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# Embryonic development of GABAergic terminals in the mouse hypothalamic nuclei involved in feeding behavior

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ABSTRACT

The inhibitory neurotransmitter gamma-amino butyric acid (GABA) plays important roles in energy balance and feeding behavior in the hypothalamus. To reveal the time course of GABAergic network formation, we examined the immunohistochemical localization of glutamic acid decarboxylase (GAD), a GABAergic neuron marker, vesicular GABA transporter (VGAT), a marker of inhibitory terminals, and $K^+$-$Cl^-$-cotransporter2 (KCC2), which shifts GABA action from excitation to inhibition, in the developing mouse hypothalamus. GABAergic terminals, seen as GAD- and VGAT-positive dots, increased in density during embryonic development. Moreover, the onset of KCC2 localization was almost concomitant with GABAergic terminal formation, and KCC2-positive profiles increased in density during development. This suggested that after the formation of GABAergic terminals, GABAergic action may change to inhibition in the hypothalamus. This maturation appears to proceed as follows: the lateral hypothalamus (LH) matures first, followed by the paraventricular nucleus (PVN) by the time of birth, while the ventromedial hypothalamus (VMH) and the arcuate nucleus (Arc) are not fully mature at the time of birth. Our findings suggest that GABAergic networks in the "feeding center" (LH) and the "exit" (PVN) may mature before birth, while those in the "satiety center" (VMH) and "higher control center" (Arc) may mature after birth.


## 1. Introduction

According to the World Health Organization, more than 10% of adults worldwide were obese in 2008, and obesity is increasing at a rapid pace (http://www.who.int/topics/obesity/en/10th Mar 2015). Obesity and excess weight are one of the biggest health issues of the 21 st century in developed countries, because they are associated with a high risk of many diseases, such as type 2 diabetes, high blood pressure, cardiac and brain infarction, and lipidosis (Freedman, 2011; Hossain et al., 2007).

To prevent a pandemic of obesity, it is necessary to clarify the mechanisms regulating food intake behavior. Generally, peripheral information on metabolic states is sent to the central nervous system (CNS), and then integrated into appetite-related nuclei for the regulation of energy balance and feeding behavior (Arora and Anubhuti, 2006; Williams and Elmquist, 2012; Yeo and Heisler, 2012). In particular, nuclei and neural circuits in the hypothalamus are considered to be the control center of energy expenditure and food intake behavior, because the hypothalamus integrates sensory information, and regulates the autonomic nervous system and hormone secretion (Gao and Horvath, 2008; Valassi et al., 2008; Yeo and Heisler, 2012). The four appetite-related nuclei in the hypothalamus, the arcuate nucleus (Arc), lateral hypothalamus (LH), paraventricular nucleus (PVN), and ventromedial hypothalamus (VMH), receive circulating peripheral signals, such as insulin, leptin, and ghrelin, and construct neural circuits within the hypothalamus (Arora and Anubhuti, 2006; Gao and Horvath, 2008; Meister, 2007; Williams and Elmquist, 2011, 2012; Yeo and Heisler, 2012). The VMH sends peripheral signals to

*Abbreviations:* III, third ventricle; AgRP, agouti-related protein; Arc, arcuate nucleus; CART, cocaine and amphetamine regulated transcript; $Cl^-$, chloride ion; $[Cl^-]_i$, intracellular chloride ion concentration; CNS, central nervous system; E, embryonic day; fx, fornix; GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; kDa, kilodalton; DMH, dorsal medial hypothalamus; KCC2, $K^+$-$Cl^-$ co-transporter 2; lat., lateral part; LH, lateral hypothalamus; magno, magnocellular region; $Na^+$, sodium ion; NPY, neuropeptide Y; $K^+$, potassium ion; P0, day of birth; P2M, 2 months old; parvo, parvocellular region; pro, recombinant protein; PB, phosphate buffer; PBN, parabrachial nucleus; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; SDS, sodium dodecyl sulfate; VGAT, vesicular GABA transporter; VMH, ventromedial hypothalamus.

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the PVN. The LH and Arc, which connect each other, receive signals from the periphery, and have projections to the PVN. The Arc contains two main neuronal groups, orexigenic and anorexigenic, which innervate one another within the Arc. The PVN, mainly the anterior and medial parts, known as the parvocellular regions (van den Pol, 1982), receives signals from both the peripheral tissue and the three other hypothalamic nuclei, and is located at the exit of neuronal circuits in the hypothalamus.

Within the neural circuits in the hypothalamus, many peptide neurons are involved in food intake behavior (Krashes et al., 2014; Valassi et al., 2008; van Swieten et al., 2014; Zeltser et al., 2012). For example, neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons are localized in the Arc and send axons to the PVN, particularly melanocortin receptor positive neurons, which innervate the pontine parabrachial nucleus (PBN) and increase food intake. In contrast, pro-opiomelanocortin (POMC)/cocaine- and amphetamine- regulated transcript (CART) neurons are the main target of leptin (Thornton et al., 1997), are inhibited by NPY/AgRP neurons within the Arc, send axons to the PVN, and anorexigenically control feeding behavior.

In the present study, we focused on neurons containing γ-amino butyric acid (GABA). GABAergic terminals are distributed in the adult hypothalamus, and inhibit neuronal activity (Arora and Anubhuti, 2006; Kelly and Grossman, 1979; Kelly et al., 1979; van Swieten et al., 2014). They also play important roles in feeding behavior and energy expenditure. Moreover, a GABA agonist was previously shown to increase feeding and body weight (Kelly et al., 1977; Kelly and Grossman, 1979; Kelly et al., 1979). Recent studies revealed the precise relationship between GABAergic innervations and feeding behavior by molecular engineering. Some of the NPY/AgRP neurons in the Arc are GABAergic (Meister, 2007; van den Pol, 2003; Yeo and Heisler, 2012). Triple knockout mice lacking NPY, AgRP, and vesicular GABA transporter (VGAT), which is responsible for the vesicular release of GABA, showed impaired feeding behavior; however, double knockout mice lacking NPY and AgRP were normal, indicating that GABAergic innervations from the Arc to the PVN are responsible for increasing food intake (Atasoy et al., 2012; Tong et al., 2008). Mice lacking leptin receptors in GABAergic neurons exhibited an increased adipose mass, strongly suggesting that leptin acts via a network of GABAergic neurons to reduce the inhibitory tone to POMC neurons (Cone and Simerly, 2011; Vong et al., 2011). Despite this obvious importance of GABAergic networks in food intake and energy expenditure, little is known about their development in the hypothalamus during the embryonic period (Altman and Bayer, 1986; Dellovade et al., 2001; McClellan et al., 2006). It is important that feeding-related neuronal circuits, including the GABAergic network of the hypothalamus, should be sufficiently developed before birth because mammals drink milk immediately after birth.

In the mature CNS, GABA mediates the hyperpolarization of the membrane potential (Macdonald and Olsen, 1994; Olsen and Tobin, 1990), whereas it acts as an excitatory neurotransmitter during development, and is involved in morphogenesis (Ben-Ari, 2002; Ben-Ari et al., 2007; McCarthy et al., 2002; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005). This developmental shift in GABA action from excitation to inhibition is considered to be a result of a negative shift in the chloride ion ($Cl^-$) reversal potential by the increased expression of $K^+$-$Cl^-$ co-transporter 2 (KCC2) (Ben-Ari, 2002; Owens and Kriegstein, 2002; Payne et al., 2003). Thus, the formation of GABAergic terminals and dominant expression of the KCC2 may be the beginning of GABAergic inhibition (Hubner et al., 2001; Lee et al., 2005; Rivera et al., 2005) and the maturation process of the GABAergic network (Kin et al., 2014; Kosaka et al., 2012; Takayama and Inoue, 2003, 2010).

To reveal the time course of GABAergic network maturation in the hypothalamus, we examined the immunohistochemical localization of three molecules involved in GABAergic transmission; glutamic acid decarboxylase (GAD), VGAT, and KCC2. GAD, a GABA-synthesizing enzyme, is a marker of GABAergic neurons, in particular their axons and terminals. VGAT, which transports GABA and glycine into synaptic vesicles in the axon terminals, is a marker of GABAergic and glycinergic terminals. KCC2 immunohistochemistry was performed to detect developmental changes in GABA action, one of the GABAergic network maturation processes. These molecules have previously been investigated in other areas (Kin et al., 2014; Kosaka et al., 2012; Takayama and Inoue, 2010), but here, we focused on the four appetite-related nuclei, the Arc, VMH, LH, and PVN (parvocellular region).

## 2. Materials and methods

### 2.1. Animals

We examined mice from C57Bl/6J strain (Japan SLC, Inc., Hamamatsu, Japan) on embryonic day (E) 13 (mating date = E0), E15, E17, on the day of birth (P0), and at 2 months of age (P2 M). At each stage, at least five mice and five fetuses, which were obtained from at least three pregnant mice, were examined. All experiments were approved by the Animal Care and Use Committees of the University of the Ryukyus (No. 5762) and were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the University of the Ryukyus. Every effort was made to minimize the number of animals used and their suffering.

### 2.2. Tissue preparation

Fetuses were removed from the uterus of pregnant mice anesthetized by intraperitoneal injection of a mixed solution (10 μl/g body weight), containing 8% Nembutal and 20% ethanol in saline. Mice at P0 and P2 M were anesthetized on ice and by injection of the same mixed solution, respectively. Fetuses and mice were fixed by transcardial perfusion with 4% paraformaldehyde in phosphate buffer (PB, 0.1 M pH 7.4), and immersed in the same fixative overnight. Brains were removed from skulls at P2 M and P0. Brains and fetuses were cryoprotected with 30% sucrose in PB for more than 2 days at 4 °C. The middle part of each forebrain was cut into 20 μm-thick coronal sections using a cryostat. The sections were mounted on gelatin-coated glass slides.

### 2.3. Establishment of rabbit VGAT antibody

Recombinant protein containing amino acid residues 1-111 of mouse VGAT (AB080232.1, GI26665358) was prepared as described previously (Sunagawa et al., 2017). Rabbits were immunized at 2-week-intervals by the subcutaneous injection of the recombinant protein (200 μg/rabbit), which was emulsified in an equal amount of complete Freund's adjuvant (DIFCO Laboratories, USA). The specific immunoglobulin G fraction binding to the recombinant protein used for the immunization was affinity-purified. The specificity of the VGAT antibody was checked by western blotting and immunohistochemistry as previously reported (Sunagawa et al., 2017). Briefly, for the western blotting, the lysed P2 fraction prepared from adult mouse whole brain was extracted with sodium dodecyl sulfate (SDS) sample buffer, subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. After treating with blocking buffer (Block One; Nacalai Tesque, Kyoto, Japan), the nitrocellulose membrane was incubated with an anti-VGAT antibody (2 μg/ml) or anti-VGAT with recombinant protein (2 μg/ml) for 2 h, and then reacted with horseradish peroxidase-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories. Inc., USA). Immunoreactivity was visualized with

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