



Distribution of gonadotropin-inhibitory hormone (GnIH) in male Luchuan piglets

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ABSTRACT

Gonadotropin inhibitory hormone (GnIH) has emerged as a novel hypothalamic neuropeptide that actively inhibits gonadotropin release in birds and mammals. Recent evidence indicates that GnIH not only acts as a key neurohormone that controls vertebrate reproduction but is also involved in stress response, food intake, and aggressive and sexual behaviors, suggesting a broad physiological role for this neuropeptide. To elucidate its multiple sites of action and potential functions, studying the detailed distribution of GnIH in different organs, except for the hypothalamus-pituitary-ovary/testis axis, is necessary. Therefore, in the present study, in different central nervous system (CNS) and peripheral organs of male Luchuan piglets, the distribution of GnIH was systemically determined using immunohistochemistry, and the expression of GnIH mRNA was investigated using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Our results demonstrate that GnIH immune reactive (GnIH-ir) neurons were widely distributed in the pig CNS, but the number and size of the GnIH-ir neurons varied and exhibited morphological diversity. In the peripheral organs, GnIH immunoreactive cells were observed in the respiratory tract, alimentary tract, endocrine organs, genitourinary tract and lymphatic organs. GnIH mRNA was highly expressed in the CNS, with the highest expression in the hypothalamus. In the peripheral organs, high GnIH mRNA levels were detected in the testis, while no GnIH expression was observed in the liver, lungs and heart et al. These results demonstrated that GnIH might play an important role in modulating a variety of physiological functions and provided the morphological data for further study of GnIH in pigs.

1. Introduction

Gonadotropin-inhibitory hormone (GnIH) is a novel hypothalamic neuropeptide that was originally discovered in 2000 in quail as an inhibitory factor for gonadotropin release (Tsutsui et al., 2000; Ubuka et al., 2012a). After over a decade of research, avian GnIH and its orthologs, which share a common C-terminal LPXRFamide (X = L or Q) motif, have been identified and characterized in various species from fish to mammals (Bentley et al., 2008; Smith and Clarke, 2010; Tsutsui et al., 2017; Ubuka et al., 2018). Importantly, as in birds, mammalian GnIH orthologs [RFamide-related peptides (RFRPs)] also act to inhibit gonadotropin release across mammalian species, including rats, hamsters, and sheep (Tsutsui, 2010; Tsutsui et al., 2010; Ullah et al., 2016).

An abundance of original research papers have demonstrated that GnIH appears to act similarly across vertebrate species to regulate reproduction in the hypothalamic-pituitary-gonadal (HPG) axis (Clarke et al., 2009; Tsutsui, 2009; Tsutsui et al., 2017). In the brain, GnIH neuronal cell bodies are found in the hypothalamus, and their fibers mostly extend into the diencephalon and midbrain (Kriegsfeld et al.,

2006; Tsutsui et al., 2007; Ukena et al., 2003). GnIH neurons project into the external layer of the median eminence, and GnIH fibers are closely associated with many other neurons, such as GnRH, POMC, NPY, orexin, and kisspeptin neurons (Ducret et al., 2009; Kriegsfeld, 2006; Qi et al., 2009; Ubuka et al., 2008). These findings suggested that GnIH neurons may not only inhibit gonadotropin release and synthesis in the pituitary but also regulate various neurons in the brain. In addition, GnIH and GPR147 are expressed in the gonads and accessory reproductive organs (Li et al., 2012; Singh et al., 2011a; Ubuka et al., 2006), indicating that they are possibly involved in autocrine/paracrine regulation of gonadal steroid production and germ cell differentiation and maturation.

Although studies on GnIH in the past decade mainly focused on its reproductive physiology, recent evidence has further indicated that GnIH is also involved in several non-reproductive functions, such as appetite regulation, energy metabolism, and anxiety and stress responses (Clarke et al., 2012; Leon et al., 2014; Tsutsui et al., 2012; Wahab et al., 2015). Multiple physiological functions suggest that GnIH might be widely distributed in many tissues. However, there have been

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Table 1
Primers and reaction conditions of semi-quantitative RT-PCR.

Primers	Sequences (5'–3')	Length (bp)
GnIH	(+)TAACATCCCAACATCTTTGTACAG (-)CGGGTGATGGAGTAAAGTAAC	442
GAPDH	(+)AGGTCGGAGTGAACGGATTG (-)CAGTCTTCTGGGTGGCAGTGAT	549

Table 2
Localization and relative score intensity of GnIH in the central nervous system and the peripheral organs of male Luchuan piglets.

Tissues	GnIH
The central nervous system	
Telencephalon	
Cerebral cortex	++
Septal region	++
Hippocampus	+
Olfactory bulb	+
Diencephalon	
Preoptic area	+++
Hypothalamic periventricular regions	+++
Suprachiasmatic nucleus	+
Supraoptic nucleus	+
Brainstem	
Midbrain	+
Pons	+
Medulla oblongata	++
Cerebellum	+
Spinalcord	+
The peripheral organs	
Male Reproductive system	
Testis	+++
Epididymis	+++
Accessory glands	+++ / ++
Endocrine system	
Thyroid gland	++
Parathyroid gland	+++ / ++
Adrenal gland	++
Immune system	
Thymus	++ / +
Lymph nodes	+
Palatine tonsil	++
Spleen	+++ / ++
Urinary system	
Kidneys	±
Urinary bladder	++
Digestive tract	
Esophagus	+++
Stomach	+++
Small intestines	++
Large intestines	++
Digestive gland	
Submandibular gland	+++
Pancreas	+++
Liver	±
Respiratory system	
Trachea	+++ / ++
Lungs	–
Cardiovascular system	
Heart	±
Large artery	–
Muscle tissue and skin	–

The intensities of signals indicated above represent a subjective consensus of sections examined from the CNS and peripheral organs collected from male Luchuan piglets (N = 3). The signals were estimated as score intensity of immunoreactivity on a scale of - to 3 + as: -, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; +/–, there was heterogeneity in the signal, e.g., some of the histological units contained signal, and others did not.

no reports demonstrating the comprehensive anatomical locations of GnIH in fish and mammals. There is also a lack of information on the distribution and biological role of GnIH in pigs. Although we have

cloned partial pig GnIH precursor cDNA and demonstrated its distribution and reproductive functions in the pig hypothalamic–pituitary–gonadal (HPG) axis (Li et al., 2012, 2013), further studies on the multiple sites of action and potential functions of pig GnIH are very important. Therefore, the distribution of GnIH in different central nervous system and peripheral organs of male Luchuan piglets was systemically determined using immunohistochemistry (Table 2), and the expression of GnIH mRNA was investigated. Taken together, the results of this study describe the comprehensive anatomical locations of GnIH in pigs and provide the morphological basis for hypothetical physiological functions of GnIH.

2. Results

2.1. Localization of GnIH in male Luchuan piglet tissues

2.1.1. Distribution of GnIH immunoreactive cells in the central nervous system

Telencephalon: In the cerebral cortex (Fig. 1A–D), intense and numerous GnIH immunoreactive cells were found in the external granular, external pyramidal and internal pyramidal layers, while dispersed labeled GnIH immunoreactive cells were found in the internal molecular, pyramidal and polymorphic layers. The immunoreactivities of the GnIH proteins were mainly localized to the astrocytes and pyramidal cells in the six layers of the cerebral cortex. In addition, clusters of distinct immunoreactive cells and fibers were observed in the septal region, the nucleus of the stria terminalis and the diagonal band (Fig. 1E and F). In the hippocampus, pyramidal cells of the stratum pyramidale exhibited moderate immunoreactivity. Multiple intense and dispersed immunoreactive cells were identified in the stratum multi-formis (Fig. 1G–I). In the olfactory bulb (Fig. 1J–L), a few immunoreactive cells were observed in the external plexiform layer, whereas cells with strong immunoreactivity were found in the mitral cell layer.

Diencephalon: Clusters of GnIH immunoreactive cells were maximally located in the preoptic area and the hypothalamic periventricular regions, such as the dorsomedial nucleus (DMN), intermediate periventricular nucleus (Ipe), paraventricular nucleus (PVN) and arcuate nucleus (Arc) (Fig. 2A–D and 2F). A few moderately immunoreactive cells were also identified in the suprachiasmatic nucleus (SCN) and supraoptic nucleus (SON) (Fig. 2E).

Brainstem: Abundant GnIH immunoreactive cells and fibers were generally restricted to the periventricular region of the brainstem. Clusters of distinct immunoreactive cells were observed in the central gray substance of the midbrain and in the dorsal raphe nucleus in the midbrain (Fig. 2G–I). The parabrachial nucleus in the pons also exhibited GnIH-positive cells (Fig. 2J). However, compared to the mid-brain and pons, the quantity and distribution of GnIH immunoreactive cells and fibers were increased in the medulla oblongata. Many intense and dispersed immunoreactive cells and fibers were located in the tegmental area. Clusters of cells with intense to moderate immunoreactivity were observed in the central gray substance of the medulla oblongata (CGMB), dorsal raphe nucleus (DR) and inferior olivary nucleus (Fig. 2K–M).

Cerebellum: Purkinje cells in the Purkinje cell layer showed intense to moderate levels of GnIH immunoreactivity (Fig. 2N and O).

Spinalcord: The quantity and distribution of GnIH immunoreactive cells were localized to the gray matter, which showed intense to moderate staining (Fig. 2P and Q).

2.1.2. Distribution of GnIH immunoreactive cells in the peripheral organs

Male Reproductive system: In the testis (Fig. 3A–C), Leydig cells in the testis interstitium as well as the spermatogenic epithelium displayed moderate to strong GnIH immunoreactivity, while the Sertoli cells were negative. In the testicular lobule (Fig. 3D and E), strong GnIH immunoreactivity in the spermatogonium was prominent. In the

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