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Wnt-2b in the intermediate hyperpallium apicale of the telencephalon is critical for the thyroid hormone-mediated opening of the sensitive period for filial imprinting in domestic chicks (*Gallus gallus domesticus*)

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ABSTRACT

Filial imprinting is the behavior observed in chicks during the sensitive or critical period of the first 2–3 days after hatching; however, after this period they cannot be imprinted when raised in darkness. Our previous study showed that temporal augmentation of the endogenous thyroid hormone 3,5,3′-triiodothyronine (T_3) in the telencephalon, by imprinting training, starts the sensitive period just after hatching. Intravenous injection of T_3 enables imprinting of chicks on days 4 or 6 post-hatching, even when the sensitive period has ended. However, the molecular mechanism of how T_3 acts as a determinant of the sensitive period is unknown. Here, we show that *Wnt-2b* mRNA level is increased in the T_3-injected telencephalon of 4-day old chicks. Pharmacological inhibition of Wnt signaling in the intermediate hyperpallium apicale (IMHA), which is the caudal area of the telencephalon, blocked the recovery of the sensitive period following T_3 injection. In addition, injection of recombinant Wnt-2b protein into the IMHA helped chicks recover the sensitive period without the injection of T_3. Lastly, we showed Wnt signaling to be involved in imprinting via the IMHA region on day 1 during the sensitive period. These results indicate that Wnt signaling plays a critical role in the opening of the sensitive period downstream of T_3.

1. Introduction

Some types of learning can only be acquired during a limited period called the sensitive period (Hess, 1959; Knudsen, 2004). A classic example is found in the filial imprinting of precocial birds (Horn, 2004; Lorenz, 1935; Matsushima et al., 2003). Newly hatched chicks (*Gallus gallus domesticus*) and ducklings follow the first conspicuous moving object which they are exposed to during a limited period soon after birth. Under natural conditions, this is usually the hen. However, under experimental conditions, chicks can be imprinted to a wide variety of objects rather than their mother, though the predisposed preference for objects with biological motion is significant (Miura and Matsushima, 2012, 2016; Vallortigara et al., 2005). While following an object, the birds learn the color and shape of the object, and become attached to it (Horn, 2004). The learning that occurs during this sensitive period is considered to exert a long-lasting influence on the development of the individual's social and emotional behaviors (Scott, 1962).

Chicks are imprintable during a restricted sensitive period that lasts for about 3 days after hatching in the case of domestic chicks (Yamaguchi et al., 2012). After the sensitive period ends, non-trained naive chicks can no longer be imprinted. Recently, we found that the thyroid hormone 3,5,3′-triiodothyronine (T_3) is a determining factor of the sensitive period. Intrinsically increasing T_3 in the brain, followed by imprinting training on day 1, enables chicks to imprint the training object (Yamaguchi et al., 2012). Moreover, even after the sensitive period ends, chicks can be imprinted by intravenous T_3 injection or exogenous T_3 injection into the intermediate medial mesopallium (IMM, comparable to the association cortex in mammals) on days 4 or 6, suggesting that T_3 recovers the sensitive period in chicks, and that the action of T_3 is mediated through the IMM (Yamaguchi et al., 2012). Indeed, the IMM is assumed to be a critical brain region for the acquisition of imprinting (McCabe et al., 1981), and also for the 3-h retention from the end of imprinting training (McCabe et al., 1982).

We recently found that another brain region, the intermediate hyperpallium apicale (IMHA), receives efferent neural projections from the IMM. When the IMHA was ablated in newly hatched chicks on day 1, they were unable to imprint, showing that the IMHA is crucial for memory acquisition during the sensitive period. When the sensitive period closed on day 4, the IMHA-ablated chicks exhibited suppression of the recovery of the sensitive period caused by exogenous T_3 injection

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into the IMM, indicating that the IMHA is critical for the recovery of the sensitive period following the action of T_3 in the IMM. However, the injection of T_3 into the IMHA did not recover the sensitive period in 4-day-old chicks, suggesting that the IMHA is not directly targeted by T_3. Accordingly, the IMHA is likely to be the region which receives and retains information from the IMM (Aoki et al., 2015). Until recently, a diffusible factor that directly acts on and modulates the IMHA to receive information from the IMM had not been identified.

One of the candidate diffusible factors is Wnt. The Wnt family are secreted glycoproteins that control gene expression and cytoskeletal reorganization, and have well-established roles in the development of the nervous system, and neuronal maintenance in vertebrates and invertebrates (Ciani and Salinas, 2005; Inestrosa and Arenas, 2010; Logan and Nusse, 2004). The Wnt proteins participate in processes such as neuronal migration, axon pathfinding, dendritic morphogenesis, and synaptic differentiation (Salinas and Zou, 2008). Beyond brain development, the role of the Wnt proteins in learning and memory has been studied using physiological approaches in mouse hippocampal slices in vitro (Budnik and Salinas, 2011; Oliva et al., 2013; Stamatakou and Salinas, 2014). However, there are only a few in vivo studies on the role of Wnt proteins in learning and memory, using behaving animals.

In this study, in order to understand the mechanism underlying T_3-mediated recovery of the sensitive period in filial imprinting, we identified the upregulated and downregulated genes following T_3 injection. We found that *Wnt-2b* mRNA level is increased in the T_3-injected chick telencephalon, including the IMHA region. We hypothesized that Wnt-2b is a diffusible factor that directly acts on the IMHA, and may modulate the efferent neural projections from the IMM. We show that Wnt-2b plays a critical role in the recovery of the sensitive period in the IMHA, which acts downstream of the IMM. The IMHA may receive and retain information from the IMM. Similar to day 4, when the sensitive period ends, Wnt signaling is also involved in imprinting via the IMHA region on day 1 during the sensitive period.

2. Material and methods

2.1. Animals

All experimental procedures were conducted according to the guidelines of the Committee on Animal Experiments of Teikyo University, Japan (approval number: 12-019), and the National Regulations for Animal Welfare in Japan (Law for the Humane Treatment and Management of Animals; after partial amendment number 68, 2005). Domestic chicks of the Cobb strain (*Gallus gallus domesticus*) were used for all experiments. A total of 142 chicks were used (microarray and real-time polymerase chain reaction [RT-PCR]: 25; in situ hybridization: 5; immunofluorescence: 3; behavioral experiment: 109). Fertilized eggs were obtained from a local supplier (3-M, Nagoya, Japan). After hatching, the chicks were placed in a dark environment or very dim lighting until they were subjected to the imprinting training (Izawa et al., 2001).

2.2. Intravenous injection of thyroid hormone

Intravenous injection of thyroid hormone was performed as previously described (Yamaguchi et al., 2012). Briefly, T_3 (Dawson et al., 1986) (Sigma-Aldrich, cat# T2877, Tokyo, Japan) was dissolved in 0.002 M NaOH and 0.9% NaCl. A single shot of 100 μl T_3 solution (10 μM) per chick was injected intravenously. The resulting concentration of T_3 in the serum was about 100 ng/ml.

2.3. cDNA microarray analysis and quantitative RT-PCR

Both cDNA microarray and quantitative RT-PCR were performed as previously described, with modifications (Yamaguchi et al., 2008a; Yamaguchi et al., 2010). Chicks were intravenously injected with T_3, and kept in a dark environment for 1 h, before the dissection. Telencephalons of 4-day-old T_3-injected chicks (n = 13) and control dark-reared chicks (n = 12) were dissected under deep anesthesia. The sex of individual chicks was determined for cDNA microarray analysis. Total RNA was extracted from these telencephalons with TRIzol (Invitrogen, Carlsbad, CA, USA). To distinguish male chicks from female chicks, we performed RT-PCR. The sequences of the primers used were as follows: protein kinase C (*PKC*) female-specific sense, 5′-AGTGCCTTGCGTTCC ATGATA-3′; *PKC* female-specific antisense, 5′-AAGGGACTGAGGGTGG GTATC-3′; *PKC* non-sex-specific sense, 5′-AGTGCCTTGCGTTCCATG ATA-3′; *PKC* non-sex specific antisense, 5′-GCCCAGGTTAGCAGCACAC TTC-3′ (Yamaguchi et al., 2012). Then, total RNAs extracted from 25 animals were separated into four groups (1, male T_3-injected chicks [n = 7]; 2, female T_3-injected chicks [n = 6]; 3, male dark-reared chicks [n = 7]; and 4, female dark-reared chicks [n = 5]). We then performed a microarray expression analysis to identify the genes that were upregulated following T_3 injection (group 1 versus group 3, and group 2 versus group 4). Probe labeling, hybridization, washing, and data collection were conducted following the manufacturer's instructions (Agilent, Santa Clara, CA, USA). We found two genes that showed a > 1.7-fold increase in expression following T_3 injection. We confirmed the upregulation of these two genes using quantitative RT-PCR, using the total RNAs mentioned above (Table 1). The relative expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Lenart et al., 2017; Simon et al., 2018). The primers used were as follows: *Wnt-2b*, 5′-GCAGGCCTTGCTCATGCT-3′ (sense) and 5′-TCTGTCTTCAGGGCATGATCTTAA-3′ (antisense); *Dio3* (type 3 iodothyronine deiodinase), 5′-ACCGGAGGGCTACAAGATCTC-3′ (sense) and 5′-TGGAGCCGGGTTTTGTACTG-3′ (antisense); and *GAPDH*, 5′-TGGAGCCCCTGCTCTTCA-3′ (sense) and 5′-GGAACAGAACTGGCCT CTCACT-3′ (antisense). Because there was no significant difference between males and females with regard to *Dio3* expression, (dark-reared male vs dark-reared female: Mann-Whitney *U* test, $p = 0.291$; T_3-injected males vs T_3-injected females: $p = 0.153$), we analyzed the data together for quantitative RT-PCR (Fig. S2). The same was true for *Wnt-2b* gene expression (dark-reared male vs dark-reared female: Mann-Whitney U test, $p = 0.291$; T_3-injected males vs T_3-injected females: $p = 1.00$), and hence, the data for both males and females were pooled and analyzed together (Fig. 1A).

2.4. In situ hybridization and cloning of Wnt-2b cDNA

In situ hybridization was performed as described (Yamaguchi et al., 2008b). Chicks were anesthetized by an intraperitoneal injection (0.40 ml) of a 1:1 mixture solution of ketamine (10 mg/ml, ketalar-10, Sankyo Co., Tokyo, Japan) and xylazine (2 mg/ml, Sigma). Four-day-old chicks were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.5) under deep anesthesia. Brains were post-fixed with the same fixative for 24 h, and immersed in 18% sucrose in PBS. Then they were frozen in a plastic mold with Tissue-Tek optimum cutting temperature compound (Sakura FineTechnical, Tokyo, Japan), placed on dry ice, and stored at −80 °C. The brain tissues were then cut into 10-μm-thick sections using a cryostat (Leica CM3050 S,

Table 1
List of the genes upregulated by T_3 injection, identified using cDNA microarray and quantitative RT-PCR.

Genes	cDNA microarray		qRT-PCR	
	♂	♀	♂	♀
	(Fold)	(Fold)	(Fold)	(Fold)
Dio3 (Type 3 iodothyronine deiodinase)	3.0	3.1	3.2	4.4
Wnt-2b (Wnt family member 2B)	2.4	1.8	3.2	3.8

Abbreviations: qRT-PCR, quantitative real-time polymerase chain reaction.