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Research paper

Distribution and morphology of gonadotropin-releasing hormone neurons in the hypothalamus of an induced ovulator – The llama (*Lama glama*)

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a decapeptide involved in the regulation of reproduction in all mammals, but the distribution of GnRH neurons within the brain varies widely among species. The objective of the present study was to characterize the number and distribution of GnRH neurons in the hypothalamus and preoptic area of llamas, an induced ovulator. The brains of female llamas (n = 4) were fixed, frozen and sectioned serially every 50 μm in the transverse (coronal) plane. Every 10th section was stained for immunohistochemical detection of GnRH-positive neuron cell bodies and fibers by incubation with *3,3′-diaminobenzidine*. The number of counted immunoreactive cells ranged from 222 to 250 (≈241 ± 13 cells in the preoptic area and hypothalamus per animal) and were localized in the medio-basal hypothalamus (44.3%), anterior hypothalamus (27%), preoptic area (14.9%), diagonal band of Broca/medial septum (13.4%), and mammillary area (0.5%). The immunoreactive cells were not localized in specific hypothalamic nuclei, but rather appeared to be distributed diffusely. The highest concentration of immunoreactive neuron fibers was in the median eminence ($P < 0.05$), but fibers were identified in most of the areas analyzed, including the neurohypophysis. The GnRH neurons within the hypothalamus displayed monopolar (33%), bipolar (39%), and multipolar (28%) morphologies. The bipolar type was most common in the medio-basal region (40%; $P < 0.05$). We conclude that GnRH neurons and fibers form a network within the anterior and medio-basal hypothalamus of llamas, suggesting the central location of mechanisms controlling reproductive processes in llamas (i.e., induced ovulation).

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide that is fundamental in the regulation of reproduction (Gibson et al., 1997). It was originally isolated from the porcine (Schally et al., 1971) and ovine hypothalamus (Amos et al., 1971) and later identified in the brains of many different species (King and Anthony, 1984). To date, three different GnRH isoforms have been discovered among species, but the isoform involved mainly in reproductive processes is Type 1, or mammalian GnRH (reviewed by Herbison, 2005).

During embryonic development, GnRH cells migrate caudally from the nasal placode into the brain in a mid-ventral direction toward the hypothalamus (Wray, 2010), but also to non-hypothalamic areas (Merchentaller et al., 1984). Consequently, the distribution of GnRH cells is not homogenous throughout the brain and varies markedly among species (reviewed by Silverman et al., 1993). Studies in rodents using immunocytochemistry and *in situ* hybridization have shown an accumulation of GnRH cells in the preoptic area surrounding the organum vasculosum of the lamina terminalis (reviewed by Silverman et al., 1993). In sheep, however, a third of GnRH immunoreactive cells were also detected in the medio-basal hypothalamus surrounding the third ventricle (Lehman et al., 1986; Caldani et al., 1988). In addition to morphological differences, there is evidence of functional differences within the GnRH system. In a study in sheep designed to determine estrogen responsive areas involved in the surge and tonic secretion of GnRH and LH, implants of estradiol in the medio-basal hypothalamus induced a surge-like release of LH whereas implants in the preoptic area did not (Caraty et al., 1998). In contrast, rodents in which the preoptic afferents to the hypothalamus were surgically sectioned (i.e., rostral to the anterior hypothalamus) failed to ovulate (Wiegand et al., 1980). The variation among species in the distribution and function of different GnRH neuronal populations (Herbison, 1998) will influence our

Abbreviations: BSA, Bovine serum albumin; GnRH, Gonadotrophin-releasing hormone; LH, Luteinizing hormone; OIF, Ovulation-inducing factor; OIF/NGF, Ovulation-inducing factor/nerve growth factor; PBS, Phosphate buffer saline

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understanding of the mechanistic pathway of the ovulatory process in different animals.

Llamas are South American camelids classified as induced ovulators (Fernandez-Baca et al., 1970). Using transrectal ultrasonography, emergence of anovulatory follicular waves were detected at regular intervals until mating with an intact or vasectomized male, which elicited ovulation within 48 h (Adams et al., 1989, 1990). Although spontaneous ovulation in alpacas has been reported a low rate (5% of ovulation; Fernandez-Baca et al., 1970), mating triggers a rise in circulating concentrations of luteinizing hormone (Bravo et al., 1990) causing ovulation and corpus luteum formation (Adams et al, 1989, 1991). Based on information from other induced ovulators (Carrol et al., 1985; Greulich, 1934), it was assumed initially that ovulation in llamas was triggered by physical stimulation of the genitalia (Fernandez-Baca et al., 1970). However, the discovery of an ovulation-inducing factor (OIF) in the seminal plasma of camelids led to the realization that systemic absorption of this seminal protein was the primary trigger for the ovulatory response in camelids within 30 h of treatment (Adams et al., 2005; Ratto et al., 2005, 2006, 2010). More recently, OIF in seminal plasma was found to be identical in amino-acid sequence and structure to nerve growth factor (Ratto et al., 2012), and that this protein acts in an endocrine fashion to elicit an LH surge and ovulation (Adams et al., 2005) through a direct or indirect effect on GnRH neuron secretion (Silva et al., 2011).

The GnRH system has not been described in camelids, and an understanding of its structural components is needed to determine the mechanistic pathways involved in controlling ovarian function in this induced ovulator. The objective of the present study was to identify the anatomical distribution and morphology of GnRH neuron cell bodies and fibers in the llama hypothalamus in an effort to identify the major GnRH pathways throughout the hypothalamus.

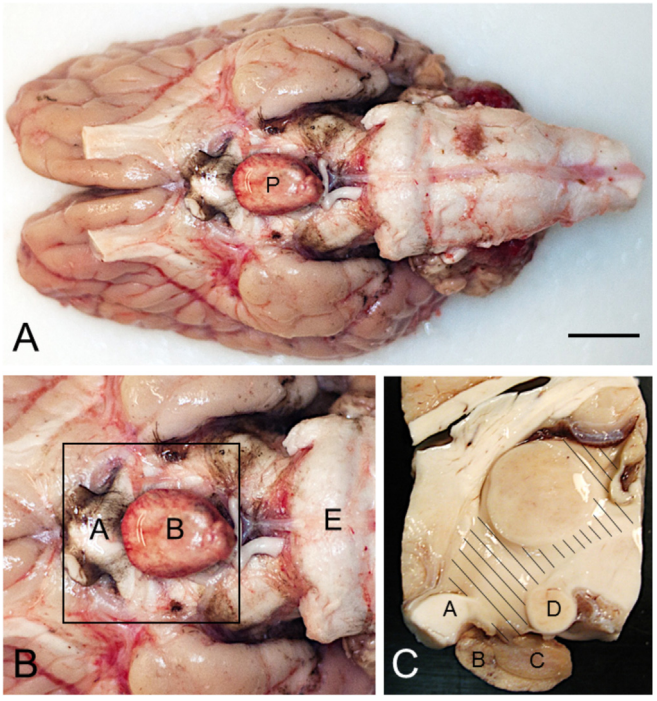

Fig. 1. Localization of the hypothalamus of the llama brain. (**a**) Ventral view of the llama brain and pituitary gland (P). (**b**) Magnification of A indicating the area that was dissected and used for immunohistochemistry (*square box*). (**c**) Median section of the llama hypothalamus showing the third ventricle (parallel lines). a = optic chiasma, b = anterior pituitary, c = posterior pituitary, d = mammillary body, e = pons. *Scale bar* 1.5 cm.

2. Materials and methods

2.1. Animals and tissue collection

Non-pregnant, non-lactating adult mature female llamas (n = 4) were euthanized during the follicular phase with an overdose of pentobarbital following the guidelines of the Animal Care Committee of the University of Saskatchewan. The head was separated from the body, perfused with 2 L of cold heparinized saline (0.9% NaCl) solution (10,000 IU heparin/L) injected through one of the common carotid arteries using a peristaltic pump over a period of 20 min, followed by 2 L of a cold 4% paraformaldehyde in phosphate buffered saline (PBS; pH = 7.4). The brain was extracted from the cranium, and the portion of mid-brain containing the preoptic area and hypothalamus was dissected as a single piece and immersed in 4% paraformaldehyde overnight at 4 °C (Fig. 1). On the following day, the fixed sample was washed 3 times in PBS and immersed in 30% sucrose in PBS at 4 °C (weekly changes over a period of two weeks) until the tissue sank. Tissues were frozen at −80 °C and sectioned in a transverse (coronal) plane every 50 µm using a cryostat microtome. Sections were stored in cryoprotectant solution (30% sucrose, 30% ethylene glycol in PBS) at −20 °C until immunohistochemistry was performed. In addition, the frozen pituitary glands of 2 of the llamas were sectioned sagittally at 20 µm increments using the cryostat microtome, mounted on poly-L-lysine coated glass slides and stained for GnRH as described below.

2.2. Immunohistochemistry

The immunohistochemical procedure was applied to unmounted (free-floating) brain sections to obtain optimal staining of thick sections (Hoffman et al., 2008). The cryoprotectant solution was removed by rinsing the sections 4 times in PBS for 15 min each. Endogenous peroxidase activity was blocked by incubating sections for 30 min in 30% hydrogen peroxide at room temperature, followed by 2 rinses in PBS. Samples were heated in a sodium citrate solution (0.1 M in distilled water; pH = 6.0) at 93 °C for 30 min to unmask antigens due to the fixation procedure. After cooling to room temperature and removing the antigen-retrieval solution, non-specific binding was blocked by incubating sections with 1% BSA and 0.3% triton X-100 in PBS (blocking buffer) for 3 h. The sections were incubated for 24 h at 4 °C in a solution of primary antibody (mouse monoclonal antibody against GnRH, SMI 41; Stemberger Monoclonals, Covance, Princeton, NJ, USA) diluted 1:5000 in 1% BSA, 0.3% triton X-100, and 0.1% sodium azide in PBS (pH = 7.4). Sections were washed 3 times for 15 min each with PBS and subsequently incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (diluted 1:200 in blocking buffer; Dako, Burlington, Ontario, Canada) for 24 h at 4 °C. The immunoreaction was revealed by incubating the sections in 3,3′-diaminobenzidine and hydrogen peroxide for 30 min, and sections were then rinsed in distilled water to stop the reaction (Hoffman et al., 2008). Immunostained sections were mounted in poly-L-lysine coated slides, cleared in xylene, and cover-slipped. A subset of immunostained sections from one animal were counter-stained for 3 min in hematoxylin before clearing and cover-slipping, to permit identification of the nucleus of neurons and glial cells. Likewise, serial non-immunostained sections from one animal were stained with Cresyl violet for identification of neurons in hypothalamic regions.

The specificity of the mouse monoclonal antibody (SMI 41) was tested by pre-adsorption with the GnRH antigen, omission of the primary antibody, or by replacing the primary antibody with an isotype antibody (Fig. 2). The median eminence was used as a possitive control, since GnRH immunoreactive fibers have been described in a variety of species (King and Anthony, 1984). For preadsorption, the primary antibody (diluted 1:5000) was preincubated with 500 ug of GnRH (ab 120184; Abcam, Cambridge, MA, USA) overnight at 4 °C, and applied onto sections following the procedure described above. No immunoreaction was detected when the the primary antibody was pre-