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DISTRIBUTION OF PREPRODYNORPHIN mRNA AND DYNORPHIN-A IMMUNOREACTIVITY IN THE SHEEP PREOPTIC AREA AND HYPOTHALAMUS

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Abstract—Endogenous opioid peptides (EOP) are important modulators in a variety of neuroendocrine systems, including those mediating reproduction, energy balance, lactation, and stress. Recent work in the ewe has implicated the EOP, dynorphin (DYN), in the inhibitory effects of progesterone on pulsatile gonadotropin releasing hormone secretion. Although DYN is involved in a number of hypothalamic functions in the sheep, little is known regarding the localization of preprodynorphin (PPD) expression and its major product DYN A (1–17). In this study, we determined the distribution of PPD mRNA and DYN A-containing cell bodies in the brains of ovary-intact, luteal ewes. To detect PPD mRNA, an ovine PPD mRNA was subcloned by reverse transcription–polymerase chain reaction from sheep hypothalamus and used to create a ^{35}S-labeled riboprobe for *in situ* hybridization. Neurons that expressed PPD mRNA and DYN A immunoreactivity were widely distributed in the ovine preoptic area and hypothalamus. PPD mRNA-expressing cells were seen in the supraoptic nucleus, paraventricular nucleus, preoptic area, anterior hypothalamus area, bed nucleus of the stria terminalis, ventromedial nucleus (VMN), dorsomedial nucleus of the hypothalamus, and the arcuate nucleus. All of these regions also contained DYN A-positive cell bodies except for the VMN, raising the possibility that PPD is preferentially processed into other peptide products in the VMN. In summary, based on the expression of both mRNA and peptide, DYN cells are located in a number of key hypothalamic regions involved in the neuroendocrine control of homeostasis in sheep.

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Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; DTT, dithiothreitol; DYN, dynorphin; ir, immunoreactive; LH, lateral hypothalamus; ME, median eminence; OT, optic tract; OVLT, organum vasculosum of the lamina terminalis; OVX, ovariectomized; PB, phosphate buffer; PBS, 0.1 M phosphate buffer with 0.9% saline; PBTX, 0.1 M phosphate buffer with 0.9% saline containing 4% normal donkey serum; POA, preoptic area; PPD, preprodynorphin; PR, progesterone receptor; PVN, paraventricular nucleus of the hypothalamus; RCA, retrochiasmatic area; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; SSC, saline–sodium citrate; TEA, triethanolamine.

Key words: sheep, estrous cycle, luteal phase, gonadotropin releasing hormone, hypothalamus.

Dynorphin A (DYN) is a member of a family of opioid peptides derived from the prodynorphin precursor. While first identified in the pituitary (Cox et al., 1975; Goldstein et al., 1979; Lowney et al., 1979), DYN has subsequently been shown to be present throughout the CNS (Goldstein and Ghazarossian, 1980; Watson et al., 1981; Weber et al., 1981; Khachaturian et al., 1982). The precursor, prodynorphin, has several possible products including DYN A 1–17, DYN A 1–13, DYN A 1–8, and DYN B. The relative concentration of preprodynorphin (PPD) products can vary in different neural tissues depending on post-translational processing specific to that area (Goldstein et al., 1979).

DYN is functionally involved in a variety of neuroendocrine systems including those mediating reproduction (Zhang et al., 2002), feeding (Baile et al., 1987), water homeostasis (Shimizu et al., 1989), lactation (Kim et al., 1997), and the stress response (Young and Lightman, 1992). In the reproductive neuroendocrine system, DYN has been implicated in mediating responses to circulating levels of endogenous gonadal steroids. Specifically, evidence has accumulated suggesting that DYN conveys the negative feedback influence of progesterone on GnRH secretion (Goodman et al., 2002) in sheep, whereas in the rat, DYN has been suggested to play a role in both the positive feedback influence of estradiol leading to the preovulatory luteinizing hormone surge (Zhang et al., 2002) and the negative feedback action of progesterone (Gallo, 1980). Consistent with the role as steroid responsive cells, DYN cells in the rat preoptic area (Simerly et al., 1996) and in the sheep preoptic area and mediobasal hypothalamus (Foradori et al., 2002) have been shown to contain progesterone receptors (PR).

Previous studies on the distribution of DYN immunoreactive perikarya and terminals have been carried out in the rat (Nakao et al., 1981; Khachaturian et al., 1982), hamster (Neal and Newman, 1989), and primate (Khachaturian et al., 1985; Abe et al., 1988). However, there have been few studies of the distribution of DYN in the sheep brain, either at the level of the peptide (Marson et al., 1987) or mRNA (Matthews et al., 1993; Iqbal et al., 2003), and those carried out have been limited in scope, focusing on the changes in PPD expression associated with stress (Matthews et al., 1993) or food restriction (Iqbal et al., 2003). The aim of the present study, therefore, was to directly compare the overall distribution of PPD mRNA and one of

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its major peptide products, DYN A 1–17, in the preoptic area and hypothalamus of breeding season ewes. To ensure the species-specific detection of DYN cell bodies, a portion of the ovine PPD was cloned using reverse transcription–polymerase chain reaction and used to produce a riboprobe for *in situ* hybridization.

EXPERIMENTAL PROCEDURES

Animals

Adult Suffolk ewes were maintained in an open barn with free access to water and fed once daily with a maintenance regimen of silage. They were moved to indoor facilities 2–3 days before experimentation. Once indoors, the animals were housed two/pen under a photoperiod similar to that occurring outdoors. All handling and experimental procedures involving animals used in this study were approved by the West Virginia University Animal Care and Use Committee which conforms to international guidelines on the ethical use of animals. Efforts were made to minimize the number of animals used and their suffering.

Experimental protocols

This study was performed during the breeding season (October through January) in 12 ewes that had demonstrated at least two normal 16–17-day estrous cycles (determined by monitoring estrous behavior with a vasectomized ram). Ewes in the mid-luteal phase (days 6–9) of the estrous cycle were used. Serum and ovaries were harvested to verify hormone levels and the presence of corpora lutea consistent with mid-luteal sheep. Based on examination of the ovaries and progesterone serum levels all ewes were judged to be in the luteal phase of the estrous cycle.

Tissue preparation

For immunocytochemistry, five animals were heparinized (two i.v. injections of 25,000 U heparin given 10 min apart), then deeply anesthetized with sodium pentobarbital (approximately 2000 mg, i.v.), and rapidly decapitated. The heads were perfused via both internal carotids with 6 l of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3) containing 0.1% sodium nitrite and 10 U/ml heparin. Following perfusion, the brain was removed and a tissue block containing the septal region, preoptic area (POA), and hypothalamus was dissected out. The tissue was stored in 4% paraformaldehyde at 4 °C overnight and then placed in 30% sucrose at 4 °C until infiltration was complete. Thick (50 μm) frozen coronal sections were cut and stored at −20 °C in a cryopreservative solution (Watson et al., 1986) until being processed immunohistochemically for DYN.

For *in situ* hybridization analysis of PPD mRNA expression, seven animals were given a lethal dose of sodium pentobarbital (2 g, i.v.), and decapitated. Following decapitation, a tissue block containing the septal region, POA, and hypothalamus was dissected out. Hypothalamic blocks were rapidly removed and placed on dry ice. After freezing, the blocks were stored at −80 °C until sectioned. Sections (20 μm) were cut on a cryostat, thaw mounted on SuperFrost plus slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at −80 °C until processed for *in situ* hybridization.

Radioimmunoassay

Progesterone was measured by duplicate aliquots of serum sample collected from each ewe just before kill using a commercially available kit (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Sensitivity of the assay averaged 0.06 ng/ml and intra- and inter-assay CV were 3.4% and 10.1%, respectively. All animals had circulating progesterone concentrations in the luteal phase range of 1.0–2.2 ng/ml.

Immunohistochemistry

DYN A was detected using a modified avidin–biotin–immunoperoxidase protocol with nickel-enhanced diaminobenzidine as chromogen (blue–black reaction product) (Adams, 1981; Estienne et al., 1990). The immunohistochemistry procedure was carried out on free-floating sections at room temperature, except for incubation with primary antibodies against DYN, which was performed at 4 °C. Sections were washed in 0.1 M PB with 0.9% saline (PBS) for several hours to remove cryoprotectant. After washing, the sections were placed in a 1% hydrogen peroxide (Sigma, St. Louis, MO, USA) for 10 min to remove endogenous peroxidase activity. The sections were then washed and incubated in PBS containing 4% normal donkey serum (Jackson Laboratories, Inc., West Grove, PA, USA) and 0.4% Triton X-100 (Sigma; PBTX) for 1 h. Sections were then incubated in rabbit polyclonal antibody against DYN A 1–17 (1:20,000; IHC 8730; Peninsula Laboratories, Inc., San Carlos, CA, USA) for 48 h in PBTX. The DYN A 1–17 antibody shows a small cross-reactivity with DYN A 1–13 (0.43%) and none with any other prodynorphin derivatives. Following incubation, sections were washed and then placed in a solution of PBSTX with biotinylated donkey anti-rabbit IgG (1:400; Jackson Laboratories, Inc.) for 1 h. The sections were washed and incubated for 1 h in avidin–biotin–HRP complex (1:400; Vector Laboratories, Burlingame, CA, USA). DYN was visualized using 3,3′-diaminobenzidine with 0.02% nickel sulfate, and 0.003% hydrogen peroxide as substrate. Immunohistochemical controls included omission of the primary antibody from the immunostaining protocol, the absence of which completely eliminated staining for the corresponding antigen.

Ovine PPD cloning

To quantify PPD mRNA expression in the sheep POA and hypothalamus by ISH we first isolated a cDNA fragment of ovine PPD using reverse transcription–polymerase chain reaction with primers specific for the 3′ region of the bovine PPD sequence (accession no. U58500; Jiang et al., 1997), bases 1719–2270. Primers used were upper: 5′-GCTCGCCTTCTGAATGCTGA-3′ and lower: 5′-CCTGGATCCACAAACCGAACC-3′. Total RNA was extracted from a fresh frozen sheep hypothalamic block collected specifically for this purpose and then 2 μg of RNA was reverse transcribed using avian myeloblastosis virus-RT (30 U; Promega, Madison, WI, USA). This was followed by PCR using *Taq* polymerase (Life Technologies, Rockville, MD, USA) for 35 cycles (Amplitron II thermocycler) with an annealing temperature of 51 °C and a 2 min extension time. A predicted 551-base pair PCR fragment was isolated from agarose (Life Technologies), ligated into the PGEM-T Easy Vector (Promega), and subcloned into JM109 competent cells (Promega). The PPD sequence was determined using T7 and SP6 primers and screened for sequence identity with the BLAST search tool (National Center for Biotechnology Information). The ovine PPD was determined to be approximately 92% homologous to the known bovine PPD sequence (U58500).

In situ hybridization

Antisense and sense RNA fragments were generated from linearized templates (2 μg) using T7 and SP6 RNA polymerases (Promega), respectively, in a transcription reaction containing: 5× transcription buffer (Promega), 5 mM rNTPS (GTP, CTP, ATP), 100 μM UTP, Rnase inhibitor (Rnasin; Promega; N211A), 100 mM dithiothreitol (DTT), and 100 μCi ^{35}S-UTP (3000 Ci/mmol; NEN) for 2 h at 37 °C. Samples were then incubated with Dnase-I (Sigma) at 37 °C for 15 min to remove template. The reaction was stopped with 0.5 EDTA. Unincorporated isotopes were removed using spin columns (Roche, Indianapolis, IN, USA). Probes were diluted in TE (Tris, EDTA; containing 10 mg/ml Torula RNA;