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Impact of social stress in early ontogenesis on food addiction and ghrelin levels in the hypothalamus of rats

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ABSTRACT

BACKGROUND: Factors that can trigger episodes of binge (compulsive) eating include psychological and physical stress. Our studies have shown that maternal deprivation in early ontogenesis leads to an increase in elements of gambling addiction in the Iowa Gambling Task test. This raises the question of the role of maternal deprivation in the development of other types of non-chemical addictions, particularly food addiction.

AIM: To study the role of ghrelin in the manifestation of food addiction elements in rats subjected to maternal deprivation in early ontogenesis.

MATERIALS AND METHODS: Wistar rats were separated from their mothers for 180 minutes daily from day 2 to day 12 after birth. Male rats aged 90–100 days were used in the experiments. To induce compulsive overeating, the animals were given a high-carbohydrate diet (a mixture based on chocolate paste) for 1 hour every third day for 1.5 months. Fifteen minutes before feeding, the chocolate paste was placed within 5 cm of the rat's reach with visual contact. After compulsive overeating was established, the number and area of ghrelin-producing neuroendocrine cells in the hypothalamus were analyzed using immunohistochemistry in intact rats and animals subjected to maternal deprivation stress.

RESULTS: It was shown that intermittent consumption of the chocolate mixture predicted overeating in rats, independent of weight gain or obesity, as a result of compulsive overeating. In studying the effect of maternal deprivation on chocolate consumption, it was found that the average daily consumption in the maternal deprivation group increased ($p < 0.001$) compared to the control group. However, there was no significant difference in standard food consumption between the maternal deprivation and control groups. The number and area of ghrelin-producing neuroendocrine cells in the lateral portion of the medial arcuate nucleus of the hypothalamus were reduced in rats after maternal deprivation.

CONCLUSIONS: It was concluded that early psychogenic stress from maternal deprivation causes a dysregulation of the ghrelin system, contributing to elements of food addiction in rats.

Keywords: food addiction; maternal deprivation; ghrelin; neuroendocrine cells.

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Влияние социального стресса в раннем онтогенезе на проявление пищевой зависимости и содержание грелина в гипоталамусе крыс

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АННОТАЦИЯ

Актуальность. Факторы, которые могут вызывать эпизоды компульсивного (приступообразного) переедания, включают психические и физические стрессы. В наших исследованиях показано, что материнская депривация в раннем онтогенезе вызывает повышение элементов игровой зависимости в тесте Iowa Gambling Task. Возникает вопрос о значении материнской депривации в формировании других видов нехимических зависимостей, в частности, пищевой.

Цель — исследование роли грелина в проявлении элементов пищевой зависимости у крыс, подверженных отлучению от матери в раннем онтогенезе.

Материалы и методы. Крыс линии Вистар со 2-го по 12-й день после рождения на 180 мин отлучали от матери, в опытах использовали самцов в возрасте 90–100 дней. При выработке компульсивного переедания животные получали в течение 1 ч диету с высоким содержанием углеводов (смесь на основе шоколадной пасты) каждый третий день в течение 1,5 мес. За 15 мин до кормления шоколадную пасту помещали в 5 см досягаемости при визуальном контакте. После выработки пищевого компульсивного переедания исследовали количество, площадь грелин-продуцирующих нейроэндокринных клеток гипоталамуса с помощью метода иммуногистохимии у интактных крыс и животных после стресса материнской депривации.

Результаты. Показано, что прерывистое потребление шоколадной смеси предсказывает переедание у крыс независимо от увеличения массы тела или ожирения, что является следствием компульсивного (приступообразного) переедания. При изучении влияния материнской депривации на потребление шоколада было показано, что среднее суточное потребление в группе материнской депривации увеличивалось ($p < 0{,}001$) относительно контрольной группы. При изучении влияния материнской депривации на потребление стандартного корма было показано, что потребление в группе материнской депривации не изменялось относительно контрольной группы. Число и площадь нейроэндокринных клеток, продуцирующих грелин, в латеральной части медиального аркуатного ядра гипоталамуса снижались у крыс после материнской депривации.

Заключение. Сделан вывод, что ранний психогенный стресс материнской депривации вызывает дисбаланс системы регуляции грелина с элементами пищевой зависимости у крыс.

Ключевые слова: пищевая зависимость; материнская депривация; грелин; нейроэндокринные клетки.

Как цитировать
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BACKGROUND

Environmental influences during early ontogenesis have a significant impact on function in sexually mature individuals [1]. Early psychogenic stress affects growth processes, metabolism, inflammatory responses, acts on neuronal proliferation, differentiation and migration [1] and leads to post-traumatic stress disorders [2, 3]. Maternal deprivation (MD) causes long-term behavioral and motivational disorders [4], depression, increased anxiety and substance abuse [5]. MD causes persistent changes in emotional reactions and addictive drug dependence in animals [6, 7]. MD involves daily deprivation (15 min to 6 h) of litters from females during the first two weeks of life and causes profound neurochemical and behavioral changes in offspring that are detectable in adulthood [8].

Ghrelin was first found in the late 20th century. It consists of 28 amino acids, is formed in the gastric mucosa and in the lateral region of the arcuate nucleus of the hypothalamus, and enters the circulation [9]. There are three ghrelin isoforms from a single precursor, i.e. acylated ghrelin, non-acylated ghrelin and obestatin, and two molecular forms of the ghrelin receptor, i.e. GHSR1a and GHSR1b. Only the GHSR1a receptor has biological activity [10]. The ghrelin receptors are located in the stomach, adrenal glands, myocardium, thyroid gland and brain — hypothalamus, pituitary gland, hippocampus, amygdala complex, brain stem and cortex [11]. Ghrelin is involved in the regulation of eating behavior [12], dependence on psychostimulants [13, 14] and alcohol [14, 15], and in stress responses [14, 16].

Studies of non-chemical forms of addiction, such as food addiction, are currently relevant. International classifications (ICD11 and DSM5) define food addiction as an independent disorder, which has the main features typical for alcohol or drug addiction [17]. Our studies showed that the ghrelin receptor antagonist [D-LYS3]-GHRP6 reduces the signs of impulsivity (risk-taking behavior) in rats by affecting dopamine and serotonin metabolism [18]. The involvement of neuroendocrine processes and a number of neurotransmitter systems, particularly opioids, serotonin, dopamine, and hormones, in the mechanisms of food addiction has been shown [19]. Compulsive (binge) overeating disorder, a form of food addiction, involves intermittent, excessive consumption of palatable food in short periods of time, and this behavior, unlike bulimia or anorexia nervosa, is not accompanied by compensatory behavior [20]. Factors that may affect episodes of compulsive overeating include various stressors (partial deprivation from food and intermittent exposure to energy-rich palatable food) [19]. Previously, we showed that higher intermittent intake of fat- and sugar-rich foods predicts overeating in rats regardless of body weight gain or obesity as a sign of compulsive (binge) overeating [21].

The aim of the study is to investigate the effects of MD stress in early ontogenesis on the signs of food addiction and ghrelin content in the hypothalamus of sexually mature rats.

MATERIALS AND METHODS

Experiments were performed on Wistar rats weighing 250–300 g (62 males and 10 females). The animals were bred in the Rappolovo nursery (Tosnensky district of the Leningrad region). Animals were kept in standard cages (40 × 50 × 20 cm) with free access to water and pelleted food in the vivarium of the FSBSI Institute of Experimental Medicine. Inverted light from 8:00 am to 8:00 pm at 22 ± 2°C was used. The experiments were performed in accordance with the ethical principles outlined in Directive of the European Parliament and the Council of the European Union 2010/63/EC of September 22, 2010, approved by the Bioethics Commission of the FSBSI Institute of Experimental Medicine (Protocol No. 2/23 of June 15, 2023).

After arrival from the nursery, experimental animals underwent a 2-week quarantine period in the vivarium of the Institute of Experimental Medicine. Female Wistar rats were kept in plastic cages (40 × 50 × 20 cm) of 5 individuals each with access to water and food *ad libitum*. One male for five females was put in each cage, the next day vaginal smears were taken from the females and examined by light microscopy. If females were found to have spermatozoa in the proestrus stage in their smears, that day was considered the pregnancy onset date. After the pregnancy onset, the animals were placed in individual cages. The gestation age was 20 ± 2 days. MD methods were used to stress the offspring.

Maternal deprivation model

From Day 2 to Day 12 of the postnatal period, the rats were placed in individual plastic cups for 180 min for 10 consecutive days. There was no eye contact with the mother. After MD and milk feeding, 20 rats were reared in standard cages, containing 5 individuals each. Twenty males aged 90–100 days and weighing 200–250 g were used in the experiment [21].

Method of compulsive overeating of high-calorie food

Experimental groups were given access to a high-carbohydrate diet (chocolate paste-based mixture) for 1 h every third day. Control animals consumed only standard pelleted rat food. The high-calorie food was a paste prepared by mixing chocolate paste, ground pelleted rat food and water in the following percentage ratio: 52 : 33 : 15. The calorie content was 3.63 kcal/g. Standard pelleted rat food was placed inside a metal mesh container that was hung from the front wall of the cage; it was removed from the cage to measure weight to determine

food intake. The paste mixture was served in a cup; the handle of the cup was inserted into the metal wall of the cage. Fifteen minutes before giving the feeder with chocolate paste, it was placed within 5 cm of the animals' reach and in full visual contact. Within 15 minutes, the cup containing chocolate paste was placed inside a metal mesh container that was hung up on the front wall of the cage. Under these conditions, the animals could see the cup with the paste. During this 15-minute period, the rats made repetitive movements of their front paws, head, and trunk aimed at obtaining the paste, but could not reach it. It resulted in a mild stressful condition. After 15 minutes, the cup was placed in a cage for free access to the rats [21]. The following day, before the overeating session, standard rodent food present in each cage was weighed to estimate food intake over 24 h. Fifteen days after the start of the chocolate diet experiment, the rats were segregated in single cages and continued to be fed it for another 30 days. The following parameters were recorded: amount of standard food eaten and amount of chocolate paste eaten per 1 h of access. The animals' weight was recorded once a week on a strictly scheduled day.

Immunohistochemistry

After the maze test, the animals were decapitated, brain was extracted and fixed in 10% formalin. After standard paraffin embedding, 3 μm thick frontal hypothalamic slices were prepared in which, after immunohistochemical reaction, neuroendocrine cells producing ghrelin were studied as part of the widest caudal part of the medial arcuate nucleus of the hypothalamus containing the largest number of neurons, 3.6 mm at the level of bregma [22]. Monoclonal mouse antibodies to GrlRG, (Cloud-Clone Corp., PRC), diluted to 1500 μg/mL were used to identify ghrelin granules. Secondary biotinylated antibodies were used from the Vectastain ABC kit (USA). The antigen was demasked by heating to 90 °C in citrate buffer (pH 5.5) for 20 min. Bound antibodies were manifested using diaminobenzidine solution. Morphometry was performed using the Imagescope program (Electronic Analysis, Russia). Morphometric parameters of ghrelin-producing neurons were established in the left and right lateral parts of the medial aspect of the arcuate nucleus (n = 8) with an area of 0.01 mm^2, where their greatest number was detected. The number and area of ghrelin-producing neuroendocrine cells of the hypothalamus in sexually mature male rats (4 rats per group): intact rats and animals after MD were studied.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 software package (GraphPad Software, USA). The distribution of the studied parameters was tested for normality by the Kolmogorov-Smirnov test. One-factor ANOVA analysis of variance was used to analyze the morphological and PCR data. Bonferroni test was used for pairwise comparison of group mean values in post-hoc analysis. Differences were considered significant at $p < 0.05$. Data in the figures are presented as mean values and standard error of the mean.

STUDY FINDINGS

The study of the effect of MD on the consumption of chocolate mixture showed that the mean daily consumption over 10 days of the experiment was increased ($p < 0.001$) in this group compared to the control. During the study of the effects of MD on the intake of standard food, it was noted that the mean daily intake over 10 days of testing in this group did not differ from the control (Figure 1).

Neuroendocrine cells containing ghrelin granules were detected in the lateral part of the medial arcuate nucleus of the hypothalamus. Part of the neuronal bodies of the arcuate nucleus of the hypothalamus and neuroglia cells were ghrelin-negative (Figure 2). Immunopositive peptide granules were also seen separately and in small groups within the outgrowths, apparently in axons, of endocrine cells. The number and body area of hypothalamic ghrelin-producing cells decreased in rats after MD (Figure 3).

DISCUSSION

The term "food addiction" is used to describe compulsive eating behavior associated with loss of control over food [1, 2]. Eating behavior can be regulated by both homeostatic (related to energy need/storage) and hedonic pathways (dopaminergic brain reward system) that control energy intake and body weight [10].

In the present study, compulsive (binge) overeating was produced using the method of overeating high-calorie food. Episodes of overeating were induced after using intermittent exposure to a carbohydrate and fat source in a restricted access compulsive overeating model. Chronic MD stress was shown to cause increased signs of compulsive overeating of high-calorie food [21].

Chronic MD stress in animals is a model of maternal neglect in humans. Analysis of data from an experimental model of MD in early ontogenesis proves a significant influence of stress on the formation of compulsive overeating [21]. Early mental stresses have long-term effects on development and socialization in children and adolescents, and on the risk of eating disorders and binge-eating. During adolescence, hormonal restructuring, imbalance of excitation and inhibition processes occur, when the important role of neurochemical intracerebral processes in the formation of compulsive overeating becomes critical [2].

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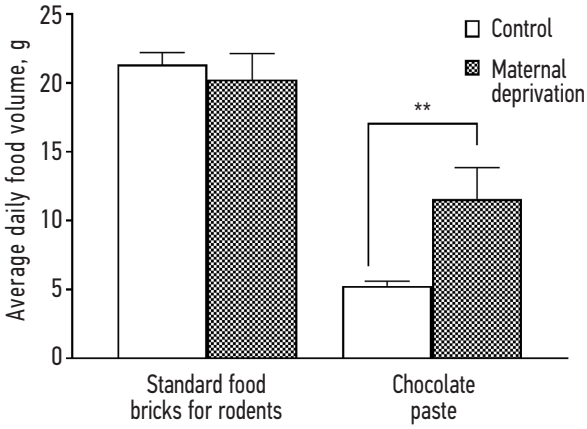

Fig. 1. The effect of maternal deprivation on the average daily consumption of standard food and chocolate paste under intermittent chocolate provision. $^{**}p < 0.001$ compared to the control group of animals

Рис. 1. Влияние материнской депривации на среднее суточное потребление стандартного корма и шоколадной пасты при выдаче шоколада в прерывистом режиме. $^{**}p < 0{,}001$ относительно контрольной группы животных

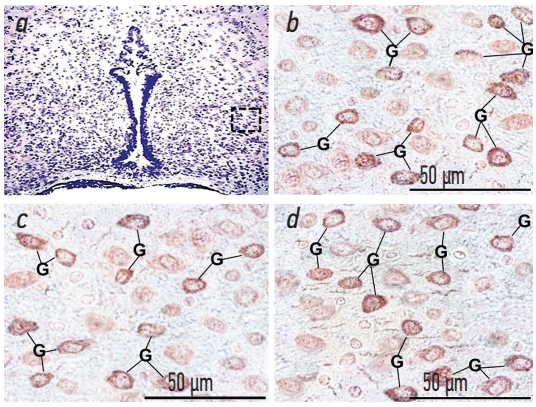

Fig. 2. Ghrelin-producing neuroendocrine cells in the lateral portion of the medial arcuate nucleus of the hypothalamus under various types of stress: *a*, the examined area of the arcuate nucleus with a surface area of 0.01 mm^2, Nissl staining; *b*, immunohistochemical detection of ghrelin granules in the control group; *c*, *d*, under maternal deprivation. G, ghrelin-producing neurons

Рис. 2. Грелин-продуцирующие нейроэндокринные клетки в латеральной части медиального отдела аркуатного ядра гипоталамуса при различных видах стрессорного воздействия: *a* — выделена исследуемая область аркуатного ядра площадью 0,01 мм^2 при окрашивании по Нисслю; *b* — иммуногистохимическое выявление гранул грелина в контроле; *c*, *d* — при материнской депривации. G — грелин-продуцирующие нейроны

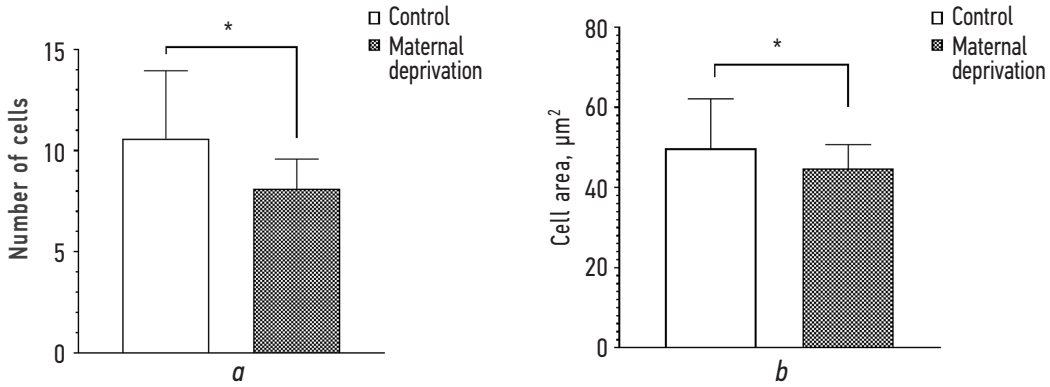

Fig. 3. The number (*a*) and area (*b*) of ghrelin-producing nturoendocrine cells (per 0.01 mm^2) of the lateral portion of the medial arcuate nucleus of the hypothalamus in rats after maternal deprivation; $^{*}p < 0.01$ compared to the control group of animals

Рис. 3. Количество (*a*) и площадь (*b*) грелин-продуцирующих нейроэндокринных клеток (на площади 0,01 мм^2) в латеральной части медиального отдела аркуатного ядра гипоталамуса у крыс после материнской депривации, $^{*}p < 0{,}01$ относительно контрольной группы животных

Experimental modeling of a number of its symptoms provides opportunities for direct study of the neurochemical mechanisms of compulsive overeating. The experiment showed involvement of neuroendocrine processes and a number of neurotransmitter systems, particularly on its formation. Opioid, dopamine, and serotonin systems were shown to be involved in the formation of positive emotions in compulsive overeating [23]. In addition to these main mediators, the ghrelin system is also involved in the mechanisms of compulsive overeating, as was shown in the present study. Ghrelin acts primarily in the hypothalamus and stimulates eating behavior aimed at regulating energy homeostasis [24]. Previously, our studies showed activation of eating behavior and weight gain in rats reared under the chronic stress in isolation [25]. The significance of ghrelin signaling in brain regions outside the hypothalamus is in its effects on learning and memory, reward and motivation, anxiety and depression. Possible targets of ghrelin action during stress appear to be corticoliberin-producing (CRH) neurons of the paraventricular nucleus of the hypothalamus. Administration of ghrelin was shown to activate these neurons [24]. The target of ghrelin action under stress also appears to be the extended amygdala system, which includes the

bed nucleus of the terminal striatum, the central nucleus of the amygdala, the nerveless substance, and the contiguous nucleus shell, being the extrahypothalamic CRH system [26]. Structures of the extended amygdala receive inputs from dopaminergic neurons of the ventral tegmental area and constitute the main functional system for the realization of emotional-motivational effects of various drugs. Blockade of CRH in the central nucleus of the amygdala, the bed nucleus of the terminal striatum, and the contiguous nucleus eliminates or significantly reduces the activating effects of addictive drugs [27].

Possible targets of ghrelin action in response to chronic stress appear to be neurons of the paraventricular nucleus of the hypothalamus [24], where a high concentration of corticoliberin is noted [28]. Ghrelin receptors have been found in the paraventricular nucleus. Ghrelin administration increased the concentration of c-FOS-protein in corticoliberin-containing neurons of the paraventricular nucleus and caused activation of the hypothalamic-pituitary-adrenal (HPA) system [28]. Ghrelin is also found in other brain structures: the bed nucleus of the terminal striatum, the central nucleus of the amygdala complex, the prefrontal cortex, the locus coeruleus, and the medulla oblongata [26]. The target of ghrelin action under stress also appears to be the extended amygdala system, which includes the central nucleus of the amygdala, the bed nucleus of the terminal striatum, the nerveless substance, the prefrontal cortex, and is the extrahypothalamic corticoliberin system [29]. Structures of the extended amygdala receive inputs from dopaminergic neurons of the ventral tegmental area of the midbrain and constitute the main functional system for the realization of emotional-motivational effects of various drugs [29]. Blockade of corticoliberin neurotransmission in the central nucleus of the amygdala, the bed nucleus of the terminal striatum, and the contiguous nucleus eliminates or significantly reduces the activating effects of addictive drugs [30]. Structures of the extended amygdala appear to be important for the realization of reinforcement mechanisms [27]. The central nucleus of the amygdala and the bed nucleus of the terminal striatum were shown to have a regulatory effect on the hypothalamus. Psychogenic stress causes activation of pathways in the prefrontal cortex, the bed nucleus of the terminal striatum, the amygdala, and then in corticoliberin-containing neurons and the hypothalamic paraventricular nucleus [27]. Ghrelin administration induces spiking activity in corticoliberin-containing neurons, increases the amount of GHSR1 RNA in the hypothalamic paraventricular nucleus, and increases serum corticosterone levels in rodents. Ghrelin can have an effect on corticoliberin-containing neurons through other neurochemical systems: orexin, neuropeptide Y, and monoaminergic neurons possessing ghrelin receptors [31].

MD has traditionally been used to describe the effects of early experience on HPA system activity [21]. Periodic MD is a powerful social chronic stressor that can activate the HPA response in rats in the hyporesponsiveness period during the first 2 weeks after birth [21]. MD can alter neuroendocrine and neurotransmitter responses to stressors and, with repeated exposure, lead to long-term increases in HPA axis reactivity, anxiety, depression, and substance abuse in adulthood [21]. The blood ghrelin concentration was shown to increase with MD stress [32] and isolation rearing after breastfeeding, which is also traditionally used to study the effect of early experience and social deprivation stress factor on the HPA system [33–35].

Ghrelin has been observed to be synthesized by endocrinocytes of the gastric mucosa (mainly) and in the lateral part of the arcuate nucleus of the hypothalamus [36]. In the present study, we confirmed the presence of ghrelin-containing cells in the lateral part of the arcuate nucleus of the hypothalamus (Figure 2). The number and area of viable hypothalamic ghrelin-producing nonendocrine cells were decreased in rats after chronic stresses of MD or rearing in social isolation. We have previously studied the effect of acute psychoemotional stress on the ghrelin system. Presentation of rats to a predator (python) resulted in a significant 8- to 12-fold decrease in the desacylghrelin level in the amygdala and hypothalamus compared to intact rats [36]. The decrease in desacylghrelin level after acute vital stress may suggest that the ghrelin system is involved in a unified stress system at the cellular level [37].

CONCLUSION

Thus, the study shows that chronic MD stresses in early ontogenesis cause elements of food dependence in animals. The number and body size of ghrelin-producing neurons in the lateral part of the medial area of the arcuate nucleus of the hypothalamus were decreased in rats after MD.

It can be concluded that early psychogenic stress of MD causes an imbalance of the ghrelin regulatory system with elements of food addiction in rats.

ADDITIONAL INFO

Authors' contributions. All authors made significant contributions to the conception and preparation of the article, and read and approved the final version before publication. Personal contribution of each author: A.A. Lebedev — idea of the work, planning of the experiment, discussion, writing and editing of the article; A.V. Droblenkov — conducting the morphological study, description of the obtained results; S.S. Purveev, G.P. Kosyakova, V.A. Lebedev, M.A. Netesa — data collection in the experiment, data processing; A.A. Bezverkhiy — assistance in implementing

morphological studies; P.D. Shabanov — design and execution of the article.

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Competing interests. The authors declare that they have no competing interests.

Ethics approval. The present study protocol was approved by the local Ethics Committee of the Institute of Experimental Medicine (protocol No. 2/23 от 2023 June 15).

ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

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