High-fat-diet exposure induces IgG accumulation in hypothalamic microglia

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SUMMARY

The mediobasal hypothalamic arcuate nucleus (ARC), with its relatively 'leaky' blood-brain barrier that allows more circulating molecules to enter the brain, has emerged as a key sensor of blood-borne signals. In both the ARC and white adipose tissue (WAT), consumption of a high-fat diet (HFD) rapidly induces infiltration of microglia (ARC) or macrophages (WAT). Animals with HFD-induced obesity (DIO) and insulin resistance additionally accumulate B cells in WAT, increasing the local production of pathogenic antibodies. We therefore investigated whether DIO mice or genetically obese *ob/ob* mice have increased IgG in the ARC, analogous to the recent observations in WAT. Following 16 weeks of exposure to a HFD, wild-type (WT) mice had significantly increased IgG-immunoreactivity (ir) signaling that was specific to the ARC and was exclusively concentrated in microglia. By contrast, IgG-ir of age-matched obese *ob/ob* mice fed standard chow had ARC IgG levels comparable with those in chow-fed WT control mice. However, following 2 weeks of HFD exposure, *ob/ob* mice also had a significant increase of IgG-ir in the ARC. In summary, our findings reveal a novel pathophysiological phenomenon, specific for the hypothalamic ARC, that is induced by exposure to a HFD and can be enhanced, but not caused, by genetic obesity.

INTRODUCTION

The brain is often considered an 'immunoprivileged' organ, with immune signals having limited access under normal conditions. However, during infection and other brain neuropathologies (e.g. neurodegenerative diseases), the blood-brain barrier (BBB) weakens and B cells can penetrate to infiltrate specific brain regions as part of a systemic immune response (Haire et al., 1973; McRae-Degueurce et al., 1988). In the hypothalamic arcuate nucleus (ARC) and median eminence complex, a differentially structured BBB allows more blood-borne signals to enter the brain (Gross, 1992), and it was recently reported that mice fed a high-fat diet [HFD; diet-induced obesity (DIO) mice] have an increased presence of proinflammatory factors in these areas (Thaler et al., 2012). There is also an increase in microglia, the resident macrophages in the brain, in the ARC of DIO mice (Thaler et al., 2012). These increased indices of inflammation are reminiscent of what occurs in visceral white adipose tissue (WAT) of DIO mice, where both macrophages and B cells accumulate (Winer et al., 2011), leading to the production of pathogenic antibodies that might be involved in the complex process leading to insulin resistance in DIO (Winer et al., 2011). Among the different types of immunoglobulin, IgG2c is predominantly found to be increased in visceral WAT in DIO mice (Winer et al., 2011), and the B cell infiltration and IgG deposition are also considered to be a proinflammatory marker. In the hypothalamus, inflammatory factors resulting from a calorie-dense diet are substantially involved in developing central leptin and insulin resistance, which will increase food intake, reduce energy expenditure, increase hepatic glucose production, and eventually cause obesity, diabetes and other metabolic syndromes such as cardiovascular disease (Obici et al., 2002; Munzberg et al., 2004; De Souza et al., 2005; Pocai et al., 2005; Posey et al., 2009; Thaler and Schwartz, 2010; Lumeng and Saltiel, 2011). Because of this similarity in inflammatory responses to a HFD in WAT and the ARC, we investigated whether IgG accumulation is another parallel process that takes place in the ARC in response to exposure to a HFD, and also whether the phenomenon could be triggered by genetically induced obesity, using *ob/ob* mice as a model.

RESULTS

HFD exposure, but not increased body weight alone, increases IgG accumulation in the ARC

In the hypothalamus of wild-type (WT) mice on standard chow [body weight (BW): 28.14±0.72 g], modest IgG-immunoreactivity (ir) was observed in the ARC, but no signal was detected in other hypothalamic areas (Fig. 1A and Fig. 2). Following 16 weeks of exposure to a HFD, DIO mice weighed significantly more (50.81±1.68 g) than control mice fed chow (36.55±1.61 g; $P<0.001$). There was a significant increase of IgG-ir in the ARC of these DIO mice relative to the chow-fed controls (Fig. 1B).

In chow-fed obese *ob/ob* mice with comparable BW to the DIO mice (48.39±1.45g; $P=0.29$ vs DIO mice), ARC IgG-ir was comparable to that of control WT mice fed chow (Fig. 1C). This implies that increased exposure to dietary lipids is the predominant cause for IgG accumulation in the ARC, rather than increased BW per se. Consistent with this, only 2 weeks of exposure to a HFD led to greater IgG accumulation in the ARC of *ob/ob* mice than what had been observed in WT mice following 16 weeks of HFD exposure (Fig. 1D).

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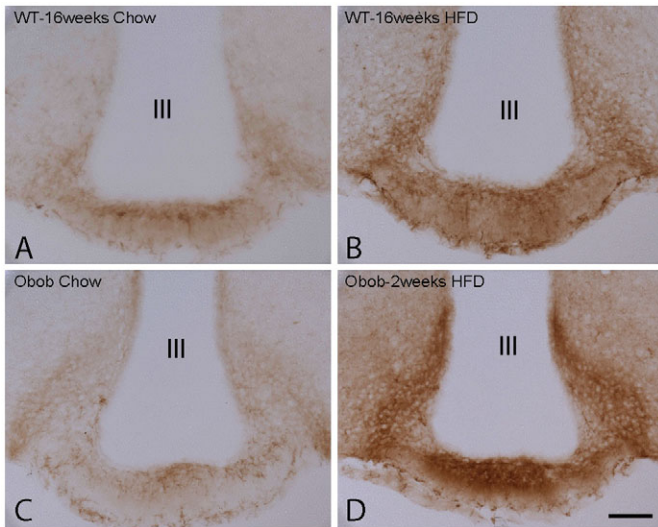*Author for correspondence (susanna.hofmann@helmholtz-muenchen.de)

Fig. 1. Sixteen weeks of HFD induces intensive IgG deposition in the ARC. Compared with chow-fed control mice (A), 16 weeks of HFD caused intensive IgG deposition in the ARC (B). This deposition pattern is not apparent in chow-fed obese *ob/ob* mice ARC (C), but can be induced by 2 weeks exposure to the HFD (D). III, third ventricle. Scale bar: 100 μm.

Hypothalamic IgG accumulation induced by HFD exposure occurs in microglia

In DIO mice, the strongest IgG-ir profile in the ARC shared a remarkable morphological similarity with microglia, consistent with the possibility that the detected IgG is colocalized with microglia. We therefore co-stained mouse brains following 16 weeks of HFD exposure for IgG along with a marker for microglia activity [ionized calcium binding adaptor molecule 1 (Iba1)] and also with the astrocyte marker glial fibrillary acidic protein (GFAP). Consistent with our earlier findings that activity of both Iba1-ir and GFAP-ir increased in the ARC in response to a HFD (Thaler et al., 2012), we found that microglia transformed from cells with small somata and finely ramified processes to an activated phenotype associated with enlarged somata and highly ramified processes in DIO mice (Fig. 3). In HFD mice, there was a clear colocalization of IgG-ir and Iba1-ir, indicating that substantial amounts of the HFD-induced IgG were located in the microglia. However, no IgG-ir was colocalized with GFAP, indicating that HFD-induced IgG
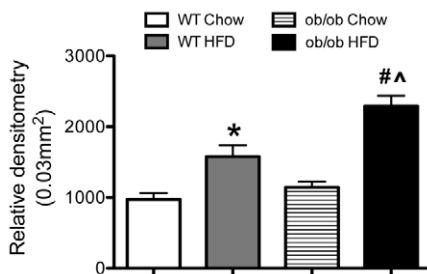

Fig. 2. Relative densitometry measurement of IgG-ir in the ARC of mice fed chow and HFD for 16 weeks, and in the ARC of *ob/ob* mice fed with chow or HFD for 2 weeks. *$P<0.01$ vs WT Chow; #$P<0.01$ vs *ob/ob* Chow; ^$P<0.01$ vs WT HFD.
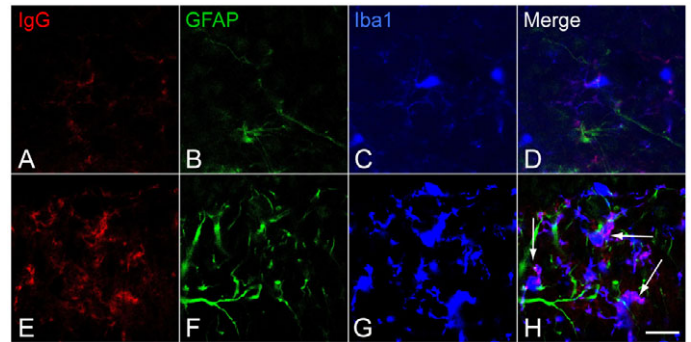

Fig. 3. Immunofluorescent co-staining of IgG, Iba1 and GFAP in the ARC of mice fed chow or a HFD for 16 weeks. In comparison to the ARC of chow-fed mice (A-D), IgG, Iba1 and GFAP immunoreactivity are all increased in the ARC of HFD mice (E-H), and only Iba1 and not GFAP is colocalized with IgG. Arrows point to the microglia co-stained with IgG. Scale bar: 20 μm.

accumulation did not occur in astrocytes (Fig. 3). We conclude that HFD exposure leads to a strong and specific deposition of IgG in microglia.

Hypothalamic IgG accumulation on a HFD mainly consists of IgG1

Specific immunofluorescent staining for IgG1, IgG2a, IgG2b and IgG3 revealed a clear increase only for IgG1 in the ARC of HFD-fed mice (Fig. 4A,D and Fig. 5). IgG2a (Fig. 4B,D), IgG2b (immunoreactivity signal not detectable; data not shown) and IgG3 (Fig. 4C,F) did not differ between chow- and HFD-fed mice (Fig. 4B,C,E,F). In summary, these data suggest that the increase of IgG in the ARC of HFD mice is mainly a consequence of the accumulation of IgG1 (but not IgG2 or IgG3) in microglia in the ARC.

DISCUSSION

When challenged with a calorie-rich diet (HFD), numerous parallel molecular events occur in peripheral tissues and the central nervous system (CNS) that are involved in the pathogenesis of the metabolic syndrome. The development of resistance to key endocrine signals maintaining metabolic homeostasis, such as leptin and insulin, represents a hallmark of this process. Intrigued by recent reports suggesting that, in visceral WAT of DIO mice, B-cell-derived IgG might be involved in the pathogenesis of insulin resistance (Winer et al., 2011), we hypothesized that a similar phenomenon might play a relevant pathogenic role in the CNS. We therefore investigated whether, when mice are exposed to a HFD, IgG would accumulate in key areas of the mouse brain known to control systemic metabolism and BW. WT mice and mice with morbid monogenetic obesity (leptin-deficient *ob/ob* mice), both fed a standard chow diet, were compared as controls. After 16 weeks, a HFD induced significant accumulation of IgG specifically in the hypothalamic ARC in WT mice, but not in other regions of the CNS. Surprisingly, this increase was not observed in *ob/ob* mice fed a standard chow diet, but was rapidly, and even more markedly, induced when *ob/ob* mice were exposed to a HFD. These novel and unexpected observations suggested that induction of IgG in the ARC occurs in DIO, and seems to be caused directly by diet exposure rather
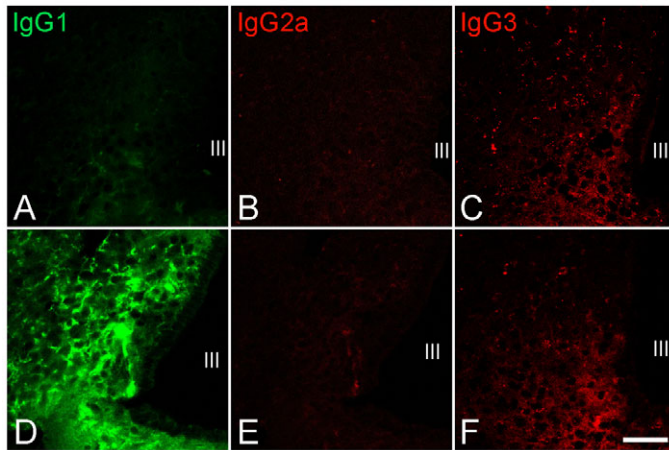
Fig. 4. Immunofluorescent staining of IgG1, IgG2a and IgG3 in the ARC of mice fed chow or a HFD for 16 weeks. (A-C) Chow-fed mice; (D-F) HFD-fed mice. Only IgG1 but not IgG2a or IgG3 immunoreactivity is increased in the ARC of HFD mice. III, third ventricle. Scale bar: 50 μm.

Fig. 5. Relative densitometry measurement of IgG1-ir, IgG2-ir and IgG3-ir in the ARC of mice fed chow or HFD for 16 weeks. *$P<0.01$ vs Chow.

than by increased body weight per se, because it was absent in genetically induced obesity.

Subtype analysis of the IgG detected in the hypothalamus of mice on a HFD revealed that, among three IgG isotypes known to be present in the ARC, only IgG1 increased with HFD exposure. Neither IgG2 nor IgG3 were increased by HFD exposure. Co-staining of the microglia marker Iba1 and the astrocyte marker GFAP identified clear colocalization of the hypothalamic IgG with microglia, with no IgG being found in astrocytes.

Microglia with IgG-like immunoreactivity have been observed in several strains of mice (including C57BL/6 used in this study) in several brain regions (Hazama et al., 2005). It is also the case that brain microglia can uptake serum IgG through Fcγ receptors, which recognize IgG1/2b (Frei et al., 1987) and cause IgG accumulation inside the microglia (Hazama et al., 2005). This raises the possibility that, in DIO mice, microglia in the ARC, where the BBB has a specialized 'leaking' structure for circulating molecules to enter brain tissue, can uptake IgG1 from the systemic circulation. However, we cannot exclude the possibility that a specific IgG1-producing B cell infiltrated into the ARC.

Our data indicate that the increased IgG1 in ARC microglia of mice on a HFD is different from what has been observed in visceral WAT, where IgG2c is the dominant isotype stimulated by a HFD (Winer et al., 2011). This indicates that different types of inflammatory response can take place in different tissues in response to HFD challenge.

As the brain's innate immune cells, microglia are important for maintaining a homoeostatic balance in a normal CNS by clearance of cell debris with their highly dynamic ramified processes and constant phagocytosis (Neumann et al., 2009). Under certain pathological circumstances, antibodies produced by pathological elements can augment this clearance function. This is well exampled in Alzheimer's disease (AD), in which antibodies against amyloid β-peptide can trigger microglial activation, and enhance phagocytosis and clearance of pre-existing amyloid through Fc receptors (Bard et al., 2000). Thus, there is the possibility that HFD-
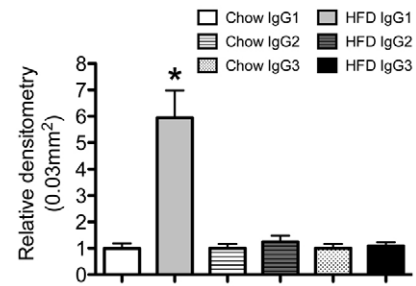
stimulated IgG in the circulation enters the microglia with the purpose of increasing microglial activity and boosts its scavenging function. This would be considered to be an immune-to-brain communication pathway.

By contrast, highly activated microglia release cytokines to recruit or stimulate more microglia and even lymphocytes, such as B cells and T cells, into the ARC (Persidsky et al., 1999; Nelson et al., 2002). Recruited B cells could therefore produce antibodies locally in the ARC. Thus, the IgG accumulation could be the cause, but also the consequence, of microglial activation, and these two processes might eventually turn into a vicious cycle. Such a process would increasingly influence the microenvironment of the ARC and possibly damage other cell types in the ARC. Consistent with this, we observed in recent studies that pro-opiomelanocortin (POMC) cells seem to decrease in number in the ARC after chronic exposure to a HFD, and this could be a consequence of neuronal damage triggered by HFD exposure (Thaler et al., 2012).

Our data suggest that activated microglia are associated with deposition of IgG during HFD exposure in mice. One remaining question is whether the newly discovered IgG deposition in the ARC would be helpful or harmful to the complex and delicately balanced neuronal-glial ARC circuits involved in metabolic control and BW regulation. Future studies will also have to address the potential of hypothalamic IgG as a target for the treatment of diet-induced disorders such as diabetes and obesity.

METHODS
Animals
All studies were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Cincinnati.

Immunohistochemical and immunofluorescent staining
WT (C57BL/6) and *ob/ob* (Jackson Labs, Bar Harbor, ME) mice that had received 2 or 16 weeks of HFD (58% fat) or chow diet (both Research Diets, D12331, New Brunswick, NJ) were deeply anesthetized with a lethal dose of sodium pentobarbital and perfused with saline, followed by a solution of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C. Brains were removed and kept in fixative at 4°C for overnight post-fixation, then equilibrated for 48 hours with 30% sucrose in 0.1 M Tris-buffered saline (TBS; pH 7.2). Brains were coronally cut in a cryostat into 30 μm sections; sections used for immunohistochemistry were collected and rinsed in 0.1 M TBS.

TRANSLATIONAL IMPACT

Clinical issue
Modern society is suffering increasingly from obesity and associated metabolic diseases, despite great efforts involving education and medication. An incomplete understanding of the pathology of metabolic syndrome limits the development of effective therapies. Among many hypotheses, hypothalamic neuropathy is currently a leading theory; that is, neuronal malfunction is thought to result in an imbalance in body homeostasis owing to disrupted communication between the brain and other body systems. It is known that neurons can only function normally in a 'clean' microenvironment, which is maintained in part by microglia, the immune cells of the brain. Although it is well known that obesity is associated with adipose tissue inflammation, whether the brain is similarly affected by obesity is less clear.

Results
Recent work from this group showed that a high-fat diet (HFD) induces signs of microglial activation in the mouse arcuate nucleus (ARC; equivalent to the infundibular nucleus in humans) of the mediobasal hypothalamus. Here, they show that 16 weeks of HFD induced a significant increase in IgG accumulation in the mouse brain, specifically in the ARC. Moreover, IgG was deposited within microglia, and was predominantly of the IgG1 subtype. IgG accumulation seems to be caused by a HFD, but not obesity per se, because IgG accumulation did not occur in genetically obese (ob/ob) mice fed normal chow. However, when ob/ob mice received a HFD for as little as 2 weeks, the IgG deposition pattern in the ARC was comparable to that in wild-type mice on a HFD for 16 weeks.

Implications and future directions
This finding advances knowledge about the pathological changes affecting the central nervous system during the development of metabolic syndrome, and provides support for the idea that hypothalamic neuropathy contributes to the condition. Notably, another group recently reported that a HFD caused infiltration of IgG2 in visceral fat in mice. The similarity between the brain and visceral fat with respect to IgG infiltration indicates a common drug target in both the brain and periphery; however, the different IgG subtypes detected suggest that different inflammatory responses might occur in each tissue. Finally, the early onset of IgG accumulation observed in ob/ob mice indicates that IgG infiltration into the brain might precede other inflammatory changes. Thus, reducing IgG levels in the brain could be an effective therapeutic approach for preventing obesity or treating the condition at an early stage.

General endogenous IgG was detected using anti-mouse IgG (H+L) antibody. IgG isotypes were detected with anti-IgG1-, -IgG2a-, -IgG2b- and -IgG3-specific antibodies. Potential host cells for IgG were detected by co-staining of IgG with the microglia activity marker Iba1 and the astrocyte marker GFAP.

For checking total IgG-ir, brain sections were incubated with horse anti-mouse IgG (a general IgG recognizing both heavy and light chains; Vector, USA) for 1 hour, rinsed and incubated in avidin-biotin complex (Vector, USA) for 1 hour, and the reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide for 7-10 minutes. Sections were mounted on gelatin-coated glass slides, dried, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped for observation by light microscopy.

For co-staining of IgG with Iba1 and GFAP, brain sections were first co-incubated with goat anti-Iba1 and rabbit anti-GFAP primary antibodies overnight at 4°C, rinsed with TBS and then incubated with biotinylated horse anti-mouse IgG, and then streptavidin-conjugated Cy3, DyLight-649-conjugated anti-goat IgG and DyLight-488-conjugated anti-rabbit IgG (Jackson ImmunoResearch, USA).

For staining of IgG isotypes, brain sections were co-incubated with goat anti-mouse IgG1 conjugated with Alexa Fluor 488, goat anti-mouse IgG2a conjugated with Alexa Fluor 555, goat anti-mouse IgG2b conjugated with Alexa Fluor 350 or goat anti-mouse IgG3 conjugated with Alexa Fluor 594 (Invitrogen, USA). After the last incubation for fluorescent antibodies for 1 hour, all sections were rinsed and mounted on gelatin-coated glass slides, dried and covered by polyvinyl alcohol mounting medium with DABCO (Sigma, USA) and observed by confocal microscopy (Zeiss-LSM710, Germany).

Analysis of the immunoreactivity profile
For each mouse with each staining profile, two to three sections in the middle portion of the ARC were selected and images were collected; both sides of the ARC were manually outlined with an area of 0.03 mm^2 on each side. The relative optic densitometry of every instance of immunoreactivity was measured by ImageJ (NIH, USA), and the mean densitometry number from each mouse was calculated. All values were then expressed as the mean ± s.e.m. from each group and data were analyzed using one-way ANOVA. Statistical significance was set at $P<0.01$.

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