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

**Running Head: Weight loss induces astrogliosis**

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**Acknowledgments:** This work was supported in part by the German Center for Diabetes Research (DZD), by the Helmholtz Alliance ICEMED-Imaging and Curing Environmental Metabolic Diseases and by the Helmholtz-Israel-Cooperation in Personalized Medicine. Elements of artwork used in the table of contents image and figure 2 were provided by Servier medical art under the creative commons licence 3.0.

**Conflict of interest statement:** The authors declare that they have no conflicts of interest.

**Word count:** 2750

**Abstract**

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. We subjected high fat diet-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. 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. 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, Astrocytes, Microglia, Hypothalamus, Inflammation, Weight loss

**Table of contents image:**

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**Main points:**

Profound weight loss induced reactive gliosis in hypothalamic, arcuate nucleus residing astrocytes, coinciding with an increase in non-esterified fatty acids.

**1. Introduction**

The central nervous system plays a major role in the regulation of metabolic balance and energy homeostasis (Brobeck, 1946)(McNay et al., 2012; Schwartz et al., 2013; Thaler et al., 2012). 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 (Simon et al., 2017), to regions governing energy expenditure, food intake (Woods, Lotter, McKay, & Porte, 1979) and glucose control (Schwartz et al., 2013). The arcuate nucleus of the hypothalamus (ARC) has been shown to be one of the core control centers regulating metabolism and energy expenditure. Here, POMC and AgRP neurons respond to signaling cues from the periphery (Cowley et al., 2001; Fan, Boston, Kesterson, Hruby, & Cone, 1997) such as leptin (Cowley et al., 2001; Faouzi et al., 2007), insulin (Belgardt & Brüning, 2010)or ghrelin (Cowley et al., 2003). In obesity, the ARC has been shown to enter an inflammation-like state, disrupting its normal homeostatic function (Thaler et al., 2012). In this situation, both ARC neurons (De Souza et al., 2005) and glial cells (Schur et al., 2015; Thaler et al., 2012; Valdearcos et al., 2017) release and respond to inflammatory signals.

Astrocytes and microglia have important regulatory function within the CNS, responding to noxious stimuli, such as physical trauma, neurodegeneration, hypoxia or cancer (Ridet, Privat, Malhotra, & Gage, 1997). 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 (Ridet et al., 1997). Previously, in mice fed a HFD the amount of reactive astrocytes and microglia in the ARC was increased (Thaler et al., 2012). Importantly, astrocytic and microglial RG occurs prior to an increase in body weight (Valdearcos et al., 2017) 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 (Fischer et al., 2017), including a reversal of RG in the ARC (Berkseth et al., 2014). Pharmacological treatment of diet-induced obese (DIO) mice with gut derived peptides, such as the GLP-1 analogue Exendin-4 (EX4) have been successful in transiently reducing body weight (Müller et al., 2012). 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 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 8 months. The diet intervention study was performed as described previously (Harrison et al., 2018). In brief, mice were divided into 5 groups with 16 animals per group: a chow control (chow), a HFD control (HFD), a diet switch group (H>C), 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 CO2 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)(Harrison et al., 2018). Following a 6 h fast, blood was collected in tubes containing 50 µL EDTA and then centrifuged at 2000 x 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: mouse monoclonal α-GFAP (Sigma-Aldrich, #G3893) diluted 1:1000 and polyclonal rabbit α-Iba1 (Synaptic system, #234003) diluted 1:500. Following 3 x 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 x 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)(Schindelin et al., 2012). 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 was 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 (Balland & Cowley, 2017; Berkseth et al., 2014; Thaler et al., 2012; Valdearcos et al., 2017). 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)(Gibson-Corley, Olivier, & Meyerholz, 2013). This method, used previously by others (Berkseth et al., 2014; Lemstra et al., 2007), 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 (Fig. 1).

**3.2. Rapid weight loss increases circulating NEFAs**

To understand how weight loss regimes would affect reactive gliosis within the ARC, we designed a diet intervention study for mice that had become DIO after 8 months of HFD feeding compared to age matched chow controls (Fig. 2A). On day 0 of the diet intervention study, the BW 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, i.p Dose etc.), or to calorie restriction that was matched to the Ex-4 animals. After 11 days of diet intervention the weight loss groups lost significant amounts of BW compared to the chow and HFD control groups: HC (-11.7 %), CR (-27.8 %) and EX4 (-30.16 %)(Fig 2B). The final BW on day 11 was significantly higher in the HFD and HC groups compared to chow, CR and EX4 (Fig. 2C). There were no differences in circulating triglycerides between the diet groups (Fig. 2D). Plasma NEFAs were unaltered in the chow, HFD and HC groups, however were significantly increased in EX4 treated mice and elevated in CR mice (Fig. 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 (Fig. 3A). Interestingly, we could not detect an increase in microglia RG between chow fed mice and any of the other study groups (Fig. 3B). This was consistent with either measuring Iba1 fluorescence intensity (Fig. 3C) or number of microglia (Fig. 3D). Weight loss did also not impact the reactive state of microglia in the ARC (Fig. 3B-D). There was also no correlation between body weight loss and RG of microglia (Fig. 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 (Fig. 4A). Astrocytic RG was significantly increased in mice that had shown a drastic weight loss due to treatment with EX4 or CR compared to the chow controls (Fig. 4B). The HC group also displayed increased astrocytic RG, however this did not reach significance (Fig. 4B). GFAP expression as measured by GFAP signal intensity showed no significant differences between all groups (Fig. 4C). Interestingly, when we matched the astrocyte RG score to the body weight loss of the animal, we saw a significant positive correlation between BW lost and RG (Fig. 4D).

**4. Discussion**

In the current study, we investigated how weight loss regimes with either a simple diet switch from HFD to chow, calorie restriction or pharmacological treatment with EX4 could affect RG within the ARC. Overall, we show that after chronic feeding of HFD for 8 months mice do not show an increased RG in microglia or astrocytes compared to chow fed controls. 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 where 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 (Gupta, Knight, Gupta, Keller, & Bruce-Keller, 2012). Consistent with our findings, they also revealed that this effect was independent of microglia (Gupta et al., 2012). 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 (Dhopeshwarkar & Mead, 1973; Smith & Nagura, 2001). Furthermore, when administered peripherally, NEFAs were shown to accumulate in astrocytes localized close to blood-brain-barrier borders (Bernoud et al., 1998).

When mice suffering from DIO are subjected to calorie restriction they lose significant amounts of fat mass and are able to normalize their body weight within several weeks (Kirchner et al., 2012). However, when mice are then allowed to feed ad-libitum, they regain the weight lost, regardless if they consume HFD or chow (Kirchner et al., 2012). This indicates that calorie restriction, although providing acute metabolic benefits, prevents a long term reduction in body weight, which is achieved by a diet switch to chow alone (Fischer et al., 2017). We reveal that calorie restriction, resulting in profound body weight loss, leads to an increase in astrocyte RG in the ARC. Whether or not this increased astrogliosis can functionally contribute to the increased susceptibility for weight regain of previously obese mice remains to be tested.

A further trait of obesity is the lacking in response to 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 (de Git & Adan, 2015)). Using EX4 treatment, which has been shown to induce leptin re-sensitization (Müller et al., 2012), 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 Ballandand colleagues who showed that despite an increase in RG after 10 days of HFD feeding, mice retained leptin sensitivity (Balland & Cowley, 2017).

Astrocyte and microglial RG in the ARC has typically been assessed in an acute to sub-chronic situation, where animals have been exposed to HFD for time spans of a 1-10 days (Balland & Cowley, 2017; Buckman et al., 2015; Thaler et al., 2012), up to several weeks (Berkseth et al., 2014; Thaler et al., 2012). In our case, mice were subjected to chronic HFD feeding for over 8 months. 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 in such that after 8 months of HFD feeding, mice showed an increased detection of GFAP in the ARC (Thaler et al., 2012). Although this seems in direct contradiction with 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) where 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 may contribute to changes in RG after 8 months of diet feeding. Overall, we observed comparable RG in astrocytes in our chow and HFD groups. This may point towards a relative lack of effect of our 58% HFD on astrocytes even after 8 months 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 et al. (Ref).

Reactive gliosis is a complex process induced by inflammatory cytokines such as interleukin 1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Johns, 2014). 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 (Pekny & Nilsson, 2005; Robel, Berninger, & Götz, 2011). It has been previously described that analyses not taking these multiple aspects into account may result in misleading results (Berkseth et al., 2014). 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 (Fig. 4A, B). Our scoring system was designed according to optimal ordinal scoring requirements, such as using 4-5 score levels, which are optimal in terms of sensitivity and reliability (Gibson-Corley et al., 2013).

Taken together, our findings suggest that mice which are chronically obese after 8 months of HFD feeding display RG levels in the ARC comparable to levels seen in age-matched chow fed mice. Furthermore, profound weight loss by calorie restriction 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 (Garcia-Caceres et al., 2019). 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.

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**Figure Legends**

**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.

**Figure 2: Weight loss by CR or EX4 results in increased circulating NEFAs.** Mice were subjected to chow or HFD feeding for 8 months (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 Fig. 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.

**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 Fig. 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). Statistical test for B-C: One-way ANOVA with Tukey’s post-hoc test. Statistical test for D: Spearman correlation. r = correlation coefficient.