Activation of Microglia in Specific Hypothalamic Nuclei and the Cerebellum of Adult Rats Exposed to Neonatal Overnutrition


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Obesity is associated with a state of chronic inflammation resulting in alterations in circulating levels of interleukins, cytokines and adipokines, largely as a result of increased production by adipose tissue, liver and cells of the immune system, which is considered to be involved in the development of many of the comorbidities associated with obesity (1,2). Moreover, proinflammatory cytokines are also produced in the hypothalamus and this process of hypothalamic inflammation is proposed to participate in the induction of excess weight gain, possibly through participation in the development of central insulin/leptin resistance (3–7). Indeed, a high-fat diet induces hypothalamic inflammation and production of cytokines, including tumour necrosis factor (TNF) α and interleukin (IL)1β and 6 (3–5,8), all of which have been shown to affect metabolism (9–12). Furthermore, the increased weight gain and leptin resistance resulting from hypothalamic inflammation is suggested to involve activation of the IκB kinase IKK(β)/nuclear factor

Much attention has been drawn to the possible involvement of hypothalamic inflammation in the pathogenesis of metabolic disorders, especially in response to a high-fat diet. Microglia, the macrophages of the central nervous system, can be activated by proinflammatory signals resulting in the local production of specific interleukins and cytokines, which in turn could exacerbate the pathogenic process. Because obesity itself is considered to be a state of chronic inflammation, we evaluated whether being overweight results in microglial activation in the hypothalamus of rats on a normal diet. Accordingly, we used a model of neonatal overnutrition that entailed adjustment of litter size at birth (small litters: four pups/dam versus normal litters: 12 pups/dam) and resulted in a 15% increase in bodyweight and increased circulating leptin levels at postnatal day 60. Rats that were overnourished during neonatal life had an increased number of activated microglia in specific hypothalamic areas such as the ventromedial hypothalamus, which is an important site for metabolic control. However, this effect was not confined to the hypothalamus because significant microglial activation was also observed in the cerebellar white matter. There was no change in circulating tumour necrosis factor (TNF) α levels or TNFα mRNA levels in either the hypothalamus or cerebellum. Interleukin (IL)6 protein levels were higher in both the hypothalamus and cerebellum, with no change in IL6 mRNA levels. Because circulating IL6 levels were elevated, this rise in central IL6 could be a result of increased uptake. Thus, activation of microglia occurs in adult rats exposed to neonatal overnutrition and a moderate increase in weight gain on a normal diet, possibly representing a secondary response to systemic inflammation. Moreover, this activation could result in local changes in specific hypothalamic nuclei that in turn further deregulate metabolic homeostasis.

Key words: Obesity, IL6, TNFα, leptin, inflammation.

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of rats (n = 6 per group) were sacrificed by decapitation and the brains rapidly
overnight at 4°C. The brains were immediately frozen and stored at −80°C. The brains
were then removed and post-fixed in the same fixative (1 mg/ml). The brains were
then processed to determine whether microglia activation was anatomically spec-
cific. Tissue sections were fixed for 20 min at room temperature in 4% para-
formaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), washed in PB and incubated in
30% methanol containing 3% hydrogen peroxide for 30 min. The sections were
washed in PB with 0.3% bovine serum albumin and 0.3% Triton X-100 (wash buffer)
and then incubated overnight at 4°C in a humid chamber with anti-MHC-II (dilution 1 : 300; Serotec, Oxford, UK) diluted in PB containing 0.3% Triton X-100 and 3% normal goat serum. Sections were
then washed twice in buffer and incubated for 2 h with horseradish peroxidase
detected goat anti-mouse immunoglobulin G (dilution 1 : 1000; Pierce, Rockford, IL, USA). After washing, the sections were incubated in avi-
din-biotin peroxidase complex (ABC; Pierce) diluted 1 : 500. Peroxidase activ-
ity was revealed with 0.01% hydrogen peroxide, using 3,3′-diaminobenzidine
(0.03%; Sigma, St Louis, MO, USA). Immunostaining was absent when the
primary antibody was omitted. The experimental groups were assayed in
parallel.

Sections were visualised with a Zeiss Axioplan microscope (Oberkochen, Germany) and a × 40 objective. Images were captured with a digital camera and
processed using Image-Pro Plus software (version 5.0, Media Cybernetics Inc, Silver Spring, MD, USA). The total number of MHC-II immunoposi-
tive cellular profiles in each hypothalamic area was counted in six sections
per rat and, in the cerebellar white matter, in nine sections per rat (n = 6).

Western blotting
The hypothalami and cerebellum were homogenised in radioimmunoprecipita-
tion assay lysis buffer and western blotting performed as described previ-
ously (22). All primary antibodies [IL6 (PreProTech, Rocky Hill, NJ, USA); F4/80 (Santa Cruz, Santa Cruz, CA, USA); phosphorylated IκBα (Cell Signaling,
Danvers, MA, USA)] were used at a dilution of 1 : 1000 and incubated over-
night at 4°C under agitation. The membranes were incubated with the cor-
responding secondary antibody conjugated with peroxidase (Pierce), visualised
by chemiluminescence (Perkin Elmer, Boston, MA, USA) and quanti-
tified by densitometry with a Gel Logic 1500 Image analysis system (Kodak,
Rochester, New York, NY, USA). Results were normalised to glyceraldehyde-
3-phosphate dehydrogenase (GAPDH) levels in each lane and then normal-
ised to control levels in each assay.

Quantitative real-time polymerase chain reaction (PCR)
Total RNA was extracted from the hypothalamus and cerebellum using the
Tri-Reagent (Invitrogen, Carlsbad, CA, USA) protocol. Real-time PCR to quan-
ify IL6 and TNFα mRNA levels was performed as described previously (23) using assay-on-demand kits (Rn01410330 and Rn01525859, respectively; Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems) in accordance with the manufacturer’s directions in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Values were normalised to the housekeeping gene GAPDH (Rn99999916). In accordance with the manufacturer’s instructions, the △ΔCT method was used to determine relative expression levels. Statistics were performed using △ACT values.

Statistical analysis

Differences between experimental groups were analysed by unpaired Student’s t-tests. Data are presented as the mean ± SEM. P < 0.05 was considered statistically significant.

Results

At PND 60, rats from litters of four pups weighed more than those from litters of 12 (control: 250 ± 4.7 g, overnourished: 289 ± 6.9; P < 0.01) and had increased serum leptin (control: 2.8 ± 0.6 ng/ml, overnourished: 4.6 ± 0.5 ng/ml; P < 0.05) and IL6 levels (control: 7.9 ± 2.7 pg/ml, overnourished: 16.8 ± 1.2 pg/ml; P < 0.01). In both experimental groups, serum TNFα levels were below the level of detection of the assay in the majority of the samples (data not shown). There was no change in total lipid (control: 329.5 ± 18.7, overnourished: 283.1 ± 16.3 mg/dl) or triglyceride (control: 94.1 ± 14.1, overnourished: 104.5 ± 8.2 mg/dl) levels.

IL6 protein levels increased in both the hypothalamus and cerebellum in males from small litters (Fig. 1A,B; P < 0.01 for both). However, no change in IL6 mRNA levels was found in either the hypothalamus (control: 100 ± 7.7, overnourished: 93.1 ± 5.0% control) or cerebellum (control: 100 ± 20.1, overnourished: 114.3 ± 18.7% control). Similarly, TNFα mRNA levels were not modified in the hypothalamus (control: 100 ± 14.0, overnourished: 122.8 ± 4.2) or cerebellum (control: 100 ± 8.4, overnourished: 114.3 ± 18.7% control).

Phosphorylated IκB levels were not affected by early overnutrition/increased adult weight in either brain area (Fig. 1C,D) and F4/80 was undetectable in the hypothalamus and cerebellum by western blotting. However, activated microglia could be detected by immunohistochemistry in both areas. Few MHC-II immunopositive cells were detected in the hypothalamus of control rats (Fig. 2A,C), indicating that there are few activated microglia in this brain area in normal rats, although, visually, more cells were found in overnourished rats (Fig. 2A,D). Quantitative analysis indicated a significant increase in the number of MHC-II positive cellular profiles in the grey matter of the hypothalamus of overnourished rats (Fig. 2E; P < 0.05). These cells appeared in the paraventricular, ventromedial (VMN), dorsomedial and arcuate nuclei, as well as in the optic chiasm (OC) and median eminence (ME). The number of MHC-II positive cellular profiles was significantly increased in the VMN, OC and ME of overnourished rats (Table 1). The density of MHC-II immunopositive cells was also increased in the cerebellum of overnourished rats (Fig. 2F) compared to controls (Fig. 2G). This increase was found primarily in the white matter and quantitative analysis showed it to be significant (Fig. 2I; P < 0.01).

Discussion

Early overnutrition as a result of a change in litter size resulted in a significant increase in bodyweight in young adults, as reported previously (24). Although these rats were only approximately 15% heavier than their controls, they already had significantly higher serum leptin and IL6 levels, suggesting that this mild increase in bodyweight is already associated with at least some degree of systemic inflammation. IL6 levels were also increased in both the hypothalamus and cerebellum. As there was no change in the mRNA levels for this cytokine in either area, the increased protein levels could reflect an increase in IL6 uptake from the circulation.

Markers of inflammation reported to be increased in obese subjects and in response to a high-fat diet, such as increased F4/80 levels or activation of the IKKβ/NF-κB pathway, as well as increased IL6 or TNFα mRNA levels (3–6,8,13), were not altered in the hypothalamus of neonatally overnourished rats. However, they did exhibit an increase in the number of activated microglia in specific hypothalamic nuclei. It is possible that the low degree of microglial activation in this experimental model is not detectable by methods such as western blotting. Similarly, changes in cytokine production in specific hypothalamic nuclei might not be detected in the assays of total hypothalamic mRNA employed in the present study. However, it is also possible that the hypothalamic inflammatory process, including microglial activation, in response to neonatal overnutrition and increased weight gain on a normal diet may
differ from that as a result of a high-fat diet, and thus may not result in activation of the IKKβ/NF-κB pathway. Indeed, microglia can be activated by a number of factors that do not necessarily result in the same response (17–19,25). Although IL6 activates microglia (25–27) systemic IL1β and TNFα injection, but not IL6, is reported to activate NF-κB, including in microglia (28). Moreover, IL6, which is considered to have both inflammatory and anti-inflammatory effects, can have anti-obesity effects at the level of the hypothalamus (11).

The underlying cause of microglial activation in these neonatally overnourished animals remains to be elucidated. Although a long-term organisational effect of increased nutrition during development may be involved, being overweight during later life may also play an important role. It is possible that the increase in circulating leptin levels, in addition to the increase in IL6 levels, could be a triggering factor because this cytokine is also known to stimulate microglial activation and release of cytokines (14,17–19). In addition, it is likely that the state of activated microglia observed in the present study, at least partially, from early changes in bodyweight and chronic exposure to associated alterations in circulating levels of inflammatory adipokines, rather than from changes in adulthood. This may help to explain the differences in patterns of hypothalamic inflammation compared to those observed with other forms of metabolic stress, such as a high-fat diet during adulthood. Inflammatory cytokines can influence neuropeptides involved in metabolic control (9–12) and microglial activation was increased in hypothalamic areas directly involved in this process. Thus, it might be speculated that the small increase in bodyweight throughout postnatal development,

| Table 1. Mean ± SEM Number of Major Histocompatibility Complex-II Positive Cells Per Tissue Section in Distinct Areas of the Hypothalamus of Adult Male Rats from Litters of 12 Pups (controls) and Rats Overnourished as a Result of Reduced Litter Size (Four Pups/Dam) from the Day of Birth. |
|-----------------|-----------------|-----------------|-----------------|
| Control         | Overnourished   | P value         | NS             |
| Optic chiasm    | 1 ± 0.3         | 31 ± 11         | P < 0.05       |
| Paraventricular nucleus | 4 ± 0.8       | 5 ± 0.9         | NS             |
| Ventromedial nucleus | 4 ± 0.5       | 8 ± 0.9         | P < 0.005      |
| Dorsomedial nucleus | 4 ± 1.0       | 8 ± 2.0         | NS             |
| Arcuate nucleus  | 3 ± 0.4         | 5 ± 1.0         | NS             |
| Median eminence  | 4 ± 1.0         | 14 ± 3.0        | P < 0.02       |

NS, Not significant.
which modifies circulating factors such as leptin, results in activation of hypothalamic microglia that in turn produce cytokines affecting the local neuroendocrine environment further exacerbating poor metabolic control and increased weight gain, as well as the response to further metabolic challenges. Indeed, animals with increased weight as a result of neonatal overnutrition are more prone to gain weight on a high-fat diet (24).

Activation of microglia was not specific to the hypothalamus because increased activation, as well as an increase in IL6 levels, was also found in the cerebellum. Moreover, in overnourished rats, more MHC-II positive cells could be seen throughout the white matter and in other brain areas such as the hippocampus (unpublished observation, S. Tapia-González, J. A. Chown) suggesting a systemic source for central microglial activation. The cerebellum is one of the brain areas with the highest density of microglia (29) and is suggested to have an increased sensitivity to circulating inflammatory substances as a result of its microvascular structure (30) and, although not directly involved in the neuroendocrine control of metabolism, it plays an important role in the integration of the somatic-visceral control of food intake and motivation for food (31) and leptin receptor levels in the cerebellum are modified by high-fat diet (32). However, cerebellar microglial activation, as well as in other brain areas, could be involved in other pathophysiological responses to weight gain and changes in circulating factors.

In conclusion, neonatal overnutrition, which is associated with a small increase in adult bodyweight even on a normal diet, results in a significant increase in microglial activation in specific hypothalamic areas, as well as an increase in IL6 levels that may be a result of increased systemic uptake. Indeed, a systemic cause for this microglial activation is supported by the fact that this phenomenon was found in diverse areas of the brain. Whether microglial activation increases as the degree of obesity increases, and whether this activation in the hypothalamus is involved in perpetuating metabolic disturbances, deserves further investigation.

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