Neuropeptide Y Is Produced by Adipose Tissue Macrophages and Regulates Obesity-Induced Inflammation

Kanakadurga Singer¹, David L. Morris¹, Kelsie E. Oatmen¹, Tianyi Wang¹, Jennifer DelProposto¹, Taleen Mergian¹, Kae Won Cho¹, Carey N. Lumeng¹,²

¹Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, Michigan, United States of America, ²Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan, United States of America

Abstract

Neuropeptide Y (NPY) is induced in peripheral tissues such as adipose tissue with obesity. The mechanism and function of NPY induction in fat are unclear. Given the evidence that NPY can modulate inflammation, we examined the hypothesis that NPY regulates the function of adipose tissue macrophages (ATMs) in response to dietary obesity in mice. NPY was induced by dietary obesity in the stromal vascular cells of visceral fat depots from mice. Surprisingly, the induction of Npy was limited to purified ATMs from obese mice. Significant basal production of NPY was observed in cultured bone marrow derived macrophage and dendritic cells (DCs) and was increased with LPS stimulation. In vitro, addition of NPY to myeloid cells had minimal effects on their activation profiles. NPY receptor inhibition promoted DC maturation and the production of IL-6 and TNFα suggesting an anti-inflammatory function for NPY signaling in DCs. Consistent with this, NPY injection into lean mice decreased the quantity of M1-like CD11c⁺ ATMs and suppressed Ly6c⁻ monocytes. BM chimeras generated from Npy⁻/⁻ donors demonstrated that hematopoietic NPY contributes to the obesity-induced induction of Npy in fat. In addition, loss of Npy expression from hematopoietic cells led to an increase in CD11c⁺ ATMs in visceral fat with high fat diet feeding. Overall, our studies suggest that NPY is produced by a range of myeloid cells and that obesity activates the production of NPY in adipose tissue macrophages with autocrine and paracrine effects.

Introduction

Obesity correlates with the induction of a chronic state of inflammation that is linked to metabolic dysfunction [1]. Clinical studies demonstrate elevations in c-reactive protein (CRP) and pro-inflammatory cytokines in obese children and adults [2,3]. A key event in obesity-induced inflammation is the alteration in the inflammatory profile of adipose tissue macrophages (ATMs) [4,5]. In the lean state, adipose tissue contains a population of resident macrophages which express markers of alternative activation (M2). With obesity, there is a shift in the features of the ATM population towards a classical activation (M1) profile driven by the appearance of CD11c⁺ ATMs. The induction of CD11c⁺ ATMs appears to be closely linked to the increase in circulating Ly6c⁻ monocytes seen with dietary obesity [6]. Compared to Ly6c⁺ monocytes, Ly6c⁻ monocytes are preferentially recruited to active areas of inflammation including atherosclerotic plaques [7] and in obesity are thought to drive M1 ATM accumulation in adipose tissue [6].

There are a wide range of triggers for adipose tissue inflammation that occur in the setting of diet induced obesity and increased adiposity. These include lipolytic signals, chemokines, chemoattractants, cytokines, and pathogen associated molecular proteins (PAMPs) such as lipopolysaccharide [4,8,9]. These signals activate a range of pro-inflammatory signaling pathways that include chemokine receptors (e.g. CCR2) and a host of innate immune pattern recognition receptors as Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs). Attenuation of many of these inflammatory signals has been shown to improve metabolic disease [10,11]. Hence, identification of the signals that trigger adipose tissue inflammation is critical. Physiologic stress signals have been shown to be activated with obesity and induce inflammation in adipose tissue [12,13]. While there are many neurohumoral factors associated with stress responses, neuropeptide Y (NPY) is a dominant hormone that is elevated in chronic stress and sympathetic nervous system activation [13].

NPY is a peptide hormone that binds to a family of 5 G-protein coupled receptors (Y1–Y5) found in multiple tissues [14]. NPY receptors are found in a broad array of tissues including those involved in metabolism such as adipose and liver. In immune cells, only Y1, Y2, and Y5 receptors have been identified [15]. The best characterized function of NPY in obesity is in the CNS where neuronal NPY stimulates orexigenic pathways via Y1 receptor activation [16]. However, there is evidence that NPY influences metabolic function in peripheral tissues as well, mostly via Y2 and...
Y5 receptors although Y1 receptors have been studied on adipocytes themselves. For example, with obesity, NPY is induced in adipose tissue where it may regulate multiple aspects of adipocyte biology. In adipocytes, NPY decreases lipolysis and promotes adipogenesis [17,18] suggesting that NPY has beneficial effects on lipid uptake and storage in fat. Consistent with this, NPY deficient mice are more obese and insulin resistant with high fat diet feeding [19]. However, in a model combining chronic stress and diet-induced obesity, NPY induction in fat correlated with insulin resistance and an increase in ATMs [20,21]. In this model, blockade of the NPY 2 receptor (Y2R) decreased ATM accumulation and insulin resistance in stress-induced obesity models [20]. The source of NPY and the pattern of NPY receptor activation in obese adipose tissue are unknown.

Relevant to ATM biology is the evidence that NPY is a potent immune mediator with both pro-inflammatory and anti-inflammatory actions on leukocytes. Exogenous NPY protects against experimental autoimmune encephalitis and decreases inflammation in experimental sepsis [22,23]. NPY can also decrease macrophage oxidative burst via Y1, Y2, and Y5 NPY receptors [15] and decrease neutrophil and T-lymphocyte tissue infiltration [24,25]. Overall, the effects of NPY on leukocytes may be highly context dependent as Y1 activation has been shown to downregulate T cell responses, but paradoxically activate antigen presenting cells [26].

Given that NPY has a role in immune cell activity and obesity, we investigated the hypothesis that NPY influences the activity of ATMs as a potential link between stress signals, obesity, and inflammation. This investigation led us to find evidence that NPY is produced by macrophages in visceral fat and that ATMs are the primary source of the increase in NPY expression seen in fat with obesity. NPY production and inflammatory regulation in myeloid cells was confirmed in bone marrow (BM) derived cells. In vitro studies and BM chimeras suggest that myeloid cell derived NPY has anti-inflammatory autocrine and paracrine effects within adipose tissue.

Methods

Animals and Animal Care

Mice used in these experiments were male C57Bl/6J, 129S-NPYtm1Rpa/J (NPY-KO), and control 129S mice (Jackson laboratories). Mice were fed ad lib either a control normal diet (ND) consisting of 4.5% fat (5001;LabDiet) or a high fat diet (HFD) of 60% of calories from fat (Research Diets, Inc., D12492) starting at 6-8 weeks of age for 16 weeks unless specified. Animals were housed in a specific pathogen-free facility with a 12 h dark cycle and given free access to food and water. All animal use was in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and approved by the University Committee on Use and Care of Animals at the University of Michigan [Animal welfare assurance number A3114-01]. Mice were treated with antibiotics (polymyxin and neomycin) before BMMP and CD11c and MHCII expression in BMDC by flow cytometry. Differentiation was confirmed by demonstrating F4/80 expression in BMMP and CD11c and MHCII expression in BMDC by flow cytometry. Cells were then placed in 10% serum media for 24 hours prior to treatment with NPY (100 nM), LPS (10–100 ng/ml), IL-4 (20 ng/ml) for 18–24 hours. 3T3-L1 cells were differentiated into adipocytes as previously described [4]. NPY ELISA studies were performed on media with the use of NPY high sensitivity kit from Bachem (S-1145).

Hepatic Triglyceride Content

Livers were weighed, snap frozen in liquid nitrogen, and stored at -80 degrees C. Frozen liver samples (~200 mg) were thawed and triglycerides were assessed as previously described [28] and quantified using Triglyceride Assay Kit (Sigma).

Bone Marrow Transplantation

Bone marrow cells were isolated from 129S wildtype and Npy−/− mice and [30] injected IV into lethally irradiated (900 Rad) 6 week old 129S wild-type recipient mice (10 millon cells/mouse). Animals were treated with antibiotics (polymyxin and neomycin) for 4 weeks post BM transplantation. Following two weeks of normal chow diet, they were started on ND or HFD diets for 8 weeks. Glucose tolerance testing was performed as described [6] performed after 6 hours of fasting with intraperitoneal injection of 0.7 g/kg of dextrose.

Statistics

Results are presented as mean ± SEM. Statistical analyses were conducted using an unpaired 2-tailed Student’s t-test, with significance set at p-value <0.05. Results with multiple groups were first assessed with ANOVA followed by confirmatory analysis, by individual t-test, and bone marrow transplant experiments assessed with two way ANOVA.

Results

Npy is Induced in SVF Cells in Visceral fat of Obese Mice

To examine the time course and depot specificity of Npy expression in mouse models of diet-induced obesity, we examined Npy expression in several fat depots from male C57Bl/6 fed a HFD diet (60% kcal from fat). Compared to ND fed mice, Npy expression was increased with HFD in a time dependent manner in visceral/epididymal white adipose tissue depots (EWAT; Figure 1A) with induction observed as early as 8 weeks on diet
(p = 0.001 by one-way ANOVA). At all time points examined, the expression of *Npy* in inguinal subcutaneous fat depots (IWAT) was significantly lower than EWAT (Figure 1B). In contrast to EWAT, HFD led to no significant changes in the expression of *Npy* in IWAT over time. No significant changes in *Npy* expression were observed in brown adipose depots (BAT) with HFD (Figure 1C), however dorsal subcutaneous fat pads (DWAT) demonstrated a trend toward *Npy* induction with HFD similar to what was seen with EWAT.

To determine the source of NPY in adipose tissue, we examined *Npy* expression in adipocyte and SVF fractions from HFD EWAT after collagenase digestion. Expression of the Y1 and Y2 receptors (*Npy1r* and *Npy2r*) (Figure 1E) were elevated in the SVF compared to adipocyte fractions in obese mice. *Npy* expression tended to be enriched in the SVF fraction, but did not reach statistical significance (Figure 1D; representative of three independent experiments). *Npy5r* expression was not detectable by RT-PCR in SVF or adipocyte fractions in lean or obese mice. To validate the production and secretion of NPY by SVF cells, NPY protein was assessed in the media from cultures of EWAT and IWAT SVF cells from obese mice and higher NPY levels were detected in EWAT conditioned media compared to IWAT (Figure 1F).

**Figure 1.** *Npy* induction within visceral fat and SVF with diet-induced obesity. *Npy* gene expression by quantitative RT-PCR in (A) epididymal fat (EWAT) and (B) inguinal fat depots (IWAT) of normal diet (ND), 8 week high fat diet (HFD), and 20 week HFD C57Bl6 mice. N = 4 mice per group. (C) *Npy* expression in various fat depots of ND and 16 week HFD fed animals. N = 4 mice per group. (D) *Npy* expression in stratified adipose tissue. EWAT from HFD C57Bl6 mice was separated into adipocyte (Ad) and stromal vascular fraction (SVF) cells after collagenase digestion. *Npy* expression was assessed by quantitative RT-PCR. N = 4 per group. (E) *Npy1r* and *Npy2r* expression in adipocytes and SVF cell fractions from lean mice. N = 4 per group. (F) NPY protein expression in cultured SVF cells from HFD C57Bl6 mouse EWAT and IWAT. Media was removed from cells after 18 hours of culture and assessed for NPY by ELISA (N = 3 wells per group). *p<0.05, **p<0.01, ***p<0.005 by t-test.

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NPY is Produced by ATMs and Bone Marrow Derived Myeloid Cells

Sympathetic nerves are a candidate source of NPY in peripheral tissues, however, our SVF preparations do not isolate intact nerves based on visual inspection and immunostaining SVF cells for the axon specific protein GAP-43 (data not shown). This suggested that NPY was produced by a non-neuronal component of the SVF that is induced with obesity. Given the increase in ATM content with obesity and reports of NPY expression in immune cells [33], we evaluated the hypothesis that ATMs are a source of NPY. EWAT SVF from lean and obese mice were separated into ATM (CD11b+ F4/80-) and non-ATM (CD11b- F4/80+) fractions by FACs and followed by RNA isolation (Figure 2A). In lean animals, Npy was expressed equally between ATM and non-ATM cells. With HFD, no significant changes were seen in the expression of Npy in non-ATM cells, but there was a significant induction of Npy in the CD11b+ F4/80- ATMs (Figure 2B). A similar induction of Npy was seen purified ATMs from db/db mice (Figure 2C). This demonstrates that ATMs are a regulated source of NPY production and are the primary source of NPY in obese fat. Further cell sorting of M1 (CD206+) and M2 ATMs (CD11c+) demonstrated similar NPY gene expression in both ATM populations suggesting that Npy expression is not a specific feature of one ATM type or another at the gene expression level (Figure 2D). Since obesity is known to induce markers of classical M1 activation of ATMs and induce the expression of the dendritic cell (DC) marker CD11c, we examined the influence of inflammatory signals on Npy induction in macrophages and DCs. BMMP and BMDCs were treated with M1 (LPS) or M2 (IL-4) skewing stimuli and Npy was assessed. Npy expression was detectable in both BMMP and BMDC by RT-PCR, however there were no significant changes in Npy mRNA expression with either LPS or IL-4 treatment (Figure 3A). However, when the NPY production in the media was quantified, Npy was induced by LPS in the media of both BMMP and BMDC. The specificity of this ELISA assay was confirmed in media from BMDCs derived from NPY KO mice where Npy was below the limit of detection of the assay. Treatment with IL-4 did not significantly alter NPY secretion by either cell type (Figure 3B). In all conditions tested, the mRNA and protein expression of NPY was higher in BMDC than BMMP. These results suggest that the increase in NPY production with obesity is tied to the generation of M1-polarized CD11c+ ATMs in obese mice and that the inflammatory induction of NPY is primarily at the level of translational or post-translational regulation.

NPY 1 Receptors are Present on Non-ATMs and ATMs

Fractionation studies of whole adipose tissue demonstrated that NPY receptors Npy1r and Npy2r are both preferentially expressed in SVF cells relative to the adipocytes in fat (Figure 1E). We next used our FACS sorted SVF populations to examine which cells within adipose tissue are potentially responsive to NPY. Npy1r and Npy2r expression was present in both ATMs and non-ATMs at similar levels and was not significantly altered by HFD (Figure 4A and 4B). Npy5r expression was not consistently detectable in either adipocyte or SVF fractions (data not shown).

Since the non-ATM population is also enriched for preadipocytes, we examined receptors in these cells by assessing receptor gene expression during the differentiation of 3T3-L1 adipocytes. 3T3-L1 pre-adipocytes had high expression of Npy1r that was suppressed during differentiation (Figure 4C). Npy mRNA and protein were undetectable at any stage of differentiation of 3T3-L1 pre-adipocytes consistent with our fractionation data (data not shown).

NPY Antagonists Promote DC Maturation and Pro-inflammatory Gene Expression

While many studies have demonstrated that macrophages and DCs respond to exogenous NPY [13,24] our initial in vitro studies did not show any alterations in macrophage polarization with NPY supplementation (data not shown). We attribute this to the basal production of NPY observed by macrophages and DC. We confirmed the expression of the Npy1r in cultured BMDCs and observed an increase in Npy1r expression in BMDCs derived from obese mice (Figure 4D). There was very low expression of Npy2r and Npy5r in these cells by RT-PCR (data not shown). Therefore, an antagonist approach was used to examine the importance of NPY signaling on myeloid cell function.

BMDCs were treated with a cocktail of specific antagonists against Y1, Y2, and Y5 NPY receptors prior to LPS stimulation (M1). Blockade of NPY signaling led to an increase in the expression of proinflammatory genes Il6, Tnfa, and Nos2 (Figure 5A) and an increase in secreted IL-6 and a trend toward increased TNFα (Figure 5B). While similar trends were seen in unstimulated DCs results were not significant. In addition, DC maturation markers H2ab1 (MHCIi), CD40, and CD86 expression in LPS treated DC with a trend towards an increase in mature CD11c+ MHCIi+ cells (Figure 5D). Overall, these results suggest that NPY has anti-inflammatory effects on DCs that blunt their maturation and inflammatory cytokine production upon LPS stimulation.

NPY Treatment Decreases Adipose Tissue and Systemic Inflammation in Lean Mice

To assess the effects of NPY on adipose tissue inflammation in vivo, we sought to simulate the induction of NPY with obesity with NPY injection. NPY or scrambled control peptide were injected I.P. into male C57 mice for 10 days. There were no changes in body weight, EWAT weight, fasting glucose, fasting insulin, liver weight, or liver triglyceride content with NPY treatment (Table S2). The total ATM content in visceral fat showed a trend towards a decrease with NPY treatment (Figure 6A). When ATMs were subdivided based on CD11c expression, NPY injection led to a significant decrease in the quantity of CD11c+ ATMs compared to controls and a trend towards a decreased in CD11c+ ATMs. Consistent with these changes, the expression of M1 genes Il6, Tnfa, and H2ab1 was decreased in NPY injected mice (Figure 6B). NPY injection led to a significant increase in adipocyte size (Figure 6C; Table S2) that correlated with an increase in Pparg (p = 0.023) gene expression (Figure 6D). To determine the mechanism of this decrease in CD11c+ ATMs, blood monocytes were examined by flow cytometry. While total monocytes were similar in control and NPY injected animals, there was a decrease in classical CD115+ Ly6c+ monocytes and an increase in non-classical CD115+ Ly6c+ monocytes (Figure 6E). In sum, this data suggests that elevated NPY suppresses CD11c+ ATM accumulation in fat by altering the quantity of circulating Ly6c+ monocytes.

Given the suppression of CD11c+ ATMs with NPY in lean mice, we examined the possibility that exogenous NPY might
Figure 2. \textit{Npy} gene expression in fat is primarily derived from ATMs in obese animals. (A) Flow cytometry analysis of SVF cells from EWAT of obese C57Bl/6 mice 18–20 wk HFD. SVF cells were stained with F4/80 and CD11b prior to analysis by flow cytometry. Fluorescent activated cell sorting (FACS) isolated purified ATM and non-ATM fractions (middle and right panels show validation plots). (B) \textit{Npy} expression in ATM and non-ATM fractions by quantitative RT-PCR (N = 5 mice per group). (C) Comparative analysis of \textit{Npy} expression in F4/80\(^+\)CD11b\(^+\) ATMs from ND, HFD, and \textit{db/db} mice (N = 3 per group). (D) \textit{Npy} expression in FACS purified M2 (CD206\(^+\)) and M1 (CD11c\(^+\)) ATMs (F4/80\(^+\)CD11b\(^+\)). \(*p<0.05, **p<0.01, ***p<0.005\) by t-test.

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Figure 3. NPY production by bone marrow derived dendritic cells (BMDC) and macrophages (BMMP). (A) Quantitative RT-PCR analysis of \textit{Npy} expression in BMMPs and BMDCs from C57Bl/6 mice with or without LPS (100 ng/ml) for 24 hours. (B) NPY protein expression by ELISA on media from BMDC or BMMP cultured for 24 hours with and without LPS (100 ng/ml) or IL-4 (20 ng/ml) treatment. N = 3 per group. \(*p<0.05, **p<0.1, \#p<0.05\) between BMMP and BMDC, by t-test.

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NPY in early obesity in mice. Mice were fed a HFD for 4 weeks prior to treatment with a similar regimen of NPY. No significant changes in body weight, fasting glucose levels, or glucose tolerance measured by GTT were detected in NPY injected mice (Figure S1, Table S3). Analysis of fasting insulin levels demonstrated a trend towards higher insulin levels in the NPY treated group compared to controls (p = 0.09; Table S3). NPY injection induced an enlargement of adipocyte cell size in the HFD mice similar to what was observed in ND fed mice. In contrast with the lean mice, NPY treatment of obese mice did not alter ATM content in EWAT, inflammatory gene expression in fat, or blood monocytes (Figure S1 B-D).

Lack of NPY in Hematopoietic Cells Potentiates Obesity-induced Inflammation in Adipose Tissue

To examine the significance of NPY production by macrophages/myeloid cells in obesity, we used BM chimeras to examine the hypothesis that loss of NPY from hematopoietic cells would increase adipose tissue inflammation. BM chimeras were generated by injecting donor BM cells from NPY KO or wildtype (WT) mice into lethally irradiated WT recipients. 6 weeks after transplantation, mice were fed either a ND or HFD for 8 weeks. HFD exposure led to significant weight gain and expansion of EWAT weight in mice reconstituted with NPY KO (KO→WT) and WT (WT→WT) marrow (Figure 7A and B). No significant differences in weight were observed between donor groups on either ND or HFD. Overall, there were more ATMs per fat pad in HFD animals (Figure 7C). When we looked at the % ATMs within the SVF there were significant differences amongst all groups (two-way ANOVA p = 0.009) with more ATMs in the HFD KO-WT group (Figure 7D). Stratification of ATMs into subtypes based on CD11c expression demonstrated an increase in the quantity of CD11c⁺ M1 and MHCII⁺ CD11c⁺ ATMs with HFD in both groups. However, obese mice that received BM from NPY KO donors had more CD11c⁺ M1 and MHCII⁺ CD11c⁺ ATMs when compared to WT (Figure 7E) similar to our in vitro antagonist studies.

*Npy* expression in the chimeras was evaluated to assess the contribution of hematopoietic compartment to adipose Npy expression. WT→WT mice demonstrated an induction of Npy in EWAT with HFD. In contrast, no increase in Npy expression with HFD was seen in NPY KO donors (KO→WT) by ANOVA (Figure 7F). *Tnfa* expression of EWAT was induced with HFD in both chimeras to similar levels (Figure 7G). Evaluation of

![Figure 4. NPY1R is expressed in pre-adipocytes.](image-url)
metabolism showed that glucose intolerance was induced in HFD animals compared to ND, but no significant differences in glucose tolerance was observed between KO→WT and WT→WT animals based on GTT (Figure S2). However, there was a trend to higher fasting insulin levels in KO→WT mice fed HFD suggesting that myeloid NPY deficiency increases insulin resistance with dietary obesity (Figure 7H). When fat cell size was analyzed in the EWAT, adipocytes in the HFD NPY KO-WT group were smaller than those in the HFD WT-WT group \( p = 0.043 \), HFD KO-WT 56.5 ± 1.7 \( \mu m^2 \), HFD WT-WT 61.3 ± 1.6 \( \mu m^2 \). This finding is consistent with the increase in adipocyte size observed with NPY injection into lean mice.

**Discussion**

In this study, we report the novel finding that the stress hormone NPY is expressed at physiologic levels by adipose tissue macrophages and participates in obesity-induced adipose tissue inflammation. While macrophage NPY expression is induced by inflammatory signals and obesity, our results support an anti-inflammatory effect of NPY signals on macrophage and DC maturation. In vitro, NPY receptor blockade on macrophages/dendritic cells promotes M1 cytokine gene expression and DC maturation. Similarly, BMT in vivo experiments showed that the loss of \( Npy \) expression exclusively in hematopoietic cells increased CD11c\(^+\) ATMs, however, a significant decrease in cytokine production was not observed. The discrepancy in these observations likely relates to the timing of NPY exposure as well as the fact that in vivo experiments involve multiple cell lines that can respond and generate NPY (e.g. adipocytes and nerves). In vivo, NPY supplementation decreased M1 ATMs in lean mice by suppressing the levels of circulating Ly6Chi monocytes, an unexpected and novel mechanism linking stress hormones and inflammation.

Our observation that myeloid cells within adipose tissue are a regulated source of NPY production is consistent with other reports of NPY expression in non-neuronal cells. Macrophage NPY expression is evident in publicly available gene expression array data [34]. NPY induction has been reported in stimulated immune cells [33], platelets and endothelial cells [35-37] and in the lung [38]. Given our observations, it is possible that macrophages in other important metabolic tissues (e.g. liver and brain) may secrete NPY and influence metabolic tissues. Preliminary studies did not identify significant \( Npy \) gene expression in microglia however, stress or inflammatory stimuli may be required for significant \( Npy \) induction, as observed in astrocytes [39]. Our studies also suggest that macrophage NPY secretion can be regulated post-transcriptionally, and therefore mRNA expression techniques may be imprecise in certain contexts.

Our data supporting an anti-inflammatory function for NPY in macrophages, support the evidence that NPY has protective functions during chronic stress in the context of anxiolysis [40] and appetite [41]. Our studies show that this effect is primarily mediated by Y1, Y2, and Y5 receptors in macrophages as gene expression analysis failed to detect the expression of the Y4 receptor in macrophages (data not shown). NPY has also been shown to play a protective role in shock, sepsis, and autoimmune conditions [42,43]. In models of endotoxemia, NPY can block monocyte induction and improve inflammatory measures [23].

Macrophage derived NPY may participate in the connections between stress and obesity. Clinical studies have shown that increased stress plays a role in weight gain and modifies the risk for diabetes, but the specific mechanisms that link stress pathways with insulin resistance remain unclear [12,44,45]. Our results show an early induction of NPY in obese adipose tissue which may
dampen the early inflammatory response to obesity. With prolonged exposure to HFD, the anti-inflammatory actions of NPY may be overwhelmed by the ongoing accumulation of M1 ATMs. While our results show an anti-inflammatory function of NPY in lean states and mild obesity, previous work suggests a pro-inflammatory role for NPY in response to the combined effects of stress and HFD [21]. Differences in the levels of NPY induction and alterations in NPY receptor number and type may explain this dichotomy. This hypothesis is consistent with recent studies showing differential NPY receptor contributions to leukocyte activation [46]. In addition, our BM chimera experiments demonstrate that ATMs are a source of NPY in fat, but may not.

Figure 6. NPY treatment decreases circulating Ly6c<sup>+</sup> monocyte and CD11c<sup>+</sup> ATM content. Lean C57Bl/6 mice were treated with NPY or NPY scramble control peptide (60 µg/kg/day) IP for 10 days. N = 4 mice per group. (A) Quantitation of ATMs by flow cytometry. EWAT SVF cells were stained for ATM markers (F4/80, CD11b, CD11c) and analyzed by flow cytometry. ATM content normalized to fat pad weight. Gene expression analysis of EWAT for (B) inflammatory genes by quantitative RT-PCR. (C) light microscopy image showing larger adipocytes with NPY injection (left panel) compared to scramble control peptide injection (right panel) (D) Gene expression analysis of EWAT for adipocyte genes by quantitative RT-PCR. *p<0.05. (E) Quantitation of Ly6c<sup>hi</sup> and Ly6c<sup>lo</sup> CD115<sup>+</sup> blood monocytes by flow cytometry (N=4 per group). Data is representative data of one of 3 replicate experiments. doi:10.1371/journal.pone.0057929.g006
NPY in Adipose Tissue Macrophages

A. Weight (g)

B. EWAT (grams)

C. ATM (F4/80+CD11b+)

D. ATM % of SVF cells

E. % ATM

F. Npy Expression (A.U.)

G. Trif Expression (A.U.)

H. Insulin (ng/ml)
not be the sole source. Understanding how factors such as NPY may influence local inflammation is key given that macrophage content and activity within adipose tissue is an important factor for insulin resistance and risk for type 2 diabetes.

Our data support a model where NPY generated from ATMs has autocrine, paracrine, and systemic effects. NPY production by macrophages is increased by HFD and dampens M1 ATM activity. The NPY produced from ATMs may also activate NPY1Rs on pre-adipocytes and adipocytes contributing to a decrease in lipolysis [17,18], which may further decrease pro-inflammatory signals. At a systemic level, NPY generated from hypertrophied adipose tissue may try to suppress the production/ trafficking of circulating classical Ly6ch high monocytes.

While we have observed an induction of CD11c+ ATMs with short-term HFD exposure (Figure 7C-D), we were not able to see a significant alteration in glucose metabolism with loss of Npy in hematopoietic cells. This may be because inflammation is only one component of the development of insulin resistance and dominates with long-term high fat diet exposure [47]. Since the metabolic phenotype of whole body NPY deficient mice is relatively mild and relates more to diet intake [48], we did not anticipate a substantial metabolic phenotype in our BMT experiment. In addition, the BM chimeras were generated on the 129SV background which is less prone to weight gain with high fat diet and relatively protected BM chimeras were generated on the 129SV background which is more prone to weight gain with high fat diet and relatively protected.

In conclusion, our findings suggest that macrophage derived NPY and its anti-inflammatory effects may serve to preserve normal adipose tissue function in the setting of obesity. Further evaluation of the triggers for this response in adipose tissue may lay the groundwork for novel treatment strategies. Future work is required to fully understand the range of signals that NPY can produce, but this study provides a unique mechanism by which ATMs may influence the adipose tissue environment.

**Supporting Information**

**Figure S1** NPY treatment in obese animals does not decrease circulating monocyte or ATM content. C57Bl6 mice were fed a HFD for 4 weeks prior to treatment with NPY or NPY scramble control peptide (60 μg/kg/day) IP for 10 days. N = 5 per group. (A) Glucose tolerance test. (B) Flow cytometry quantitation of F4/80+CD11b+MHCII+ ATMs. (C) Gene expression analysis of EWAT for inflammatory genes by quantitative RT-PCR. *p<0.05. (D) Quantitation of Ly6ch high and Ly6clo CD115+ blood monocytes by flow cytometry (N = 4 per group), by t-test. (TIF)

**Table S1** Gene primer sequences used for quantitative RT-PCR. (DOCX)

**Table S2** Summary of metabolic parameters of lean (ND) mice injected with NPY for 10 days. N = 4 per group. Data presented ± SEM. *p<0.05 by t-test. Control vs NPY injection (DOCX)

**Table S3** Summary of metabolic parameters of HFD-fed mice injected with NPY for 10 days. N = 5 per group. Data presented ± SEM. *p<0.05 by t-test. *p<0.05 Control vs NPY injection (DOCX)

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Dr. Lumeng is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

**Author Contributions**

Conceived and designed the experiments: CNL KS. Performed the experiments: KS DLM KEO TW JD TM KWC. Analyzed the data: KS CNL. Wrote the paper: KS CNL.

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