Brief Communication

MOLECULAR METABOLISM

Profound weight loss induces reactive astrogliosis in the arcuate nucleus of obese mice

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ABSTRACT

Objective: Obesity has been linked to an inflammation like state in the hypothalamus, mainly characterized by reactive gliosis (RG) of astrocytes and microglia. Here, using two diet models or pharmacological treatment, we assessed the effects of mild and drastic weight loss on RG, in the context of high-fat diet (HFD) induced obesity.

Methods: We subjected HFD-induced obese (DIO) male C57BL/6J mice to a weight loss intervention with a switch to standard chow, calorie restriction (CR), or treatment with the Glp1 receptor agonist Exendin-4 (EX4). The severity of RG was estimated by an ordinal scoring system based on fluorescence intensities of glial fibrillary acidic protein, ionized calcium-binding adapter molecule 1 positive (Iba1), cell numbers, and morphological characteristics.

Results: In contrast to previous reports, DIO mice fed chronically with HFD showed no differences in microglial or astrocytic RG, compared to chow controls. Moreover, mild or profound weight loss had no impact on microglial RG. However, astrocyte RG was increased in CR and EX4 groups compared to chow fed animals and strongly correlated to body weight loss. Profound weight loss by either CR or EX4 was further linked to increased levels of circulating non-esterified free fatty acids.

Conclusions: Overall, our data demonstrate that in a chronically obese state, astrocyte and microglial RG is indifferent from that observed in age-matched chow controls. Nonetheless, profound acute weight loss can induce astrocyte RG in the hypothalamic arcuate nucleus, possibly due to increased circulating NEFAs. This suggests that astrocytes may sense acute changes to both the dietary environment and body weight.

Keywords Reactive gliosis; Obesity; Astrocyte; Weight loss; Hypothalamus; Inflammation

1. INTRODUCTION

The central nervous system plays a major role in the regulation of metabolic balance and energy homeostasis [1]. It modulates the body's supply and demand for energy at a number of different levels ranging from complex behavioral circuits such as reward or motivation [2], to regions governing energy expenditure, food intake [3], and glucose control [4]. The arcuate nucleus of the hypothalamus (ARC) has been shown to be one of the core control centers regulating metabolism and energy expenditure. Here, proopiomelanocortin (POMC) and agouti related neuropeptide (AgRP) neurons respond to signaling cues from the periphery [5,6] such as leptin [5,7], insulin [8], or ghrelin [9]. In obesity, the ARC has been shown to enter an inflammation-like state, disrupting its normal homeostatic function [10]. In this situation, both ARC neurons [11] and glial cells [10,12,13] release and respond to inflammatory signals.

Astrocytes and microglia have important regulatory functions within the central nervous system (CNS), responding to noxious stimuli, such as physical trauma, neurodegeneration, hypoxia, or cancer [14]. In these situations, astrocytes and microglia become activated in a process known as reactive gliosis (RG). RG is characterized by morphological changes such as increased cell size, enlarged, lengthened processes, and an increase in proliferation [14]. Previously, in mice fed a high-fat diet (HFD) the number of reactive astrocytes and microglia in the ARC was increased [10]. Importantly, astrocytic and microglial RG occurs prior to an increase in body weight [13], suggesting that diet content is one of the main driving factors for RG in obesity. Switching mice from HFD to standard chow results in normalization of body weight and amelioration of associated metabolic disturbances after several weeks [15], including a reversal of RG in the ARC [16]. Pharmacological treatment of diet-induced obese (DIO) mice with gut derived peptides, such as the GLP-1 analog Exendin-4 (EX4), have been successful in transiently reducing body weight [17]. How pharmacologically aided weight loss affects and possibly ameliorates RG has so far not been examined. Here, using a diet switch to chow, calorie restriction (CR) and EX4

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Abbreviations: ARC, Arcuate nucleus; POMC, proopiomelanocortin; AgRP, agouti related neuropeptide; RG, reactive gliosis; CNS, central nervous system; DIO, diet-induced obesity; HFD, high-fat diet; EX4, exendin-4; CR, calorie restriction; HC, diet switch; NEFA, non-esterified fatty acid; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; FA, fatty acid

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treatment, we tested how these mild to drastic weight loss regimes may impact RG after chronic DIO in mice.

2. METHODS

2.1. Animals

Male C57BL/6JRj (Janvier Labs, Le Genest-Saint-Isle, France) were kept under a 12-h light/dark cycle at an ambient temperature of 22 ± 2 °C and with free access to food and water. Mice were fed either a chow (Altromin, #1314) or a 58% high-fat diet (HFD) that is enriched in sucrose (Research Diets, D12331). To induce DIO, mice were ad-libitum fed HFD for 22 weeks. The diet intervention study was performed as described previously [18]. In brief, mice were divided into 5 groups with 8 animals per group: a chow control (chow), a HFD control (HFD), a diet switch group (HC), a calorie restricted group (CR), and an Exendin-4 treated group (EX4). Following 11 days of weight loss, mice were fasted for 6 h and then sacrificed by CO_2 and transcardial perfusion. All studies were based on power analyses to assure adequate sample sizes and approved by the State of Bavaria, Germany.

2.2. Plasma analysis

Plasma was collected from a separate cohort subjected to the same diet intervention (data not shown) [18]. Following a 6 h fast, blood was collected in tubes containing 50 µL EDTA and then centrifuged at 2000× *g* and 4 °C for 10 min. Plasma was collected and stored at −80 °C until further testing. Plasma triglycerides and non-esterified fatty acids (NEFA) were measured using the LabAssay™ triglyceride colorimetric assay and the NEFA-HR colorimetric assay, respectively (Fujifilm WAKO chemicals, Neuss, Germany).

2.3. Tissue preparation and immunohistochemistry

Mice were transcardially perfused with 7.5 mL ice-cold phosphate buffered saline (PBS), followed by 7.5 mL of freshly prepared 4% paraformaldehyde (PFA). Brains were harvested and post-fixed in 4% PFA overnight. After rinsing with PBS, brains were placed in a 30% sucrose 0.1 M tris-buffered-saline (TBS) solution for 48 h in preparation for cryo-sectioning. Brains were mounted in OCT compound and 30 µm sections were cut and collected at −20 °C. Sections were then stained using the free-floating approach. Samples were washed with TBS containing 0.1% Tween 20 (TBS-T), blocked for 1 h in a 0.25% gelatin and 0.5% Triton X 100 in 1x TBS buffer. Brain sections were incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. Primary antibodies were: mouse monoclonal α-glial fibrillary acidic protein (GFAP) (Sigma–Aldrich, #G3893) diluted 1:1000 and polyclonal rabbit α-ionized calcium-binding adapter molecule 1 (Iba1) (Synaptic system, #234003) diluted 1:500. Following 3 × 10 min washing with TBS-T, sections were stained with goat α-mouse Alexa Fluor 568 (Thermo Fisher Scientific, #A11004) and goat α-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, #A11008) diluted 1:1000 in blocking buffer. Following a final 3 × 10 min wash with TBS-T, sections were mounted with Vectashield® antifade medium containing DAPI (Vectashield, Burlingame, USA).

2.4. Imaging and image analysis

Images were obtained using a Leica TCS SP5 confocal laser scanning microscope (Leica microsystems, Wetzlar, Germany). Fluorophores were excited using 405 diode, 488 argon, and DPSS 561 laser lines. Fluorescence was detected using PMT and hybrid detectors. Identical acquisition settings were used for all images recorded. Fluorescence images were analyzed using the ImageJ based software Fiji (Fiji Is Just ImageJ) [19]. Images were analyzed in a blinded fashion. A total of eight mice per group were analyzed, averaging two brain sections per mouse. The ARC was defined by drawing a region of interest (ROI) based on the DAPI staining and the known structure of the ARC. Cells were either manually counted, average fluorescence intensity of the ROI was measured, or activation scores were assigned.

2.5. Statistical analyses

Statistical testing and graphing were performed using GraphPad Prism 8.0.2 (GraphPad Software, Inc. La Jolla, USA). One-way ANOVAs with Tukey's post-hoc testing were used to test for differences between treatment groups. The ordinal RG scores were assessed by non-parametric ANOVAs (Kruskal–Wallis) comparing all groups to the chow control. Spearman correlation analyses were used to calculate the association of the activation scores with body weight loss. P-values lower than 0.05 were considered significant. Significances were indicated as follows: *$p < 0.05$, **$p < 0.01$ or ****$p < 0.0001$. All results are presented as means ± SEM.

3. RESULTS

3.1. An ordinal scoring system for reactive gliosis

The reactive state of astrocytes and microglia is typically measured by fluorescence intensity of GFAP/Iba1 or by cell number [10,13,16,20]. In an attempt to improve this quantification and to accurately assess the degree of RG in $Iba1^+$ microglia and $GFAP^+$ astrocytes, we designed a scoring system ranging from 1 (for a resting state) to 5 (fully activated state) [21]. This method, used previously by others [16,22], takes into account both relative amounts of Iba1 and GFAP protein based on staining intensity as well as changes in morphology, which is a key factor in regards to analyzing RG (Figure 1).

3.2. Rapid weight loss increases circulating NEFAs

To understand how weight loss regimes affect reactive gliosis within the ARC, we designed a diet intervention study for mice that had become DIO after 22 weeks of HFD feeding compared to age matched chow controls (Figure 2A). On day 0 of the diet intervention study, the body weight was 49.0 ± 4.7 g for DIO mice and 32.3 ± 1.5 g for the chow controls. Three groups of DIO mice were then switched to chow diet, to exendin-4 treatment (daily, s.c., 0.18 mg • kg-1), or to CR that was matched to the Ex-4 animals. After 11 days of diet intervention, the weight loss groups lost significant amounts of body weight compared to the chow and HFD control groups: HC (−11.7%, $p < 0.0001$), CR (−27.8%, $p < 0.0001$) and EX4 (−30.16%, $p < 0.0001$) (Figure 2B). The body weight before and after the study revealed no significant changes within the chow, HFD and HC groups, however significant decreases within the CR ($p < 0.0001$) and EX4 groups ($p < 0.0001$) (Figure 2C). There were no differences in circulating triglycerides between the diet groups (Figure 2D). Plasma NEFAs were unaltered in the chow, HFD, and HC groups; however, they were significantly increased in EX4 treated mice (chow: $p < 0.0067$, HFD: $p < 0.0180$, HC: $p < 0.0205$) and elevated in CR mice (chow: $p < 0.0572$, HFD: $p < 0.113$, HC: $p < 0.135$) (Figure 2E).

3.3. Chronic HFD feeding and weight loss do not modulate microglial reactivity

To examine the effects of moderate or rapid weight loss on the reactive state of microglia in the ARC, we performed immunofluorescence staining for microglia marker Iba1 in brain sections of mice undergoing weight loss interventions (Figure 3A). Interestingly, we could not detect an increase in microglia RG between chow fed mice and any of the other study groups (Figure 3B). This was consistent with either

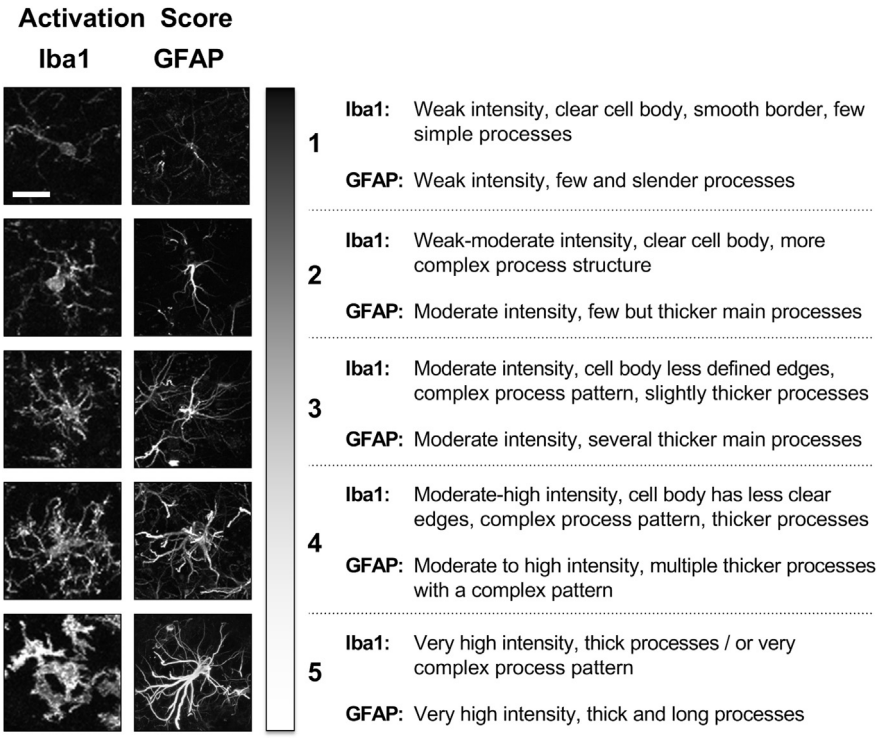

Figure 1: An ordinal activation score allows for a precise evaluation of reactive gliosis in microglia and astrocytes. Iba1+ microglia and GFAP + astrocytes were analyzed according to their staining intensity, cell body form, and process complexity and thickness. The resulting descriptions were ranked from 1 (resting) to 5 (severe reactive gliosis). Representative microscopy images for each assigned activation score are depicted. Scale bar: 20 μm.

measuring Iba1 fluorescence intensity (Figure 3C) or number of microglia (Figure 3D). Weight loss did also not impact the reactive state of microglia in the ARC (Figure 3B–D). There was also no correlation between body weight loss and RG of microglia (Figure 3E).

3.4. Rapid weight loss induces RG in astrocytes but not in microglia

The degree of astrocyte RG following a diet intervention in DIO mice was measured by analyzing brain sections stained for GFAP (Figure 4A). Astrocytic RG was significantly increased in mice that had shown a drastic weight loss due to treatment with EX4 ($p < 0.0253$) or CR ($p < 0.0383$) compared to the chow controls (Figure 4B). The HC group also displayed increased astrocytic RG, however this did not reach significance ($p < 0.334$) (Figure 4B). GFAP expression as measured by GFAP signal intensity showed no significant differences between all groups (Figure 4C). Interestingly, when we matched the astrocyte RG score to the body weight loss of the animal, we saw a significant positive correlation between body weight lost and RG ($p < 0.0045$) (Figure 4D). This correlation was specific to body weight loss as we could not detect any correlation of RG to absolute body weight (Figure 4E).

4. DISCUSSION

In the current study, we investigated how weight loss regimes with either a simple diet switch from HFD to chow, CR, or pharmacological treatment with EX4 could affect RG within the ARC. Overall, we show that after chronic feeding of HFD for 22 weeks, mice do not show an increased RG in microglia or astrocytes compared to chow fed controls. Similar to Baufeld et al., who report unperturbed astrocytosis in the hypothalamus of human individuals with BMI <25 vs. BMI >30, we found no correlation for GFAP fluorescence intensities or GFAP scores with body weight [23]. However, when chronically DIO mice undergo profound weight loss, astrocytes display an increase in RG which is not seen in microglia. This finding coincides with an increase in circulating NEFAs seen in the weight loss groups. This is in line with a study in which cultured astrocytes exposed to saturated fatty acids (FA) such as palmitic acid, lauric acid and stearic acid were shown to directly trigger the release of inflammatory cytokines [24]. Consistent with our findings, they also revealed that this effect was independent of microglia [24].

Lipolysis due to CR and subsequent increase in circulating NEFAs is well understood and has been shown in mice [25]; however, a direct link to RG remains to be tested. The possibility that circulating NEFAs may induce RG in astrocytes is supported by a number of factors. NEFAs may easily cross the blood-brain-barrier and gain access to metabolically relevant hypothalamic centers [26,27]. Furthermore, when administered peripherally, NEFAs were shown to accumulate in astrocytes localized close to blood-brain-barrier borders [28]. However, it must be stated that although the evidence is indicative, this mechanism remains speculative. CR is known to induce hormonal and metabolic changes that include decreased leptin [29]or IGF-1 levels [30], increased adiponectin [31] or FGF21 levels [32] or increased insulin sensitivity [33]. Next to increased NEFA levels, RG after an acute CR could in theory be influenced by any of these factors. Future studies should delineate the impact of NEFA and CR-linked hormonal and metabolic changes on RG. Ideally, such studies should include additional CR models, for example a lean, never obese group, which is then subjected to CR.

When mice suffering from DIO are subjected to CR, they lose significant amounts of fat mass and are able to normalize their body weight within

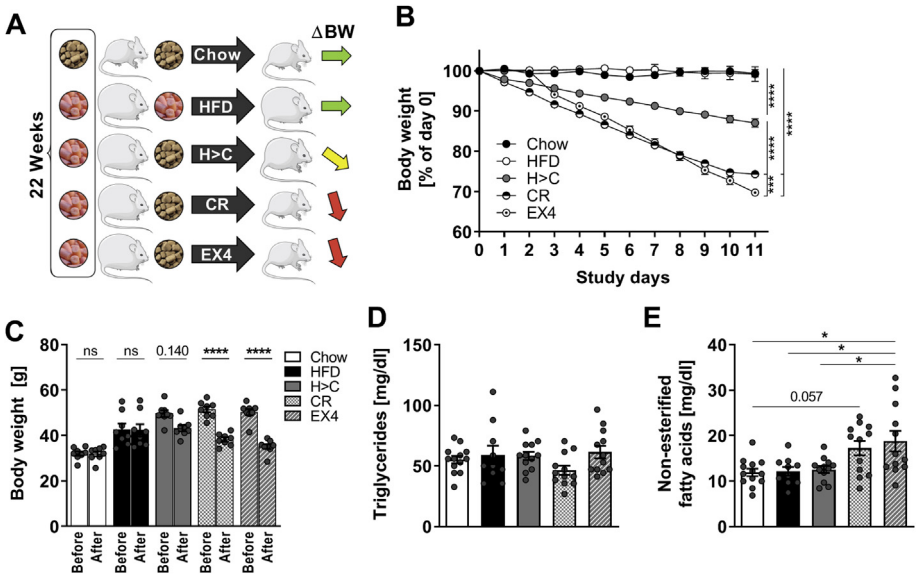

Figure 2: Weight loss by CR or EX4 results in increased circulating NEFAs. Mice were subjected to chow or HFD feeding for 22 weeks (A). Groups of obese HFD-fed mice were then switched to chow diet and either fed ad libitum (HC), treated daily with EX4 (s.c., 0.18 mg kg^{-1}) or calorie restricted to the average food intake of the EX4 group (A). Colored arrows indicate the change in body weight. Body weight change in %, n = 8 mice per group (B). Average body weights of the groups before and after the intervention (C). Fasting triglycerides (D) and non-esterified fatty acids (E) were measured at the end of the treatments in an additional cohort of mice, consisting of n = 10–13 mice per group. Statistical test: One-way ANOVA with Tukey's post-hoc test. *p < 0.05, ***p < 0.001, ****p < 0.0001 or specific p values displayed.

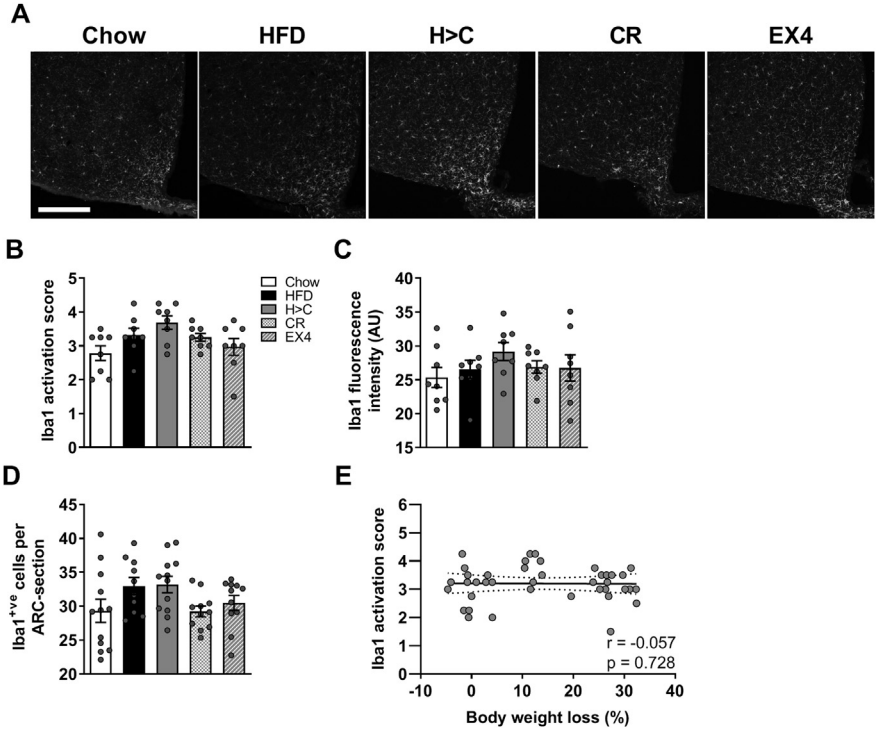

Figure 3: Microglial RG is unchanged in chronic obesity and after profound weight loss. Brain sections of mice after diet intervention were stained for Iba1 and examined using confocal microscopy. Scale bar: 200 μm (A). Brain sections were, assigned a microglia activation score as defined in Figure 1, n = 8 (B). The average Iba1 fluorescence intensity was measured in the ARC (C). Iba1+ cells were manually counted within the ARC (D). The microglial activation score was correlated to body weight loss in a linear correlation (E). Statistical test for B–D: One-way ANOVA with Tukey's post-hoc test. Statistical test for E: Spearman correlation. r = correlation coefficient.

several weeks [34]. However, when mice are then allowed to feed ad-libitum, they regain the weight lost, regardless if they consume HFD or chow [34]. This indicates that CR, although providing acute metabolic benefits, prevents a long term reduction in body weight, which is achieved by a diet switch to chow alone [15]. We reveal that CR, resulting in profound body weight loss, leads to an increase in astrocyte RG in the ARC. Whether or not increased astrogliosis can functionally contribute to the increased susceptibility for weight regain

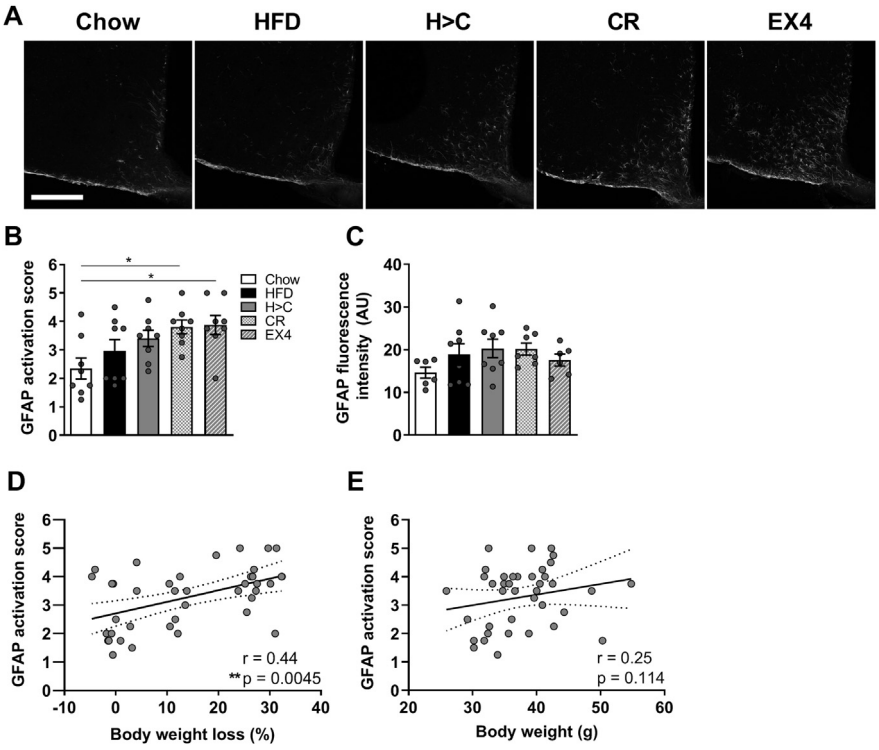

Figure 4: Weight loss is correlated to increased astrocyte RG. Brain sections of mice after diet intervention were stained for GFAP and examined using confocal microscopy. Scale bar: 200 μm (A). Brain sections were assigned an astrocyte activation score as defined in Figure 1, n = 8 (B). The average GFAP fluorescence intensity was measured in the ARC (C). Astrocyte activation scores based on GFAP staining showed a positive correlation to body weight loss (D). Astrocyte activation scores showed no correlation to absolute body weight. Statistical test for B–C: One-way ANOVA with Tukey's post-hoc test. Statistical test for D–E: Spearman correlation. r = correlation coefficient.

of previously obese mice remains to be tested. Indeed, we did not examine if the observed RG after CR would be reverted if mice were refed a HFD or normal chow and allowed to regain the lost body weight. We would assume, however, that acute refeeding of HFD would induce RG in the ARC, as reported previously [10,16,20].

A further trait of obesity is the inability of exogenously administered leptin to decrease food intake and body weight, known as leptin resistance. An inflammation like state in the ARC has been linked to leptin resistance on numerous occasions (reviewed by [35]). Using EX4 treatment, which has been shown to induce leptin re-sensitization [17], we found increased RG after significant weight loss. This indicates that although a general inflammation state in the ARC is linked to leptin resistance, RG does not seem to be responsible for this resistance. This is in line with recent work by Balland and colleagues who showed that despite an increase in RG after 10 days of HFD feeding, mice retained leptin sensitivity [20].

Astrocyte and microglial RG in the ARC has typically been assessed in an acute to sub-chronic situation, in which animals have been exposed to HFD for time spans of a 1–10 days [10,20,36], up to several weeks [10,16]. In our case, mice were subjected to chronic HFD feeding for over 22 weeks. Consistent with our findings, Baufeld et al. report a subdued microglia phenotype in the hypothalamus of mice chronically exposed to HFD, which may serve as protective mechanism to preserve neuronal homeostasis [23]. We were unable to reproduce the finding that HFD feeding induces RG in the ARC of mice. Our results differ from those of Thaler and colleagues who found that after 8 months of HFD feeding, mice showed an increased detection of GFAP in the ARC [10]. Although this seems to be in direct contradiction to our findings, there are differences between these studies in respect to the types of chow or HFD used. Thaler et al. fed their mice a 60% HFD (D12492; Research Diets) with 90% of the fat content coming from lard (i.e. 40% saturated FAs, 60% unsaturated FAs). The carbohydrate component made up 20% of total calories and consisted of 63% maltodextrin and 37% sucrose. We fed a 58% HFD (D12331; Research Diets) in which 90% of the fat was derived from coconut oil, which consists mainly of saturated FAs. 25% of the calories in the 58% HFD were from carbohydrates, with 52% sucrose and 48% maltodextrin. The overall composition of the chow diets used in our (Altromin #1314) and the study of Thaler et al. (PMI Nutrition International; 3.34 kcal/g) appeared to be similar. Nonetheless, slight differences in fat or carbohydrate composition were present, and these may contribute to changes in RG after chronic diet feeding. Overall, we observed comparable RG in astrocytes in our chow and HFD groups. This may point toward a relative lack of effect of our 58% HFD on astrocytes even after 22 weeks of exposure. On the other hand, we may also observe a more pronounced effect of our chow-diet on astrocyte RG as compared to the earlier studies by Thaler and colleagues [10].

Reactive gliosis is a complex process induced by inflammatory cytokines such as interleukin 1, interleukin-6 and tumor necrosis factor alpha [37]. Both astrocyte and microglial RG are characterized by morphological changes such as thickening of cellular processes, cell body size increase and upregulation of certain proteins such as GFAP in astrocytes or Iba1 in microglia [38,39]. It has been previously described that analyses not taking these multiple aspects into account may result in misleading results [16]. The authors revealed that despite not seeing differences in counting of microglia between lean and DIO mice, when they incorporated multiple RG features into the analysis by

the use of a scoring system, differences became apparent. Our work is in agreement with these previous findings, as although no difference was seen in GFAP intensity, we could detect differences when using an activation scoring system (Figure 4A,B). Our scoring system was designed according to established ordinal scoring requirements, such as using 4–5 score levels, which are optimal in terms of sensitivity and reliability [21].

5. CONCLUSION

Taken together, our findings suggest that mice which are chronically obese after 22 weeks of HFD feeding display RG levels in the ARC comparable to levels seen in age-matched chow fed mice. Furthermore, profound weight loss by CR or EX4 treatment results in an increase in ARC related astrocytic RG, which coincides with an increased concentration of circulating NEFAs. The role of hypothalamic glia in regulating metabolism and sensing hormonal and nutrient cues is clearly established [40]. However, whether or not reactive gliosis plays a role in chronic obesity and its comorbidities, or rather in the acute adaptation to the dietary environment, remains to be fully understood.

AUTHOR CONTRIBUTIONS

LH, KP, SCS, and PTP designed and performed the experiments, analyzed and interpreted data. LH and PTP drafted the manuscript. LH, KP, SCS, and PTP co-conceptualized the project, and reviewed the manuscript.

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CONFLICT OF INTEREST

None declared.

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