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Central Interaction Between L-Ornithine and Neuropeptide Y in the Regulation of Feeding Behavior of Neonatal Chicks

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Ornithine has been identified as a potential satiety signal in the brains of neonatal chicks. We hypothesized that brain nutrient signals such as amino acids and appetite-related neuropeptides synergistically regulate food intake. To test this hypothesis, we investigated the interaction between neuropeptide Y (NPY) and ornithine in the control of feeding behavior in chicks and the associated central and peripheral amino acid metabolic processes. Five-day-old chicks were intracerebroventricularly injected with saline, NPY (375 pmol), or NPY plus ornithine (2 or 4 μmol) at 10 μl per chick, and then subjected to *ad libitum* feeding conditions; food intake was monitored for 30 min after injection. Brain and plasma samples were collected after the experiment to determine free amino acid concentrations. Co-injection of NPY and ornithine significantly attenuated the orexigenic effect induced by NPY in a dose-dependent manner. Central NPY significantly decreased amino adipic acid, asparagine, γ-aminobutyric acid, leucine, phenylalanine, tyrosine, and isoleucine levels, but significantly increased lysine levels in the brain. Co-injection of NPY and ornithine significantly increased ornithine and proline levels in all examined brain regions, but decreased diencephalic tryptophan and glycine levels compared with those of the control and NPY-alone groups. Co-injection of NPY and high-dose ornithine significantly decreased methionine levels in all brain regions. Central NPY significantly suppressed the plasma concentrations of amino acids, including proline, asparagine, methionine, phenylalanine, tyrosine, leucine, isoleucine, glycine, glutamine, alanine, arginine, and valine, and this reduction was greater when NPY was co-injected with ornithine. These results suggest that brain ornithine interacts with NPY to regulate food intake in neonatal chicks. Furthermore, central NPY may induce an anabolic effect that is modified by co-injection with ornithine.

Key words: central nervous system, feeding behavior, L-ornithine, neonatal chick, neuropeptide Y

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Introduction

Ornithine is a free amino acid that is not incorporated into polypeptides during protein synthesis. However, ornithine is well known to play an important role in the urea cycle for the disposal of excess nitrogen such as ammonia (Rodwell, 2000). The functions of endogenous brain ornithine and its metabolism in therapeutic applications have been extensively reviewed (Slotkin and Bartolome, 1986; Seiler and Daune-Anglard, 1993). Although brain ornithine levels appear to be relatively lower than those in other tissues (Daune-Anglard *et al*., 1993), the enzyme arginase, which catalyzes the formation of ornithine from arginine, is highly active in the brain (Sadasivudu and Indira, 1974). Suenaga *et al*. (2008) reported that intracerebroventricular (ICV) injection of L-arginine proportionally increased both the arginine and ornithine concentrations in the telencephalon and diencephalon of chicks 10 min post-injection, suggesting that arginine was

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metabolized by arginase in the chick brain. There are two major pathways of ornithine metabolism in the brain that involve two different enzymes: ornithine α-ketoglutarate aminotransferase, which catalyzes the transamination of ornithine to glutamate and proline, and ornithine decarboxylase, which catalyzes the conversion of ornithine to polyamines (Seiler and Daune-Anglard, 1993). The central functions of ornithine, glutamate, and proline in attenuating stress via the induction of sedative and hypnotic effects have been elucidated in neonatal chicks (Furuse, 2015). Ornithine itself has also been proposed to play an important role in the induction of sedative and hypnotic effects, but not through polyamine metabolites (Kurauchi *et al*., 2010). Moreover, ICV injection of ornithine, but not arginine, significantly inhibited food intake in a dose-dependent manner in neonatal chicks (Tran *et al*., 2016). L-ornithine was shown to exert attenuation effects on the stress response, which is different from its actions on neural circuits in controlling food intake behavior due to the non-responsiveness of stress-related receptors (Tran *et al*., 2016). More importantly, a time-dependent increase in endogenous ornithine levels in the brain was observed following refeeding after fasting in chicks maintained in an acute satiety state (Tran *et al*., 2016). This implies that endogenous ornithine may physiologically inhibit feeding behavior in neonatal chicks.

Feeding behavior is tightly regulated by neuronal, metabolic, and endocrine signals to the central nervous system (CNS), particularly the hypothalamus (Schwartz *et al*., 2000; Richards, 2003). Appetite regulation encompasses complex interactions between neurotransmitters, including neuropeptides and classical amino acid neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) (Stanley *et al*., 2011). Therefore, we hypothesized that there could be collaboration between acute feeding signals such as amino acids and appetite-related neuropeptides in neonatal chicks.

Since the first report of the presence of neuropeptide Y (NPY) in the CNS of chickens using antibodies against porcine NPY (Kuenzel and McMurtry, 1988), accumulating evidence has indicated that NPY is one of the most potent stimulators of food intake in chickens (Kuenzel and McMurtry, 1988; Furuse *et al*., 1997; Bungo *et al*., 2000; Dodo *et al*., 2005; Saneyasu *et al*., 2011) among the very few appetite-stimulatory signals as compared with anorexigenic neuropeptides (Cline and Furuse, 2012; Tran *et al*., 2019). Indeed, there is considerable evidence to support the important physiological functions of NPY in feeding regulation in chickens. Leibowitz (1989) observed NPY gene expression in brain regions involved in appetite regulation. Furthermore, previous studies have reported a direct correlation between increased brain NPY levels and food intake in chickens. Fasting or feeding restriction enhances hypothalamic NPY gene expression in chickens (Boswell *et al*., 1999a, b; Wang *et al*., 2001). Zhou *et al*. (2005) also indicated that the NPY content of the hypothalamic infundibular nucleus and paraventricular nucleus (PVN) gradually increased with fasting time in broiler chickens; subsequent re-feeding restored the NPY levels in the PVN to pre-fasting levels. In addition, most appetite-related factors have been reported to exert their effects via actions on the NPY pathway (Herzog, 2003). Given that both NPY and ornithine act in the CNS to regulate feeding in neonatal chicks, it is possible that the potent orexigenic effects induced by NPY interact with the ornithine signaling pathway.

The proportional increase in brain ornithine levels following central administration of its precursor L-arginine was found to be accompanied by alterations in amino acid concentrations in the chick brain (Suenaga *et al*., 2008). In addition, previous studies have reported the influence of NPY on both central and peripheral amino acid metabolism (Eltahan *et al*., 2017; Tran *et al*., 2021). Therefore, the aim of the current study was to examine the involvement of ornithine signaling pathway in the orexigenic effect induced by NPY by determining food intake after direct central co-injection of NPY and ornithine, and investigating the involvement of free amino acid metabolism in the central and peripheral systems of chicks.

Materials and methods

Animals and food

Fertilized eggs (layer-type Julia strain *Gallus gallus domesticus*) were purchased from a local hatchery (Tsuboi Hatchery, Kumamoto, Japan) to obtain experimental chicks. The eggs were placed in an incubator (Rcom Maru Deluxe MAX 380; Autoelex Co., Ltd., Korea) at an incubation temperature of 37.5 °C with 60% relative humidity, and auto-turning was completed every hour until day 18. After removing eggs with undeveloped and dead embryos, the eggs were transferred into hatching trays from embryonic day 19 to prepare for hatching. After hatching, one-day-old chicks were housed in groups (15–20 chicks/cage) in metal cages (50 × 35 × 33 cm) at a temperature of 30 ± 1 °C under continuous lighting. Food (Adjust diets; Toyohashi Feed and Mills Co. Ltd., Aichi, Japan; metabolizable energy > 12.55 MJ/kg, protein > 23%) and water were provided *ad libitum.* Sex identification was performed by distinguishing feathers when 2-day-old and male chicks were selected for use in the experiments. On the day of the experiment, 5-day-old male layer chicks were randomly assigned to treatment groups based on their body weight to ensure uniform treatment.

This study was performed in accordance with the guidelines for animal experiments of the Faculty of Agriculture of Kyushu University (registration number A20-043-4), and complied with Law No. 105 and Notification No. 6 of the Japanese government.

Preparation of drugs and ICV injection

NPY (porcine) was purchased from the Peptide Institute (Osaka, Japan). Porcine NPY was used because it has high affinity to chicken NPY receptors (Lundell *et al.*, 2002). L-Ornithine monohydrochloride was purchased from Wako Pure Chemical Industries (Osaka, Japan). All drugs were dissolved in 0.85% saline solution containing 0.1% Evans Blue dye and kept on ice during the experimental period.

The drug solution and saline were injected into the left lateral ventricle of the chicks using a microsyringe according to a previously described method for ICV injection (Davis *et al*., 1979);

this injection method was confirmed to not cause stress (Koutoku *et al*., 2005). Injection of Evans Blue saline solution alone was used as a control. At the end of the experiment, the chicks were anesthetized with isoflurane, euthanized, and their brains were removed. Successful injections were confirmed by visualizing the location of Evans Blue dye. Data from chicks without dye in the lateral ventricle were excluded from the analysis.

Experimental design

The effect of the co-injection of NPY with different doses of ornithine on food intake was investigated. Chicks were ICV-injected with saline, NPY [375 pmol as per Tachibana *et al*. (2006)], NPY plus ornithine (2 µmol), or NPY plus ornithine (4 µmol) at 10 µl per chick.

After injection, chicks were given free access to food and water. Food intake was measured for 30 min after ICV injection. After 30 min of feeding, three brain regions (the diencephalon, telencephalon, and brainstem) were identified and dissected immediately after euthanasia according to the chicken brain atlas of Kuenzel and Masson (1988) and a schematic drawing of the chick brain by Chowdhury *et al*. (2014). The dissected brain regions were preserved in microtubes, frozen in liquid nitrogen, and stored at –80 °C. Jugular blood samples were collected in microtubes containing a drop of heparin solution (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) before harvesting the plasma by centrifugation for 4 min at 10,000 ×*g* (MX-307; TOMY Seiko Co. Ltd., Tokyo, Japan); the plasma was stored at −80 °C until free amino acid analysis.

Amino acid analysis

Amino acid concentrations in the brain and plasma samples were analyzed using high-performance liquid chromatography (HPLC), according to the method described by Boogers *et al*. (2008), with some modifications. 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 on ice for 30 min for deproteinization. The tissue homogenates were centrifuged at 20,000 ×*g* for 15 min at 4 °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. Plasma filtrates were obtained by centrifuging the plasma samples at 12,000 ×*g* for 10 min at 4 °C using Amicon Ultra centrifugal filters (Merck Millipore Ltd., Cork, Ireland). Standard solutions (10 µl), brain tissue filtrates (20 µl), and plasma filtrates (10 µl) were dried under reduced pressure using a centrifugal evaporator (CVE-3000; EYELA, Tokyo, Japan). The dried residues were first dissolved in 10 µl of 1 M sodium acetate–methanol–triethylamine (2:2:1), re-dried under reduced pressure, converted to their phenylthiocarbamoyl derivatives by dissolution in 20 µl of methanol-distilled water–triethylamine–phenylisothiocyanate (7:1:1:1), and allowed to react for 20 min at room temperature. The reacted samples were dried again under reduced pressure and dissolved in 200 µl of Pico-Tag Diluent (Waters, Milford, USA). These diluted samples were filtered through a 0.20-µm filter unit (Millipore). The same method was applied to standard solutions 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 e2695 separation module, a 2487 dual-wavelength ultraviolet detector, and an Empower 2 chromatography manager; Waters, Milford, USA]. Because the Pico-Tag method cannot identify the L- and D-forms of each amino acid, the nomenclature for each amino acid was used in the results. The samples were equilibrated with buffer A [70 mM sodium acetate (pH 6.45, with 10% acetic acid)–acetonitrile at 975:25] and eluted with a linear gradient of buffer B (water–acetonitrile–methanol at a 40:45:15 ratio) at 0,3%, 6%, 9%, 40%, and 100%, with a flow rate of 1 ml/min at 46 °C. Free amino acid concentrations were determined by measuring the absorbance at a wavelength of 254 nm. The concentrations of free amino acids are expressed as pmol/mg wet tissue in the brain and as pmol/µL in the plasma.

Statistical analysis

Data on brain and plasma free amino acid concentrations and food intake were compared among groups using one-way analysis of variance and the Tukey–Kramer post-hoc test. The experimental data in each group were first subjected to a Thompson rejection test to eliminate outliers ($P < 0.01$), and the remaining data were used for analysis. Statistical analysis was conducted using a commercially available package, StatView (version 5, SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Changes in food intake following co-injection of ornithine and NPY

The effect of co-injection of NPY with different doses of ornithine on food intake 30 min post-injection is shown in Fig. 1. Co-injection with ornithine significantly attenuated the orexigenic effect induced by NPY in a dose-dependent manner ($P < 0.001$). There was no significant difference in food intake between the control and the NPY plus ornithine groups.

Changes in free amino acid concentrations in the brain following co-injection of ornithine and NPY

Changes in free amino acid concentrations in different regions of the brain induced by NPY and ornithine are shown in Tables 1, 2, and 3. Co-injection of ornithine and NPY significantly ($P < 0.0001$) increased ornithine levels in a dose-dependent manner and proline levels in all brain regions compared with those of the control and NPY-alone groups. The co-injection of ornithine and NPY significantly decreased the concentrations of tryptophan ($P < 0.0001$) and glycine ($P < 0.01$) in the diencephalon. Tryptophan was not detected in the telencephalon or in the brainstem. Co-injection of high-dose ornithine and NPY significantly decreased the concentrations of methionine in all examined brain regions compared with those of the control and NPY-alone groups. The GABA concentration was significantly decreased in the NPY

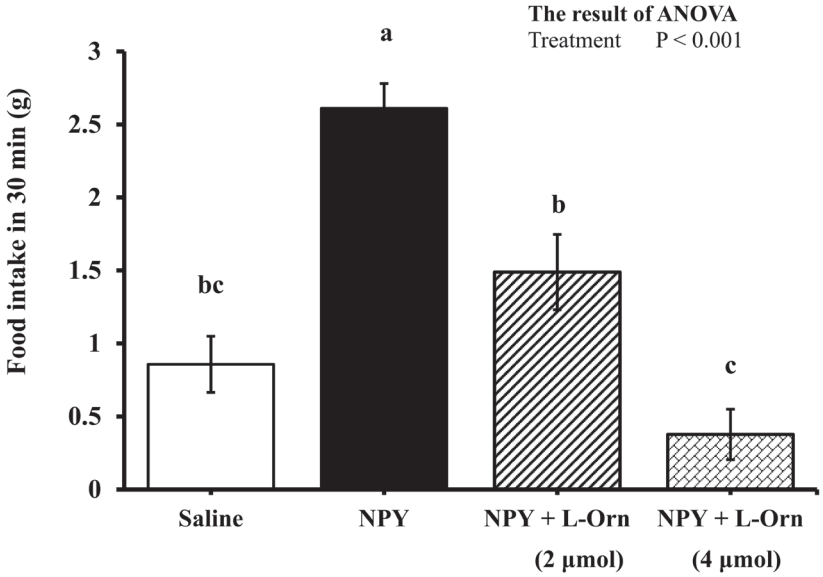

Fig. 1. **Food intake (g) of chicks following central administration of neuropeptide Y (375 pmol) and L-ornithine when subjected to *ad libitum* feeding for 30 min.** Groups with different letters (a, b) are significantly different ($P < 0.05$). Values are presented as the mean ± SEM (n = 9–10 per group). NPY, neuropeptide Y; L-Orn, L-Ornithine.

Table 1. **Effects of intracerebroventricular injection of neuropeptide Y (NPY) and ornithine on diencephalic amino acid concentrations in neonatal chicks after 30 min of *ad libitum* feeding**

Amino acids	Control	NPY			P
		Ornithine (0 µmol)	Ornithine (2 µmol)	Ornithine (4 µmol)	
Essential amino acids					
Arginine	226 ± 8	209 ± 10	233 ± 12	229 ± 8	NS
Lysine	599 ± 32^{a}	781 ± 116^{ab}	906 ± 74^{ab}	989 ± 88^{b}	< 0.05
Histidine	255 ± 18	252 ± 14	278 ± 19	264 ± 18	NS
Leucine	239 ± 11^{a}	219 ± 4^{ab}	208 ± 9^{ab}	196 ± 7^{b}	< 0.01
Isoleucine	77 ± 6	66 ± 4	65 ± 4	66 ± 5	NS
Valine	221 ± 6	215 ± 5	204 ± 6	214 ± 6	NS
Methionine	106 ± 4^{a}	98 ± 3^{a}	96 ± 3^{a}	81 ± 5^{b}	< 0.001
Threonine	645 ± 21	699 ± 36	673 ± 20	633 ± 14	NS
Tryptophan	38 ± 1^{a}	35 ± 2^{a}	27 ± 1^{b}	29 ± 0.5^{b}	< 0.0001
Phenylalanine	116 ± 3^{a}	102 ± 3^{b}	106 ± 2^{ab}	107 ± 3^{ab}	< 0.05
Glycine	1413 ± 29^{ab}	1462 ± 21^{a}	1349 ± 19^{b}	1371 ± 20^{b}	< 0.01
Nonessential amino acids					
Ornithine	44 ± 2^{a}	35 ± 2^{a}	890 ± 97^{b}	2046 ± 162^{c}	< 0.0001
Proline	314 ± 6^{a}	285 ± 9^{a}	384 ± 7^{b}	414 ± 11^{b}	< 0.0001
GABA	8241 ± 189	7832 ± 262	7786 ± 212	7697 ± 128	NS
Amino adipic acid	106 ± 4^{a}	83 ± 3^{b}	87 ± 5^{b}	80 ± 3^{b}	< 0.0005
Asparagine	301 ± 5^{a}	277 ± 7^{b}	288 ± 5^{ab}	271 ± 3^{b}	< 0.005
Tyrosine	123 ± 2^{a}	106 ± 4^{b}	106 ± 3^{b}	103 ± 6^{b}	< 0.005
Glutamic acid	8630 ± 111	8041 ± 229	8397 ± 178	8258 ± 107	NS

Values are presented as mean concentration ± SEM (pmol/mg wet tissue); n = 7–10 per group. Different superscript letters indicate significant differences at $P < 0.05$. Abbreviations: GABA, γ-aminobutyric acid; NS, not significant.

Table 2. **Effects of intracerebroventricular injection of neuropeptide Y (NPY) and ornithine on telencephalic amino acid concentrations in neonatal chicks after 30 min of *ad libitum* feeding**

Amino acids	Control	NPY			P
		Ornithine (0 μmol)	Ornithine (2 μmol)	Ornithine (4 μmol)	
Essential amino acids					
Arginine	213 ± 8	199 ± 8	231 ± 15	214 ± 12	NS
Lysine	729 ± 40^{a}	952 ± 138^{ab}	1249 ± 102^{b}	1231 ± 118^{b}	< 0.005
Histidine	310 ± 15	339 ± 19	339 ± 12	311 ± 19	NS
Leucine	271 ± 10^{a}	262 ± 11^{ab}	240 ± 8^{ab}	225 ± 11^{b}	< 0.05
Isoleucine	108 ± 5^{a}	98 ± 4^{ab}	93 ± 3^{ab}	86 ± 5^{b}	< 0.05
Valine	252 ± 6	254 ± 8	246 ± 8	252 ± 9	NS
Methionine	121 ± 4^{a}	119 ± 6^{a}	113 ± 4^{a}	93 ± 4^{b}	< 0.0005
Threonine	789 ± 45	842 ± 45	899 ± 39	785 ± 19	NS
Phenylalanine	137 ± 7	134 ± 6	132 ± 6	125 ± 6	NS
Glycine	1247 ± 30	1336 ± 40	1265 ± 43	1264 ± 33	NS
Nonessential amino acids					
Ornithine	58 ± 2^{a}	52 ± 3^{a}	1646 ± 170^{b}	2991 ± 174^{c}	< 0.0001
Proline	313 ± 3^{a}	303 ± 5^{a}	381 ± 6^{b}	378 ± 9^{b}	< 0.0001
GABA	4034 ± 79^{a}	3922 ± 91^{ab}	3782 ± 103^{ab}	3679 ±53^{b}	< 0.05
Amino adipic acid	269 ± 12^{a}	238 ± 7^{ab}	238 ± 13^{ab}	216 ± 8^{b}	< 0.05
Asparagine	346 ± 7^{a}	328 ± 3^{ab}	335 ± 6^{a}	312 ± 4^{b}	< 0.005
Glutamic acid	12507 ± 111	12348 ± 115	12406 ± 105	12242 ± 182	NS

Values are presented as mean concentration ± SEM (pmol/mg wet tissue); n = 7–10 per group. Different superscript letters indicate significant differences at $P < 0.05$. Abbreviations: GABA, γ-aminobutyric acid; NS, not significant.

plus ornithine group in the telencephalon ($P < 0.05$) and brain stem ($P < 0.005$) compared with that of the control group. The concentrations of amino adipic acid (AAA) were significantly reduced in all NPY-treated groups in the diencephalon ($P < 0.001$) and in the NPY plus high-dose ornithine group in the telencephalon ($P < 0.05$). Conversely, the concentrations of lysine were significantly increased in the NPY-alone and NPY plus ornithine groups in the telencephalon ($P < 0.005$) and diencephalon ($P < 0.05$) compared with those in the control group. The diencephalic concentrations of tyrosine ($P < 0.005$) were significantly lower in all NPY-treated groups than those in the control group. Tyrosine levels were not altered in the brainstem and were undetectable in the telencephalon. Diencephalic phenylalanine level was significantly reduced by injection of NPY alone ($P < 0.05$). The concentrations of leucine were significantly ($P < 0.05$) reduced in the NPY plus high-dose ornithine group in all brain regions. The isoleucine concentration was significantly decreased by co-injection of NPY plus ornithine in the telencephalon ($P < 0.05$) and brainstem ($P < 0.0001$). Asparagine levels were significantly ($P < 0.01$) decreased by NPY, but were restored by co-injection of ornithine (2 μmol) and NPY in the diencephalon. The NPY plus ornithine (4 μmol) group had the lowest asparagine levels in the telencephalon and brainstem ($P < 0.005$).

Changes in free amino acid concentrations of the plasma following co-injection of ornithine and NPY

The changes in plasma free amino acid concentrations induced by NPY and ornithine are shown in Table 4. The plasma concentrations of amino acids, including proline, asparagine, methionine, leucine, isoleucine, glycine, glutamine, alanine, arginine, valine, tyrosine, and phenylalanine, were significantly lower ($P < 0.0001$) in the NPY-treated groups than in the control group. Co-injection of NPY and ornithine significantly decreased the plasma concentrations of almost all amino acids, including the above-mentioned amino acids plus ornithine, serine, and histidine ($P < 0.0001$), in a dose-dependent manner. The levels of several amino acids, including lysine ($P < 0.05$), glutamate, hydroxyproline, taurine, cystathionine ($P < 0.01$), and threonine ($P < 0.0001$), were significantly reduced in the NPY plus ornithine (4 μmol) group compared with those of the control group. Plasma GABA levels were undetectable.

Discussion

Ornithine plays an important role in the control of food intake, representing a potential inhibitory signal in the neonatal chick brain (Tran *et al*., 2016). The present study revealed the possibility that ornithine functionally interacts with the NPY-induced physiological stimulation of feeding. The results showed that ornithine significantly suppressed NPY-induced food intake in

Table 3. **Effects of intracerebroventricular injection of neuropeptide Y (NPY) and ornithine on brainstem amino acid concentrations in neonatal chicks after 30 min of *ad libitum* feeding**

Amino acids	Control	NPY			P
		Ornithine (0 μmol)	Ornithine (2 μmol)	Ornithine (4 μmol)	
Essential amino acids					
Arginine	335 ± 12	319 ± 18	329 ± 22	339 ± 16	NS
Lysine	979 ± 62	1352 ± 207	1398 ± 141	1459 ± 155	NS
Histidine	214 ± 10	238 ± 8	227 ± 10	227 ± 11	NS
Leucine	287 ± 11^{a}	266 ± 7^{ab}	264 ± 12^{ab}	246 ± 7^{b}	< 0.05
Isoleucine	104 ± 3^{a}	103 ± 1^{a}	90 ± 2^{b}	86 ± 4^{b}	< 0.0001
Valine	295 ± 7	290 ± 7	286 ± 8	293 ± 5	NS
Methionine	79 ± 3^{a}	81 ± 3^{a}	71 ± 3^{a}	53 ± 2^{b}	< 0.0001
Threonine	765 ± 34	803 ± 57	781 ± 28	784 ± 31	NS
Phenylalanine	138 ± 5	126 ± 2	129 ± 4	134 ± 6	NS
Glycine	2985 ± 97	2890 ± 104	2856 ± 55	2919 ± 58	NS
Nonessential amino acids					
Ornithine	63 ± 2^{a}	56 ± 3^{a}	995 ± 215^{b}	2861 ± 402^{c}	< 0.0001
Proline	231 ± 7^{a}	231 ± 7^{a}	259 ± 11^{a}	322 ± 12^{b}	< 0.0001
Aspartate	2929 ± 57	2923 ± 14	2967 ± 45	3073 ± 43	NS
GABA	3768 ± 66^{a}	3703 ± 74^{ab}	3501 ± 53^{b}	3705 ± 65^{ab}	< 0.005
Asparagine	300 ± 6^{ab}	307 ± 7^{a}	292 ± 4^{ab}	279 ± 6^{b}	< 0.005
Tyrosine	195 ± 19	207 ± 18	194 ± 18	176 ± 18	NS
Glutamic acid	8200 ± 45	8129 ± 57	8078 ± 56	8093 ± 71	NS

Values are presented as mean concentration ± SEM (pmol/mg wet tissue); n = 7–10 per group. Different superscript letters indicate significant differences at $P < 0.05$. Abbreviations: GABA, γ-aminobutyric acid; NS, not significant.

a dose-dependent manner. Ornithine concentrations in all examined brain regions were also dose-dependently increased in the NPY plus ornithine groups compared with those of the control and NPY-alone groups. In contrast, ornithine levels were not altered by the central injection of NPY in all examined brain regions compared with those of the control group. Tran *et al*. (2016) reported that brain ornithine levels increased in a time-dependent manner following refeeding. However, in the present study, ornithine level in the brain was not altered when central NPY elicited robust feeding in the chicks. Turton *et al*. (1997) suggested that NPY may stimulate feeding by inhibiting inhibitory factors. One possibility is that NPY may inhibit satiety signaling via interacting with central ornithine to induce feeding. Conversely, a previous study in rats showed that intragastric injection of ornithine reduced body weight and food intake together with the activation of hypothalamic proopiomelanocortin (POMC) neurons (Konishi *et al*., 2015). Activation of POMC neurons was found to increase the production of melanocortins, which are anorexigenic neuropeptides present in both mammals (Parker and Bloom, 2012) and neonatal chicks (Cline and Furuse, 2012; Tran *et al*., 2019). The possible relationship between satiety signals from central ornithine and POMC neurons in chicks should be further elucidated. Accordingly, it is implied that the central feeding-related NPY pathway interacts either directly or indirectly with the ornithine mediation of food intake.

In the present study, the central orexigenic effect of NPY was accompanied by a reduction in the concentrations of several brain free amino acids, including AAA, tyrosine, phenylalanine, leucine, isoleucine, asparagine, and GABA, compared with those of the control group. Since alterations in brain amino acids were observed under feeding conditions, the impact of feeding itself was considered to play a potential role. Tran *et al*. (2016) examined the changes in brain amino acid concentrations influenced by regulated appetite when comparing the conditions of fasting and refeeding after fasting, with the findings supporting the robust increase in food intake by central NPY observed in the current study. Re-feeding after fasting increased the concentrations of brain ornithine, arginine, proline, and AAA in a time-dependent manner (Tran *et al*., 2016). However, the stimulation of feeding by central NPY was accompanied by a reduction in diencephalic AAA and asparagine, without any changes in the brain ornithine, arginine, and proline concentrations. Furthermore, changes in brain amino acids following central NPY administration were mainly observed as a reduction in concentration. This suggests that the alterations in brain amino acids following central administration of NPY were likely due to the effect of NPY.

Lysine and its metabolite AAA were differentially altered by central NPY. Two pathways are involved in lysine metabolism

Table 4. **Effects of intracerebroventricular injection of neuropeptide Y (NPY) and ornithine on plasma amino acid concentrations in neonatal chicks after 30 min of *ad libitum* feeding**

Amino acids	Control	NPY			P
		Ornithine (0 μmol)	Ornithine (2 μmol)	Ornithine (4 μmol)	
Essential amino acids					
Arginine	360 ± 19^{a}	286 ± 20^{b}	237 ± 22^{bc}	167 ± 10^{c}	< 0.0001
Lysine	1223 ± 83^{a}	1171 ± 143^{a}	1072 ± 87^{ab}	762 ± 98^{b}	< 0.05
Histidine	259 ± 12^{a}	228 ± 9^{ab}	198 ± 11^{b}	154 ± 12^{c}	< 0.0001
Leucine	672 ± 16^{a}	532 ± 33^{b}	472 ± 37^{b}	299 ± 26^{c}	< 0.0001
Isoleucine	329 ± 9^{a}	251 ± 17^{b}	220 ± 19^{b}	125 ± 14^{c}	< 0.0001
Valine	704 ± 11^{a}	627 ± 20^{b}	500 ± 15^{c}	397 ± 20^{d}	< 0.0001
Methionine	151 ± 9^{a}	116 ± 7^{b}	103 ± 11^{b}	51 ± 6^{c}	< 0.0001
Threonine	914 ± 35^{a}	871 ± 46^{a}	788 ± 34^{a}	609 ± 34^{b}	< 0.0001
Phenylalanine	285 ± 12^{a}	228 ± 9^{b}	193 ± 13^{b}	128 ± 12^{c}	< 0.0001
Glycine	392 ± 17^{a}	335 ± 8^{b}	274 ± 11^{c}	210 ± 10^{d}	< 0.0001
Nonessential amino acids					
Ornithine	161 ± 13^{a}	121 ± 12^{ab}	91 ± 10^{bc}	71 ± 6^{c}	< 0.0001
Serine	625 ± 31^{a}	576 ± 14^{ab}	508 ± 16^{bc}	433 ± 21^{c}	< 0.0001
Glutamine	1754 ± 63^{a}	1417 ± 60^{b}	1214 ± 59^{b}	945 ± 54^{c}	< 0.0001
Proline	937 ± 27^{a}	817 ± 33^{b}	683 ± 32^{c}	527 ± 25^{d}	< 0.0001
Cystathionine	93 ± 4^{a}	90 ± 4^{a}	88 ± 2^{ab}	75 ± 3^{b}	< 0.005
Asparagine	438 ± 9^{a}	359 ± 19^{b}	298 ± 16^{c}	214 ± 15^{d}	< 0.0001
Taurine	251 ± 22^{a}	226 ± 11^{ab}	213 ± 9^{ab}	176 ± 11^{b}	< 0.01
Alanine	1084 ± 41^{a}	857 ± 31^{b}	765 ± 36^{b}	589 ± 28^{c}	< 0.0001
Tyrosine	223 ± 10^{a}	178 ± 5^{b}	153 ± 10^{b}	98 ± 7^{c}	< 0.0001
Glutamic acid	115 ± 3^{a}	112 ± 3^{a}	105 ± 4^{ab}	94 ± 4^{b}	< 0.005
Hydroxyproline	171 ± 4^{a}	176 ± 12^{a}	150 ± 6^{ab}	125 ± 4^{b}	< 0.0005

Values are presented as mean concentration ± SEM (pmol/μl) n = 8–10 per group. Different superscript letters indicate significant differences at $P < 0.05$.

in animals. The pathway of lysine metabolism through the intermediate saccharopine is predominant in the liver but is not very active in the brain (Hutzler and Dancis, 1968). The second pathway is through L-pipecolic acid (L-PA), a major metabolic intermediate of lysine in mammals (Giacobini *et al*., 1980) and the chick brain (Nomura *et al*., 1978). The level of AAA, which is the end product of lysine metabolism, increased in the brain shortly after refeeding (Tran *et al*., 2016), suggesting that L-PA was quickly produced from lysine in the brain, as reported by Chang (1978). The present study showed that AAA concentrations were significantly reduced, whereas lysine levels were increased in the diencephalon and telencephalon of NPY-treated groups. This suggests that the metabolism of lysine into L-PA and AAA was restrained during the central exogenous administration of NPY. Moreover, our results suggest that the observed central alterations of lysine by NPY and NPY plus ornithine administration were due to a brain-specific mechanism, since these differed from those observed in the plasma. In addition, we found a significant reduction in the concentration of GABA in the brain following co-injection of NPY and ornithine. Acuna-Goycolea *et al*. (2005) reported that NPY inhibited the expression of glutamic acid decarboxylase 67 in the arcuate nucleus and reduced glutamatergic synaptic activity in GABA neurons via presynaptic inhibition. Accordingly, the reduction in GABA concentration in the NPY plus ornithine groups in the present study may be interpreted as inhibition of glutamic acid decarboxylase activity to in turn influence GABA biosynthesis. Depletion of GABA in the brain may inactivate its receptors for synaptic transmission. $GABA_A$ and $GABA_B$ receptors mediate the central effects of L-PA on the feeding behavior of chicks (Takagi *et al*., 2003a, b). Taken together, the central action of the L-PA pathway in the regulation of feeding behavior may be abolished in the presence of either NPY or NPY plus ornithine. The influence of exogenous ornithine on lysine metabolism in the brain found in the present study is unknown; however, the central pathways of ornithine and L-PA in the regulation of feeding behavior may be distinct from one another. Certainly, ornithine inhibits feeding behavior through additional mechanisms other than by acting on GABA receptors (Tran *et al*., 2016). The present results showed that the concentration of asparagine synthesized from aspartate by aspar-

agine synthetase was significantly reduced in the diencephalon of chicks treated with NPY compared with that of the control group; however, aspartate concentrations were not altered in any of the treatment groups. Erwan *et al*. (2013) reported that oral administration of aspartate increased aspartate concentrations in the telencephalon and diencephalon without any effect on food intake in neonatal chicks. Thus, it is possible that asparagine, but not aspartate, is involved in the orexigenic effects of NPY. A previous study indicated that the asparagine concentration was significantly decreased after 3 h of fasting in the diencephalon of chicks (Hamasu *et al*., 2009).

The identified alterations in free amino acid concentrations in the brain by co-injection of NPY and ornithine may provide several clues to reveal the interaction between NPY and ornithine in the control of food intake. To elucidate how central ornithine is involved in the effect of NPY, brain amino acid concentrations were compared between chicks co-injected with NPY and ornithine and chicks injected with either saline or NPY alone. The first point to highlight is that the dose-dependent elevated concentrations of ornithine were accompanied by an increase in proline levels in all examined brain regions in the NPY plus ornithine groups. This was not caused by changes in plasma proline levels, since NPY, with or without ornithine, induced a substantial reduction of proline levels in the plasma. Furthermore, proline does not readily cross the blood–brain barrier (Davis *et al*., 1979). Based on these facts, our results suggest that NPY may enhance the activity of ornithine aminotransferase and pyrroline-5-carboxylate reductase in the brain, as both enzymes contribute to the production of proline from ornithine. Proline has been shown to decrease food intake in neonatal chicks (Haraguchi *et al*., 2007). Accordingly, the suppression of NPY-elicited feeding by central ornithine in the current study may be partly ascribed to the elevation in brain proline levels. However, the results also indicated that the increase in proline concentration following a dose-dependent increase in brain ornithine was not dose-dependent. Therefore, exogenous ornithine is likely to be further metabolized to other metabolites.

In addition to proline, ornithine is converted into glutamate by ornithine α-ketoglutarate aminotransferase. Although changes in brain glutamate concentrations were not observed in the NPY plus ornithine groups, a significant reduction in the diencephalic glycine concentration was detected in the NPY plus ornithine groups along with a reduction in methionine concentrations in all examined brain regions of chicks treated with NPY plus high-dose ornithine compared with those of the control or NPY-alone groups. Methionine is likely converted to L-cystathione via a condensation reaction with serine; L-cystathione is subsequently degraded to homoserine and cysteine by hydrolysis. Cysteine is a rate-limiting precursor of glutathione synthesis in neurons (Meister and Anderson, 1983). Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine. Dringen and Hamprecht (1996) observed that the intracellular content of glutathione in astroglia-rich primary cultures derived from neonatal rat brains was quickly resynthesized following re-feeding with its constitutive amino acids, including glutamate, cysteine, and glycine, after 24-h incubation in minimal medium lacking amino acids and glucose. Accordingly, it is possible that the robust elevation of ornithine concentrations in the NPY plus ornithine groups observed in the present study may have enhanced the concentration of glutamate in the brain, thereby stimulating the production of glutathione. Although glutathione is well known to serve as a major protectant against oxidative stress in the brain (Cooper and Kristal, 1997), it also acts as a neurotransmitter and neuromodulator (Janáky *et al*., 1999). Paterson *et al*. (2001) observed suppression of food intake, accompanied by weight loss and a reduction of glutathione in the neocortex and thalamus of rats that were deficient in sulfur-containing amino acids such as methionine. In chicks, ICV injection of glutathione was reported to induce sleep-like behavior in a dose-dependent manner under acute stressful conditions (Yamane *et al*., 2007a). More importantly, Yamane *et al*. (2007b) reported that glutathione also dose-dependently suppressed food intake. According to Dringen and Hamprecht (1996), ornithine and other amino acids, including aspartate, asparagine, and proline, can serve as precursors for the glutamate moiety of astroglial glutathione in rats. Taken together, these findings suggest that glutathione may be a putative candidate that contributes to the suppression of NPY-elicited feeding by central ornithine. However, the relationship between endogenous ornithine and glutathione remains obscure, and the determination of glutathione concentration following exogenous ornithine administration in the chick brain may help to better elucidate the related mechanism.

On the other hand, co-injection of NPY and ornithine at different doses significantly decreased the tryptophan concentration in the diencephalon compared with that of the control and NPY-alone groups. Tryptophan is a nutritionally essential amino acid that functions as a precursor of several compounds via two major pathways: serotonin (5-HT) and kynurenine (Furuse, 2015). Melatonin and 5-HT are products of the serotonin pathway, whereas kynurenic acid (KYNA) is generated from L-kynurenine. It is postulated that the reduction in tryptophan concentration in the NPY plus ornithine group may be due to tryptophan catabolism. Bungo *et al*. (2008) reported that central injection of tryptophan reduced the food intake of neonatal layer chicks over 30 min of feeding, which is the same experimental time period employed in the current study. This previous study further demonstrated that hypophagia induced by central tryptophan is involved in the serotonergic system. Central 5-HT has been confirmed to induce anorexia in chicks (Denbow *et al*., 1982, 1986; Sashihara *et al*., 2002). The synthesis of 5-HT depends on the availability of its precursor tryptophan in the brain (Schaechter and Wurtman, 1990). However, our preliminary results indicated that monoamine concentrations were not affected by NPY and ornithine. Therefore, it is premature to ascribe the suppression of the orexigenic effect of NPY by ornithine to the metabolism of tryptophan in the serotonin pathway. According to Maddison and Giorgini (2015), more than 95% of tryptophan is metabolized via the KYNA pathway. Furthermore, KYNA appears to

have a stronger sedative effect than tryptophan under conditions of social isolation stress (Yoshida *et al*., 2012). Thus, a relationship between ornithine and tryptophan metabolism in KYNA has been postulated. Two pathways, the metabolism of tryptophan to kynurenine and the metabolism of ornithine to polyamines, were shown to potentiate each other via the transcription factor aryl hydrocarbon receptor in mouse brain cells (Rothhammer *et al*., 2018). The decarboxylation of ornithine is the first step in the biosynthesis of polyamines. Mondanelli *et al*. (2017) found that the polyamine spermidine increased kynurenine production. The central effects of KYNA in neonatal chicks include decreasing active wakefulness and increasing sleeping posture (Yoshida *et al*., 2013), which may partly occur in conjunction with the central effects of ornithine. Taken together, these findings suggest that the catabolism of tryptophan may potentially contribute to the interaction between NPY and ornithine in the regulation of feeding behavior in neonatal chicks. However, the roles of each tryptophan metabolic pathway need to be further clarified.

In the present study, the concentrations of amino acids in the plasma exhibited marked changes after a short period of feeding following central injection of NPY and ornithine in neonatal chicks. Circulating levels of amino acids are relatively constant during the adult period, whereas plasma levels of amino acids during the neonatal period are characterized by dynamic changes under catabolic conditions (Wu, 2009). Therefore, these results support the notion that dynamic changes in amino acids in the plasma reflect the important roles of nutrients in the growth and development of animals during the neonatal period (Wu, 2009). The concentrations of several brain amino acids showed a good correlation with their changes in the plasma, including leucine, isoleucine, tyrosine, and phenylalanine, whereas others, including proline, lysine, ornithine, methionine, and asparagine, showed different patterns of change between the brain and the peripheral circulation. Notably, central administration of NPY significantly decreased the levels of many proteinogenic amino acids, including proline, asparagine, methionine, leucine, isoleucine, tyrosine, phenylalanine, glutamine, alanine, arginine, valine, and glycine, compared with those of the control group. Moreover, NPY did not affect the plasma concentrations of non-proteinogenic amino acids such as taurine, cystathionine, and ornithine. It has been suggested that central NPY stimulates the accumulation of amino acids required for protein synthesis. The reduction in the concentrations of almost all detected amino acids was greater in chicks co-injected with NPY and ornithine when food intake was reduced in a dose-dependent manner. This is likely due to the accumulation of amino acids for protein synthesis stimulated by central NPY and the reduction in protein supply to break down into amino acids. The declining pattern of almost all altered amino acids in the plasma was dose-dependent, with a marked reduction observed in chicks treated with NPY plus high-dose ornithine (4 μmol). The suppression in the concentrations of several non-proteinogenic amino acids, including taurine, cystathionine, and ornithine, in the NPY plus ornithine groups may be caused by the reduction in the concentrations of their precursors such as cysteine and arginine.

Evidence supports the hypothesis that central NPY stimulates protein synthesis. Tachibana *et al*. (2006) reported that ICV injection of NPY significantly reduced plasma glucose and triacylglycerol concentrations, but increased non-esterified fatty acid concentrations under conditions of food deprivation. Consequently, this study further demonstrated that central NPY altered the utilization of metabolic fuels from carbohydrates to lipids/proteins. Therefore, the reduction in plasma amino acid concentrations observed in all NPY-treated groups in the present study was likely due to the accumulation of protein synthesis rather than to the gluconeogenic phenomenon observed in chicks during starvation (Maruyama *et al*., 1976). White *et al*. (1994) reported that low-protein diets increased NPY gene expression in the basomedial hypothalamus of rats, whereas carbohydrate or fat restriction did not influence NPY gene expression. Therefore, hypothalamic NPY may be a signal of protein homeostasis.

In summary, the present study showed that the co-injection of ornithine attenuated the orexigenic effects of NPY in a dose-dependent manner. Our results imply that there may be an interaction between the central regulation of food intake by NPY and acute satiety signals such as ornithine in the brain through several postulated metabolic pathways in the brain. Changes in plasma amino acid concentrations following central administration of NPY suggest the anabolic effect of NPY on the peripheral stimulation of protein synthesis.

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Author contributions

PVT and MF designed this study. PVT, MZE, YT, YW, GH, and VSC performed the experiments. PVT and MF wrote the paper. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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