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Distribution of proglucagon mRNA and GLP-1 in the brainstem of chicks

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Abstract

Glucagon-like peptide-1 (GLP-1), structurally similar to glucagon, synthesized from the precursor proglucagon, is a well known anorexigenic peptide in the brain of several animal species. However, there are no previous reports concerning GLP-1-containing neurons in the chick brain. The aim of the present study was to investigate the distribution of proglucagon mRNA and GLP-1-immunoreactive (GLI) perikarya in various regions of the chick brain. We detected proglucagon mRNA in the brainstem, and to a lesser extent in the telencephalon. In the brainstem, a study using immunohistochemistry revealed that GLI perikarya were present in the nucleus motorius nervi facialis pars dosalis, nucleus motoris dorsalis nervi vagi and nucleus tractus solitarii. Furthermore, we found that proglucagon mRNA expression in the brainstem decreased after 24 h fasting. The present findings support the idea that endogenous GLP-1 is involved in feeding behavior of chicks.

Keywords: Brainstem; Chick; Feeding; GLP-1; Proglucagon

1. Introduction

Glucagon-like peptide-1 (GLP-1) is one of the anorexigenic peptides in the brain of mammals (Turton et al., 1996). Intracerebroventricular (ICV) injection of GLP-1 inhibits feeding behavior of chicks (Furuse et al., 1997a,b; Tachibana et al., 2001), implying that GLP-1 is a common anorexigenic peptide over the species. However, no reports on GLP-1-containing neurons were available in the chick brain. Discovery of GLP-1-containing neurons in the chick brain would increase in physiological significance of the peptide.

Several studies on the distribution of GLP-1 were reported in the mammalian brain. Perikarya containing mRNA of proglucagon, which are the precursors of glucagon, glicentin, oxyntomodulin, GLP-1 and glucagon-like peptide-2 (GLP-2) (Drucker, 1998), are observed in the rat brain (Larsen et al., 1997; Merchenthaler et al., 1999). An immunohistochemical study revealed GLP-1-like immunoreactive (GLI) perikarya in the nucleus of the solitary tract (NTS) of the rat brainstem (Larsen et al., 1997). Since the majority of neural sites involved in feeding regulation in birds are located in the brainstem (Kuenzel, 1994), it can be expected that GLP-1-containing perikarya are located in the brainstem of chicks.

The purpose of the present study was to determine whether GLP-1 is present in the chick brain and whether proglucagon mRNA expression in the brain is related to feeding. First, we investigated the distribution of proglucagon mRNA in the brain using reverse transcription-

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polymerase chain reaction (RT-PCR). Then, we immunohistochemically investigated the distribution of GLI perikarya in the chick brainstem. Finally, we investigated whether proglucagon mRNA expression in the brainstem is changed by food deprivation.

2. Materials and methods

2.1. Animals

Day-old male layer chicks (*Gallus gallus*) were purchased from local hatcheries (Ghen Gifu, Japan) and kept in a room at 28 °C under continuous lighting. The birds were given free access to a commercial diet (Nihon Nosan Kogyo Kanagawa, Japan and Toyohashi Feed and Mills Aichi, Japan) and water except where noted elsewhere. Experiments were performed in accordance with the guidelines for animal experiments in the Faculty of Agriculture and Graduate Course of Kyushu University, and in accordance with the laws (No.105) and notifications (No.6) of the Japanese government.

2.2. Experiment 1: Distribution of proglucagon mRNA in the chick brainstem

Experiment 1 was performed to examine distribution of proglucagon mRNA in the chick brain. Five chicks (4 days old) were anesthetized by intraperitoneal administration of pentobarbital (Dainippon Pharmaceutical Osaka, Japan) under ad libitum feeding conditions. The brain was rapidly removed, weighed and divided into 5 parts: telencephalon, diencephalon, optic lobe, cerebellum and brainstem according to a previous report (Kuenzel and Masson, 1988). Briefly, the telencephalon was separated from diencephalon by cutting the junction. Then the cerebellum was removed by the cutting cerebellar peduncle. After removal of the optic lobe, the diencephalon and brainstem were divided at the level of the caudal end of the medial eminence. The samples were immediately treated with RNAlater Reagent (Sigma, St. Louis, MO) and stored at −80 °C until analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and the 1st-strand cDNA was synthesized from 5 μg of DNase I (Invitrogen)-treated total RNA using the Omniscript RT kit (QIAGEN GmbH, Hilden, Germany) with oligodT primer. PCR was performed with 2 μg of the cDNA using ReadyMix RED Taq PCR REACTION MIX with $MgCl_2$ (Sigma). Proglucagon cDNA was amplified with the following primers: sense, 5′-GTTCAAGGCAGCTGGCAAAATCCT-3′; antisense, 5′-TCCTCGTCCATTCACTAACCAAGC-3′. As an internal standard, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified, using the following primers: sense, 5′-ATGGGCAGATGCAGGTGCTGAGTA-3′; antisense, 5′-GTGGAAGAATGGCTGTCACCATTG-3′. PCR reactions were performed as follows: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 10 min. The PCR products (proglucagon, 396 bp; GAPDH, 624 bp) were analyzed by ethidium bromide (Sigma) staining on a 2% (w/v) agarose gel. The electrophoresis pattern was visualized with ultraviolet illumination and digitally captured with a digital photography system (B-box, DS-400, BIOCRAFT Tokyo, Japan).

2.3. Experiment 2: Distribution of GLI perikarya in the chick brainstem

Experiment 2 was done to investigate the distribution of GLI perikarya in the chick brainstem. Five chicks (7 days old) were pretreated with ICV injection of colchicine (40 μg/10 μl/chick, Wako Pure Chemical Industries, Osaka, Japan) according to the previous method by Davis et al. (1979). Colchicine was dissolved in a 0.1% Evans Blue solution, which was prepared in a saline solution. Twenty-four hours after injection, each chick was anesthetized with pentobarbital and transcardially perfused with Ringer solution followed by Zamboni's fixative. Then, the brain was removed and post-fixed with Zamboni's fixative at 4 °C. The colchicine injection was confirmed by observation of the presence of Evans Blue dye in the lateral ventricle. Next, 60-μm serial coronal sections were made from just caudal to the *tractus septomesencephalicus* (rostral end of the hypothalamus) to the caudal end of the cerebellum. After treatments with hydrogen peroxide (Wako Pure Chemical Industries) and Triton X-100 (Wako Pure Chemical Industries), the sections were incubated with rabbit anti-GLP-1 antiserum (1:2000, Affiniti Research Products Limited, Exeter, UK) at 4 °C for 48 h. The antiserum did not cross-react with other proglucagon-derived peptide. The incubated sections were treated with goat anti-rabbit IgG (1:500, ICN Immuno Biomedicals, USA) at 4 °C for 24 h, and were then incubated with rabbit peroxidase–antiperoxidase (1:500, ICN Immuno Biomedicals, USA) at 4 °C for 3 h. Immunoreactivity was visualized using diaminobenzidine tetrahydrochloride (Dhojin Chemical Institute, Kumamoto, Japan) and nickel ammonium sulfate (Wako Pure Chemical Industries). After dehydration and dealcoholization, the specimens were coverslipped with mounting medium. In all brain regions, pre-incubation of anti-GLP-1 antiserum with chicken GLP-1 (Peptide Institute, Osaka, Japan) abolished the detection of GLI (data not shown). The specimens were observed using a light microscope (OPTIPHOT, Nikon, Tokyo, Japan). Immunoreactive cells with dendrites were identified as GLI perikarya. The brain structures were identified using a chick brain atlas (Kuenzel and Masson, 1988). In the present study, the nucleus *tractus solitarii* (the chicken homologue of the mammalian NTS) is abbreviated as NTS, although the brain atlas abbreviates as S (Kuenzel and Masson, 1988).