Cognitive Impairment Due to Leptin Withdrawal in Rat Offspring of Dams with Maternal Diet-Induced Obesity

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Background: Obesity during pregnancy is a potential threat to the health and neurodevelopment of the offspring. This study investigated the effect of maternal diet-induced obesity (DIO) on the cognitive abilities of the offspring in rats.

Material/Methods: Female Sprague-Dawley rats were fed a high-fat diet to induce obesity, and the leptin levels in dams and offspring were evaluated using ELISA. The effect of DIO on the learning and memory in offspring was measured using electrophysiology and the Morris water maze test. In addition, the expression of molecules related to synaptic plasticity was investigated. Furthermore, the effect of leptin on neuronal cells was investigated, and the influence of leptin on the regulation of calcium current activity was evaluated in vitro.

Results: Results showed that DIO dams had increased leptin levels during gestation, and offspring had drastically decreased leptin levels after delivery. The cognitive ability of offspring with maternal DIO was mildly impaired after delivery. Furthermore, long-term potentiation in DIO neonatal offspring was lower than in the control group at 2–3 weeks old; decreased expression of the leptin receptor was accompanied by N-methyl-D-aspartate receptor (NMDAR) downregulation during neonatal development. In addition, it was demonstrated that leptin enhanced NMDAR activity and promoted calcium current activity in a concentration-dependent manner.

Conclusions: The results indicated that the neonatal offspring of DIO dams showed cognitive impairment during neonatal development, which may be attributed to leptin withdrawal.

MeSH Keywords: Mild Cognitive Impairment • Obesity • Receptors, Leptin

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Background

Early studies suggested that an adverse intrauterine environment has an adverse effect on postnatal development, which has been referred to as the “developmental origins of health and disease,” also known as “fetal programming” [1,2]. For example, offspring born to dams who are obese have a higher risk of becoming obese themselves. The National Health and Nutrition Examination Survey found that 50% of nonpregnant women of childbearing age in the United States were overweight or obese, suggesting that increasing numbers of babies were being born to mothers who were obese during pregnancy [3]. Being an obese mother is a high-risk factor during the critical stages of intrauterine development. It not only increases the risk of offspring developing vascular diseases later in life, but also increases the risk of obesity and impaired glucose tolerance during adolescence [4,5]. Obesity and high-fat diets induce changes in hippocampal glutamate metabolism and neurotransmission in mothers [6]. However, the effect of an intrauterine environment characterized by maternal diet-induced obesity on the cognitive behavior of the fetus has been under-investigated [7]. The mechanism of maternal obesity and the precise biological processes mediating cognitive ability and hippocampal development programming are not adequately understood.

Evidence from animal studies has also suggested that obesity induced by a high-fat diet (HFD) results in higher concentrations of serum leptin [8,9], which is a neural neurotrophic factor that not only regulates food intake but can also adjust neurogenesis. A study by Witte et al. suggested that inefficient leptin signaling can partially contribute to a decrease in memory performance through changes in the hippocampal structure [10]. Furthermore, previous studies found that leptin-insensitive rodents exhibited impaired hippocampal long-term potentiation (LTP) and spatial memory [11]. Leptin enhances N-methyl-D-aspartate receptor (NMDAR) functioning, which is widely known to be a synaptic consistency check needed for certain types of learning and controlling synaptic plasticity and memory formation; it also facilitates the induction of LTP [12]. In addition, the direct administration of leptin into the hippocampus has been shown to improve memory processing in rodents and patients with post-operative cognitive dysfunction [13,14]. Since HFDs have been shown to increase leptin concentration during maternal gestation and after the fetus leaves the intrauterine environment, fluctuations in leptin concentration may theoretically account for the neurochemical and functional changes observed in the hippocampus. Therefore, this study investigated the role of leptin in regulating the cognition and learning ability of rat pups with obese dams.

In the previous hypothesis study, we reported that a decline in leptin can affect newborn brain development, exerting neurotrophic actions and acting as a cognitive enhancer during the development of the hippocampus [15]. However, it is still unclear whether leptin is associated with cognitive impairment in newborns. Therefore, the present research investigated the effect of leptin on the cognitive ability of newborn rat pups in the context of maternal high-fat-diet-induced obesity.

Material and Methods

All animal experiments described in this paper were approved by the Ethics Committee for Animal Procedures of the Third Military Medical University and were carried out in accordance with Institutional Animal Care regulations.

Animals and dietary protocol

Female SD rats (8 weeks old) were purchased from the Animal Experiment Center of the Third Military Medical University. The rats were kept under controlled light and humidity conditions. After 1 week of acclimatization, the rats were randomly divided into 2 groups and were fed according to the method described by Castro [16]. In brief, the rats were fed one of the following diets: a standard diet (SD) (Control; n=18, 51% carbohydrate, 4% fat, and 21% protein); a high-fat diet (HFD) (DIO; n=18, 40% ground commercial rat chow, 40% full-fat sweetened condensed milk (Nestle), 7% sucrose, and 13% water) for 2 months. They and were then mated with males. Once this group was greater than 20% of the body weight of the control group, the mice were considered obese. As shown in Figure 1A, once the offspring were delivered, the HFD diet was replaced by the SD diet in the DIO group. The offspring were weaned at 2 weeks of age.

Neuron culture

Rat hippocampal neuron cells from newborn rats were cultured. In brief, hippocampi were dissected from the newborn rats, then incubated in 2.0 mL of 2.5% trypsin (0.25% final concentration) for 15 min at 37°C. The trypsin was removed and cells were washed twice with Hank’s Balanced Salt Solution. Finally, the cells were suspended in 2 mL of growth medium consisting of neurobasal (Invitrogen) supplemented with 2% FBS (Atlas Biologicals), B27 plus antioxidants (Invitrogen), and L-glutamic acid (50 μM, Sigma). The cultures were maintained at 37°C in a 95% (vol/vol) air, 5% (vol/vol) CO2 humidified incubator, and half of the media was changed every 2 days.

Assessment of glucose tolerance and leptin

Glucose tolerance was determined at day 15 of pregnancy using the intraperitoneal glucose tolerance test (IPGTT). Food was removed 4 h prior to lights-out, and baseline samples...
from tail nicks were collected at lights-out into heparin-coated tubes for glucose analysis. Rats were then intraperitoneally injected with 2 g/kg of glucose, and blood glucose was measured. The blood sample was taken from the tail vein for the measurement of leptin and glucose. The plasma concentrations of leptin were determined using the Rat Leptin ELISA kit (Santa Clara, CA, USA). After starting the diet, rats were weighed once every 10 days.

**Water maze test**

Learning and memory ability were detected via the Morris water maze test in the offspring of dams treated with either a high-fat diet or a standard diet, when the offspring were 2–3 weeks old and 4–5 weeks old (n=12). The method of the Morris water maze test is described in Cai et al. [17] Briefly, the rats underwent a 5-day testing phase with a submerged platform in a circular black pool (diameter: 200 cm) filled with opaque water. During the procedure, the platform location remained constant, and the starting point was varied between 4 constant locations on the pool rim. The acquisition phase consisted of 4 trials in which the rats had a maximum of 60 s to find the platform, followed by a 10-s rest time on the platform after each trial. The time to find the platform were monitored and recorded semi-automatically by a tracking system connected to an image analyzer (HVS Image, Hampton, UK).

**Electrophysiology experiments**

Rat hippocampal slices (400 μm) from offspring 1–2, 2–3, 3–4, and 4–5 weeks old were prepared using standard techniques and perfused with an ice-cold artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgSO₄, and 10 mM D-glucose. Recordings from the stratum pyramidale of area CA1 were obtained using electrodes (4–6 MΩ) containing 219 mM glycerol, 2.5 mM KCl, 1.2 mM CaCl₂, 7 mM MgCl₂, 26 mM NaHCO₃, 1.2 mM NaH₂PO₄, and 11 mM D-glucose. MF-field excitatory postsynaptic potentials (fESPs) were evoked by electrical stimulation at a frequency of 0.2 Hz by a bipolar stimulating electrode placed on Schaffer collateral/commissural axons in the stratum radiatum of CA1. Glass recording electrodes were placed in 400 μL ASCF in the middle of the stratum radiatum to record field potential responses.

**Real-time quantitative PCR**

Hippocampal tissue from the offspring was obtained through a high-speed rotating centrifuge. Then, RNA was extracted using Trizol and stored at –80°C. Quantitative real-time PCR was performed with 50 ng cDNA using custom-designed gene expression assays for NMDA2A, NMDA2B, and leptin receptor according to the kit’s instructions (Takara).

**Western blot analysis**

Antibodies against all proteins were purchased from Abcam (Cambridge, UK). The homogenate was centrifuged at 12 000 rpm for 5 min at 4°C, and the supernatant was stored at –80°C. Equal amounts of protein for each sample (20 μg) were separated by SDS-PAGE and subsequently transferred to PVDF membranes. The PVDF membranes were then blocked with 5% nonfat milk for 2 h at room temperature and incubated with antibodies overnight at 4°C, followed by 3 rinses with TBST buffer. The membranes were then incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. After washing with buffer, the proteins were detected by enhanced chemiluminescence reagents.

**Immunohistochemical staining**

Offspring were anesthetized at 2–3 weeks and 4–5 weeks with 10% chloral hydrate (3 mL/kg) and perfused through the ascending aorta with 0.9% NaCl (300 mL), followed by 4% paraformaldehyde. Next, the bilateral hippocampus was removed and then fixed and dehydrated in 30% sucrose solution with paraformaldehyde. Sections were frozen at –24°C after the specimens were sunk, and the 20-μm-thick sections were washed in PBST 3 times and incubated in 30% H₂O₂ for 20 min. The sections were cultured in goat serum after being washed 3 times in PBST. Primary antibodies against the detected proteins were applied overnight. Then, the samples were incubated in the horseradish-peroxidase-labeled secondary antibodies for 30 min at 37°C followed by rinsing with PBS. Sections were then visualized with DAB under a light microscope.

**Immunofluorescence staining of cell cultures**

The leptin-treated cells were washed with Tris-buffered saline and fixed in a 10% formalin neutral buffer solution for 10 min. Then, the cells were blocked for 1 h at room temperature with 5% (vol/vol) goat serum in TBS. The cells were then incubated at 4°C overnight with the primary antibody, and then exposed to fluorescence-labeled secondary antibodies for 2 h at room temperature. A fluorescence microscope was used to observe the results.

A laser scanning confocal microscope (LSCM) was used to measure changes in Ca²⁺. Neural cells were incubated with the Ca²⁺ sensitive dye fura-3 AM. The samples were incubated with a working solution away from light at 37°C for 30 min, washed 3 times with Hanks’ solution, and incubated a second
Figure 1. Successful construction of the DIO model; change in weight and leptin concentration in dams and offspring. (A, B)

Experimental timeline including the DIO and control groups. (C, D) Weight gain in maternal rats and offspring. Body weight increased significantly in the maternal DIO group after 40 days compared to the control group. At birth, the weight of the pups in the experimental group was significantly higher than in the control group. (E) IPGTT in pregnant control and DIO rats. Glucose levels (mg/dl) increased at 30 min, 60 min, and 90 min. (F) IPGTT was measured; AUC was significantly reduced in the DIO group compared to the control group during pregnancy. (G, H) Leptin concentrations in DIO dams and offspring. Data are expressed as mean ±SD. (* p<0.05, ** p<0.01).
time at 37°C for 30 min. The fluorescence intensity was determined by LSCM.

Data analysis

All data are expressed as mean ±SD. The data were analyzed using the t test with the SPSS 18.0 statistical package; p<0.05 was used as the criterion for statistical significance.

Results

Successful construction of the DIO model and measurement of leptin concentrations in dams and offspring

The experimental timeline for the DIO and control groups is shown in Figure 1A, 1B. Both groups of rats were fed the diets for 100 days. Beginning at day 40, the experimental rats were significantly heavier than rats in the control group (p<0.05; Figure 1C). In addition, the newborn offspring of the DIO rats were significantly heavier than those from the control group (p<0.05; Figure 1D). Results of the glucose tolerance test showed that area under the curve (AUC) of blood glucose was significantly higher in the DIO group compared to the control rats (AUC glucose: 1038.5±21.21 vs. 916.6±25.43; p<0.05) (Figure 1E, 1F). These results demonstrate that the DIO model was successfully developed through diet-induced obesity and glucose intolerance during pregnancy. Furthermore, although leptin concentrations increased quickly during gestation in both groups, leptin concentration in the DIO dams was significantly higher than in the control group (Figure 1G; DIO leptin: 14.79±0.65 ng/mL vs. C leptin: 8.51±0.935 ng/mL; p<0.01). Interestingly, the leptin concentrations in the DIO offspring decreased dramatically following birth and returned to the same levels as the pups from control dams by day 28 (Figure 1H; DIO leptin: 13.74±5.11 ng/mL to 2.42±0.96 ng/mL; C leptin: 7.125±0.92 ng/mL to 2.27±1.19 ng/mL).

Slight impairment of learning and memory in DIO offspring

Learning and memory behaviors in rat offspring were assessed through the Morris water maze test. The mean escape latency...
starting from the fourth quadrant during 5 days of test trials was lower in the control group compared to the young offspring (2–3 weeks old) of DIO dams (Figure 2A; Control_time: 9.56±1.12 vs. DIO_time: 22.4±1.08; p<0.05). However, no significant differences were observed in the older (4–5 weeks old) offspring (Figure 2B).

To determine whether there were differences in hippocampal plasticity between the 2 groups, electrophysiological experiments were undertaken to detect long-term potentiation (LTP) in younger and older offspring. Figure 2C, 2D show that LTP induction in hippocampal slices was similar between the offspring in the control group and the DIO group. However, LTP maintenance was impaired in the DIO offspring; significant differences were observed at 2–3 weeks old in DIO pups (Control_2–3: 135.96±10.66; DIO_2–3: 125.87±13.70, p<0.05) (Figure 2C). However, there were no significant differences among adult offspring (Figure 2D).

**Leptin receptor expression and NMDAR in vivo and in vitro**

We hypothesized that the activation of leptin in neuronal cells plays an important role in hippocampal plasticity. Therefore, we further investigated the expression of the leptin receptor and the change in NMDAR signaling pathways in the hippocampi of the offspring.

Figure 3A shows that a maternal high-fat diet decreased the expression of the leptin receptor in the hippocampi of the offspring during the developmental period, accompanied by
downregulation of the NMDA receptor. Decreased expression of NMDAR2A and NMDAR2B was confirmed by Western blot analysis and RT-PCR during the early period (Figure 3B, 3C).

The offspring of DIO dams showed a decrease in leptin receptor expression in the DG region and slightly decreasing trends in the CA1 and CA3 regions of the hippocampus (Figure 3D). However, leptin receptor expression levels in 4-week-old rats were not different between groups. NMDAR expression in the offspring of DIO dams decreased initially; however, immunostaining for the NMDAR of 4-week-old offspring showed no difference (Figure 3E, 3F). As shown in Figure 4A–4D, cells co-cultured with leptin exhibited a significant increase in expression of the leptin receptor, NMDA2A, and NMDA2B.

**Leptin regulation of neural cell calcium current via the NMDAR signaling pathway**

To determine the effect of leptin on the plasticity of the neural cells, we measured the impact of leptin on intracellular calcium ion concentrations in the neural cells. We found that leptin decreased intracellular calcium concentrations and promoted calcium inflow in a concentration-dependent manner (Figure 5A). A high leptin concentration (200 nM) promoted calcium outflow and maintained a lower intracellular value (Figure 5B; RFI: 98.98±1.67 to 13.11±1.61; *p<0.001). A low leptin (50 nM) concentration decreased the intracellular calcium concentration initially, and then promoted accelerated calcium inflow, where adding D-AP5 (NMDAR inhibitor) decreased sensitivity to leptin.
Leptin is a cytokine secreted by lipids at a high rate during pregnancy, which influence neuronal excitability and synaptic plasticity by modulating ion channel activity, such as NMDAR, in the hippocampus [18]. We found that the cognitive ability of offspring with maternal DIO was impaired, likely the result of the dramatic reduction of leptin concentration after birth. Furthermore, we found that NMDAR expression was downregulated at the CA1, CA3, and DG areas of the hippocampi in neonatal offspring with maternal DIO. In addition, the in vitro studies indicated that leptin regulated the NMDAR-induced Ca\(^{2+}\) response of neurons in a concentration-dependent manner. These findings illustrate the importance of the modulation of NMDA receptor function by leptin to synaptic plasticity during development.

As opposed to using a gene knockout mouse model, in this study we induced obesity through a high-fat diet in SD rats, which is a more accurate reflection of the daily dietary habits of humans. We confirmed that a maternal high-fat diet contributed to hyperleptinemia and impaired maternal glucose tolerance, which is consistent with the findings of other studies [19]. In the present study, the offspring of dams with maternal DIO showed a dramatic decrease in leptin following delivery when compared to the control group. This sudden decrease of leptin may have a negative influence on hippocampus development.

Cognitive ability was investigated through the Morris water maze test and electrophysiology. The results indicated that cognitive ability was impaired in offspring with maternal DIO. In the Morris water maze experiment, adolescent DIO offspring took longer to reach the platform at the fourth starting point \((p<0.05)\), with a lower LTP value for offspring with maternal DIO at 1–3 weeks, compared to the control group. However, the}
there were no significant differences among offspring 4–6 weeks old. These results support the idea that hippocampal learning deficits observed might be linked to leptin withdrawal.

High-fat diets evoking alterations in synaptic plasticity might be linked to leptin withdrawal in offspring. Leptin affects the synaptic plasticity of the hippocampus in 2 ways. The first is associated with leptin-enhanced NMDAR synaptic transmission, with a consequent NMDAR-induced intracellular calcium increase, which is traditionally regulated through the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathways [20]. As shown in Figure 5A–5C, leptin can transiently decrease intracellular Ca\(^{2+}\) concentrations and then promote Ca\(^{2+}\) influx through the N-methyl-D-aspartate receptor in a concentration-dependent manner, which can effectively enhance LTP and synaptic plasticity. It was also found that the leptin-induced enhancement of NMDAR responses was reduced by D-AP5, indicating that leptin can induce a Ca\(^{2+}\) current through NMDAR signaling pathways. Others have shown that spinal leptin can regulate NMDAR expression through the Janus tyrosine kinases/STATs family signaling pathways to alter spinal nerve responses [21]. In our study, a reduction of NMDAR2B and NMDAR2A expression was observed in the hippocampi of offspring with maternal DIO during leptin withdrawal, and a higher expression in neural cells was observed following leptin treatment.

**Conclusions**

The main finding of this study is that maternal diet-induced obesity during gestation caused mild cognitive impairment in offspring as a result of leptin withdrawal after delivery. In addition, NMDA signal pathway signals in hippocampal neurons were reduced in offspring with maternal DIO. This study sheds new light on the hypothesis that leptin withdrawal in offspring can mediate the activity and expression of the NMDA receptor, further resulting in impaired synaptic plasticity during development. However, the specific signaling pathways and molecular mechanisms involved in these effects of leptin require further study.

**Conflicts of interest**

None.
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