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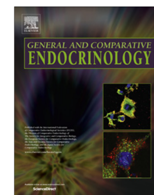

Evidence that histaminergic neurons are devoid of estrogen receptor alpha in the ewe diencephalon during the breeding season

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ABSTRACT

In sheep as in rat, it has been highly suggested that neuronal histamine (HA) participates to the estradiol (E2)-induced GnRH and LH surges, through H1 receptor. With the aim of determining if E2 could act directly on HA neurons, we examined here whether HA neurons express estrogen receptor alpha (ERα) in the ewe diencephalon during the breeding season. We first produced a specific polyclonal antibody directed against recombinant ovine histidine decarboxylase (oHDC), the HA synthesizing enzyme. Using both this anti-oHDC antibody and an anti-ERα monoclonal antibody in double label immunohistochemistry, we showed that HA neurons do not express ERα in diencephalon of ewes with different hormonal status. This result diverges from those obtained in rat, in which around three quarters of HA neurons express ERα in their nucleus. This discrepancy between these two mammal species may reflect difference in their neuronal network.

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1. Introduction

In mammals, it is well documented that induction of GnRH preovulatory surge is dependent of ovarian estradiol (E2) acting at cerebral level (Christian and Moenter, 2010). In sheep, the major site of action of E2 for the GnRH surge induction is located in the mediobasal hypothalamus (Caraty et al., 1998) in which an estrogen receptor alpha (ERα) containing cell population is widely distributed. As GnRH neurons are located in preoptic area (Caldani et al., 1988), E2 may act indirectly via interneurons to produce its effect. Nevertheless, the neural pathways whereby E2 controls GnRH release are not fully understood.

Histamine (HA) is a neurotransmitter synthesized in one step by histidine decarboxylase (HDC) from histidine. HA can affect LH secretion via a stimulation of GnRH release (for review see Knigge and Warberg, 1991). In rats, it was strongly suggested that estrogen-sensitive histaminergic neurons are involved in the GnRH and LH surges through H1 histaminergic receptors (H1R) activation (Fekete et al., 1999). In sheep, HA could also contribute to the LH preovulatory surge via H1R. Administration of diphenhydramine, an H1R antagonist, decreased the LH surge in E2-treated ovariectomized (OVX) ewes (Van Kirk et al., 1989). Alexander et al. (1995) suggested that HA modulates the E2-induced LH surge by affecting the secretion of GnRH. It might therefore be asked whether E2 could act directly on ewe histaminergic neurons or not. Tillet et al. (1998) showed that histaminergic neurons extended as a continuum from the ventral tegmental area to the caudal part of the infundibular nucleus (IN) in the ewe. The distribution of ERα in the ewe (Lehman et al., 1993) overlaps that of histaminergic neurons in a region located between the mediobasal hypothalamus (MBH) and the mammillary bodies (MB).

The aim of this study was to examine if histaminergic neurons express ERα in the ewe. We produced a rabbit polyclonal antibody directed against recombinant ovine HDC (oHDC) to label sheep histaminergic neurons and we used it to perform double labeling with an anti-ERα monoclonal antibody in the ewe diencephalon.

2. Materials and methods

Unless otherwise stated, reagents were the highest available grade from Sigma–Aldrich (Saint-Quentin Fallavier, France).

Abbreviations: GnRH, gonadotropin-releasing hormone; IN, infundibular nucleus; kDa, kilodalton; LH, luteinizing hormone; LHA, lateral hypothalamic area; MB, mammillary bodies; MBH, mediobasal hypothalamus; MR, mammillary recessus; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TRIS, 2-amino-2-hydroxymethyl-1,3-propanediol.

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2.1. Animals and experimental design

The immunization experiments for producing anti-ovine HDC antibodies were conducted using young New Zealand male rabbits.

Immunohistochemical (IHC) and in situ hybridization (ISH) experiments were carried out from mature Ile-de-France ewes ($n = 9$) treated and slaughtered during the breading season between November 1st and December 1st. The ewes were divided into three groups. In A group ($n = 3$) ewes were ovariectomized and slaughtered 14 days later. In B group ($n = 3$), ewes were treated with an intravaginal progesterone-releasing controlled internal drug release dispenser (CIDR, Inter AG, Hamilton, New Zealand). After 14 days, the progesterone implants were removed and the ewes were slaughtered 9 days later, during the mid-luteal phase. In C group ($n = 3$), ewes were treated with progesterone as in B group and were slaughtered 60 h later, during the late follicular phase or the preovulatory surge. The phase of the oestrous cycle was confirmed for ewes from B and C groups by determining LH (Montgomery et al., 1985), E2 (Ben Saïd et al., 2007) and progesterone (Saumande et al., 1985) blood levels by RIA. All animals were decapitated in an official slaughterhouse, by a licensed butcher, according to the French laws. The experimental procedures were performed in accordance with local animal regulations (Authorization No. A37801 of the French Ministry of Agriculture).

2.2. Preparation of polyclonal anti-HDC antibody

2.2.1. Cloning of a partial ovine HDC (oHDC) cDNA

Total RNA from ewe mammillary bodies was extracted according to Chomczynski and Sacchi (1987), except that two phenol:chloroform extractions were added before isopropanol precipitation (Pillon and Bruneau, 2003). Total RNA was reverse-transcribed using the Superscript II RNAse H^- reverse transcriptase (Invitrogen, Cergy Pontoise, France) in the presence of a mix of oligodT and random hexanucleotide primers. First-strand cDNA was amplified with Platinum Taq DNA polymerase (Invitrogen) as advised by the manufacturer, using a Minicycler heating block (MJ Research, VWR International, Fontenay-sous-Bois, France). The PCR program was made up of a first cycle of 95 °C for 2 min, 55 °C for 1 min and 72 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. A final step at 72 °C for 15 min was performed to ensure a complete elongation of the strands. PCR primers (5′ primer: ATGATGGAG CCTGAKGARTAC, and 3′ primer: CTTGTACTTGTCCTTGACCCA) derived from the alignment of human, rat and mouse HDC cDNA sequences (Genbank accession number M60445, M29591, AF109137, respectively) and were expected to amplify a 927 base pairs (bp) DNA fragment (not including primers). Amplified products were blunt-ended by T4 DNA polymerase treatment, and inserted into the EcoRV restriction site of the pKS bluescript vector (Stratagene, La Jolla, USA). They were sequenced and then, a consensus sequence was deduced. The 927 bp oHDC cDNA sequence shows 98% similarity with the 22–948 bp corresponding fragment of the bovine HDC (bHDC) cDNA sequence (Genbank accession number BT020686) which was included in Genbank subsequently to our work. The genbank accession number for the 927 bp oHDC is JX035810.

2.2.2. Expression of recombinant oHDC in E. coli and purification

The cDNA containing the partial coding sequence of the oHDC (o309HDC) was extracted from the pKS-oHDC between the BamHI and HindIII restrictions site of the polylinker and inserted into the same restriction sites of the pQE30 expression vector (Qiagen, Courtaboeuf, France). The resulting construct (pQE30-oHDC) was expected to encode a fusion protein of 38.7 kDa (346 aa) that contains 19 extra aminoacids (including a hexa-His-tag) at the N-terminus of the oHDC sequence. *E. coli* BL21 (DE3) transformed by pQE-oHDC were grown in Luria Broth (LB) medium with ampicillin at 37 °C until OD_{600nm} reached 0.5. Incubation was then continued for 4 h after addition of 1 mM isopropyl β-D-1-thiogalactopyranoside. Bacterial cells were harvested by centrifugation at 2000*g* for 30 min. The pellet was suspended in 20 mM TRIS–HCl, pH 8.0, 200 mM NaCl. As the protein was localized in the insoluble fraction, inclusion bodies were purified and solubilized according to Fawzi et al. (1996). Soluble proteins were purified by Ni–NTA (Qiagen). The final preparation had the expected molecular weight (SDS–PAGE, MS/MS analysis) and was >95% pure (SDS–PAGE, Coomassie blue staining). Analysis by MS/MS showed a total of eleven peptides with seven different peptides. The peptide sequences of the 116 non redundant amino acids were assigned to bHDC (gi|59857737). They represented 37.5% of the translated coding sequence. The observed yield was 60 mg/2 L culture medium.

2.2.3. Immunization of rabbit with oHDC

Six rabbits, whose preimmune serum samples were kept, received a first set of four intradermal injections of 100 μg of recombinant sheep HDC in saline solution, emulsified in complete Freund adjuvant (v/v), for the first injection, and in incomplete Freund adjuvant (v/v), for the next three injections. Two boosters of 100 μg of HDC in saline solution were administered intravenously (i.v.) after i.v. administration of 0.2 ml of 2.5% promethazine (Phenergan®, DB PHARMA, La Varenne Saint Hilaire, France) at 4 weeks intervals. Five and seven days after the second booster, blood was collected. The two rabbits, whose sera showed the best result in western blot experiments, were retained. They were given a second set of four intradermal injections of 100 μg HDC in saline solution emulsified in incomplete Freund (v/v) adjuvant followed by three i.v. boosters. The serum 573 was selected for the following experiments.

2.3. Expression of ovine recombinant aromatic amino acid decarboxylase (oAADC)

With the aim of checking that the 573 anti-oHDC antibodies do not cross-react with oAADC, we produced a recombinant fragment of oAADC containing the homologous region of the recombinant oHDC for western blot experiment. A partial cDNA containing 1011 bp of the coding sequence of the oAADC (nucleotides 178–1011, Genbank accession numbers: XM_004007692), that contains the homologous region of the partial oHDC cDNA except those coding for the first ten amino acids of oAADC was inserted into the pET28a expression vector (Novagen, VWR International SAS, Fontenay sous bois, France) between the EcoRI and Hind III restriction sites. The resulting construct (pET28a-oAADC) encoded a fusion protein of 337 amino acids (o337AADC). The conditions of growth of *E. coli* BL21 (DE3) transformed by pET28a-AADC and of induction of recombinant o337AADC were identical to that described for recombinant oHDC except that the antibiotic used was kanamycin.

2.4. Characterization of oHDC antibody

2.4.1. Characterization by Western blot

SDS–PAGE was performed according to Laemmli (1970) in a Mini-PROTEAN II apparatus (Bio-Rad Life Science, Marnes la Coquette, France). Liquid electrotransfer of the proteins from SDS–PAGE gels to hybond C+ membranes (GE Healthcare, Velizy Villacoublay, France) was performed in a mini trans-Blot cell (Bio-Rad Life Science) for around 20 h in an ice bath under 30 V. The transfer buffer was 25 mM TRIS, 192 mM glycine, 20% v/v methanol pH 8.3. Gels were stained with Biosafe Coomassie stain

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