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Sex differences in urocortin 1 dynamics in the non-preganglionic Edinger–Westphal nucleus of the rat

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ABSTRACT

Women have higher vulnerability to stress and stress-induced diseases than men. Estrogen may be involved in the control of sex-dependent stress adaptation via estrogen receptors α and β (ERα/β). Urocortin 1 (Ucn1) in the npEW plays an important role in stress adaptation. We hypothesize that the activity of npEW-Ucn1 neurons differs between sexes and is related to estrogen signalling. We here indicate by immunocytochemistry the absence of ERα and the presence of ERβ in the npEW-Ucn1 neurons. Q-RT-PCR of the npEW confirmed this notion, demonstrating that in male rats ERβ mRNA was almost 5 times higher than in females in di-estrus. Furthermore, Ucn1 mRNA in males was nearly 10 times and 1.6 times higher than in females in di- and pro-estrus, respectively, indicating a sex-dependent difference in Ucn1 biosynthetic activity. Since, at the same time, immunocytochemistry revealed that the amount of Ucn1 peptide stored in the cell bodies of the npEW-Ucn1 neurons did not differ between males and females, as judged on the basis of the number and immunosignal density of these neurons, we propose that the rate of axonal Ucn1 transport and, possibly, the strength of Ucn1 secretion, are dependent on sex to the same degree as is Ucn1 biosynthesis.

1. Introduction

Classically, in rat the endocrine responses to stressors are considered to depend on activation of the hypothalamo–pituitary–adrenal (HPA-) axis (for reviews, see Carrasco and Van de Kar, 2003; de Kloet et al., 2005; Jacobson, 2005). A midbrain nucleus proposed to play an important role in stress adaptation, is the Edinger–Westphal nucleus (EW; Weninger et al., 2000; Kozicz, 2007). The EW is traditionally known as a cholinergic, preganglionic parasympathetic nucleus projecting to the ciliary ganglion. However, more recently evidence has accumulated for the existence of an anatomically and functionally distinct, non-cholinergic neuron population within the EW (Saper et al., 1976; Loewy et al., 1978; Loewy and Saper, 1978; Burde et al., 1982; Vasconcelos et al., 2003; Ryabinin et al., 2005; Laursen and Rekling, 2006) that produces urocortin 1 (Ucn1). This 'non-preganglionic EW' (npEW; Ryabinin et al., 2005; Weitemier et al., 2005; Kozicz, 2007) is the main production site in the brain of urocortin 1 (Ucn1), a peptide belonging to the corticotropin-releasing factor (CRF) neuropeptide family and binding to the CRF2 receptor (CRF2) with a higher affinity than CRF itself (Vaughan et al., 1995; Kozicz et al., 1998, 2002; Bittencourt et al., 1999). The npEW-Ucn1 neurons have been strongly implicated in stress adaptation, because they exhibit conspicuous activity changes in response to acute and chronic stressors (Weninger et al., 2000; Gaszner et al., 2004; Korosi et al., 2005; Kozicz, 2007), Ucn1-null mice reveal an impaired HPA-axis response to repeated restraint and cold stress (Zalutskaya et al., 2007) and CRF2-deficient mice are hypersensitive to stressors (Bale et al., 2000) (for review see Kozicz, 2007).

Evidence suggests that the regulatory mechanisms underlying the stress response in animals and man are sex-dependent. In both rat and human, females seem to be more vulnerable to stress than males possibly because of a stronger negative adrenal glucocorticoid feedback (Dalla et al., 2005; Kudielka and Kirschbaum, 2005; Louvart et al., 2006). This sex difference in stress sensitivity may explain why stress-related disorders like anxiety and major depression occur about twice as often in females as in man (Weissman and Olfson, 1995; Kessler, 2003; Gorman, 2006). Also, female wildtype and CRFR2-null mice show more pronounced depressive-like behavior than males (Bale and Vale, 2003).

Estrogen may play an important role in sex-dependent stress adaptation, because stress-susceptibility is highest in periods of a low or declining estrogen titer (Seeman, 1997; Payne, 2003). For example, in rats in di-estrus, when the estrogen titer is low, anxiety- and depression-like behavior is high (Frye and Walf, 2004; Walf et al., 2009a,b). Estrogen might influence the stress response by binding two types of estrogen receptor (ER), ERα and ERβ. These receptors are closely associated with Ucn1, because their

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distributions overlap in the paraventricular and supraoptic nuclei and, as is of special relevance to this study, in the EW (Kozicz et al., 1998; Bittencourt et al., 1999; Mitra et al., 2003). Vanderhorst et al. (2005) described moderate immunostaining in the EW for ERα whereas Mitra et al. (2003) and Sheng et al. (2004) reported strong immunoreactivity in this nucleus for ERβ. Haeger et al. (2006) found that estrogen in the PVN may differentially regulate Ucn1 mRNA expression, depending on the type of estrogen receptor involved: estrogen increases Ucn1 gene transcription through ERα and decreases it via ERβ. However, the nature of the relationship between estrogen and Ucn1 in the npEW is not known.

On the basis of the observations described above, we hypothesize that the activity of the npEW-Ucn1 neurons differs between sexes and is in some way related to estrogen. To test this hypothesis, we have examined in rat (1) what type of estrogen receptor is present in the npEW, (2) if such receptor(s) occur(s) in npEW-Ucn1 neurons, (3) if the degree of expression of these receptors differs between males and females in different phases of the estrus cycle (pro-estrus, when estrogen is high and di-estrus, when estrogen is low), and (4) if the amounts of Ucn1 and of Ucn1 mRNA in the npEW-Ucn1 neurons differ between sexes and between pro- and di-estrus.

2. Materials and methods

2.1. Animals

10 male and 20 female (10 in pro-estrus, 10 in di-estrus) Wistar-R Amsterdam rats were housed in standard plastic cages (40 cm × 25 cm × 20 cm; 5/cage), in a temperature- and humidity-controlled environment, on a 12-h light/12-h dark cycle (lights on 6:00 a.m.). They were allowed *ad libitum* to access tap water and rodent chows. After sacrifice, vaginal smears were taken to determine the phase of the estrus cycle. All procedures were conducted at the Anatomy Department of Pécs University, Pécs, Hungary, in accordance with the Declaration of Helsinki and the animal use guidelines of the Medical Faculty Advisory Committee for Animal Resources of Pécs University, based on the Law of 1998, XXVIII, for Animal Care and Use in Hungary. We did everything to minimize the number of animals used and their suffering.

2.2. Fixation

Rats were deeply anaesthetized with nembutal (100 mg/kg body weight), their chest cavity opened, and perfused transcardially with 50 ml 0.1 M sodium-phosphate-buffered saline (PBS; pH 7.4) followed by 250 ml of 4% ice-cold paraformaldehyde with 2% acrolein. After perfusion, animals were decapitated and their brains removed, postfixed for 24 h at 4 °C, and stored in autoclaved 0.1 M PBS, at 4 °C. Then they were transferred into 30% sucrose in 0.1 M PBS, and when completely submerged, frozen on dry ice. Thirty micrometer-thick coronal sections at the level of the superior colliculus (Bregma −5.0 to −7.0 mm; see Paxinos and Watson, 2001) were saved in sterile antifreeze solution, at −20 °C, until further processing. All subsequent staining procedures were carried out simultaneously, to assure that sections of all experimental groups were treated in the same way.

2.3. Immunocytochemistry

After 4× 15 min rinses in 0.1 M PBS, sections were consecutively treated with 1% sodium borohydride, for 30 min, 0.1 M PBS (15 min rinse) and 0.5% Triton X-100 (Sigma–Aldrich, Zwijndrecht, The Netherlands) in 0.1 M PBS, for 30 min, to facilitate antigen penetration. After an additional 15 min rinse in 0.1 M PBS, sections were incubated in 2% normal goat serum (#005-000-121; Jackson Immunoresearch Labs, West Grove, PA, USA) in 0.1 M PBS, for 30 min, to block non-specific binding sites. Sections were incubated in polyclonal (rabbit) Ucn1-antiserum (1:30,000; gift from Dr. W.W. Vale, The Salk Institute, San Diego, CA, USA), ERα-antiserum (1:5000; H-222; gift from Dr. G. Greene, Ben May Department for Cancer Research, University of Chicago, Chicago, IL, USA) or ERβ-antiserum (1:2000; #80424; gift from Dr. S.E. Alves, Merck Research Laboratories, Rahway, NJ, USA), in 2% normal goat serum, for 48 h at 4 °C, followed by 3× 15 min rinses in 0.1 M PBS, and incubated in secondary anti-rabbit antiserum (Vector ABC Elite Kit; PK-6101; Vector Labs, Burlingame, CA, USA) for 1 h. After a 3× 15 min rinses in 0.1 M PBS, sections were incubated in ABC reagent supplied with the ABC Elite Kits (Vector Labs), for 1 h. Immunostaining was visualized with 10 mg 3,3′-diaminobenzidine (DAB; D 5637; Sigma–Aldrich) in 50 ml Tris buffer (TBS; pH 7.6), for 10 min. The reaction was controlled under a stereomicroscope, and stopped in Tris buffer.

For immunofluorescent labeling, after 4× 15 min rinses in 0.1 M PBS, sections were treated with 1% sodium borohydride, for 30 min, and 0.5% Triton X-100 in 0.1 M PBS, for 30 min, and then incubated in 2% normal donkey serum (NDS) in 0.1 M PBS, for 30 min, and in (a mixture of) primary antisera in 2% NDS, for 48 h at 4 °C. Antisera used were polyclonal (goat) anti-Ucn1 (R-20; sc-1825; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:250, and polyclonal (rabbit) anti-ERβ (1:3,000). The secondary antisera Cy^2-conjugated anti-goat IgG (1:80) and Cy^3-conjugated anti-rabbit IgG (1:100) (Jackson Immunoresearch Labs) were applied for 3 h. Following several rinses in 0.1 M PBS, sections were mounted on glass slides, coverslipped with anti-fade Vectashield (Vector Labs) and studied with a Leica Microsystems TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

2.4. Antisera characterizations

Both Ucn1 sera have been generated against whole rat Ucn1 (AA 1–40). The high specificity of each serum has been confirmed by preadsorption with synthetic Ucn1 (0.1 and 1.0 μg/ml diluted antiserum), which completely abolished immunoreactivity in the rat midbrain (for details see Bittencourt et al., 1999; Turnbull et al., 1999). The ERα-antiserum has been raised against a peptide consisting of AA 586–600 of the rat estrogen receptor (C1355, synthesized by Macromolecular Resources, Fort Collins, CO, USA). The peptide was conjugated with keyhole limpet hemocyanin, and injected into rabbits. The high binding specificity of the resulting polyclonal antiserum was demonstrated by its ability to recognize authentic *in vitro* translated ER, binding to recombinant transfected ER and to a ca. 65 kDa immature rat uterine cytosolic protein that was also recognized by a previously defined anti-estrogen receptor antiserum to the hinge regions of the rat ER (ER715; for details see Furlow et al., 1990; Friend et al., 1997). For the development of the ERβ-antiserum, rabbits had been immunized with the peptide (EARSLEHTLPVNRETLKRK)-8-MAP, representing AA 64–82 of ERβ-485 (accession no. U57439; spanning exons 2 and 3); serum had been collected and titrated by Research Genetics (Huntsville, AL, USA). Antisera had been affinity-purified over Amino-Link columns (Pierce Chemical, Rockford, IL, USA) conjugated with the immunogen, and eluted with 0.1 M glycine (pH 3.0). Eluates had been dialyzed against PBS and kept frozen in aliquots at −80 °C. Previous Western blot analyses showed that the ERβ 485 antiserum recognizes a protein that migrates at about 60 kDa on SF9 cell blots, at 55 kDa on human ovary and testes blots, and at 70 kDa on whole tissue extracts of rat and mouse brain. The serum labels cos-7 cells transfected with ERβ and labeling is abolished both in these cells and in the mouse hippocampus by preadsorption with the