Effects of Hypothalamic Arcuate Nucleus NPY5 Receptor Signaling Pathway on Feeding Regulation in Normal Rats and Obesity Rats

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Abstract: Objective: In this study, we mainly observed the expression of NPY5 receptor in the hypothalamic arcuate nucleus (ARC) in normal rats and obesity rats, and the effect of the NPY5 receptor agonist [D-Trp34] NPY and NPY5 Receptor antagonist CGP-71683 on Food Intake in the normal and obesity rats. Methods: Immunohistochemistry was used to observe the expression of NPY5 receptor in ARC of normal rats and obesity rats. RT-PCR was used to observe the expression of NPY5 receptor mRNA in ARC of normal and obesity rats. The NPY5 receptor agonist [D-Trp34] NPY and NPY5 receptor antagonist CGP-71683 were injected into the ARC via intraventricular catheters to observe the food intake of rats. Results: The results of immunohistochemistry and RT-PCR experiments showed that the expression of NPY5 receptor was observed in the hypothalamus ARC, and the expression of NPY5 receptor in obesity rat was significantly higher than that in normal rats. Compared with the normal saline group, the food intake of the normal rats and obesity rats significantly increased during the 0-4h after the injection of NPY into the ARC. Similarly, the food intake of the normal rats and obesity rats significantly increased during the 0-4h after the injection of NPY5 receptor agonist [D-Trp34] NPY into the ARC. The NPY5 receptor antagonist CGP-71683 could block the NPY and NPY5 receptor agonist [D-Trp34] induced the feeding. After injection of NPY and NPY5 receptor agonist [D-Trp34] in the ARC of obesity rats, the food intake was significantly higher than in normal rats during 0-4 h. Conclusion: The NPY5 receptor in the hypothalamus ARC is involved in the regulation of feeding behavior and may be related to the formation of obesity.

Keywords: NPY; Hypothalamic Arcuate Nucleus; Obesity Rats

Introduction
The prevalence of obesity in the world is increasing. National Nutrition Survey and Association (NHANES) from 1999–2002 year data report shows that in the 20-year-old adults, 29.8% were overweight, 30.4% were obese, 4.9% were extremely obese [1]. At present, most researches about the mechanism of feeding regulation focus on neuromodulation, neuronal afferent pathways, information integration of the hypothalamus center, and efferent neural pathway could coordinate and regulate the feeding behavior. The literature reports that the hypothalamus is a key brain area involved in the regulation of food intake [2].

Neuropeptide Y (NPY), one of the most abundant neuropeptides in the brain, is mainly expressed in the central nervous system and is composed of 36 amino acids [3]. Recent studies have shown that NPY is an important signaling molecule in the feeding regulation network [3,4]. The NPY receptor receptors (Y1, Y2, Y4, Y5, Y6) have been found in mammals, and the distribution of these five receptors is different in the brain [5,6]. Previous studies have shown that among the five receptors mentioned above, NPY is involved in regulating energy balance mainly through Y1, Y2, and Y5 receptor signaling pathways [7,8]. Based on the above findings, Y1, Y2, and Y5 receptor signals play an important role in obesity susceptibility.

NPY neurons are mainly expressed in the hypothalamic arcuate nucleus (ARC), and their nerve fibers can be projected to the hypothalamic paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the hypothalamic ventromedial nucleus (VMH), and other brains area. In addition, studies have shown that 90% of NPY neurons co-express AGRP [11]. Under the condition of negative energy balance such as lower leptin level, hypoglycemia state and hypoinsulinemia,
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the expression of NPY mRNA in ARC increased. Central injection of NPY can inhibit heat production in rats, increase food intake, and promote the formation of fat in rats[12].

NPY neurons respond to changes in food intake during obesity. Studies have confirmed that the NPY5 receptor in ARC is involved in the regulation of feeding behavior[13]. The NPY5 receptor may be a pharmacological target for obesity treatment. Therefore, the purpose of this experiment was to investigate the role of NPY5 receptors in feeding regulation in ARC. To achieve this goal, we mainly observed the expression of NPY5 receptors in ARC and observed effect of intra-ARC injection of NPY5 receptor agonist [D-Trp34] NPY and NPY5 receptor antagonist CGP-71683 on food intake in normal rats and obesity rats.

1 Experimental materials and methods

1.1 Animals

Healthy male Sprague-Dawley rats, weighing 180-200 g. All rats were housed in room temperature is 25±2°C and 12 h: 12 h of day and night cycling light, given a standard laboratory diet, free access to food and drinking water. All animal experiments were strictly conducted according to the "Qingdao University Laboratory Animal Protection and Use Management Measures.”

1.2 Establishment of obesity rats model

The rats were given a standard laboratory diet for 7 days to adapt to the experimental environment. The rats were then given high-fat diet, single-cage rearing. Record the amount of food and spread, and weigh once a week. After 2 weeks, according to the order of weight gain, the middle 1/3 rats were selected as the normal control group giving basal diet. The remaining rats continued to be fed with high-fat diet. At the 8th week, they were re-ordered according to weight gain. The upper 1/3 of the rats were used as obesity rats, and the remaining rats were not used in this experiment.

1.3 Fluorescent Immunohistochemistry Experiment

3 normal rats and 3 obesity rats were randomly selected and rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and fixed on the operating table. Then injection of 250 ml 0.9% saline and 250 ml 4% paraformaldehyde for perfusion fixation. The brains of rats were decapitated and consecutively sectioned using a cryostat. The thickness of the slices was 15 μm. All the slices were placed in a refrigerator at -20°C. The ARC sections were selected for immunofluorescence staining of NPY5 receptors and then the experimental results were observed under a fluorescence microscope.

1.4 PT-PCR

(1) RNA extraction: normal rats and obesity rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and the brain was decapitated. Bilateral hypothalamic ARC was isolated and ARC total RNA was extracted. (2) Reverse transcription (RT): In a 25 μl reaction system, cDNA was reverse-transcribed using 2.5 μg RNA as a template. (3) Polymerase chain reaction: The target gene and β-actin cDNA sequence acquire from the gene library. Upstream primer sequence of β-actin cDNA: 5'-CATCATACTCGCAATGAGC-3'; Downstream primer sequences: 5'-GACAGCCTGTTGGCATATA-3'; Expected fragment size is 156 bp. The β-actin cDNA sequence amplification conditions were: 95 °C 60 s, 55 °C 40 s, 2 °C 40 s, 30 cycles. NPY-5R Upstream Primer Sequence: 5' -CATCTGAAGTCTTGGC -3'; Downstream primer sequences : 5'-ATCCAAAGACAGAGGGTCAGG -3'; Expected fragment size is 170 bp. NPY-5R amplification conditions: 92 °C 60 s, 58 °C 50 s, 72 °C 30 s, 35 cycles. All genes were pre-denatured at 95°C for 5 min, and expanded at 72°C for 7 min. The PCR product was electrophoresed on a 1.5% agarose gel and compared with the standard molecular weight of the DNA. Image and image analysis were performed using a Biometragel imaging system.

1.5 Brain cannula implantation

Obesity rats and normal rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and placed on a stereotaxic apparatus. A stainless steel cannula was placed in the ARC according to the Paxinos & Watson rat brain map[14]. And then secured with screws and dental cement, stainless steel tube probes closed the catheter. The scalp incision was made and the animals were placed in separate cages. The rats were free to drink water. Rats intraperitoneal antibiotics prevent infection. Rats can recover for at least 7 days.

1.6 Drug administration and food intake measurement

This experiment is divided into 2 parts: in the first part, 25 rats were randomly divided into 5 groups: (1) NS group; (2) NPY (3 nmol); (3) NPY5 receptor agonist [D-Trp34] NPY (3 nmol); (4) NPY (3 nmol) + NPY5 receptor antagonist CGP-71683 (12 nmol); (5) NPY5 receptor agonist [D-Trp34] NPY (3 nmol) + NPY5 receptor antagonist CGP-71683 (12 nmol). In the second part, 25 obesity rats were randomly divided into 5 groups: (1) NS group; (2) NPY (3 nmol); (3) NPY5 receptor agonist [D-Trp34] NPY (3 nmol); 4) NPY (3 nmol) + NPY5 receptor antagonist CGP-71683 (12 nmol); (5) NPY5 receptor agonist [D-Trp34] NPY (3
nmol) + NPY5 receptor antagonist CGP-71683 (12 Nmol). After a recovery period of 7 days, the drugs administration into the ARC of each group in normal and obesity rats. The rats were then given a previously weighed food and the remaining amount of food was measured within 4 hours. Calculate the cumulative food intake of normal rats and obesity rats during 0-4 h.

1.7 Histological verification
In order to detect the accuracy of the positioning, administration indole sky blue solution in the ARC at the end of the experiment. Then the rats were anesthetized, fixed by perfusion, and the brain was taken for decapitation. A 50 μm coronal frozen section was made and the position of the drug injection was observed under a microscope. Experimental data where the injection site is not in the ARC is not used in this experiment.

1.8 Statistical analysis
The experimental data were expressed in mean ± SD and statistical analysis was performed using SPSS 17.0 software. T test or one-way ANOVA was used for comparison between the two groups. P <0.05 was considered statistically significant.

2 Results
2.1 Fluorescent immunohistochemical experiment
Immunohistochemistry results showed that the expression of NPY5 receptor was observed in ARC of normal rats (Fig. 1A) and obesity rats (Fig. 1B). Compared with normal rats, the number of NPY5 receptors in the ARC of obesity rats increased (Fig. 1).

![Fig. 1 Expression of NPY5 receptor in ARC of normal and obesity rats. A: Expression of NPY5 receptor in ARC of normal rats; B: Expression of NPY5 receptor in ARC of obesity rats. Scale: 100μm.](image)

2.2 Expression of NPY5 receptor mRNA in ARC of normal rats and obesity rats
The results of RT-PCR showed that the expression of NPY5 receptor mRNA in the hypothalamus of obesity rats was significantly higher than that of normal rats (Fig. 2).

![Fig. 2 The expression of NPY5 receptor mRNA in the ARC of normal and obesity rats](image)
2.3 Effects of NPY5 receptor agonist [D-Trp34] NPY and NPY5 receptor antagonist CGP-71683 on feeding of normal rats and obesity rats

To investigate whether the NPY5 receptor influences food intake in normal rats and obesity rats, we investigated the effects of microinjection of NPY5 receptor agonist [D-Trp34] NPY and NPY5 receptor antagonist CGP-71683 into the ARC on food intake in normal rats and obesity rats. The experimental results showed that compared with the NS group, after microinjected of NPY in ARC, the food intake increased significantly during the 0-4h in normal and obesity rats (P<0.05, Fig. 3B). Similarly, compared with the NS group, after microinjected of NPY5 receptor agonist [D-Trp34] NPY in ARC, the food intake increased significantly during the 0-4h in normal and obesity rats(P<0.05, Fig. 3B). However, the injection of NPY5 receptor antagonist CGP-71683 alone had no significant effect on the food intake of rats (P>0.05, Fig. 3B). Compared with the NPY5 receptor agonist [D-Trp34] NPY group, administration of the NPY5 receptor agonist [D-Trp34] NPY and the NPY5 receptor antagonist CGP-71683 mixture into the ARC, the food intake of normal and obesity rats was significantly reduced during the 0-4h (P>0.05, Fig. 3B).

This indicates that NPY5 receptor antagonist CGP-71683 blocks NPY receptor agonist [D-Trp34] NPY-induced food intake.

After NPY was injected microinjected into the ARC of obesity rats, the food intake at 0-4h was significantly higher than that of normal rats (P>0.05, Fig. 3A). At the same time, the NPY injection of NPY5 receptor agonist [D-Trp34] in the ARC of obesity rats was significantly higher than that of normal rats (P>0.05, Fig. 3A). This may be related to increased expression of NPY5 receptors in the ARC of obesity rats.

3 Discussion

The hypothalamus is the main brain area involved in regulating energy balance. In the hypothalamus, a series of hormone and visceral afferent signals are processed [15]. Previous studies have shown that NPY interacts with other neuropeptides in the central regulatory mechanism of feeding [15,16]. NPY immunopositive neurons are mainly expressed in ARC [17].

This experiment is mainly to study the role of NPY5 receptor in feeding regulation in ARC. In the experiments we found that selective activation of NPY5 receptors in ARC significantly increased food intake in rats. As we all know, the hypothalamus maintains physiological through the regulating energy balance and feeding. The experimental results of electrical damage the hypothalamus indicate that the hypothalamic lateral area (LHA) is a “starvation center” and the hypothalamic ventromedial nucleus (VMH) is a “satisfactory center” [18]. Recent studies have found that hypothalamic nuclei and neural pathways are important factors in regulating food intake. At the same time, ARC is a key brain area that regulates feeding.

Fig.3 Effect of Injection of NPY and [D-Trp34] NPY in the ARC on Food intake in normal and obesity Rats. *P<0.05, compared with the NS group; tP< 0.05 , compared with the NPY group; tP<0.05, compared with the normal rats.

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behavior.

Most diet-induced obesity studies have shown that SD rats can exhibit different susceptibility to obesity. NPY is abundant in the mammalian central nervous system and is a strong pro-feeding factor. Central injection of NPY can result in excessive feeding and reduced energy expenditure in rats\(^{19,20}\). In addition, a large number of studies have shown that NPY mainly participates in the regulation of energy balance through NPY1 receptor, NPY2 receptor, and NPY5 receptor\(^{21-24}\). Therefore, in this study we observed the expression levels of NPY5 receptor in ARC in normal rats and obese rats. It is well known that weight is maintained by the balance between energy intake and energy expenditure. NPY served as an ingestion regulator, but there is no significant difference in plasma NPY levels between normal and obese rats. It is suggested that peripheral NPY signals are not as sensitive to changes in nutritional status and are not important to feeding regulation. This suggests that NPY in the hypothalamus may play a more important role in the pathogenesis of obesity than peripheral NPY. Our experimental results showed that the expression of NPY5 receptor in the hypothalamus ARC in obese rats was significantly higher than that in normal rats, and NPY5 receptor agonist [D-Trp34] NPY was injected into the ARC of normal rats and obese rats could increased food intake significantly, and obese rats was more pronounced. It is precisely because in NPY5 receptor mRNA expression that promotes energy intake, visceral adipose tissue accumulation, and body weight gain in rats, thereby inducing obesity;

Microinjection of the NPY5 receptor agonist [D-Trp34] NPY into the ARC of normal and obesity rats promotes food intake in rats. It suggested that NPY-induced food intake is at least partially mediated by activation of the NPY5 receptor. In this experiment, we also observed the effect of NPY5 receptor antagonist CPG-71683 on feeding behavior. The experimental results show that intra-ARC injection of NPY5 receptor antagonist CPG-71683 can eliminate the promoting effect of NPY5 receptor agonist [D-Trp34] NPY on food intake, which indicates that [D-Trp34] NPY is specific for the activation of NPY5 receptor. The NPY5 receptor agonist [D-Trp34] NPY has a weaker promote effect on food intake than the NPY, possibly because the NPY5 receptor agonist [D-Trp34] NPY in the regulation of feeding.

In conclusion, this experiment demonstrated that NPY neurons in ARC play an important role in regulation of feeding. The regulation of feeding behavior in ARC is at least partly through the NPY5 receptor signaling pathway. In addition, the food intake of obesity rats was higher than that of normal rats, which may be due to the expression of NPY5 receptor increased in obesity rats. The above studies provide a new idea for the treatment of obesity.

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