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L-Ornithine is a potential acute satiety signal in the brain of neonatal chicks

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HIGHLIGHTS

- Regulated appetite altered amino acid concentrations in all brain regions studied.
- L-Ornithine increased by re-feeding in all brain regions except the cerebellum.
- Central administration of L-ornithine inhibited food intake in chicks.

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ABSTRACT

Recently, we observed that neonatal chicks exhibit feeding behavior characterized by frequent food intake and short resting intervals, with changes detected in the brain amino acid and monoamine concentrations. In this study, we aimed to clarify further the relationship between the appetite of neonatal chicks and brain amino acid metabolism. In Experiment 1, changes were investigated in free amino acids in the brain under conditions of regulated appetite induced by fasting and subsequent short-term re-feeding. Chicks (5 days old) were distributed into four treatment groups — namely, fasting for 3 h, and fasting for 3 h followed by re-feeding for 10, 20 or 30 min. Brain samples were collected after treatment to analyze free amino acid concentrations. Amino adipic acid and proline in all brain parts as well as arginine and ornithine in all brain parts – except mesencephalic arginine and cerebellar ornithine – were increased in a time-dependent manner following re-feeding. In Experiment 2, we further examined the effect of exogenous administration of some amino acids altered in association with feeding behavior in Experiment 1. We chose L-arginine and its functional metabolite, L-ornithine, to analyze their effects on food intake in chicks. Intracerebroventricular injection (2 μmol) of L-ornithine, but not L-arginine, significantly inhibited food intake in neonatal chicks. In Experiment 3, we found that central injection of L-ornithine (2, 4, and 6 μmol) dose-dependently suppressed food intake in chicks. These results suggested that L-ornithine may have an important role in the control of food intake as an acute satiety signal in the neonatal chick brain.

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1. Introduction

Amino acids have been conventionally reviewed in nutrition as playing an important role in protein synthesis and as providing an energy source. However, their physiological functions within the central nervous system have also attracted much interest. Brain amino acids have been shown to contribute to feeding behavior in both mammalian and avian species. In rats, the primary excitatory neurotransmitter glutamate and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) were reported to mediate the stimulatory and inhibitory effects, respectively, on food intake in rats within the lateral hypothalamus [1,2]. A decline in food intake was observed when a mixture of amino acids was injected into the hypothalamus [3]. Furthermore, studies in neonatal chicks showed that central administration of several amino acids, such as L-leucine, L-proline, and L-tryptophan, influenced food intake [4–6]. Therefore, central amino acids are suggested to be potential modulators of feeding behavior. Recently, we reported that neonatal chicks exhibit feeding behavior characterized by its frequency, with accompanying changes in the brain amino acid and monoamine concentrations [7]. However, the reason for the alteration in amino acids in the chick brain in connection with feeding behavior was not clarified. Thus, the first aim of the current study was to further clarify the relationship between regulation of appetite and brain amino acids in neonatal chicks.

We also found that changes in concentrations of several amino acids in the mesencephalon in neonatal chicks were associated with their

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frequent food intake [7]. Among these altered amino acids, L-arginine is one which is classified as an essential amino acid for birds because birds are lacking in carbamyl phosphate synthetase, which is a necessary enzyme for the synthesis of citrulline, a precursor of arginine, from ornithine in the liver and kidney [8]. L-Arginine has been documented for decades to have diverse physiological functions in mammals. Among 17 examined amino acids, oral administration of L-arginine was found to inhibit food intake in rats with the involvement of intact neurons in the area postrema [9]. In neonatal chicks, central L-arginine induced sedative and hypnotic effects under a condition of acute social separation stress [10]. However, its function in the feeding behavior of neonatal chicks is still unknown. L-Arginine exerts its metabolic roles through the production of diverse metabolites including nitric oxide (NO), L-ornithine, polyamines, L-proline, L-glutamine, creatine and agmatine [11]. Among these metabolites, L-ornithine was found to have an important role in the central effect on stress attenuation through L-arginine; meanwhile, the contribution of NO and agmatine was thought to be negligible [12]. In the present study, L-arginine and L-ornithine were time-dependently increased in most of the brain regions after re-feeding. Therefore, the second aim was to elucidate whether central administration of L-arginine and its metabolite L-ornithine affects feeding behavior in neonatal chicks.

2. Materials and methods

2.1. Animals and food

Day-old male layer chicks (Julia strain; *Gallus gallus domesticus*) purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) were housed in groups in metal cages at a constant temperature of 30 ± 1 °C under continuous light. Food (AX and Adjust diets; Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were provided *ad libitum*. AX diet [Commercial starter diet (metabolizable energy: 12.77 MJ/kg and protein: 24%; food ingredients: grain 61% (mainly maize), defatted meal 25% (soybean meal and maize gluten meal), fish meal 9%, rice bran 1% and others 4%)] was used in Experiment 1 and replaced by Adjust diet (metabolizable energy: >12.55 MJ/kg, protein: >23%) in Experiment 2 and 3 because the commercial supply of AX diet ceased. There was virtually no other difference in composition between the two diets that was of any significance.

From 3 days old, chicks were gradually isolated step by step to minimize isolation stress (20 chicks/cage, 10 chicks/cage and 2 chicks/cage in 1-, 2- and 3-days old, respectively), and they were placed in individual cages 24 h before starting the experiments. On the day of experiments, chicks (5 days old) were randomly assigned to treatment groups based on their body weight so that the average body weight between the groups was as uniform as possible.

This study was performed in accordance with the guidelines for animal experiments in the Faculty of Agriculture of Kyushu University, and complied with Law No. 105 and Notification No. 6 of the Japanese government.

2.2. Preparation of drugs

In Experiments 2 and 3, L-ornithine monohydrochloride and/or L-arginine hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). The drugs were dissolved in 0.85% saline containing a 0.1% Evans Blue solution.

2.3. Experimental design

In Experiment 1, the influence of regulated appetite induced by fasting and short-term re-feeding on the concentrations of brain amino acids was determined. Chicks (5 days old) were distributed into four treatment groups: 3 h fasting; 3 h fasting and 10 min re-feeding; 3 h fasting and 20 min re-feeding; and 3 h fasting and 30 min re-feeding. After fasting and re-feeding treatments, birds were immediately decapitated following the exposure to isoflurane (Mylan Inc., Tokyo, Japan). The brains were dissected and the telencephalon, diencephalon, brain stem, mesencephalon, and cerebellum were collected and stored in Eppendorf tubes. The five brain regions were identified as described elsewhere [13,14]. Then these brain samples were frozen in liquid nitrogen and stored at −80 °C until they were analyzed for free amino acid concentrations.

In Experiment 2, the effect of intracerebroventricular (i.c.v.) injection of L-arginine and L-ornithine on cumulative food intake was investigated. Chicks (5 days old) were intracerebroventricularly injected with 2 μmol of L-arginine or 2 μmol of L-ornithine.

In Experiment 3, the effect of i.c.v. injection of L-ornithine on cumulative food intake was investigated. Chicks (5 days old) were intracerebroventricularly injected with 2, 4, or 6 μmol of L-ornithine. Saline was used as a control in Experiments 2 and 3.

The drugs were injected intracerebroventricularly into the left lateral ventricle of the chicks in a volume of 10 μl using a microsyringe according to the method as described elsewhere [15]. This injection method does not cause stress [16], and feeding is the same with or without the i.c.v. injection [17]. In Experiments 2 and 3, chicks fasted for 3 h but were given free access to water during fasting before the i.c.v. injection. After the injection, chicks were given free access to food and water. Cumulative food intakes were measured at 10, 20, and 30 min post injection. At the end of the experiments, chicks were decapitated following anesthesia with isoflurane. The brains were removed and the location of the Evan Blue dye was confirmed. Data from chicks without dye in the lateral ventricle were excluded from the study.

2.4. Amino acid analysis

Amino acid concentrations in brain samples were analyzed by high-performance liquid chromatography (HPLC) according to a method described elsewhere with some modifications [18]. The brain tissues were homogenized in ice-cold 0.2 M perchloric acid solution containing 0.01 mM ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), and then left for deproteinization on ice for 30 min. The tissue homogenates were centrifuged at 20,000 *g* for 15 min at 0 °C. The collected supernatants were filtered through a 0.20 μm filter unit (Millipore, Bedford, USA) and adjusted to pH 7 with 1 M sodium hydroxide. Each 20-μl sample of the brain was dried under reduced pressure at −100 kPa (Centrifugal Vaporizer, CVE-200D, Eyela, Japan). The dried residues were first dissolved in 10 μl of 1 M sodium acetate–methanol–triethylamine (2:2:1) and re-dried under reduced pressure, and then converted to their phenylthiocarbamoyl derivatives by being dissolved in 20 μl of methanol–distilled water–triethylamine–phenylisothiocyanate (7:1:1:1) and being allowed to react for 20 min at room temperature. The reacted samples were dried again under reduced pressure and then dissolved in 200 μl Pico-Tag Diluent (Waters, Milford, USA). These diluted samples were filtered through a 0.20-μm filter unit (Millipore, Bedford, USA). The same methods were applied to standard solutions which were prepared by diluting a commercially available L-amino acid solution (type ANII, type B, L-asparagine, L-glutamine and L-tryptophan; Wako, Osaka, Japan) with distilled water. The solution samples containing the derivatives were applied to a Waters HPLC system (consisting of a Pico-Tag free amino acid analysis column (3.9 mm × 300 mm), an Alliance 2690 separation module, a 2487 dual-wavelength UV detector and an Empower 2 chromatography manager; Waters, Milford, USA). Because this Pico-Tag method cannot identify L- and D-forms of each amino acid, we simply used the nomenclature of each amino acid in the results of Experiment 1. They were equilibrated with buffer A (70 mM sodium acetate (pH 6.45 with 10% acetic acid)–acetonitrile (975:25)) and eluted with a linear gradient of buffer B (water–acetonitrile–methanol (40:45:15) (0, 3, 6, 9, 40 and 100%)) at a flow rate of 1 ml/min at 46 °C. Free amino acid and dipeptide concentrations were determined by absorbance at a

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