



Evidence for a novel functional role of astrocytes in the acute homeostatic response to high-fat diet intake in mice

Laura B. Buckman^{1,4}, Misty M. Thompson^{1,5}, Rachel N. Lippert^{1,6}, Timothy S. Blackwell², Fiona E. Yull³, Kate L.J. Ellacott^{1,*}

ABSTRACT

Objective: Introduction of a high-fat diet to mice results in a period of voracious feeding, known as hyperphagia, before homeostatic mechanisms prevail to restore energy intake to an isocaloric level. Acute high-fat diet hyperphagia induces astrocyte activation in the rodent hypothalamus, suggesting a potential role of these cells in the homeostatic response to the diet. The objective of this study was to determine physiologic role of astrocytes in the acute homeostatic response to high-fat feeding.

Methods: We bred a transgenic mouse model with doxycycline-inducible inhibition of NFκB (NFκB) signaling in astrocytes to determine the effect of loss of NFκB-mediated astrocyte activation on acute high-fat hyperphagia. ELISA was used to measure the levels of markers of astrocyte activation, glial-fibrillary acidic protein (GFAP) and S100B, in the medial basal hypothalamus.

Results: Inhibition of NFκB signaling in astrocytes prevented acute high-fat diet-induced astrocyte activation and resulted in a 15% increase in caloric intake ($P < 0.01$) in the first 24 h after introduction of the diet.

Conclusions: These data reveal a novel homeostatic role for astrocytes in the acute physiologic regulation of food intake in response to high-fat feeding.

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Keywords Astrocyte; Glia; High-fat diet; Nuclear factor-kappaB; Food intake; Hypothalamus

1. INTRODUCTION

Obesity is a leading public health problem worldwide and is caused by dysregulation of energy homeostasis, the balance between food intake and energy expenditure. The central nervous system (CNS) controls body weight by homeostatic regulation of food intake and energy expenditure. Genetic and pharmacologic studies in rodents have identified a number of CNS circuits critical for the regulation of energy homeostasis [1,2]. However, to date the vast majority of research on the central regulation of energy homeostasis has focused on the contribution of neurons, with the potential contribution of non-neuronal CNS cells, including glia, only beginning to be appreciated [3–6]. Studies from our laboratory and others demonstrate that in rodents high-fat feeding resulting in obesity causes CNS inflammation [5,7,8]

and astrocyte activation, known as reactive astrogliosis [5,9–11]. Astrocytes are the most abundant glial cell type in the CNS and are essential for regulation of the CNS microenvironment and neuronal synaptic plasticity [12]. In the arcuate nucleus of the medial basal hypothalamus (MBH) chronic obesity-associated reactive astrogliosis has been implicated in modulating synaptic organization of the melanocortin circuitry [10], thus contributing to obesity by modulating the tone of melanocortin neurons. In addition to being elevated by chronic high-fat feeding, CNS inflammation and astrocyte activation are also evident in the rodent hypothalamus at only 24 h after the introduction of a high-fat diet [5]. During the initial 24 h period after the introduction of a highly-palatable high-fat diet mice undergo a period of voracious food intake, known as hyperphagia, before homeostatic mechanisms prevail to restore energy intake to an isocaloric level. The increase in

¹Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, 702 Light Hall, 2213 Garland Ave, Nashville, TN 37232, USA ²Division of Allergy, Pulmonary and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, 1161 21st Ave. South, Suite T-1217 MCN, Nashville, TN 37232, USA ³Department of Cancer Biology, Vanderbilt University Medical Center, 691 Preston Research Building, 2220 Pierce Ave, Nashville, TN 37232, USA

⁴ Present address: Division of Infectious Disease, School of Medicine, University of North Carolina at Chapel Hill, 2049 A Genetic Medicine Building, Chapel Hill, NC 27599, USA.

⁵ Present address: Department of Neurology, University of Tennessee Health Science Center, 415 Link Building, 855 Monroe Avenue, Memphis, TN 38163, USA.

⁶ Present address: Max Planck Institute for Metabolism Research, Gleueler Strasse 50, 50931, Cologne, Germany.

*Corresponding author. Present address: Biomedical Neuroscience Research Group, University of Exeter Medical School, Hatherly Laboratories, Prince of Wales Rd, Exeter, Devon EX4 4PS, UK. Tel.: +44 1392 726029. E-mail: K.Ellacott@exeter.ac.uk (K.L.J. Ellacott).

Abbreviations: CNS, central nervous system; GFAP, glial-fibrillary acidic protein; HFD, high-fat diet; MBH, medial basal hypothalamus; NFκB, nuclear factor kappa B

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hypothalamic astrocyte activation during this period suggests a potential contribution of these cells in this homeostatic response; however, the physiologic significance of acute astrocyte activation after high-fat feeding is not clear.

The objective of this study was to determine the contribution of astrocytes to the acute homeostatic response to high-fat feeding. In other CNS disorders, inflammation is both protective and detrimental depending on the context [13]. We hypothesized that high-fat diet-induced activation of inflammatory signaling pathways in astrocytes is part of a protective homeostatic response, which restrains food intake in response to the diet. If this hypothesis is correct then inhibiting inflammatory signaling in astrocytes should attenuate this homeostatic response resulting in increased food intake during the initial acute hyperphagic response to the high-fat diet. In CNS injury, the nuclear-factor kappa B (NFκB) transcription pathway in astrocytes plays a key role in the production of proinflammatory cytokines and the development of reactive astrogliosis [14,15]. To determine the contribution of astrocytes to the acute homeostatic response to high-fat feeding we bred a mouse model with doxycycline-inducible inhibition of the NFκB transcription pathway under the control of the astrocyte specific glial-fibrillary acidic protein (GFAP) promoter. Using this model we examined how preventing astrocyte activation by inhibiting the NFκB transcription pathway specifically in astrocytes of adult mice altered the acute homeostatic response to a high-fat diet.

2. MATERIALS AND METHODS

2.1. Animals

Animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Vanderbilt University. Animals were housed at 21 ± 2 °C and fed a standard chow diet (13% kcal from fat; Purina 5010, PMI Nutrition International, MO) unless stated otherwise. Male C57BL/6J mice (Stock # 000664) were purchased from the Jackson Laboratory (Bar Harbor, ME). Double transgenic mice with astrocyte targeted expression of a tetracycline-inducible inhibitor of NFκB signaling were generated by crossing IκBα-dominant negative mice [16–21] on the FVB background with C57BL/6J mice expressing the reverse tetracycline-controlled transactivator under the control of the human glial-fibrillary acidic protein (GFAP) promoter [GFAP-rTA^{M2}; Jackson Laboratory, ME (stock number 014098)]. Hereafter these animals will be referred to as IκB-DN⁺ mice. Single transgenic littermates expressing only the IκBα-dominant negative transgene (IκB-DN⁻) were used as controls. F1 hybrid mice were used for all studies with transgenic animals. Male mice (8–10 weeks of age) were used for all feeding studies and, to reduce wastage, female mice were used for verification of transgene expression. The tetracycline-inducible IκBα-dominant negative mouse has been extensively characterized and has previously been successfully used in studies examining the importance of NFκB transcription pathway in disease pathology in mouse models of cancer [16,19,20] and lung inflammation [17,18].

2.2. Acute high-fat feeding studies

Mice were individually housed and their food intake measured daily for 7 days to obtain basal caloric intake before being switched to a high-fat diet (HFD; 60% of total calories from fat; Research Diets Inc., NJ). Food intake was measured every 24 h for the next 7 days. For the molecular studies (including immunohistochemistry), the animals were euthanized 24 h after introduction of the HFD. For the studies with the IκB-DN⁺ mice, the animals were switched to water containing 2 g/L doxycycline hyclate (Sigma–Aldrich, MO) and 20 g/L Splenda[®] brand

sweetener (to mask the bitter taste of the doxycycline) 7 days before the introduction of the HFD to induce expression of the transgene.

2.3. Tissue collection for molecular analyses

Animals were deeply anesthetized and transcardially perfused with 0.9% saline. The brain was removed and sectioned using a 1-mm acrylic brain block. The MBH was dissected from a single 2 mm coronal slice. Coordinates for the MBH slice were: +1.0 to +3.0 mm interaural, as referenced by Paxinos and Franklin [22], with the medial basal section of the slice corresponding to the MBH being dissected. All tissues were rapidly frozen on dry ice and placed at -80 °C until used for molecular analyses.

2.4. Ex vivo molecular analyses

2.4.1. Primary cell culture

Primary neural cells were isolated from hypothalamii dissected from adult IκB-DN⁻ and IκB-DN⁺ mice using a trypsin based neural tissue dissociation kit, according to the manufacturer's instructions (Miltenyi Biotech Inc., CA). The culture procedure was modified from one described in the literature for culture from adult animals [23]. Cells isolated from one hypothalamus were distributed equally across three wells of a 6-well culture dish containing poly L-lysine (Sigma–Aldrich, MO) coated glass coverslips. Cells were maintained in an incubator at 37 °C in 5% CO₂ in culture media [Dulbecco's Modified Eagle Medium (DMEM), high-glucose, containing 1% penicillin-streptomycin and fetal bovine serum (FBS)]. For the first 1-week after culture the cells were maintained in culture media containing 20% FBS before being switched to media containing 15% FBS in week 2 and 10% FBS in week 3. After 2-weeks of culture, 1 μg/ml doxycycline hyclate (Sigma–Aldrich, MO) was added to the culture media to induce transgene expression. The cells were maintained in culture for a total 3-weeks before use.

2.4.2. In vitro cell stimulation and immunocytochemistry

Primary neural cells were switched to culture media containing 1% FBS 24 h prior to stimulation with 5 μg/ml lipopolysaccharide (LPS), a potent activator of NFκB signaling. After 1 h of stimulation, the media was removed and the cells fixed with cold 100% methanol. After washing in 0.01M phosphate buffered saline (PBS; pH 7.4) cells were incubated with 1.5% FBS diluted in PBS containing 0.01% Triton-X100 (PBS-T) for 1 h at room temperature to block non-specific binding. The cells were then incubated overnight at 4 °C with antibodies against the p65 subunit of NFκB (cat # sc-372; Santa Cruz Biotechnology Inc., CA) and GFAP (cat # MAB360; Millipore Inc, MA), diluted 1:200 and 1:1,000 respectively, in 1.5% FBS in PBS-T. After washing with PBS the primary antibody binding was detected after incubation with the following secondary antibodies for 1 h at room temperature: donkey anti-rabbit Alexa 488 (p65) and donkey anti-mouse Alexa 594 (GFAP) (Life Technologies, CA), both diluted 1:500 in PBS-T. After washing with PBS, the coverslips were mounted onto glass slides with mounting media containing the nuclear marker DAPI (Pro-long Gold, Life Technologies, CA) and the staining visualized under fluorescence using a Zeiss Axiolmager Z1 (Zeiss, Germany). Activation of NFκB signaling was assessed by the ability of LPS to induce translocation of p65-immunoreactivity from the cytoplasm to the nucleus. The images shown are representative of two independent experiments.

2.4.3. Verification of transgene induction using RT-PCR

RNA was extracted from brain, liver, and pancreas using Trizol according to the manufacturer's instructions (Life Technologies, CA).

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After DNase treatment (Life Technologies, CA), cDNA was synthesized from 1 μg of RNA using the iScript kit according to the manufacturer's instructions (BioRad Inc., CA). Expression of the $\text{I}\kappa\text{B-DN}$ transgene was detected using PCR with the following primer set: Forward – 5' CCTGGCTGTTGTGCGAATACC 3'; Reverse – 5' GGTGATGGTATGATGACCGG 3'. As a positive control for the integrity of the cDNA, GAPDH expression was detected using the following primer set: Forward – 5' CCATGACAACCTTGGCATTG 3'; Reverse – 5' CCTGCTTCAC-CACCTTCTTG 3'.

2.4.4. Glial-fibrillary acidic protein (GFAP) immunohistochemistry

After 24 h of HFD access mice were deeply anaesthetized before undergoing transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in PBS. Control animals were maintained on standard laboratory chow. Immunohistochemistry for GFAP was performed as previously described [9]. The images shown are representative of three animals per group.

2.4.5. Measurement of medial basal hypothalamus protein levels by ELISA

MBH tissues were homogenized on ice in RIPA buffer (Sigma–Aldrich, MO) containing protease inhibitor cocktail (Cat no. P8340, Sigma–Aldrich, MO). S100B and GFAP protein concentrations from MBH homogenates were measured using commercially available ELISAs according to the manufacturer's instructions (Millipore Inc., MO).

2.5. Statistical analyses

Data are expressed as mean \pm standard error of the mean (S.E.M.). An unpaired *t*-test was used to assess differences between two groups (animals on standard chow vs. high-fat diet). Two-way ANOVA was used to assess differences between groups of animals of different genotypes on different diets. All data analyses were performed using GraphPad Prism version 5.04 (GraphPad Software, San Diego, CA), and a significance level of 0.05 was used for statistical inference.

3. RESULTS

3.1. Astrocyte activation was acutely induced in the medial basal hypothalamus of wild-type mice following introduction of a high-fat diet

Initially, we examined changes in markers of astrocyte activation in the MBH of wild-type mice 24 h after HFD was introduced to the animals; the time point at which the peak hyperphagic response is documented. As predicted, switching wild-type mice from standard chow to highly-palatable HFD resulted in hyperphagia; in the first 24 h after introduction of the HFD the mean caloric intake of the mice increased by 75% (Figure 1A; $P < 0.001$). This increased caloric intake was accompanied by statistically significant increases in MBH levels of markers of astrocyte activation, S100B (Figure 1B; 40% increase, $P < 0.01$) and GFAP (Figure 1C; 102% increase, $P < 0.05$). Immunohistochemistry for GFAP reinforced the data from the ELISA, indicating an increased intensity and subtle changes in the morphology of GFAP-immunoreactive cells in the MBH 24 h after introduction of a HFD (Supplementary Figure 1). These findings support published work demonstrating acute hypothalamic astrocyte activation in rodents after 24 h of high-fat feeding [5].

3.2. Inhibition of astrocyte activation increased high-fat diet induced hyperphagia

To determine the physiologic significance of MBH astrocyte activation in response to acute high-fat feeding in mice, we blocked $\text{NF}\kappa\text{B}$ mediated astrocyte activation using a transgenic mouse model. We

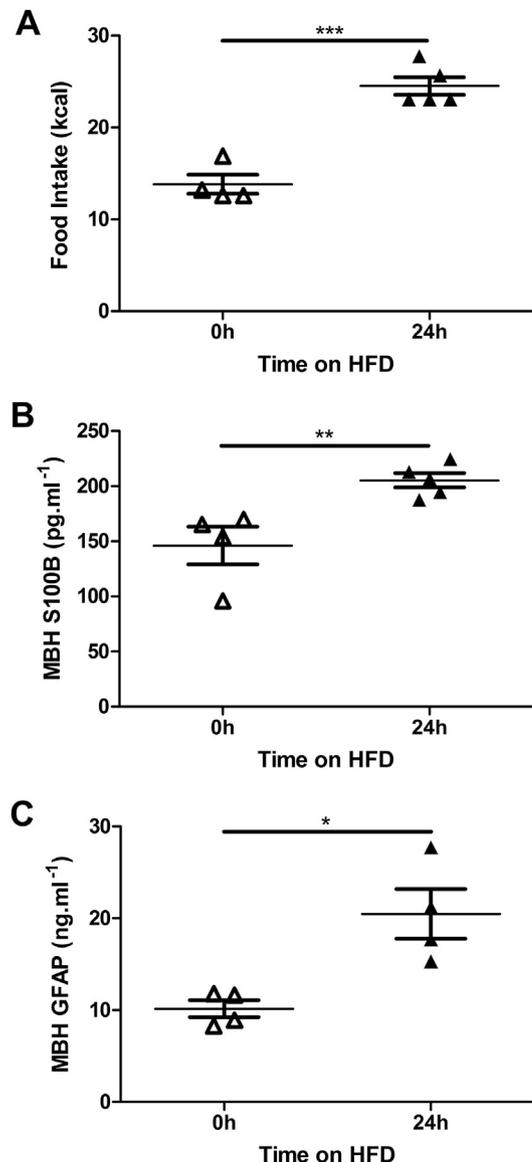


Figure 1: Astrocyte activation was acutely induced in the medial basal hypothalamus of wild-type mice following introduction of a high-fat diet – 24 h of high-fat feeding resulted in hyperphagia (A) accompanied by increased levels of S100B (B) and glial-fibrillary acidic protein (GFAP; C) in the medial basal hypothalamus (MBH). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 4–5$ /group.

bred mice with doxycycline-inducible expression of a dominant-negative form of the $\text{NF}\kappa\text{B}$ inhibitor $\text{I}\kappa\text{B}\alpha$ under the control of the astrocyte-specific GFAP promoter. We verified that transgene expression was present only in the brains in the $\text{I}\kappa\text{B-DN}^+$ animals after treatment with doxycycline using PCR (Figure 2A). No expression was detected in either control $\text{I}\kappa\text{B-DN}^-$ mice or $\text{I}\kappa\text{B-DN}^+$ mice in the absence of doxycycline. Furthermore, the expression of the dominant-negative transgene was not detected in cDNA from the liver or pancreas of any animals (Figure 2A and Supplementary Figure 2). An experimental indicator of activation of the $\text{NF}\kappa\text{B}$ signaling cascade is translocation of the p65 subunit of the $\text{NF}\kappa\text{B}$ complex from the cytoplasm to the nucleus. LPS is a potent activator of $\text{NF}\kappa\text{B}$ signaling. To verify the functional activity of our transgene, we used immunocytochemistry to examine whether induction of transgene expression in primary neural cells cultured from $\text{I}\kappa\text{B-DN}^+$ mice prevented LPS-

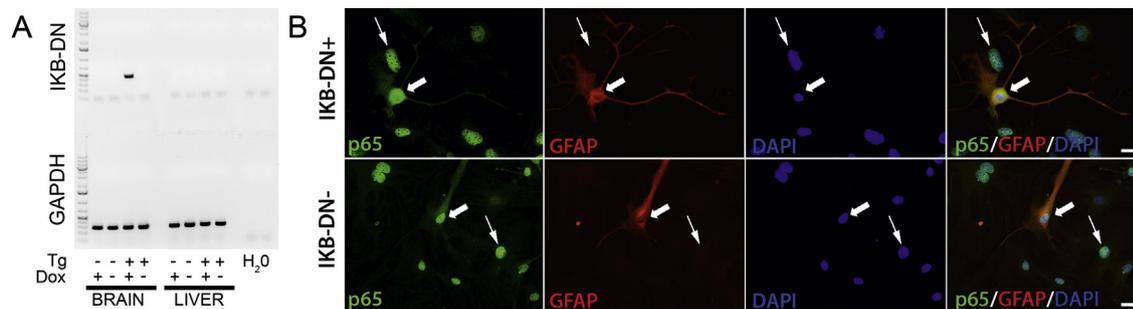


Figure 2: Brain IκB-DN transgene expression was induced upon exposure to doxycycline and inhibited NFκB signaling only in glial-fibrillary acidic protein (GFAP) immunoreactive cells – (A) Expression of the IκB-DN transgene was only detectable in cDNA made from brains of IκB-DN⁺ mice treated with doxycycline. Transgene expression was absent in liver cDNA from the same animals. Transgene expression was not detected in brain or liver cDNA from IκB-DN⁻ mice regardless of doxycycline treatment. As a positive control, GAPDH was detected in all samples verifying the integrity of the cDNA. (B) Lipopolysaccharide (LPS) failed to induce translocation of p65 from the cytoplasm to the nucleus in GFAP-immunoreactive cells isolated from IκB-DN⁺ mice (upper panels – wide arrows). LPS induced nuclear translocation of p65 in non-GFAP immunoreactive cells isolated from IκB-DN⁺ mice (upper panels – narrow arrows). LPS induced nuclear translocation of p65 in all cell types in IκB-DN⁻ mice regardless of GFAP-immunoreactivity (lower panels – all arrows). Tg = transgene; Dox = doxycycline exposure. Scale bars = 20 μm.

induced nuclear translocation of p65 in GFAP-immunoreactive cells. Following LPS treatment, p65-immunoreactivity remained localized in the cytoplasm of all GFAP-immunoreactive cells isolated from IκB-DN⁺ mice (Figure 2B, upper panels), indicating successful inhibition of NFκB signaling in these cells. The inhibition of NFκB signaling was specific to GFAP-immunoreactive cells isolated from IκB-DN⁺ mice as LPS induced nuclear localization of p65 immunoreactivity in all non-GFAP immunoreactive cells. In the absence of transgene expression, in neural cells isolated from IκB-DN⁻ mice, LPS treatment resulted in localization of p65-immunoreactivity to the nucleus in all cells, including those that were positive for GFAP-immunoreactivity (Figure 2B, lower panels). Together these data verify that the ability of the transgene to inhibit the activity of the NFκB signaling cascade in our model is restricted to GFAP-immunoreactive cells.

S100B and GFAP expression are positively regulated by activation of the NFκB signaling cascade [24,25]. We verified the successful inhibition of astrocyte activation *in vivo* in our transgenic mouse model by

measuring levels of S100B and GFAP in the MBH. IκB-DN⁺ mice did not show the HFD-induced increase in MBH S100B (Figure 3A; $P > 0.05$) and GFAP (Figure 3B; $P > 0.05$) seen in control IκB-DN⁻ littermates 24 h after introduction of the HFD (S100B - Figure 3A; $P < 0.05$, GFAP - Figure 3B; $P < 0.01$). There was a statistically significant effect of genotype on baseline S100B (Figure 3A; $P_{(\text{genotype})} < 0.01$) and GFAP (Figure 3B; $P_{(\text{genotype})} < 0.001$) expression in IκB-DN⁺ mice on standard chow; however, the reduction in S100B and GFAP levels in IκB-DN⁺ animals was not associated with any overt differences in standard chow intake prior to introduction of the HFD (Figure 3C). Inhibiting the NFκB transcription pathway in astrocytes resulted in a 15% increase in peak caloric intake 24 h after introduction of HFD in IκB-DN⁺ animals, compared with littermate controls (IκB-DN⁻; Figure 3D; $P < 0.01$). The increased food intake was only seen at 24 h after introduction of the HFD after which point there was no statistically significant difference between the groups (Figure 3C). Together these data support our hypothesis that acute astrocyte

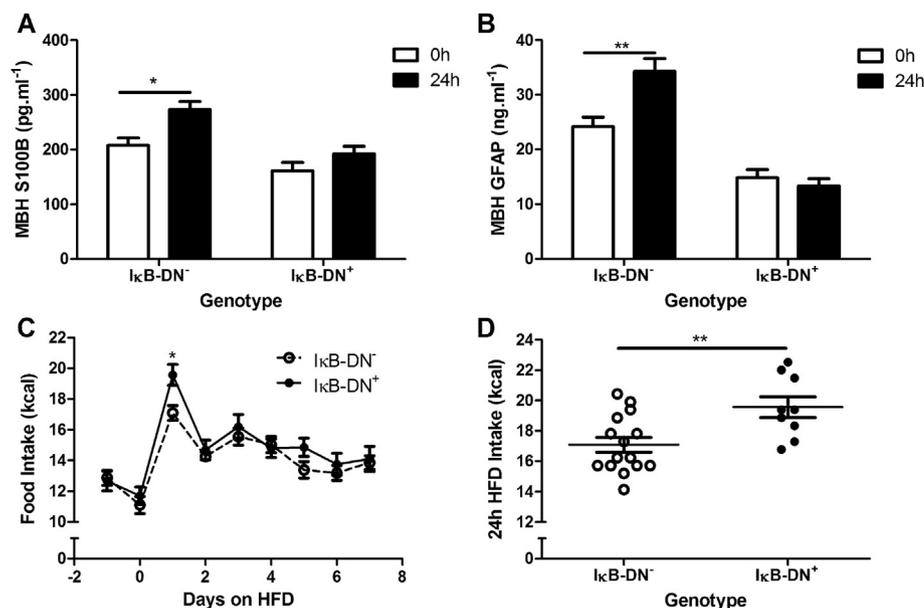


Figure 3: Inhibition of astrocyte activation increased high-fat diet induced hyperphagia – Inhibiting astrocyte activation by inducing expression of a dominant negative form of the inhibitor of NFκB signaling, IκBα, under the control of the glial-fibrillary acidic protein (GFAP) promoter (IκB-DN⁺ mice) prevented the high-fat diet induced increase in markers of astrocyte activation, S100B (A) and GFAP (B) in the medial basal hypothalamus (MBH) 24 h after introduction of the diet. This was associated with an increase in high-fat diet intake at 24 h (C and D). * $P < 0.05$, ** $P < 0.01$. $n = 3-15/\text{group}$.

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activation 24 h after introduction of a HFD is part of a homeostatic response that restrains food intake.

The $\text{I}\kappa\text{B-DN}^+$ mice and their $\text{I}\kappa\text{B-DN}^-$ littermate controls showed lower overall caloric intake on both standard chow and in response to introduction of the HFD (Figure 3C and D), compared with the wild-type mice used to generate the data in Figure 1. To test whether this was due to doxycycline exposure we performed an acute high-fat feeding study in $\text{I}\kappa\text{B-DN}^-$ mice that were not exposed to the drug. In the absence of doxycycline treatment the peak HFD intake of $\text{I}\kappa\text{B-DN}^-$ mice at 24 h was 17.2 ± 0.6 kcal (Figure 4) compared with 17.1 ± 0.49 kcal in the $\text{I}\kappa\text{B-DN}^-$ mice exposed to doxycycline (Figure 3C and D). This suggests that the genetic background of the animals (C57BL6/J in the case of the wild-type mice used to generate the data in Figure 1, compared with C57BL6/J X FVB F1 hybrids in the case of the transgenic mice used to generate the data in Figure 3) likely contributed to the differences in caloric intake seen between the studies.

4. DISCUSSION

Dysfunction in the homeostatic regulation of food intake is a key contributor to the pathogenesis of obesity. As such, an improved understanding of how food intake is regulated, particularly in response to highly-palatable calorically dense foods, is necessary for the development of effective therapeutic interventions. The presence of hypothalamic inflammation and astrocyte activation acutely after initial exposure to a highly-palatable calorically dense HFD [5] suggests a potential contribution of these cells to the homeostatic response to the diet; however, the physiologic significance of acute astrocyte activation after high-fat feeding is not clear. In this study, we provide evidence that astrocyte activation is part of an acute homeostatic response to introduction of a HFD that restrains food intake and that the absence of HFD-induced astrocyte activation is associated with exaggerated hyperphagia. These findings are significant as they are amongst the first to provide direct experimental evidence for a role of astrocytes in the regulation of food intake.

We focused on astrocyte activation in the MBH in this study, as it is likely to be one of the principal brain regions mediating the effects seen. It has previously been shown that the hypothalamus exhibits rapid changes in glial cell activity in response to high-fat feeding [5] and the presence of a circumventricular site, the median eminence, within this area makes it

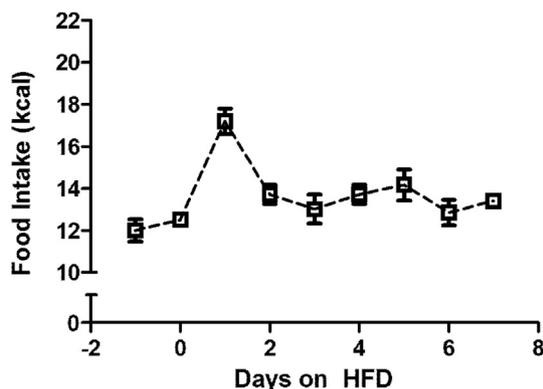


Figure 4: Doxycycline treatment did not influence the peak hyperphagic response to high-fat diet in $\text{I}\kappa\text{B-DN}^-$ mice — In order to control for potential effects of doxycycline on food intake we examined the response to introduction of a high-fat diet in control $\text{I}\kappa\text{B-DN}^-$ mice in the absence of doxycycline. The pattern and magnitude of food intake was similar to the $\text{I}\kappa\text{B-DN}^-$ mice treated with doxycycline (see Figure 3C). $n = 6$.

highly sensitive to changes in hormonal and nutritional inputs. Furthermore, the MBH contains critical neuronal circuitry mediating feeding behavior including the well-characterized melanocortin neurons in the arcuate nucleus. Indeed, astrogliosis associated with chronic high-fat feeding has been implicated in modulating the synaptic organization of melanocortin neurons in this brain region [10]. Like the MBH, caudal brainstem areas such as the nucleus of the solitary tract and dorsal vagal complex also mediate feeding behavior [26] and are in close proximity to another circumventricular organ, the area postrema. To date no one has reported inflammation or glial activation in the brainstem in response to high-fat feeding; but the involvement of this or indeed other brain areas, such as nuclei of the mesolimbic dopaminergic system that mediate reward, cannot be excluded in our study as the transgenic mouse model used targeted all GFAP-expressing cells and not those exclusively in the MBH.

While GFAP is principally expressed in the central nervous system, peripheral sites of expression including the liver [27], pancreas [28] and enteric nervous system [29] have been documented. We did not detect any expression of the $\text{I}\kappa\text{B-DN}$ transgene in the liver or pancreas in our doxycycline-treated $\text{I}\kappa\text{B-DN}^+$ animals, likely due to the low level of expression of GFAP in these tissues relative to the brain where astrocytes constitute up to 50% of the cellular mass; however, a potential contribution of non-CNS GFAP-positive cells in mediating the altered acute feeding response to HFD cannot be completely ruled out. Alterations in synaptic plasticity, exemplified by rapid rewiring of melanocortin neurons in the arcuate nucleus, have been observed acutely in response to introduction of a HFD [30]. In other neuroendocrine axes, astrocytes modulate synaptic plasticity contributing to the regulation of tone within neuronal circuits [31]. A likely mechanism by which astrocytes contribute to a reduction in acute HFD-induced food intake is *via* modulation of the activity of neurons that regulate food intake in the arcuate nucleus or another brain region. This neuronal modulation may occur *via*: 1) the action of astrocyte-derived cytokines or other biochemicals such as ketone bodies; 2) astrocyte ensheathment of neurons physically hindering synaptic connectivity; 3) astrocyte mediated alterations in neurotransmitter reuptake at the synapse; and/or 4) astrocyte induced alterations in blood–brain barrier permeability which change humoral and nutritional inputs to the CNS from the periphery. In a recent study Le Foll and colleagues presented evidence that astrocytes in the ventral medial hypothalamus play a role in modulating food intake in response to a high-fat diet *via* the action of ketone bodies [4]. An important mechanistic question that remains unresolved in the field relates to what is causing the high-fat diet induced inflammation and astrocyte activation in the CNS. Candidates include saturated fatty acids [32], endoplasmic reticulum stress [33], neuronal injury [5] or neurogenic neuroinflammation [34].

5. CONCLUSION

In summary, we have discovered that astrocytes mediate a previously undescribed homeostatic response to consumption of a high-fat diet that functions to acutely restrain food intake. This has implications for our understanding of the cellular mechanisms by which food intake is regulated by the CNS.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2014.10.001>

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