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Effect of Stereotaxic Surgery of the Third Ventricle on Growth Performance in Neonatal Chicks

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Feeding behavior and energy metabolism are precisely regulated by humoral and/or neural factors in the central nervous system. In particular, nuclei, such as the arcuate nucleus, ventromedial hypothalamic nucleus, and lateral hypothalamic area located near the third ventricle of the hypothalamus are the centers of feeding and energy metabolism in various vertebrate species, including chickens. In this study, we evaluated the effects of cannulation of the third ventricle on chick growth and feeding behavior in the neonatal stage, to develop a method for local and chronic central nervous system-mediated energy metabolism. Referring to the chick brain atlas, a guide cannula was inserted into the third ventricle of the chick under anesthesia immediately after hatching using a stereotaxic instrument. The chicks that recovered from anesthesia were bred for 11 days under normal feeding management conditions, and then feed intake amount, body weight gain, and metabolic tissue weight were measured. The effects of direct stimulation of the third ventricle with 2-deoxy-D-glucose on the expression level of the immediate-early gene, *cFOS*, and feed intake in 5-day-old chicks were also evaluated. There were no differences in feed intake, body weight gain, and metabolic tissue weight between 11-day-old cannulated and control chicks. The expression of *cFOS* mRNA in the ventromedial hypothalamic nucleus was higher than that in the amygdala after the third ventricular administration of 2-deoxy-D-glucose. Additionally, direct third ventricular injection of 2-deoxy-D-glucose attenuated the feeding behavior of chicks for a while. Overall, we speculate that the technique is effective for local and/or chronic stimulation of the nucleus near the third ventricle of the chick hypothalamus, which is important for feed and energy metabolism regulation.

Key words: energy metabolism, growth performance, neonatal chick, stereotaxic surgery, third ventricle

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Introduction

Feeding behavior and energy metabolism regulate animal growth. It is well known that these metabolic regulations in animals are maintained in an optimal state via transmission of humoral and neural signals to the central nervous system (CNS), which processes and integrates the metabolic information (Cone, 2005; Wynne *et al*., 2005; Lam, 2010; Sánchez-Lasheras *et al*., 2010). Various nutrients, hormones, and neurotransmitters act on the nucleus of each brain region, regulating feeding behavior, metabolism, reproductive behavior, and migratory behavior in avian species (Denbow, 1999; Boswell, 2005; Scanes, 2009). Similar to mammals, it has been recognized that the ventromedial hypothalamic nucleus (VMH) is the satiety center and the lateral hypothalamic area (LH) is the feeding center in chickens (Kuenzel, 1994). In these regions, inhibition of the neuronal activities by electrophysiological techniques causes hypothalamic dysfunction, resulting in overeating or anorexia (Sonoda and Kojima, 1984). Hence, the bidirectional regulation of energy consumption varies depending on the nucleus in the hypothalamus, and the analysis of local information regulation mechanisms is important. Such analysis methods can help understand the local and chronic actions of the stimulating nuclei, but these methods require the insertion of a cannula into a chicken under anesthesia using a brain stereotaxic device. Therefore, only chickens grown to a certain degree in which these treatments can be easily performed are often used.

In poultry production, it is important to analyze nutritional

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and metabolic regulation of weight gain between the embryonic and neonatal stages, as weight gain at 1 week after hatching has been shown to correlate with productivity at market age (Gonzales *et al*., 2003). Hence, the CNS network analysis of metabolic control mechanisms in neonatal chicks is also important because the mechanisms significantly change depending on the main energy source (i.e., a shift from lipid to carbohydrate metabolism) between the embryogenesis and neonatal stages. Therefore, the importance of nutritional–physiological brain function analysis during this period, in particular, glucose and insulin interactions, has been indicated (Tokushima *et al*., 2003). To analyze feeding behavior and metabolic regulation during the neonatal period, the method of Davis *et al*. (1979), which is a single administration into the lateral ventricle (i.e., near the telencephalon), is often applied. Analysis using the third ventricular guide cannula at this critical period can enable the analysis of the regulation of the local and/or chronic metabolic network system through the chick hypothalamus. However, there are only a few reports regarding local analysis of feeding behavior and energy metabolism using cannulation with surgery in neonatal chicks.

Therefore, in this study, to examine the application of guide cannula manipulation in the analysis of neonatal feeding behavior and energy metabolism, we investigated the effects of 1) stereotaxic surgery in the third ventricle on the growth performance in neonatal chicks and 2) direct stimulation of the third ventricle on hypothalamic neuronal activity and short-term feeding behavior in neonatal chicks.

Materials and methods

Animals

Fertilized eggs of layers (Momiji) were obtained from a local hatchery (Goto Hatchery Inc., Gifu, Japan) and incubated at a temperature of 37.8℃ and relative humidity of 60% for hatching. The hatched male chicks were reared in electronically heated group housing cages (60 cm×40 cm×30 cm; floor space: 0.24 m^2) or individual cages (25 cm×10 cm×30 cm; floor space, 0.025 m^2) with free access to a commercial standard diet (JA Higashinihon Kumiai Shiryou Co., Ltd., Gunma, Japan; ME 2.9 Mcal/kg, CP 22%) and water until the end of the experiment.

The chicks were divided into the following groups: the test group, which was subjected to cannulation, and the control group, which was subjected to anesthesia treatment (anesthesia group), and no treatment (intact group). All experiments were conducted in accordance with the regulation of the Animal Experiment Committee of Nippon Veterinary Life Science University (No. 27K-35).

Stereotaxic Surgery in Neonatal Chicks

The newly hatched chicks were anesthetized via intraperitoneal administration of tribromoethanol (Nacalai, Kyoto, Japan) solution (2-2-2-tribromoethanal, 160 mg/kg body weight [BW] and 2-methyl-2-2-butanol, 96 mg/kg BW). After sufficiently confirming the effect of anesthesia, the chicks were fixed on a brain stereotaxic apparatus (Model 900; David Kopf Instruments, CA, USA). Thereafter, the scalp was incised, a hole was made in the skull using a drill (Urawa Kogyo Co., Ltd., Saitama, Japan), and a guide cannula (Bio Research Center, Aichi, Japan) was inserted form the reference plane (X axis, mediolateral; Y axis, anteroposterior; and Z axis, height; Fig. 1A) with reference to a chick brain atlas (Pulles *et al*., 2007). The inserted guide cannula was fixed with dental cement (Matukaze, Kyoto, Japan). In this experiment, chicks subjected to anesthesia were used as the control. After confirming that the inserted guide cannula was firmly fixed, the chicks were returned to a brooder maintained at 34℃ and allowed to recover from anesthesia.

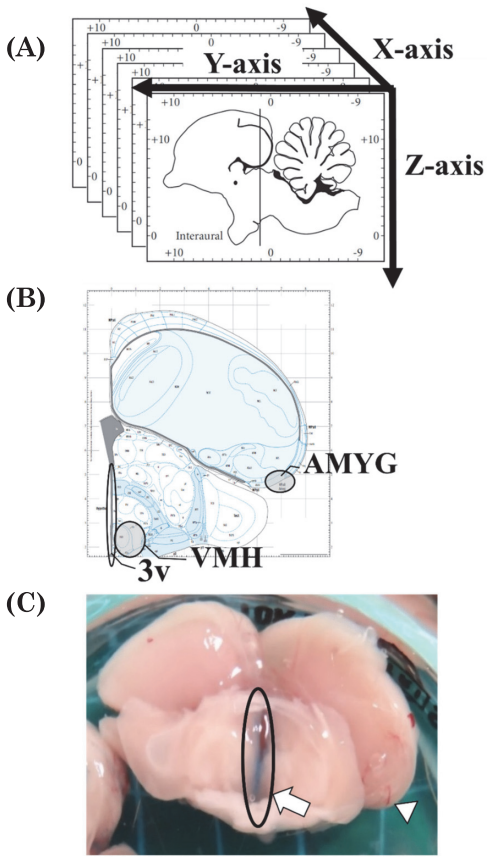

Fig. 1. **Insertion site of a guide cannula into the third ventricle was based on the stereotaxic planes that Puelles *et al*. (2007) reported.** (A) A schematic diagram of the 3D coordinates of the target: X axis, mediolateral; Y axis, anteroposterior; Z axis, height. (B) The coronal brain atlas represents VMH, arcuate hypothalamic nucleus, including the ventromedial hypothalamic nucleus; AMYG, amygdala; and the 3v, third ventricle. (C) A photograph of a coronal brain slice. Circled area (staining blue) represents the injection site of the stain solution. Arrows (VMH) and arrowhead (AMYG) indicate sample collection areas.

Growth Performance of Neonatal Chicks After the Stereotaxic Surgery

The chicks that recovered from anesthesia were maintained at 31.3℃±0.6℃, with 24 h lighting, and free access to a commercial starter diet and water during the experimen-

tal period. For 3 days after hatching, the chicks were raised in experimental group cages, and then moved to single cages at 4 days of age and acclimatized. Feed efficiency was also calculated from the measured BW and feed intake of chicks at 5–11 days of age. At the end of day 11, 10 μL of saline containing 0.1% Evans blue dye (Sigma, MO, USA) was administered to the chicks via the guide cannula using a microsyringe equipped with an inter cannula. Thereafter, the chicks were decapitated, and the whole brain, liver, heart, pancreas, pectoral muscle, and leg muscle were collected and weighed. The collected whole brain was sliced into 1 mm thick sections using a brain matrix (Brain Science Idea Co., Ltd., Tokyo, Japan), and the site where the guide cannula was attached was identified.

Brain Microinjection and Neuronal Activity Marker Gene Expression

Guide cannulation was performed, and growth performance was determined in neonatal chicks after stereotaxic surgery. To 5-day-old chicks, 10 μL of 2-deoxy-D-glucose (2-DG) (Sigma) solution (50 mM 2-DG/0.85% saline) was administered using a microsyringe equipped with an internal cannula. The dose of 2-DG applied here was used according to the report of Saito *et al*. (2011). The chicks were decapitated at 30 min after injection and the whole brain was collected. The collected whole brain was sliced into 1 mm thick sections using a brain matrix. After confirming Evans blue dye staining around the third ventricle, the VMH and amygdala (AMYG) were excised (Fig. 1B), snap frozen in liquid nitrogen, and stored at −80℃ until further analysis. From the collected VMH and AMYG, total RNA was extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan). Briefly, tissue samples were homogenized using BioMasher II (Nippi, Tokyo, Japan) and total RNA was extracted according to the manufacturer's protocol. The absorbance of the total RNA solution was measured using a nanodrop (Thermo Fisher Scientific, MA, USA), and then the concentration of RNA was calculated, and the purity was confirmed. Thereafter, total RNA (700 ng) was subjected to DNase treatment using the DNase treatment kit (Invitrogen, CA, USA) and cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan).

Quantitative Polymerase Chain Reaction

The synthesized cDNA was subjected to quantitative polymerase chain reaction (PCR) analysis on a Real-time PCR System 7500 (Applied Biosystems, CA, USA). Briefly, following denaturation at 95℃ for 30 s, the PCR was carried out with 20 μL of reaction mixture containing 1×SYBR Premix EX Taq (Takara Bio Inc., Shiga, Japan) and 0.4 μM of each primer under the following condition: 95℃ for 5 s and 60℃ for 34 s. Ribosomal protein S17 (*RPS17*) was used as the endogenous control. To normalize the data, ΔC_T was calculated for each sample by subtracting the C_T of *RPS17* from the C_T of the gene of interest. The specific primer sequences used to amplify each target were as follow: *cFOS*, 5′-GGGGACAGCCTCACCTACTA-3′ (sense primer) and 5′-GTCGGGACTGGTGGAGATG-3′ (antisense primer); *RPS17*, 5′-AAGCTGCAGGAGGAGGAGAGG-3′ (sense primer) and 5′-GGTTGGACAGGCTGCCGAAGT-3′ (antisense primer). For relative quantification, ΔC_T of the control group was subtracted from the ΔC_T of each experimental sample to generate $\Delta\Delta C_T$. The $\Delta\Delta C_T$ was then used to calculate the approximate fold difference, $2^{-\Delta\Delta CT}$. The results are expressed as the gene of interest mRNA/*RPS17* mRNA ratio.

Brain Microinjection and Feeding Behavior

At 5 days of age, after being deprived of feed for 3 h (to intensify hunger), 2-DG solution or saline solution of the same concentration used in the above gene expression analysis was administered to the chicks via the guide cannula placed in the third ventricle. The amount of feed intake for 60 min after third ventricular micro injection was measured by monitoring the reduction in the pre-weighed amount of feed. At the end of the experiment, Evans blue staining solution was injected into the chicks. The chicks were decapitated, and the site of guide cannula insertion was confirmed by dye staining. The chicks with no Evans blue dye in the third ventricle were excluded from the analysis.

Statistical Analysis

Growth performance, neuronal activity marker expression, and feeding behavior of neonatal chicks with guide cannula were analyzed using the Tukey–Kramer test. All analyses were performed using the commercially available package JMP Version11 (SAS Institute, NC, USA). The results are represented as mean±standard error of the mean (SEM). Differences were considered significant when the P value was less than 0.05.

Results

Neonatal Growth Performance After Stereotaxic Surgery

Evans blue staining solution was injected into chicks via a guide cannula to check the guide cannula attachment area. When the guide was attached in the direction of 0 mm along the X axis, 1.6 mm along the Y axis, and 10 mm along the Z axis, clear staining was observed around the chick hypothalamic third ventricle (Fig. 1). We confirmed that chicks under tribromoethanol anesthesia and with guide cannula attached recovered after one day, and there were no obvious abnormalities in behavior, such as walking, resting, eating, and drinking. Thereafter, each chick was raised on a commercial starter diet under similar feeding conditions. There was no difference in BW gain 5 days after hatching in each treated chick (Table 1). Additionally, there was no difference in BW gain, feed intake, and feed efficiency of chicks from 6 to 11 days after hatching (Table 1). Furthermore, there were no significant differences in the whole brain, heart, liver, pectoral muscle, pancreas, or leg muscle weights collected at the end of the study (Table 2).

Immediate-early Gene Expression and Feeding Behavior After Local Micro Injection of 2-DG

In chicks injected with a 2-DG solution, the expression of VMH *cFos* mRNA was higher than that of intact chicks, but there was no difference in AMYG *cFos* mRNA expression (Fig. 2). When 2-DG solution was administered to the third ventricle of chicks at 5 days of age, the amount of feed intake

Table 1. **Body weight gain, feed intake, and feed efficiency of chicks with a guide cannula in the third ventricle during 11 days after hatching**

	Intact (5)	Tribromoethanol (5)	Cannula (5)	P value
Body weight gain (P1–P5) (g)	18.96±1.52	17.9±1.73	15.84±1.67	0.421
Body weight gain (P6–P11) (g)	45.32±5.15	40.68±5.08	49.34±2.26	0.500
Feed intake (P6–P11) (g)	75.06±5.50	69.54±6.72	80.02±2.48	0.403
Feed efficiency (P6–P11)	0.60±0.03	0.59±0.03	0.62±0.01	0.727

The values represent the mean±SEM; $n=5$ chicks. P1–P5, 1 to 5 days after hatching; P6–P11, 6 to 11 days after hatching.

Table 2. **Tissue weight in 11-day-old chicks with a guide cannula in the third ventricle**

	Intact (5)	Tribromoethanol (5)	Cannula (5)	P value
Whole brain (g)	1.40±0.03	1.39±0.04	1.42±0.02	0.707
Heart (g)	0.76±0.06	0.82±0.10	0.94±0.03	0.248
Liver (g)	3.69±0.25	3.40±0.38	3.64±0.18	0.746
Pectoral muscle (g)	3.27±0.16	3.26±0.26	3.27±0.21	0.999
Leg muscle (g)	0.47±0.04	0.45±0.04	0.51±0.03	0.930
Pancreas (g)	6.01±0.36	6.06±0.39	6.21±0.39	0.540

The values represent the mean±SEM; $n=5$ chicks.

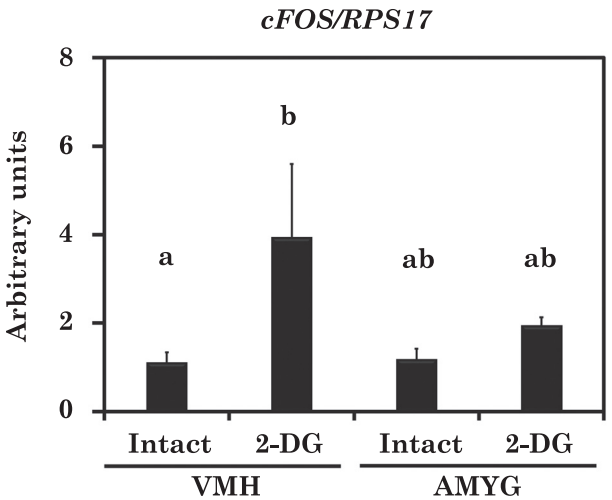

Fig. 2. **Levels of *cFos* mRNA expression in VMH, arcuate hypothalamic nucleus, including the ventromedial hypothalamus; and AMYG, amygdala after 2-DG, 2-deoxy-D-glucose administration to the 3v, third ventricle-cannulated chicks.** The values are the mean±SEM. The number of chicks used: intact, 5; cannulated, 3. The means with different letters are significantly different at $P<0.05$.

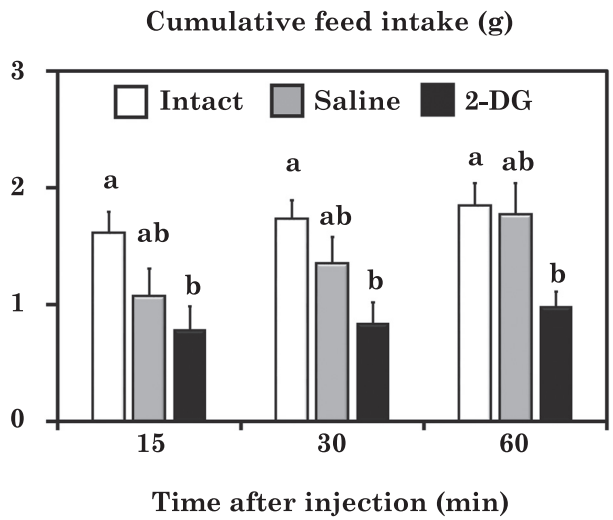

Fig. 3. **Cumulative feed intake of fasted chicks. 3v, third ventricle, injected with saline or 2-DG, 2-deoxy-D-glucose.** The values are the mean±SEM. The number of chicks used: intact, 5; saline, 5; 2-DG, 4. The means with different letters are significantly different at $P<0.05$.

after 15 min was lower in both the saline- and 2-DG-administered groups than in the intact group. However, the difference in the amount of feed consumed in the saline group after 30 min gradually became nonsignificant from that in the intact group, whereas the amount of feed consumed in the 2-DG treatment group was low throughout the experimental period (Fig. 3).

Discussion

In this study, we investigated the effects of direct cannulation of the third ventricle using a stereotaxic surgery on growth performance and feeding behavior in neonatal chicks. According to the chick brain atlas (Pulles *et al*., 2007), a guide cannula was attached to the third ventricle of anesthetized chicks. The chicks that recovered from anesthesia and in which the guide cannula was attached did not exhibit

abnormal behaviors such as difficulty walking. The volume of anesthetics has been recommended for various experimental animals depending on body size, age, and surgical procedure, and pentobarbital and tribromoethanol are commonly used anesthetics for stereotaxic surgeries (Papaioannou and Fox, 1993; Cooley and Vanderwolf, 2005). When tribromoethanol was used as an anesthetic, serious adverse effects, such as peritoneal adhesion and inflammation in the peritoneal cavity have been reported to be caused by improper storage or additional dosage of the anesthetic (Meyer and Fish, 2005; Hill *et al*., 2013). Therefore, the concentration of tribromoethanol solution (2-2-2-tribromoethanal, 160 mg/kg BW and 2-methyl-2-2-butanol, 96 mg/kg BW) used in this study was adjusted just before the day of surgery, and a single dose was administered intraperitoneally. Immediately after administration (~15 min), the effects of anesthesia, such as muscle relaxation were confirmed. The chicks recovered from the effects of anesthesia without any particular problems; additionally, abnormal behavior in postoperative chicks could not be confirmed.

With reference to the brain atlas of Pulles *et al*. (2007), if a guide cannula is placed in the direction of 0 mm along the X axis, 1.6 mm along the Y axis, and 10 mm along the Z axis from the reference plane, the tissue around the third ventricle was stained with Evans blue dye (Fig. 1C). Furthermore, the fact that clear Evans blue staining could not be confirmed in other ventricles, such as the lateral ventricle suggests that the stereotaxic surgery performed in this study could directly control the activity of neuronal signaling around the third ventricle of the chick hypothalamus. The intraventricular administration method devised by Davis *et al*. (1979) is frequently used to analyze feeding behavior and metabolism regulation in neonatal chicks. Therefore, it is apparent that various neuropeptides, neurotransmitters, and nutrients act on feeding behavior of chicks in a stimulatory or inhibitory manner, and a unique feeding regulation mechanism in the neonatal stages has been clarified (Furuse 2002, 2007; Furuse *et al*., 2007; Bungo *et al*., 2011; Tachibana and Tsutsui, 2016). In addition to the method of lateral ventricular administration reported by Davis *et al*. (1979), the fact that Evans blue dye stained the region only around the third ventricle in this study implied the possibility of application of this method for the direct analysis of hypothalamic feeding behavior and energy metabolism in neonatal chicks. In the present study, because it was considered necessary to examine the effects of anesthesia by cannulation and chronic indwelling on the initial growth of chicks thereafter, the cannula-implanted chicks were reared under general feeding conditions until 11 days after hatching. There was no difference in weight gain, feed intake, and tissue weight among any group until the end of the study. Although analysis in adult chickens using a cannula has been conducted with a recovery period of approximately one week after surgery (Marmo *et al*., 1978; Denbow *et al*., 1983), and the results of this study showed that the analysis of feeding behavior after cannulation to the third ventricle could be conducted for at least 5 days after surgery.

In this study, we demonstrated that it is possible to activate the nucleus around the third ventricle of the chick through guide cannulation. 2-DG administration to the third ventricle of chicks significantly increased *cFOS* mRNA expression in the VMH 30 min after administration compared with that of the control group, but not in the AMYG. The AMYG is located near the lateral ventricle and is a nerve region that responds to emotions, such as anxiety and fear (Montag-Sallaz *et al*., 1999). The results of this trial suggested that the stimulation of 2-DG via guide cannula was specific to the third ventricle, even if glucose analogues of major nutrients in the CNS were administered. Neurons in the CNS are stimulated and activated by sensory and behavioral stimuli, and the *cFos* and *Arc/Arg3.1* gene or protein expression levels are often used as markers when activated (Montag-Sallaz *et al*., 1999). We also examined the gene expression level of *cFos* as an indicator of neuronal activation, because analysis of *cFos* mRNA and/or protein expression as an index has been used in studies on avian feeding behavior and energy metabolism (Thayananuphat *et al*., 2007; McConn *et al*., 2014; Nagarajan *et al*., 2014). Based on these facts, guide cannulation of the third ventricle employed in this study might be a direct or first stimulation of the neurons localized in the VMH located nearby.

Finally, in the present study, when 2-DG was administered via a chick guide cannula at a concentration that affected *cFos* gene expression, the subsequent chick feed intake was significantly lower than that in the saline group until 60 min after injection (Fig. 3). 2-DG, a non-metabolic sugar analogue, is rapidly taken up into cells via the glucose transporter and is phosphorylated to 2-DG6 phosphate by hexokinase, but the reaction in the Krebs cycle and electron transport system does not proceed. It has been shown in various animal species that the administration of 2-DG into the brain reduces the intracellular glucose utilization rate and induces feeding behavior (Smith and Epstein, 1969; Sakata *et al*., 1987). Considering glucose sensitivity in hypothalamic feeding regulation and energy metabolism, there are two glucose-responsive neurons that are activated or inhibited by the concentration of glucose. It is known that the neurons inhibited by glucose are glucose-sensitive neurons and are localized at high frequency in the LH, and the neurons activated by glucose are present in the VMH as glucose-receptive neurons (Oomura and Yoshimatsu, 1984).

Chickens are considered hyperglycemic animals with a blood glucose level more than twice that of mammals, and the weak mechanism of insulin-responsive glucose uptake compared with that in mammals is one of the reasons for the characteristic (Simon, 1989). In addition, Denbow *et al*. (1982) have also suggested the existence of chicken-specific hypothalamic glucose sensitivity because the administration of 2-DG to the lateral ventricle of 5–9-week-old male broilers under 24 h fasting conditions did not affect feed intake. The difference from previous findings suggests that cellular localization of glucose receptors in the hypothalamic nucleus differs depending on the type of chicken or the stage of growth. Hence, some hypotheses were made about the

feeding suppression effect of 60 min of 2-DG administration into the third ventricle of layer chicks. Firstly, it might presumably be because the initial reaction of 2-DG by direct injection into the third ventricle may have been observed. Similar to mammals, the chicken VMH is one of the satiety centers because dysregulation of the VMH displayed hyperphagia (Sonoda, 1983). Hence, it was also thought to be the result of initial activation of the satiety center, but not the feeding center. Secondly, there is a possibility of influence by the timing of 2-DG administration. In mice, the response to 2-DG has been reported to show a biphasic response with increased and suppressed feeding depending on the administration time (Larue-Achagiotis and Le Magnen, 1979). Lastly, there is a need to consider 2-DG dosage. The brain constantly monitors glucose concentration, and when there is a high concentration of glucose, such as that immediately after a meal, it leads to the activation of the firing of the pro-opiomelanocortin (POMC) neuron and anorexigenic neuron located near the third ventricle (Claret *et al*., 2007). Claret *et al*. (2007) also reported that these anorexigenic effects induced by the activation of POMC are promoted via the first enzyme in glycolysis, hexokinase. Therefore, the feeding behavior of chicks might have changed when lower concentrations of 2-DG were administered. In this study, 2-DG was administered to investigate the activation of the nucleus of the hypothalamus, but it is suggested that these differences in reactivity need to be studied focusing on chicken type, age, and nutritional conditions.

Stereotaxic surgeries are essential for chronic *in vivo* treatment, such as morpholino-oligo analysis (Wellman *et al*., 2015) and optogenetic analysis (Coutinho *et al*., 2017). This means that they can be applied to functional analysis with arbitrarily controlled gene expression and protein expression in the chick hypothalamus. In other words, clarifying the mechanism of interaction between initial chicken growth and neuronal development, in which brain function is evaluated, can contribute to improve productivity via promotion of the chick brain functions.

Taken together, the present study results showed that (1) properly treated chicks with a guide cannula in the third ventricle could grow without any abnormal behavior and (2) the nucleus near the third ventricle could be specifically stimulated. The technique might be useful for local and/or chronic analyses of brain function in feeding behavior and metabolism regulation in neonatal chicks.

Conflicts of Interest

The authors declare that there are no conflicts of interest that would prejudice the impartiality of this research.

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