High fat induces acute and chronic inflammation in the hypothalamus: effect of high-fat diet, palmitate and TNF-alpha on appetite-regulating NPY neurons

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▶ **To cite this version:**

Ps Dalvi, Ja Chalmers, V Luo, Dyd Han, L Wellhauser, et al.. High fat induces acute and chronic inflammation in the hypothalamus: effect of high-fat diet, palmitate and TNF-alpha on appetite-regulating NPY neurons. International Journal of Obesity, 2017, 41 (1), pp.149-158. 10.1038/ijo.2016.183 . hal-03108269

HAL Id: hal-03108269
https://cnrs.hal.science/hal-03108269

Submitted on 16 Nov 2022

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Accepted Article Preview: Published ahead of advance online publication

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Cite this article as: P S Dalvi, J A Chalmers, V Luo, D-YD Han, L Wellhauser, Y Liu, D Q Tran, J Castel, S Luquet, M B Wheeler, D D Belsham, High-fat induces acute and chronic inflammation in the hypothalamus: Effect of HFD, palmitate and TNF-α on appetite-regulating NPY neurons, *International Journal of Obesity* accepted article preview 24 October 2016; doi: 10.1038/ijo.2016.183.

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Accepted manuscript

Received 24 May 2016; revised 18 August 2016; accepted 18 September 2016; Accepted article preview online 24 October 2016

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High-fat induces acute and chronic inflammation in the hypothalamus: Effect of HFD, palmitate and TNF-α on appetite-regulating NPY neurons.

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Abbreviated title: Hypothalamic inflammation over time in NPY neurons

Disclosure: The authors have nothing to disclose.

Keywords: hypothalamus; neuroinflammation; palmitate; TNF-alpha; neuropeptide Y; cytokines; transcription; signal transduction.

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Abstract

Background: Consumption of dietary fat is one of the key factors leading to obesity. High-fat diet (HFD)-induced obesity is characterized by induction of inflammation in the hypothalamus; however, the temporal regulation of proinflammatory markers and their impact on hypothalamic appetite-regulating neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons remains undefined.

Methods: Mice were injected with an acute lipid infusion for 24 h or fed a HFD over 8-20 weeks. Characterized mouse NPY/AgRP hypothalamic cell lines were used for *in vitro* experimentation. Immunohistochemistry in brain slices, or qRT-PCR in cell lines, was performed to determine changes in expression of key inflammatory markers and neuropeptides.

Results: Hypothalamic inflammation, indicated by TNF-α expression and astrocytosis in the arcuate nucleus, was evident following acute lipid infusion. HFD for 8 weeks suppressed TNF-α, while significantly increasing HSP70 and ciliary neurotrophic factor, both neuroprotective components. HFD for 20 weeks induced TNF-α expression in NPY/AgRP neurons, suggesting a detrimental temporal regulatory mechanism. Using NPY/AgRP hypothalamic cell lines, we found that palmitate provoked a mixed inflammatory response on a panel of inflammatory and endoplasmic reticulum (ER) stress genes, whereas TNF-α significantly upregulated IκBα, NF-κB, and IL-6 mRNA levels. Palmitate and TNF-α exposure predominantly induced NPY mRNA levels. Utilizing an IKKβ inhibitor, we demonstrated that these effects potentially occur via the inflammatory IKKβ/NF-κB pathway.

Conclusions: These findings indicate that acute lipid and chronic HFD feeding *in vivo*, as well as acute palmitate and TNF-α exposure *in vitro*, induce markers of inflammation or ER stress in the hypothalamic appetite-stimulating NPY/AgRP neurons over time, which may contribute to a dramatic alteration in NPY/AgRP content or expression. Acute and chronic HFD feeding *in vivo* temporally regulates arcuate TNF-α expression with reactive astrocytosis, which suggests a time-dependent neurotrophic or neurotoxic role of lipids.

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Introduction

Obesity has emerged as a major health problem not only in the industrialized countries, but also in the developing nations. Sedentary lifestyles, reduced physical activity, accompanied by increased consumption of dietary fats, have contributed to the worldwide obesity pandemic [1]. Food intake and energy expenditure are tightly regulated by the hypothalamus. Hypothalamic appetite-regulating neurons, such as neuropeptide Y (NPY), agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) neurons, are responsible for sensing information on nutritional status by integrating central and peripheral signals[2]. Recent studies have shown that consumption of dietary fats induces hypothalamic resistance to insulin and leptin, which contributes to hyperphagia, obesity, and type 2 diabetes (T2DM) [3, 4]. In rodent genetic and diet-induced obesity models, the functional resistance to insulin and leptin in the hypothalamus develops partly through activation of inflammation in the mediobasal hypothalamus [3-5]. Additionally, research using rodent strains sensitive to diet-induced obesity has demonstrated an altered hypothalamic NPY/AgRP content or expression [6, 7], however, the exact role of inflammation in these neurons in obesity is poorly understood. Another study using postmortem human hypothalamii indicated that the expression of AgRP and NPY is correlated well with body weight changes or body mass index [8], yet the contribution of neuroinflammation in regulation of these neuropeptides still remains unstudied.

In recent years, a number of studies have implicated several signal transduction pathways involved in the induction of inflammation, such as suppressor of cytokine signaling-3 (SOCS3) [5], c-Jun N-terminal kinase (JNK) and I kappa B kinase (IKK) [4]. Further, several studies have provided considerable evidence that the inflammatory response to dietary fat is mediated by toll-like receptor (TLR) signaling, activation of NF-κB, and production of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α [9, 10]. Among these inflammatory markers, a role for TNF-α in the pathogenesis of T2DM and obesity has been demonstrated[11]. In diet-induced obese rats, the expression of TNF-α and other proinflammatory cytokines is remarkably increased in the hypothalamus [4, 12], and TNF-α has been found to modulate hypothalamic neuropeptides involved in appetite regulation [13]. However, the temporal activation of TNF-α and other proinflammatory markers by acute and chronic high-fat diet (HFD) in the hypothalamic mediobasal areas that are critical for energy homeostasis remains uncharacterized.

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In the present study, we confirmed acute upregulation of TNF-α expression and astrocytosis in the arcuate nucleus (ARC) within 24 h of Intralipid infusion, which corroborates a previous study using HFD[12]. Within the first 8 weeks of chronic HFD feeding, the TNF-α expression was reduced; however, the expression of heat shock protein 70 (HSP70) that protects from oxidative stress, and also the expression of ciliary neurotrophic factor (CNTF) that reduces neuronal injury during inflammation, was increased, suggesting an effective neuroprotective response in the ARC of the hypothalamus. The TNF-α expression increases following long-term HFD feeding for 20 weeks indicating that TNF-α-induced inflammation may have a time-dependent neurotrophic or neurotoxic role. Using well-established mouse hypothalamic cell lines, we report evidence of increased inflammatory and ER stress markers with acute treatment of the saturated fatty acid palmitate and TNF-α. We also report that appetite-stimulating NPY is significantly upregulated by palmitate and TNF-α in the neuronal cells. These *in vivo* and *in vitro* findings collectively suggest that acute and chronic HFD exposure regulates hypothalamic inflammation by temporal activation of TNF-α that may further modulate appetite by positively regulating NPY, suggesting a dysregulation of energy homeostasis feedback in a high fat environment.

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Methods

Animals

Animal experiments were performed with approval of the Animal Care Committee of the University Paris Diderot-Paris 7 and the University of Toronto. Male C57BL/6 mice (Janvier, Le Genest St Isle, France) were housed individually. Male CD-1 mice (Charles River, Canada) were maintained on an *ad libitum* standard rodent chow diet. C57BL/6 mice were used for acute Intralipid infusion, and CD-1 mice were fed with a diet containing 60% kcal fat (HFD, 5.49 kcal/gm, F3282; Bio-Serv, NJ, USA) for periods ranging from 8-20 weeks. 10 to 12 week old animals were used at the commencement of the experiments. Once the mice were switched to the HFD feeding, body weight was monitored weekly.

Intralipid Infusion

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Catheter implantation and lipid perfusion were carried out as previously described [14]. Infusions started after a 7-10 days recovery period. Intralipid® was obtained from Sigma-Aldrich (St. Louis, MO). Mice were infused with saline solution for 3-4 days for habituation to the infusion device. Mice were then divided into 2 groups: one received saline and the other a triglyceride emulsion Intralipid at a rate of 0.1-0.3 µl/min for 6 h. The pump settings allowed a constant flow rate of ~0.1 µl/min. The animals were sacrificed 24 h following infusion.

Glucose tolerance test:

Glucose tolerance was assessed by intraperitoneal (i.p.) glucose tolerance test (IPGTT) after 7 weeks of treatment. Mice were fasted for 6 h, and the tests were carried out at 2:00 pm. Glucose (1.5 mg/g body weight) was administered by i.p. injection. Blood samples were drawn from the tail vein at 0, 10, 30, 60, 90, and 120 min after glucose administration. Blood glucose levels were measured using a Glucometer (Bayer).

Immunofluorescence

Mice were anesthetized and perfused transcardially with ice-cold phosphate-buffered saline followed by freshly prepared 4% paraformaldehyde solution. The brains were removed, post-fixed in 4% paraformaldehyde, serially cryoprotected in sucrose, snap-frozen in and stored at -80°C. Frozen brains were sliced in 20 µm sections (Leica CM1510S), as previously described[15]. Sections blocked in 5% normal donkey or rabbit serum (Vector Laboratories, Burlington, ON) were incubated overnight at 4ºC with specific primary antibodies: rabbit anti-AgRP (1:200; catalog no. #H003-57, Phoenix Pharmaceuticals, Burlingame, CA), rabbit anti-NPY (1:200; catalog no. #H049-03, Phoenix Pharmaceuticals), chicken anti-NSE (1:100; catalog no. #Ab 39369, Abcam, Cambridge, MA), chicken anti-GFAP (1:500; catalog no. #Ab 4674, Abcam), goat anti-TNF-α (1:50; catalog no. #SC1350, Santa Cruz, Inc., Santa Cruz, CA), rabbit anti-HSP70 (1:100; catalog no. Ab31010, Abcam), and rabbit anti-CNTF (1:1000; catalog no. Ab46172, Abcam). Immunofluorescence was performed with a combination of Alexa Flour 555- or Alexa Flour 488-labeled anti-rabbit, anti-goat, or anti-chicken secondary antibodies (1:500; Life Technologies Inc.) and nuclear stain TOPRO (1:1000; catalog no. T3605(Life Technologies Inc.) was used to identify cell nuclei.

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Images from immunostained sections were captured on a Zeiss LSM 510 confocal upright microscope outfitted with a color digital camera and AxioVision 3.0 imaging software. For the quantification of cells, every second section throughout the ARC was taken to visualize the distribution of immuofluorescence (total 4-5 sections/mouse). For each of the sections, bilateral images were captured in a single plane of focus at x40 magnification and a 100 μm^2 box was placed in the center of the selected hypothalamic ARC region. The intensity of immunofluorescence was measured in a blinded manner using ImageJ software. The intensity was determined as the signal to background intensity ratio. The results were expressed as relative intensity of GFAP, TNF-α, CNTF, HSP70 or NSE in hypothalamic ARC AgRP- or NPY-expressing neurons for each group of mice.

Cell Models

The clonal, embryonic mouse hypothalamic mHypoE-44 and mHypoE-46 cell lines, and the adult mouse hypothalamic mHypoA-NPY/GFP cell line, were generated and culture conditions described previously by our laboratory [16-18]. Cell lines were in 60 mm dishes (Sarstedt, Montreal, QC)and allowed to reach 70-80% confluence. TNF-α, sodium palmitate and the IKKβ inhibitor, PS1145 were purchased from Sigma. Sterile hypure water was used as the vehicle for TNF-α and sodium palmitate. Sodium palmitate was solubilized in sterile hypure water by heating the mixture to 65°C followed by gentle vortexing, and immediately added to culture medium pre-heated to 37°C containing 5% FBS to act as a carrier. The inhibitor PS1145 was prepared in dimethyl sulfoxide (DMSO), and the concentration of DMSO was ~0.01% for treatments. Cells were pre-treated with PS1145 1 hour prior to administration of TNF-α or sodium palmitate.

Quantitative RT-PCR

RNA was isolated from cell using the purelink RNA mini columns and on column DNAse (Ambion). A total of 1 ug of RNA was then used to synthesize cDNA with the high capacity cDNA archive kit (Applied Biosystems). Quantitative RT-PCR was conducted using gene specific primers (Supplemental Table 1) and platinum SYBR green qPCR supermix-UDG w/ROX according to the manufacturer's instructions. cDNA samples (25 ng) and dose curve (Range of 50 ng to 1.56 ng) were loaded in triplicate for each gene. Samples were run on a 384-

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well block ABI 7900HT fast real-time PCR system at 50°C for 2 min, 95°C for 2 min, and 40 repeats of 95°C for 15 sec to 60°C for 1 min, followed by melting curve analysis at 95°C for 15 sec to 60°C for 15 sec then 95°C for 15 sec. and analyzed using SDS 2.4 software (Applied Biosystems).

Statistical analysis

Data are presented as the mean ± SEM. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Two groups were compared using two-tailed Student's t tests. For more than two groups, statistical analysis was performed using one-way or two-way ANOVA, and statistical significance was determined by post hoc analysis using Bonferroni test, Holm-Sidak or Student's *t*-test with $P < 0.05$.

Results

Temporal expression of inflammatory markers

To test whether acute treatment with fatty acids induces hypothalamic inflammation, we used Intralipid, an emulsion of fatty acids containing 7-14% palmitic acid together with a mixture of linoleic and oleic acids. We injected Intralipid via carotid artery cannulation for its direct delivery into the brain and these lipids readily diffuse across the blood-brain barrier to access hypothalamic neurons[19]. We found that TNF-α immunoreactivity was significantly increased within 24 h post fatty acid exposure in the hypothalamus (Figures 1A-C). We observed no change in the immunoreactivity of either CNTF or HSP70 (Figure 1C). We found that the number of GFAP-positive astrocytes was singificantly upregulated (Figure 1D).

To further understand the effect of prolonged exposure to saturated fats on hypothalamic inflammation and putative neuroprotective responses, mice were fed a HFD for 8 weeks. HFD–fed mice exhibited significantly increased body weight gain over time (Figure 1E), however we found that the cumulative food intake during the 8th week of feeding was significantly decreased in HFD-fed mice (Figure 1F). To test the efficacy of the HFD feeding on induction of glucose intolerance, we performed IPGTTs. When glucose was administered via an i.p. injection, HFD-fed mice demonstrated significantly increased glucose excursion and area under the glucose

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curve compared with chow diet-fed mice (Figures 1G and H), suggesting dysregulation of glucose homeostasis in the HFD-fed mice. Further, we found that the immunofluorescence of TNF-α was significantly diminished at this stage (Figures 1I-K). On the other hand, we found a significant increase in the expression of HSP70 (Figure 2A-C) and CNTF (Figure 2D-F), the prominent components of the neuroprotective response to neuronal injury. We also found that the number of GFAP-positive astrocytes remained singificantly upregulated (Figure 2G). In a separate cohort of mice fed with HFD for 8 weeks, gene expression analysis revealed significantly elevated hypothalamic levels of IκBα, without any change in the levels of IL-6 and NPY, however, the levels of AgRP and NF-κB, an activator of TNF-α, were significantly suppressed (Figure 2H).

In mice fed a HFD for 20 weeks, the expression of TNF-α was found to be significantly elevated in the ARC NPY/AgRP neurons (Figures 3A-C). To further demonstrate that the hypothalamic inflammation was clearly evident in the ARC neurons, and not only limited to the glia, we used immunofluorescence for neuron-specific enolase (NSE), marker of mature neurons. We detected that TNF-α and NSE were clearly co-localized in the ARC (Figure 3D).

Astrocytosis marker analysis

During neuronal injury, astrocytes can be activated and mobilized to the site of the injury where they can further proliferate to give rise to reactive astrocytosis[20, 21]. Using immunohistochemistry to detectimmunoreactivity of GFAP, which is regarded as a sensitive and reliable marker expressed by most reactive astrocytes that are responding to CNS injuries [22], we found that there was a significant upregulation of GFAP expression by 17% in the hypothalamic ARC by 24 h following Intralipid infusion in mice (Figures 1A-D). This result suggests an acute effect of fatty acids in promoting astrocyte accumulation in this brain region. Interestingly, the level of hypothalamic astrocytosis was found to be elevated by 135%-290% following extended HFD feeding at 8 weeks compared with that in chow-fed controls (Figures 1I-K and 2A-G).

Temporal palmitate-induced gene expression

Intralipid contains 7-14% palmitate and the HFD used to chronically feed the mice contained 14.1% saturated fat. Therefore, to further understand the role of saturated fatty acid-

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induced acute inflammation in appetite-regulating NPY/AgRP neurons, we used the saturated fatty acid palmitate to treat immortalized mouse hypothalamic cell lines, expressing NPY and AgRP, for the *in vitro* experimentation[16]. Expression of pro-inflammatory markers, such as IκBα, NF-κB p50 subunit, IL-6, TNF-α; NF-κB p65 subunit RelA; pro-apoptotic proteins, such as CHOP, p53, FADD, glucose-regulated protein-78; components of the neuroprotective response to neuron injury, such as HSP70, HSP72; and appetite-regulating genes, such as AgRP and NPY, were assessed by qRT-PCR. Palmitate treatment (4 h) in the hypothalamic mHypoE-44 and -46 neuronal cells induced a mixed inflammatory response, while robustly increasing mRNA levels of CHOP, a marker of endoplasmic reticulum (ER) stress implicated in apoptosis, and significantly upregulated NPY mRNA levels without any change in AgRP mRNA (Figures 4A and B). Of interest, we did not observe any change in the level of TNF-α mRNA in either cell line; however, IκBα and NF-κB mRNA levels decreased, whereas proinflammatory marker IL-6 mRNA levels changed in a cell-specific manner.

Prolonged exposure to palmitate for up to 33 h demonstrated that NPY (Figures 5A and B) and CHOP (Figures 5C and D) mRNA levels in both mHypoE-44 and -46 cell lines remain elevated, whereas AgRP mRNA levels again did not change (data not shown). By inhibiting the IKKβ/NF-κB cascade through pre-treatment with the IKKβ inhibitor PS1145 (20 μM), we demonstrated that the palmitate-mediated increase in NPY mRNA levels was abolished, indicating palmitate acts through the IKKβ/NF-κB pathway to regulate NPY expression (Figures 6A and B). On the other hand, CHOP mRNA levels were unchanged, suggesting an IKKβ/NF-κB -independent mechanism forits upregulation (Figures 6C and D).

Temporal TNF-α-induced gene expression

Based on the acutely increased expression of TNF-α *in vivo*, we further investigated the potential role of TNF-α on acute inflammation in the NPY/AgRP neuronal cell models. Besides the mHypoE-46 cell line[16], we used another well-characterized adult-derived mouse NPY/AgRP cell line, mHypoA-NPY/GFP[18]. We found that when treated with TNF-α, both the hypothalamic cell lines demonstrated a robust inflammatory response indicated by an upregulation of IκBα, NF-κB, IL-6, and RelA mRNA levels within 6 h following TNF-α treatment (Figures 4C and D). This upregulation was found to be independent of the IKKβ/NF-κB cascade given that PS1145

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pretreatment did not abolish the effect of TNF-α (Figures 6H and J). Treatment with TNF-α in the mHypoE-46 cells, but not in the mHypoA-NPY/GFP cells, upregulated NPY mRNA levels within 6 h, which was reversed by pretreatment with PS1145, indicating involvement of the inflammatory IKKβ/NF-κB pathway, similar to that found with palmitate (Figures 6E and F).

Discussion

Activation of an inflammatory response in the hypothalamus is well-documented in rodent models of diet-induced obesity [3, 4, 23], however, the underlying mechanisms remain largely unstudied. Further, the temporal regulation of TNF-α and other markers of neuron injury or protection are not yet described. We report that the expression of TNF-α, HSP70, and CNTF in the hypothalamus is temporally regulated by Intralipid infusion or HFD feeding in mice. A rapid increase in TNF-α expression in the ARC following an acute infusion of Intralipid suggests a primary response that most likely limits or reverses the injury caused by high fatty acid exposure in favour of neuroprotection, despite being a proinflammatory cytokine [24, 25]. The neuroprotective role of TNF-α is also demonstrated in TNF-α receptor-deficient mice that develop increased neuronal lesions in response to ischemic injury [26]. The suppression of TNF-α expression and increase in HSP70 and CNTF that were observed with extended exposure to HFD feeding at 8 weeks suggest possible neuroprotective or even neuroregenerative responses that can be triggered to further prevent or limit injury and promote repair mechanisms in the hypothalamus[17, 27, 28]. Eventually, chronic HFD feeding at 20 weeks causes an increase in TNF-α expression in the NPY/AgRP neurons, suggesting exhaustion of hypothalamic protective mechanisms and development of chronic inflammation, favouring appetite and neuropeptide dysregulation inclined towards energy intake.

Acute Intralipid infusion induces TNF-α expression in the hypothalamic neurons within 24 h. Although some previous studies have evaluated the abilities of fatty acids to induce cytokine expression in the hypothalamus [3], this is the first description of TNF-α induction in the mouse hypothalamus within a short period of time. Our finding is consistent with the previously observed HFD-induced acute increase in TNF-α mRNA expression that was accompanied with reactive gliosis in the hypothalamus of rats after one day of HFD feeding[12]. The same study found that the acutely increased TNF-α mRNA expression eventually subsided only to rise again

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after 4 weeks of HFD feeding [12]. The rapid onset of inflammation in the mediobasal hypothalamus manifests neuron injury that may recruit neuroprotective measures to prevent further damage of the ARC neurons. This initial neuroprotective response represents an important defence mechanism to protect neurons involved in appetite regulation and energy homeostasis. As such, it has already been demonstrated that chronic HFD feeding significantly reduces the number of hypothalamic POMC neurons that play a major role in protecting against excess weight gain[12], and their loss is sufficient to cause obesity in mice [29].

Although, the exact role of Intralipid-induced acute TNF-α in the hypothalamus remains to be investigated, Amaral *et al*. evaluated the capacity of TNF-α to modulate hypothalamic neuropeptides involved in appetite regulation and energy homeostasis [13], in which they found that direct TNF-α injection in the hypothalamus acutely upregulates both orexigenic NPY and anorexigenic POMC expression within 2 h post-injection[13]. Acute TNF-α infusion has also been shown to cause hypothalamic insulin and leptin resistance within 4 h post-infusion[30]. We did not investigate whether Intralipid or the Intralipid-induced increase in the TNF-α expression causes neuronal insulin resistance in these mice; however, studies have demonstrated that a short exposure to fatty acids results in a significant reduction in insulin signaling in the CNS[31, 32]. Overall, based on the past and present evidence we predict a possible path towards the development of insulin resistance via TNF-α induction following acute Intralipid treatment in mice, and therefore, further studies are needed to understand the mechanistic role of TNF-α in this process.

The HFD used contained as much as 6-fold more saturated fat than the control diet. This is comparable to the amount of saturated fat present in most Western diets [33]. Chronic feeding of rats with a similar diet has been shown to cause high levels of TNF-α, IL-1β, and IL-6 in the hypothalamus, predominantly in the median eminence and ARC[3]. It was found that elevated levels of pro-inflammatory cytokines, such as TNF-α, induce cell apoptosis that can be prevented by over-expression of HSP70 [34]. In the brain, the heat shock proteins serve a neuroprotective role [35]. HSP70 is well-studied and is involved in the repair mechanism after environmental stressors, such as heat, ischemia, ultraviolet irradiation, and oxidative stress [35]. In the present study, the increase in HSP70 that was observed following extended exposure to HFD feeding supports

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itsneuroprotective role in the hypothalamus. Another study found that the mRNA expression of hypothalamic HSP72, a member of the HSP70 family, was increased within 3 days of HFD exposure in rats and was detected in the POMC neurons that are critical for energy balance regulation[12]. The induction of heat shock proteins following HFD warrants further study on their potential therapeutic neuroprotective roles.

Studies have documented the ability of glial-derived CNTF to promote cell survival and differentiation in a number of neuronal and glial cell types [36, 37]. Here we show that CNTF expression does not change in the hypothalamic ARC following acute Intralipid infusion, but in 8 week HFD feeding conditions it significantly increases in the ARC of mice. Our immunofluorescence data indicate that an increase in CNTF expression was paralleled by an increase in hypothalamic astrocyte expression, suggesting that chronic HFD feeding in mice is associated with increased CNTF expression via astrocytosis in the hypothalamus. This finding supports the notion that CNTF is a novel, glial-derived modulator of hypothalamic neuropeptidergic neurons in chronic HFD-fed mice[38]. Studies have demonstrated a neuroprotective effect of CNTF via stimulation of gene expression, cell survival or differentiation [37]. Recently, we and others have shown that CNTF induces neurogenesis in the adult hypothalamus and may contribute to the control of energy homeostasis[17, 28]. Other studies have also clearly demonstrated that administration of CNTF or its recombinant analog Axokine reduces food intake [39-41]. Thus, the increased expression suggests that CNTF could potentially enhance neurogenesis in the hypothalamus, as a part of reactive repair mechanism to counter the detrimental effects of HFD exposure on neurons.

We found that long-term HFD feeding in mice induced increased levels of TNF-α expression in NPY/AgRP neurons. Studies on enteric neurons have suggested that during inflammation TNF-α upregulation induces NPY, and NPY-TNF-α crosstalk aggravates inflammatory signaling [42]. In support of this previous finding, our study demonstrates a high level of crosstalk between NPY-TNF-α in the NPY/AgRP neurons in favour of excessive energy accumulation in HFD-fed mice. However, we found that in chronically HFD-fed mice the cumulative food intake decreased over time suggesting a compensatory response to surplus energy balance, but the neuronal circuits involved and the exact role and contribution of the

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TNF-α-mediated inflammation in the arcuate NPY/AgRP neurons in the chronic positive energy balance despite reduced food intake warrants further investigation. To further enhance our understanding of the acute effect of palmitate or TNF-α on the markers of inflammation or neuropeptide expression, we selected three different cell lines that endogenously express NPY/AgRP[16, 18]. Accordingly, we found that both palmitate and TNF-α induce inflammatory markers in these neuronal cells with simultaneous upregulation of NPY mRNA levels. Palmitate also induced a robust increase in the CHOP mRNA, a marker of endoplasmic reticulum (ER) stress implicated in apoptosis. Indeed, saturated fatty acids induce ER stress via activation of TLR signaling in the hypothalamus [3]. Furthermore, in diet-induced obese mice, CHOP and other markers of ER stress were linked in the hypothalamus to an induction of central leptin and insulin resistance [43]. Previously, using the mHypoE-44 cell line, it was found that palmitate treatment induced ER stress through a c-Jun N-terminal kinase (JNK)-dependent pathway and activation of the ER stress markers eIF2 alpha and X-box binding protein-1 to induce insulin resistance [44]. However, whether CHOP activation in these neuronal cells induces leptin or insulin resistance or apoptosis remains to be studied.

TLR4 acts as a predominant molecular target for saturated fatty acids in the hypothalamus, triggering the intracellular signaling network that induces an inflammatory response via upregulation of TNF-α, and determines the resistance to anorexigenic or susceptibility to orexigenic signals[3]. The findings from this study indicate that acute treatment of TNF-α induces robust inflammatory response by upregulating IκBα, NF-κB and IL-6 mRNA levels in the hypothalamic neuronal models. These components are a part of the IKKβ/NF-κB cascade activated by TLR4 [9, 10]. With the IKKβ inhibitor PS1145, we demonstrated that the palmitate-mediated increase in NPY mRNA levels was abolished, indicating palmitate and TNF-α act through the IKKβ/NF-κB pathway to regulate NPY expression. However, the exact downstream mechanisms responsible for the change in NPY mRNA expression remain to be determined. Further studies are also warranted in other neuronal cell types to understand the role of activated TNF-α together with robust astrocytosis in the neurodegeneration of anorexigenic neurons, such as POMC during chronic HFD feeding conditions[12, 45]. Clearly, there will likely be a coordinated activation of pathways in each neuronal cell type to achieve the final detrimental result of chronic obesity.

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In conclusion, we report that, in mice, acute exposure to fatty acids induces expression of inflammatory cytokine TNF-α in the mediobasal hypothalamus that is accompanied with reactive astrocytosis. The longer-term HFD exposure at 8 weeks does not induce TNF-α expression, but gives rise to induction of markers of neuroprotection and neuroregeneration. Although the defence mechanisms appear to limit the neuronal injury initially, recovery appears to be transient as chronic inflammation rebounds with extended HFD feeding. Reactive astrocytosis remains persistent with the HFD feeding model most probably due to neuronal injury, damage, or loss in the hypothalamus critical for energy homeostasis. The *in vitro* data suggests that TNF-α may play an important role in palmitate- and TNF-α-induced acute or longer-term stimulation of NPY via activation of the inflammatory IKKβ/NF-κB cascade. These findings suggest a chronic positive energy balance mechanism after prolonged exposure to HFD or saturated fatty acids, such as palmitate, mediated through the increased expression of the appetite-stimulating neuropeptide, NPY, and this could ultimately lead to the difficulties experienced with weight regulation and management.

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Acknowledgements

We acknowledge funding from the Canadian Institutes for Health Research (CIHR) (DDB, MBW), Canadian Diabetes Association (DDB, MBW), and Canada Research Chairs Program (DDB). Scholarship support through the Banting and Best Diabetes Research Centre (PSD, LW), CIHR Fellowship (LW), NSERC Studentships (PSD, DQT), and an Ontario Graduate Scholarship (PSD, DQT) is much appreciated.

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Figure legends

Figure 1: Effect of acute Intralipid treatment at 24 h post-infusion in C57BL/6 mice on hypothalamic arcuate nucleus (ARC) reactive astrocytosis and expression of TNF-α, a marker of inflammation, and effect of high fat diet (HFD, 60% kcal) feeding for 8 weeks in CD-1 mice on body weight gain, glucose tolerance, ARC reactive astrocytosis and expression of TNF-α. (A, B, I, J) Representative images showing expression of immunofluorescence for GFAP (green) and TNF-α (red) in the hypothalamic ARC in C57BL6 mice at 24 h following acute infusion with saline (A) or Intralipid (B), or in CD-1 mice fed with chow (I) or HFD (J) for 8 weeks. (A-D) Inflammation in the ARC, indicated by significant upregulation of astrocytosis and TNF-α expression (A-D) without any change in CNTF or HSP70 expression (C) was evident within 24 h following Intralipid infusion in C57BL/6 mice, whereas the number of GFAP-positive cells was significantly upregulated (D). (E, F) Analysis revealed that 8 weeks of HFD feeding had significant effect on body weight gain (E), while the cumulative food intake was significantly decreased in the HFD-fed mice during the 8th week of HFD-feeding (F). (G) Intra-peritoneal glucose tolerance test (IPGTT) was completed during the 8 week HFD feeding period and was performed in mice that were fasted for 6 h. (H) AUC for the IPGTT was calculated from 0 to 120 min (units are millimoles per liter per minute). (I-K) TNF-α expression was downregulated while astrocytosis remained significantly upregulated at 8 weeks of HFD feeding in CD-1 mice. Nuclei are stained with DAPI (blue). Quantification of intensity of immunofluorescence (C, K) of CNTF, HSP70, GFAP and TNF-α in the ARC from C57BL/6 mice infused with saline or Intralipid (C), or from CD-1 mice fed either chow or HFD for 8 weeks (K), and quantification of the number of GFAP-positive astrocytes in the hypothalamic arcuate nucleus (D). The dashed boxes indicate the region used for quantification of intensity of CNTF, HSP70, GFAP and TNF-α immunofluorescence, and GFAP-positive astrocyte number. Data in the bar graph are expressed as mean ± SEM (n = 5-6 animals/group); *P < 0.05 versus control.

Figure 2: Effect of high fat diet (HFD, 60% kcal) feeding for 8 weeks in CD-1 mice on the markers of reactive astrocytosis, neuroprotective response to neuron injury, and proinflammatory and appetite regulating genes in the hypothalamic arcuate nucleus (ARC). (A-G) Representative images showing expression of immunofluorescence for GFAP (A, B, D, E), HSP70 (A, B), and

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CNTF (D, E) in the hypothalamic ARC in CD-1 mice fed with chow (A, D) or HFD (B, E) for 8 weeks. Immunofluorescence (A, B, D, E) shows expression of GFAP (green), HSP70 (red) and CNTF (red). Nuclei are stained with DAPI (blue). Quantification of immunofluorescence intensity (C, F) of GFAP (C, F), HSP70 (C), and CNTF (F) in the ARC. (G) Quantification of the number of GFAP-positive astrocytes in the hypothalamic arcuate nucleus. (H) Quantification of levels of mRNA encoding proinflammatory (IκBα, NF-κB and IL-6) and appetite-regulating genes (AgRP and NPY) in the whole hypothalamus. The dashed boxes indicate the region used for quantification of intensity of CNTF, HSP70, and GFAP immunofluorescence, and GFAP-positive astrocyte number. Total RNA was isolated from the whole hypothalamus and was used as a template for real-time RT-PCR with primers specifically designed to amplify mRNA encoded by specific genes. Data in the bar graph are expressed as mean ± SEM (n = 5-6 animals/group); *P < 0.05, **P < 0.01, and ***P < 0.001 versus control.

Figure 3: Effect of high fat diet (HFD, 60% kcal) feeding for 20 weeks on mouse hypothalamic arcuate nucleus (ARC) neurons. (A-D) Representative images showing expression of double-labelled immunofluorescence images for AgRP (green) (A, B) or NPY (green) (C, D) and TNF-α (red) (A-D) in the hypothalamic ARC following chow (A, C) or HFD (B, D) feeding for 20 weeks in CD-1 mice: HFD feeding induces hypothalamic inflammation as indicated by increased expression of TNF-α, a marker of inflammation, in the AgRP and NPY neurons of the ARC. (E) Graphical representation showing quantification of the number of neurons co-expressing AgRP or NPY and TNF-α in the ARC of CD-1 mice: HFD feeding significantly increases the number of AgRP and NPY neurons co-expressing TNF-α. The dashed boxes indicate the region used for quantification of intensity of AgRP, NPY, and TNF-α immunofluorescence, and TNF-α-positive AgRP and NPY neuronal number. (F) Representative images showing double-labelled immunofluorescence for TNF-α (red) and neuron-specific enolase (NSE, green) in the ARC of the hypothalamus. Nuclei are stained with DAPI (blue). Data in the bar graph are expressed as mean ± SEM (n = 5-6 animals/group); *P < 0.05 versus control.

Figure 4: Gene expression analysis by real-time RT-PCR for the hypothalamic markers of inflammation, ER stress, neuroprotective response to neuron injury, and appetite regulation following (A,B) palmitate or (C,D) TNF-α treatment in the hypothalamic NPY-expressing

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neuronal cell lines mHypoE-44, mHypoE-46, and mHypoA-NPY/GFP. (A,B) The cells were treated with palmitate or (C,D) TNF-α and total RNA was isolated at 4 h (palmitate) or 6 h (TNF-α) and used as a template for real-time RT-PCR with primers specifically designed to amplify mRNA encoded by specific genes, n=3-4. The mRNA levels were quantified using the Ct method and normalized to the internal control Histone 3A. All results shown are relative to corresponding control mRNA levels and are expressed as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus control. Statistical analysis was performed using two-way ANOVA followed by post hoc analysis using Holm-Sidak *t*-test.

Figure 5: Effect of prolonged exposure of palmitate on NPY, AgRP, and CHOP mRNA levels in the hypothalamic mHypoE-44 and mHypoE-46 cell lines. The cells were treated with palmitate (50μM) and total RNA was isolated at the indicated time points over 33 h time period and used as a template for real-time RT-PCR with primers specifically designed to amplify mRNA encoded by specific genes. (A, B) NPY, (C, D) AgRP, and (E, F) CHOP mRNA levels were quantified using the Ct method and normalized to the internal control Histone 3A in the mHypoE-44 (A, C, E) and mHypoE-46 (B, D, F) neuronal cell lines, n = 3-4. All results shown are relative to corresponding control mRNA levels at each time point and are expressed as mean as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus control. Statistical analysis was performed using two-way ANOVA followed by post hoc analysis using Bonferroni test.

Figure 6: (A-D) Effect of pre-treatment with the IKKβ inhibitor PS1145 on palmitate-mediated regulation of NPY and CHOP mRNA levels in the hypothalamic mHypoE-44 and mHypoE-46 cell lines. (E-J) Effect of pre-treatment with the IKKβ inhibitor PS1145 on TNF-α-mediated regulation of NPY, IκBα, and IL-6 mRNA levels in the hypothalamic mHypoA-NPY/GFP and mHypoE-46 cell lines. The cells were pre-treated with PS1145 (20 μM) 1 h prior to treatment with palmitate (50 μM) or TNF-α (50 ng/ml) and total RNA was isolated for at 4 h (palimitate) or 6 h (TNF-α) and used as a template for real-time RT-PCR. (A,B) NPY, (C,D) CHOP, (E,F) NPY, (G,H) IκBα, and (I,J) IL-6 mRNA levels were quantified using the Ct method and normalized to the internal control Histone 3A in the cell lines indicated, n = 3-4. All results shown are relative to corresponding control mRNA levels at each time point and are expressed as

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mean as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus control. Statistical analysis was performed using two-way ANOVA followed by post hoc analysis using Bonferroni test.

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Figure 1

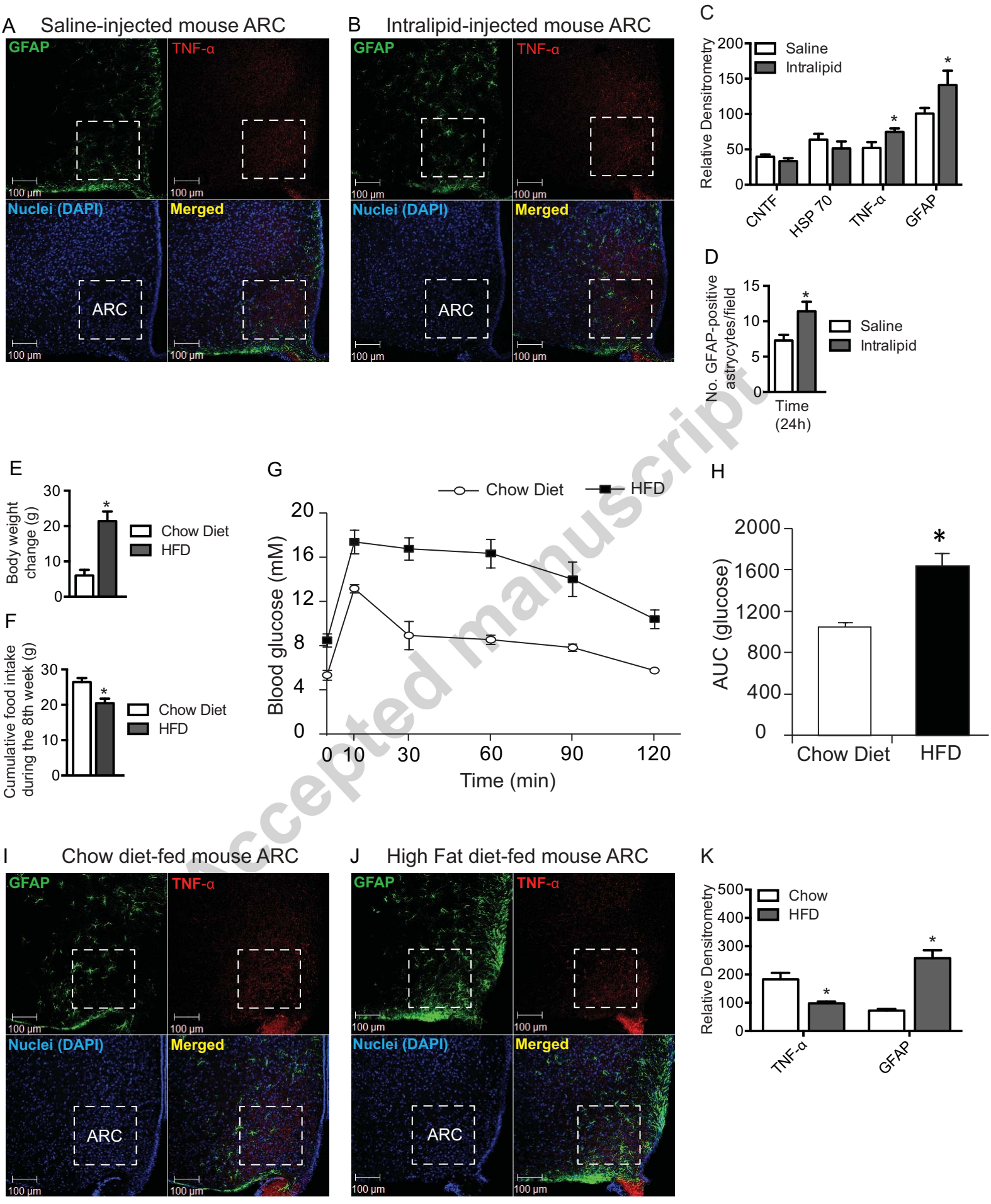

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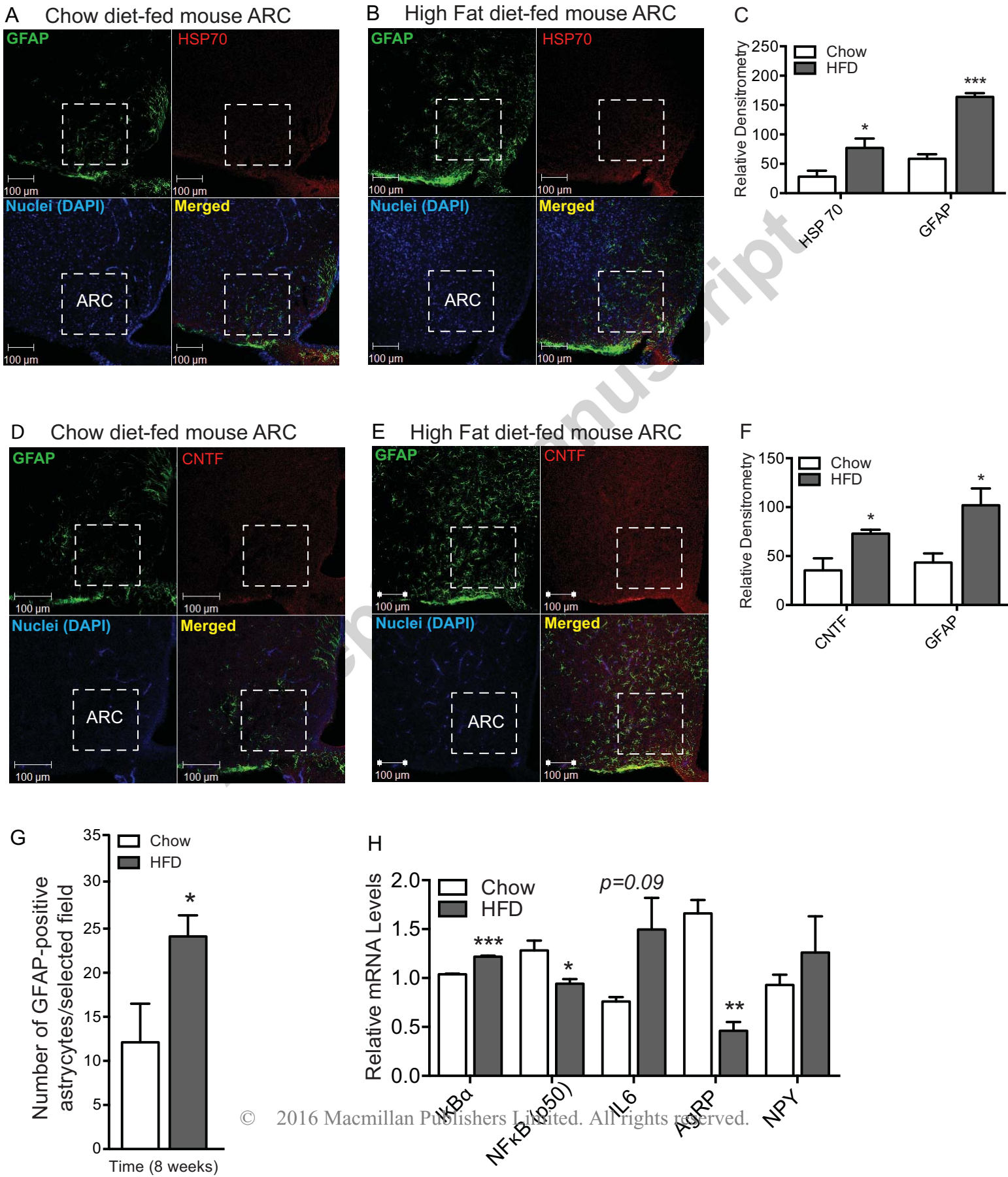
Figure 2



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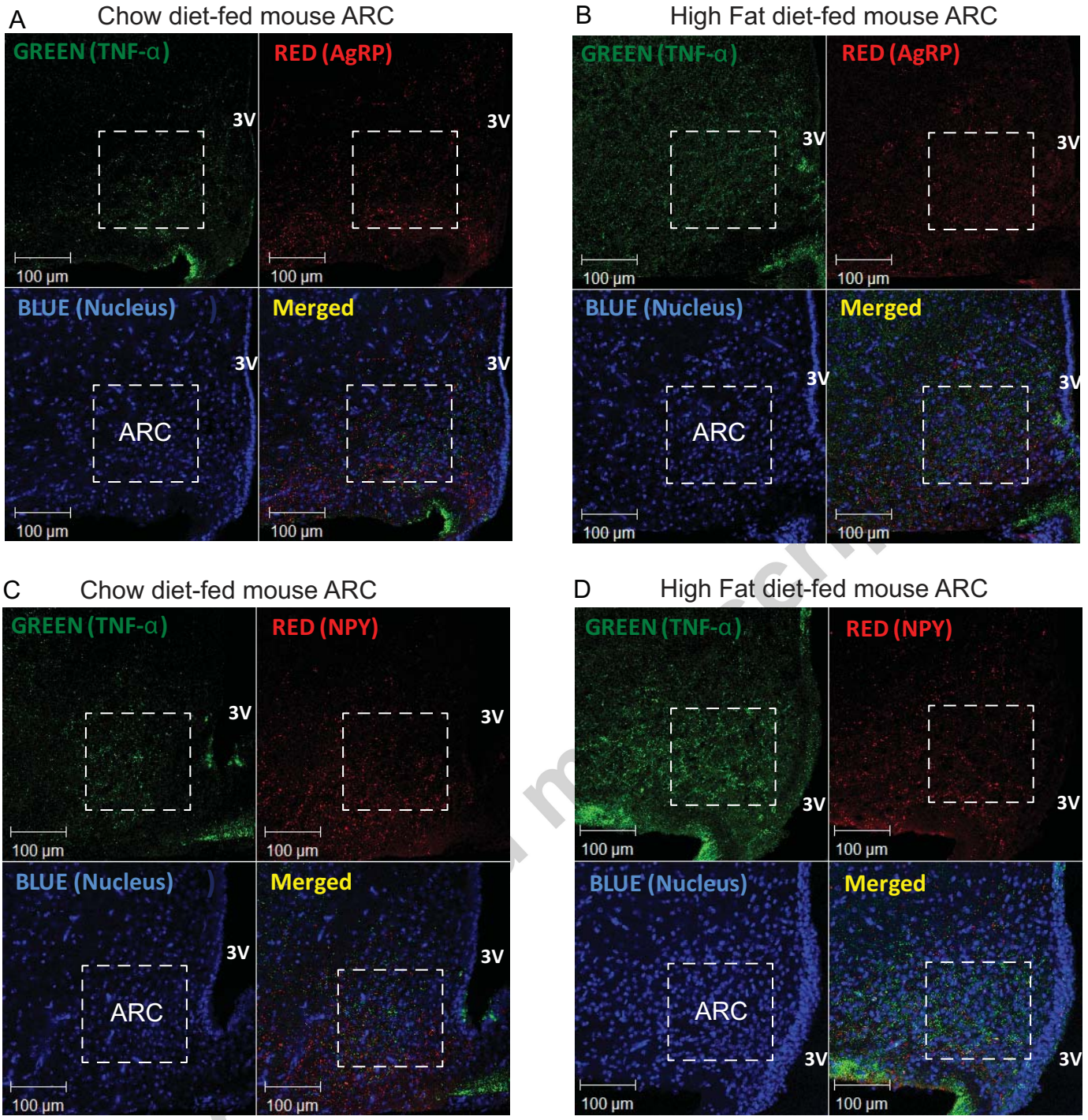

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Figure 4

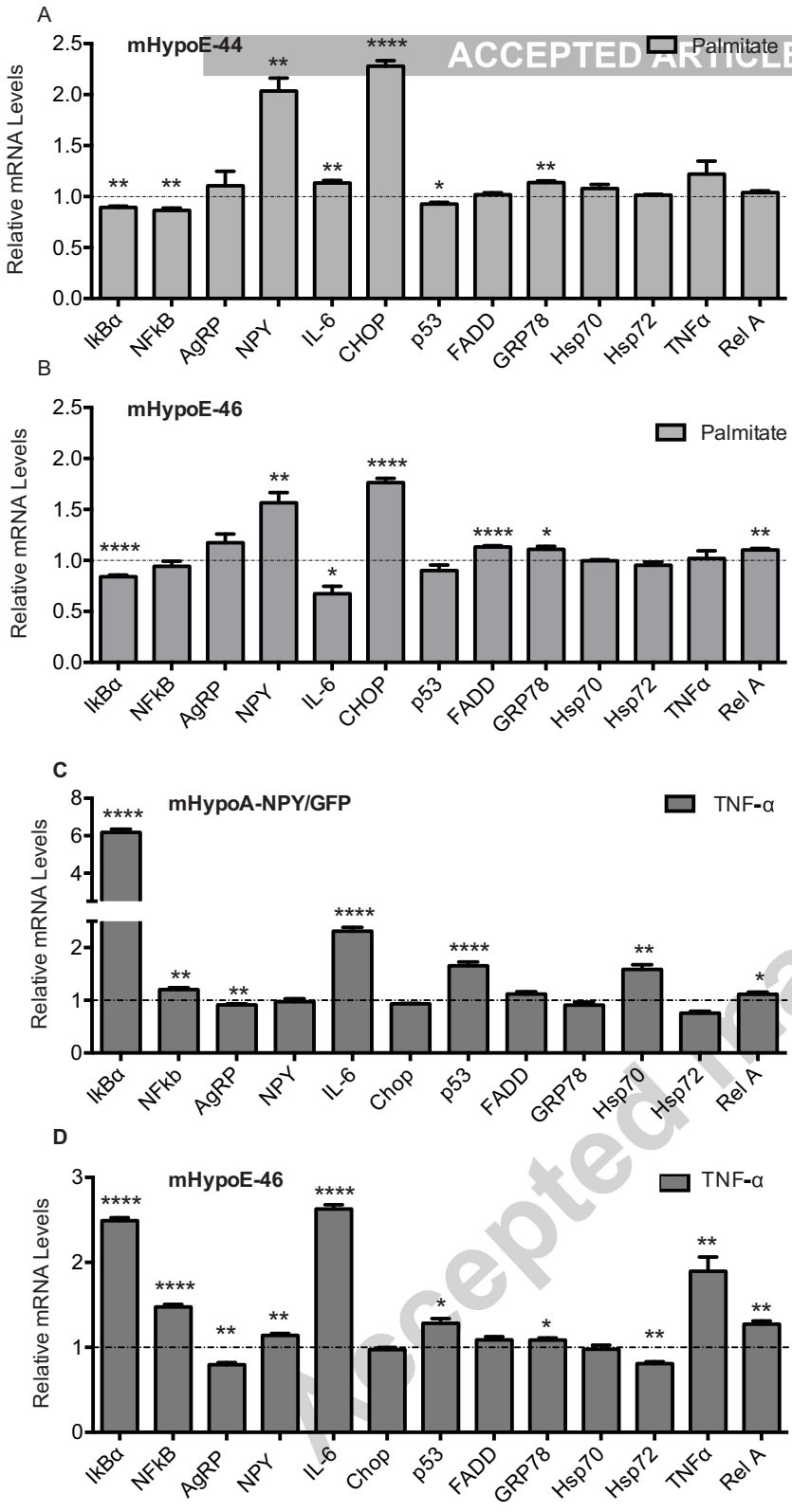

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Figure 5

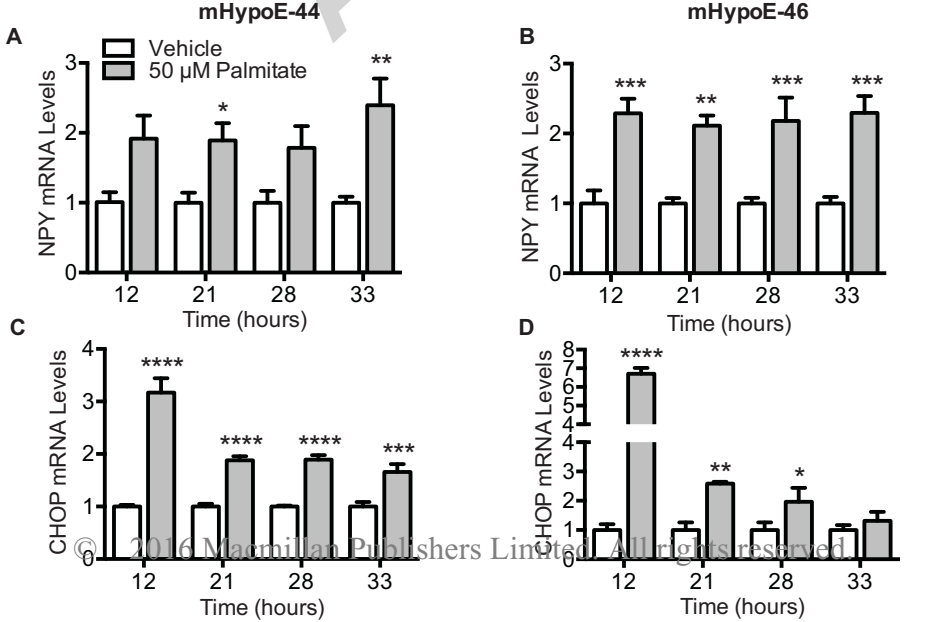

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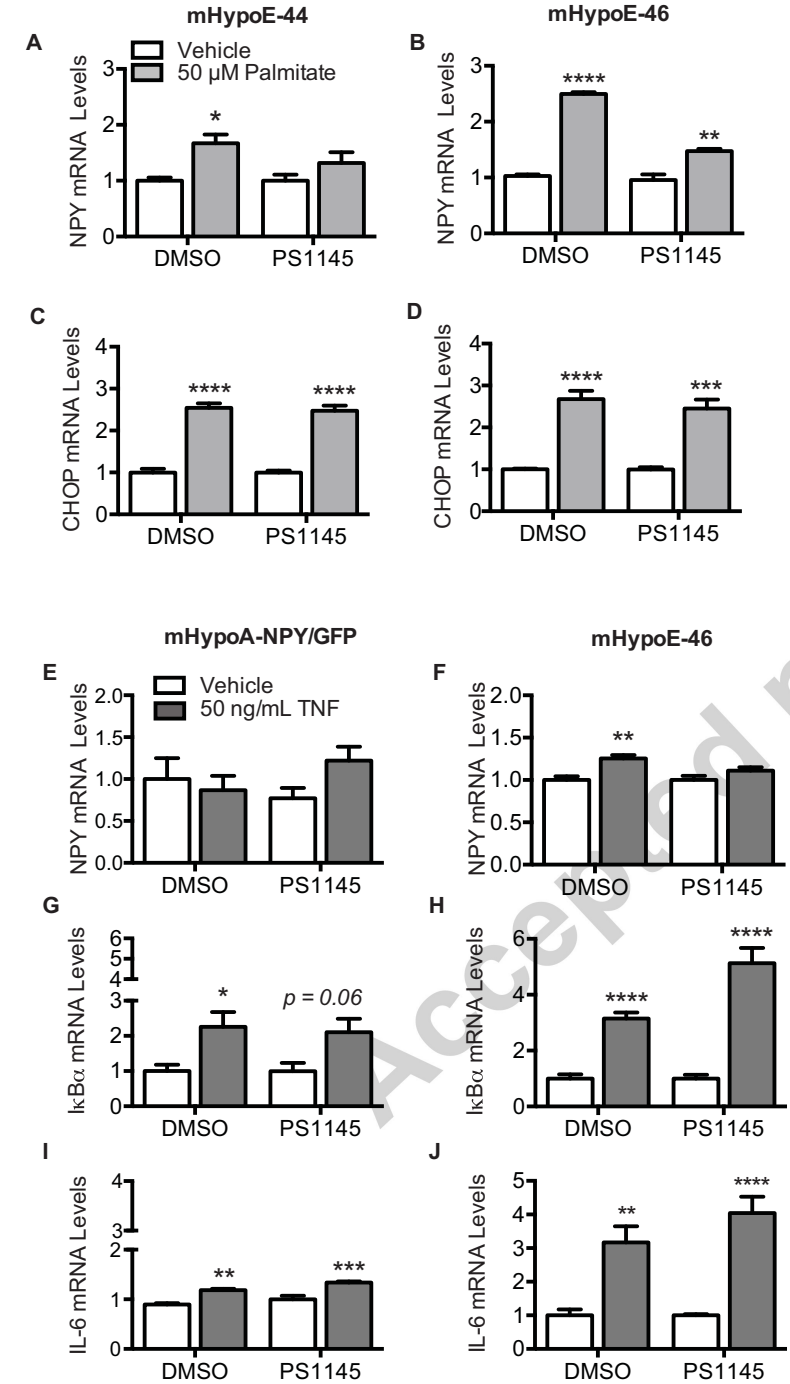
Figure 6



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