSH in the ARC after NTX treatment (Fig. 5A–5C). These results suggested that NTX might affect POMC processing. However, no differences in expression of the processing enzymes (*i.e.*, *Pcsk1*, *Pcsk2*, and *Cpe)* were observed in the ARC of NTX- vs saline-treated mice (Fig. 5D–5F). β-EP and α-MSH levels were also significantly lower in the remaining MBH

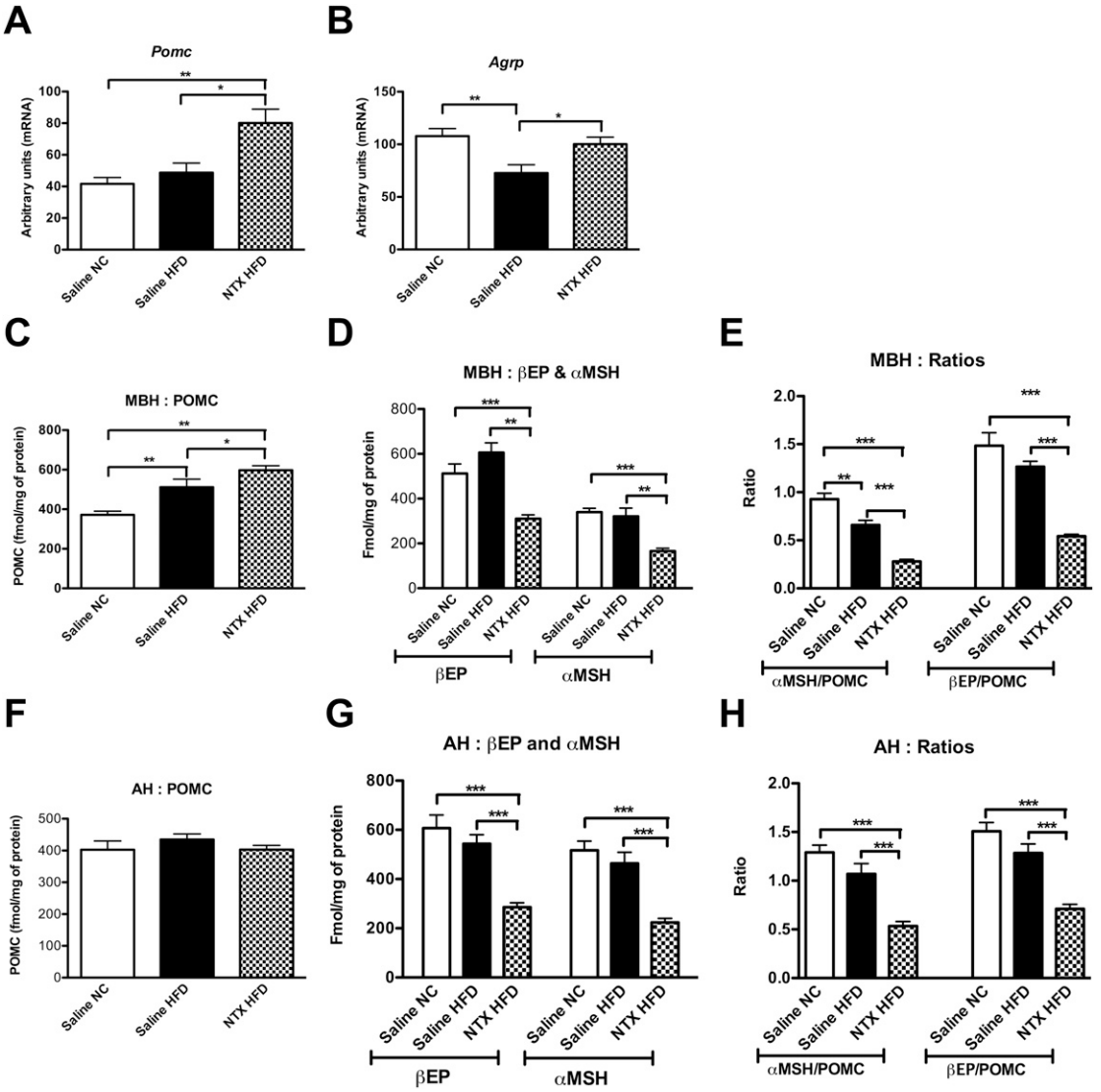

Figure 2. Effects of NTX or saline infusion for 7 d on hypothalamic mRNA and peptide levels after animals were switched to an HFD as compared with animals fed NC. Saline HFD, solid bars; NTX HFD, hatched bars; Saline NC, open bars. (A) *Pomc* mRNA levels in the MBH. (B) AgRP mRNA levels in the MBH. (C, D) POMC, β-EP, and α-MSH peptide levels in MBH. (E) Ratios of β-EP to POMC and α-MSH to POMC in the MBH. (F–H) POMC, β-EP, and α-MSH peptide levels and their ratios in the AH. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

after the ARC dissection and in the AH (data not shown). Expression of the α-MSH degrading enzyme, *Prcp,* did not change significantly in the ARC but did increase in the MBH minus the ARC ($P = 0.03$; Fig. 5G and 5H). PRCP protein levels also increased significantly in the MBH minus the ARC after NTX treatment (Fig. 5I).

D. Effects of NTX on β-EP Processing in the Hypothalamus (Experiment 2)

HPLC analysis showed that processing of β-$EP_{1–31}$ to β-$EP_{1–27}$ and β-$EP_{1–26,}$ which have decreased opioid activity, was decreased in the MBH and AH after NTX treatment (Fig. 6).

3. Discussion

In this study, we examined the effects of opioid antagonism with NTX on *Pomc* and *Agrp* gene expression and POMC peptide processing in the hypothalamus in conjunction with changes

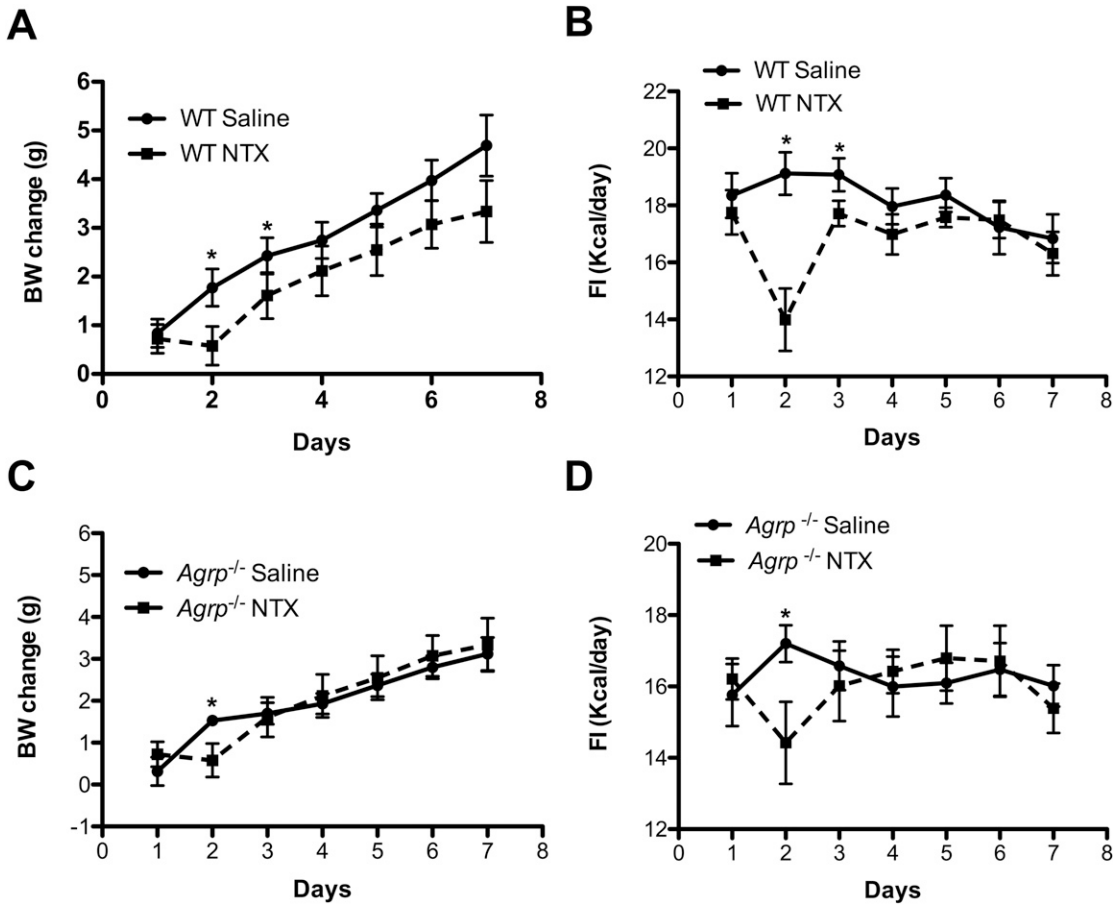

Figure 3. Effects of NTX (dashed lines) or saline (solid lines) infusion for 7 d on cumulative BW change and daily food intake in (A, B) WT mice or (C, D) $Agrp^{-/-}$ after being switched to an HFD. *$P < 0.05$. FI, food intake.

in energy balance. We confirm clear stimulatory effects of NTX on hypothalamic *Pomc* in mice fed low- and high-fats diets, yet only transient decreases in food intake and BW gain were noted in mice fed the HFD. The effects on *Pomc* expression were accompanied by an increase in POMC prohormone levels and a decrease in the levels of the processed peptides α-MSH and β-EP. Arcuate expression of the POMC processing enzymes *Pcsk1*, *Pcsk2*, and *Cpe* was not altered by NTX treatment. However, *Prcp* expression was increased by NTX treatment, which could serve to increase α-MSH inactivation. NTX treatment also resulted in increased hypothalamic *Agrp* expression, which could attenuate the effects of *Pomc* stimulation on energy balance. However, the effects of NTX on food intake and BW change were not enhanced in *Agrp*-null mice.

Although NTX interacts with opioid receptors in many brain regions and can potentially affect feeding behavior at multiple levels, we focused on the melanocortin system, given its crucial role in regulating energy balance. The POMC-derived peptide β-EP can exert opioid receptor–mediated effects on energy balance and can also cause autoinhibition of POMC neurons and decrease MSH production, resulting in increased food intake and weight gain, at least acutely. β-EP acutely stimulates food intake after intracerebroventricular injection and attenuates the effects of α-MSH on food intake, but these effects were not sustained [10]. Unexpectedly, specific genetic deletion of β-EP yielded a mildly obese phenotype [33], although, under certain conditions, the hedonic aspects of feeding were decreased [34]. However, deletion of the μ opioid receptor did protect from diet-induced obesity [35], and treatment with opioid receptor antagonists can inhibit food intake. NTX, which has high affinity for μ opioid receptors, inhibits food intake and weight gain in rodents fed a highly palatable diet. NTX also decreases food intake in humans in the short term, but when used alone has not been highly effective in producing weight

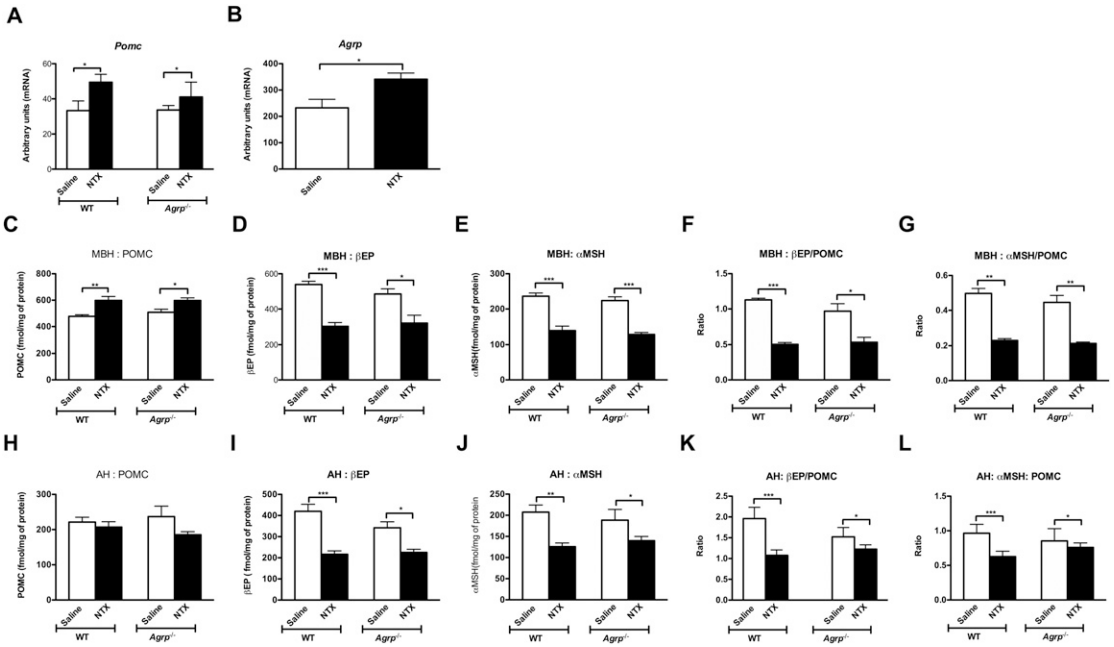

Figure 4. Effects of NTX (solid bars) or saline (open bars) infusion for 7 d on hypothalamic mRNA and peptide levels in WT or *Agrp*$^{-/-}$ animals after being switched to an HFD. (A) *Pomc* mRNA levels in the MBH of WT or *Agrp*$^{-/-}$ mice. (B) *Agrp* levels in the MBH of WT mice. (C–E) POMC, β-EP, and α-MSH peptide levels in the MBH of WT or Agrp$^{-/-}$ animals. (F, G) Ratios of β-EP to POMC and α-MSH to POMC in the MBH. (H–J) POMC, β-EP, and α-MSH peptide levels in the AH of WT or *Agrp*$^{-/-}$ mice. (K, L) Ratios of β-EP to POMC and α-MSH to POMC in the AH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

loss [20]. However, effectiveness increases when used in combination with bupropion, a dopamine and norepinephrine reuptake inhibitor, and this is the basis for the US Food and Drug Administration–approved weight loss combination of NTX and bupropion [36–38]. It has been postulated that stimulation of POMC by bupropion can be enhanced by combination therapy with NTX [17]. This has been confirmed by electrophysiology studies showing that bupropion stimulates POMC neurons and that NTX potentiates this stimulation by blocking β-EP–mediated POMC autoinhibition [17]. NTX stimulates the release of β-EP and MSH from the perifused hypothalamus of rats *in vitro* and stimulates POMC mRNA levels in the rat hypothalamus after 1 week of infusion [18, 39]. Nevertheless, the effect of NTX on energy balance is quite modest despite the known stimulatory effects of NTX on POMC.

In the current study in mice, NTX treatment induced an increase in *Pomc* mRNA levels in the MBH after 1 week of infusion. This was accompanied by a consistent increase in POMC prohormone levels in the MBH and marked decrease in levels of the processed peptides α-MSH and β-EP. The marked decrease in the ratio of α-MSH and β-EP to POMC is consistent with selective release of the processed peptides and/or an effect on POMC processing. In a previous study in the rat, we showed that NTX stimulated the release of β-EP and MSH from the perifused hypothalamus *in vitro* when NTX was added to the perifusion media or administered *in vivo* 60 minutes before removal of the hypothalamus for perifusion [19]. However, when the hypothalamus was perifused after 1 week of *in vivo* NTX treatment, β-EP and MSH release was no longer elevated [19]. This would be consistent with the early effects of NTX on food intake reported in the current study that were not sustained at 1 week. To further explore a potential processing mechanism, we measured arcuate expression of the POMC processing enzymes *Pcsk1*, *Pcsk2*, and *Cpe* and found no changes induced by NTX treatment. However, it is still possible that cellular levels of these enzymes are selectively altered in POMC neurons and may not be reflected in whole arcuate measurements. In addition, potential effects on protein levels or enzyme activity are still possible. Thus, at this point, it remains unclear if the relative changes in levels of the POMC prohormone vs the processed

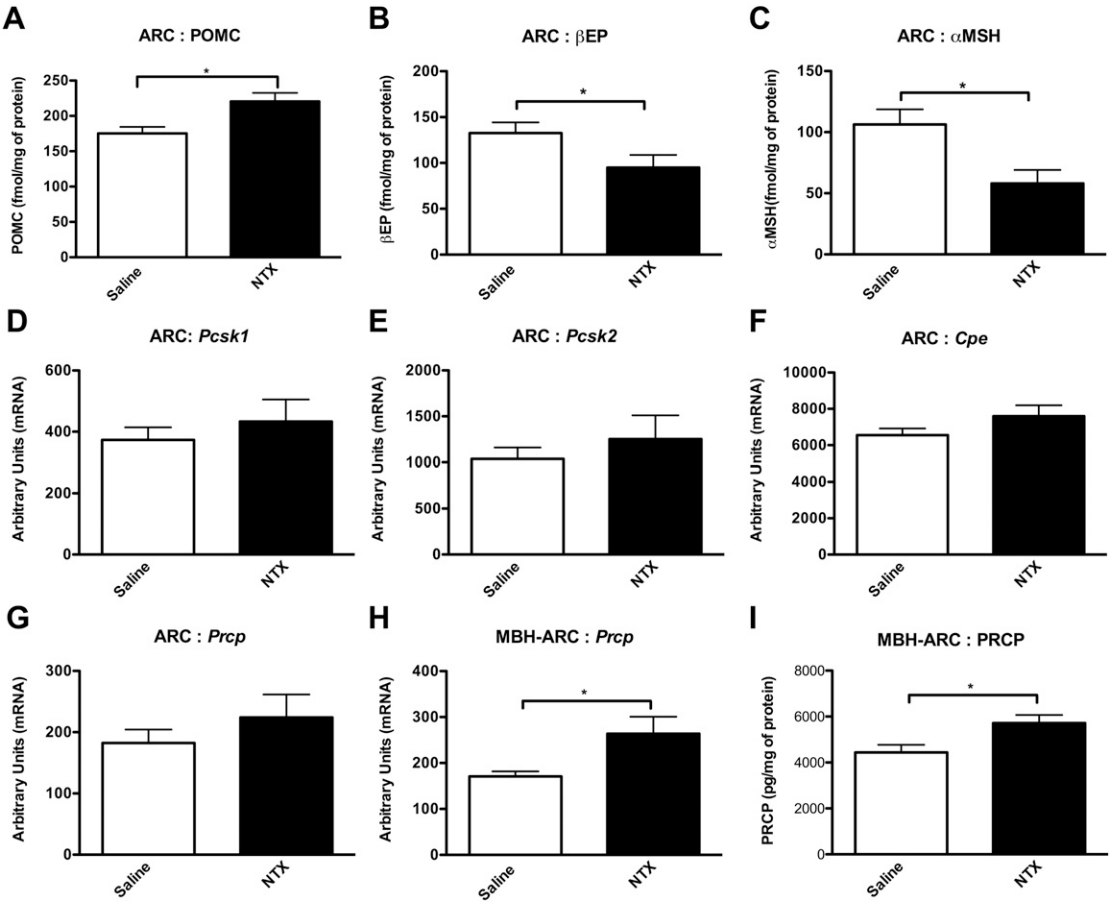

Figure 5. Effects of NTX (solid bars) or saline (open bars) infusion for 7 d on POMC peptides and processing enzymes and on *Prcp* in the ARC. (A–C) POMC, β-EP, and α-MSH levels in the ARC. (D–F) Pcsk*1*, *Pcsk2*, and *Cpe* mRNA levels in the ARC. (G) *Prcp* mRNA levels in the ARC. (H, I) *Prcp* mRNA and protein levels in the MBH minus the ARC. $^*P < 0.05$.

peptides are indicative of a change in POMC processing. Of note in humans, cerebrospinal fluid β-EP levels increased after 2 and 7 days of NTX treatment and there was a decrease in the ratio of β-EP to POMC [40], which is the inverse of the changes measured in the hypothalamus.

In addition, we provide evidence that NTX affects β-EP processing and possibly α-MSH inactivation. Processing of β-EP$_{1–31}$ to β-EP$_{1–27}$ and β-EP$_{1–26}$ (which have reduced opioid activity) was reduced in the MBH and AH by NTX treatment. This effect on β-EP processing is similar to what has been reported in the rat [18, 39]. However, we did not see any changes in the levels of *Pcsk2* and *Cpe*, the enzymes involved in the processing of β-EP$_{1–31}$ to β-EP$_{1–27}$ and β-EP$_{1–26}$. We also show that *Prcp* expression in the hypothalamus was increased as a result of NTX treatment. PRCP is a serine protease that cleaves peptides like α-MSH with a penultimate proline and leads to the generation of α-MSH$_{1–12}$, which is ineffective in reducing food intake [21]. Increased PRCP activity thus could lead to increased inactivation of α-MSH, which could then reduce the effectiveness of NTX as an obesity treatment.

AgRP neurons also express μ opioid receptors and there is evidence that these neurons can be regulated by opioids [14]. We show that NTX treatment resulted in an increase in *Agrp* expression in the MBH. A similar tendency for NTX to cause an increase in plasma AgRP levels has been reported in humans [40]. We speculated that *Agrp* deletion might cause more robust and sustained effects of NTX on energy balance. Hence, we used an *Agrp* KO mouse model to potentially enhance the NTX effects on weight loss and food intake. However, our results showed no difference in food intake or BW change between the *Agrp* KO animals and WT controls after NTX treatment. This does preclude, however, a role for AgRP in attenuating the effects of NTX on feeding, given

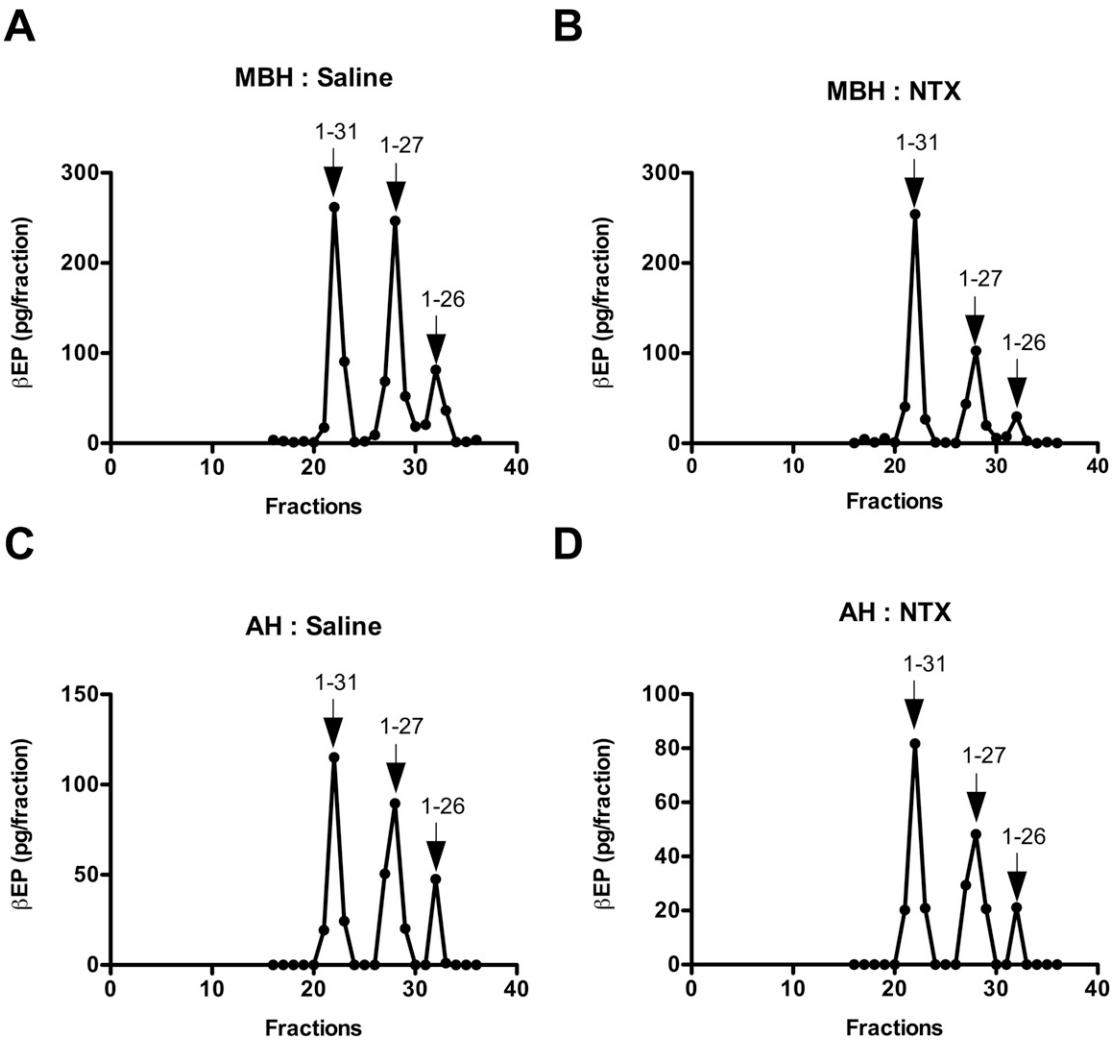

Figure 6. HPLC elution profiles of the β-EP immunoactivity in pooled extracts of MBH from mice treated with (A) saline or (B) NTX for 7 d. HPLC elution profiles of the β-EP immunoactivity in AH of mice treated with (C) saline or (D) NTX for 7 d. Arrows indicate the elution positions of synthetic peptides β-$EP_{1–31}$, β-$EP_{1–27}$, and β-$EP_{1–26}$.

the known developmental compensation that occurs with embryonic deletion of *Agrp*. However, $Agrp^{-/-}$ mice exhibit an age-dependent lean phenotype [41], and Quinones *et al.* [42] showed that mice having p53 deletion in AgRP neurons are sensitive to diet-induced obesity. It remains to be determined if conditional *Agrp* deletion or selective deletion of MOR from AgRP neurons would enhance the effect of NTX. Thus, it remains possible that selective AgRP inhibition might enhance the effects of NTX on food intake and BW change [43].

We conclude that despite clear stimulatory effects of NTX on *Pomc* expression in the hypothalamus of mice fed low- and high-fat diets, only modest transient decreases in food intake and BW were seen in mice fed the HFD. Our findings suggest that effects of NTX treatment on POMC processing and possibly α-MSH inactivation, as well as stimulatory effects on AgRP neurons (which also produce NPY and GABA), could mitigate the effects of NTX on energy balance.

Acknowledgments

We thank Dr. Anne White, Faculties of Life Sciences and Medical and Human Sciences at the University of Manchester, United Kingdom, for providing the antibodies and the proopiomelanocortin (POMC) standard used for the POMC assay.

Financial Support: This work was supported by National Institutes of Health grant RO1-DK08003 (S.L.W.) and the Dr. Robert C. and Veronica Atkins Foundation (S.L.W.).

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Disclosure Summary: The authors have nothing to disclose.

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