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DISTRIBUTION OF RETINOIC ACID RECEPTOR-α IMMUNOREACTIVITY IN THE HUMAN HYPOTHALAMUS

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Abstract—Retinoids, a family of molecules that is derived from vitamin A, are involved in a complex signaling pathway that regulates gene expression and controls neuronal differentiation in the central nervous system. The physiological actions of retinoids are mainly mediated by retinoic acid receptors. Here we describe the distribution of retinoic acid receptor α (RARα) in the human hypothalamus by immunohistochemistry. RARα immunoreactivity showed a widespread pattern throughout the hypothalamus, with high density in the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), supraoptic nucleus (SON), infundibular nucleus and medial mamillary nucleus. No staining was observed in the sexually dimorphic nucleus of preoptic area, tuberomamillary nucleus and lateral hypothalamic area. RARα was co-localized with vasopressin (AVP) neurons in the SCN, PVN and SON, and co-localized with corticotropin releasing hormone (CRH) neurons in the PVN. These findings provide a neurobiological basis for the participation of retinoids in the regulation of various hypothalamic functions. As shown earlier, the co-localization of RARα in CRH neurons suggests that retinoids might directly modulate the hypothalamus-pituitary-adrenal axis in the PVN, which may have implications for the stress response and its involvement in mood disorders. Functional studies in the other sites of RARα localization have to follow in the future.

Key words: RARα, hypothalamus, immunohistochemisty, AVP, CRH.

Retinoic acid, a biologically active derivative of vitamin A, plays a crucial role in the development and homeostasis of vertebrates (Chambon, 1996). Retinoids exert their pleiotropic effects through retinoic acid receptors (RARs), which belong to the superfamily of steroid/thyroid hormone receptors (Chambon, 1996; Lane and Bailey, 2005). Three subtypes of RARs, encoded by distinct genes, have so far been identified and characterized: RARα, RARβ and RARγ (Germain et al., 2006; Bremner and McCaffery, 2008). Different subtypes of RARs can form heterodimers with retinoid X receptors and retinoid target genes by binding to the Retinoic acid (RA) Response elements in the promoter region (Lane and Bailey, 2005; Germain et al., 2006). The wide distribution of RARs in mammals corresponds with their many different roles in physiological and pathological processes (Dolle et al., 1990; Leroy et al., 1991; Ruberte et al., 1991; Giguere, 1994).

RARα, the first discovered subtype of the RARs, is widely distributed, including in the brain, skin, muscle, heart and kidney (Giguere et al., 1987; Petkovich et al., 1987; Leroy et al., 1991). In agreement with its distribution, RARα has been reported to regulate epidermal cell growth and differentiation and is involved in various skin disorders, such as psoriasis and cancer (Darwiche et al., 1996; Torma et al., 2000; Hatoum et al., 2001). In addition, RARα plays an important role in renal development, renal injury and many renal diseases (Argiles et al., 1994; Mendelsohn et al., 1999; Hara et al., 2001; Liebler et al., 2004; Schaier et al., 2004; Yamauchi et al., 2005). RARα also regulates muscle cell migration, differentiation and proliferation (Halevy and Lerman, 1993; Neuville et al., 1999; Day et al., 2006). Moreover, RARα is expressed in several regions of the rodent brain, such as the cortex and hippocampus, and the function of RARα in these regions has been extensively studied (Krezel et al., 1999; Zetterstrom et al., 1999; McCaffery et al., 2006; Chai et al., 2009).

However, the expression and function of RARα in the hypothalamus has been rarely studied, and no study is available on the distribution of RARα in the human brain. Our previous study showed the expression of RARα in the paraventricular nucleus (PVN) of the human hypothalamus and its involvement in affective disorders (Chen et al., 2009). In the present study, we systematically investigated RARα expression in human hypothalamus by immunohistochemistry as a prerequisite for future studies on the role of retinoids in the adult hypothalamus in health and disease.

EXPERIMENTAL PROCEDURES

Subjects

In the present study we used five human hypothalami obtained *via* the rapid autopsy procedure of the Netherlands Brain Bank (NBB) at the Netherlands Institute for Neuroscience (NIN). Permission was obtained for a brain autopsy and for the use of human brain

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Abbreviations: AVP, vasopressin; CRH, corticotropin releasing hormone; DBB, diagonal band of Broca; DMN, dorsomedial nucleus; FO, fornix; INF, infundibular nucleus; ir, immunoreactive; MMN, medial mamillary nucleus; NBM, nucleus basalis of Meynert; NTL, lateral tuberal nucleus; OT, optic tract; OX, optic chiasma; PVN, paraventricular nucleus; PeVN, periventricular nucleus; RA, Retinoic acid; RARα, retinoic acid receptor α; SCN, suprachiasmatic nucleus; SDN, sexually dimorphic nucleus of preoptic area; SON, supraoptic nucleus; TBST, triton X-100; TMN, tuberomamillary nucleus; VMN, ventromedial nucleus; 3V, third ventricle.

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Table 1. Clinico-pathological data

Number	NBB number	Gender	Age (y)	PMD (h:min)	Fixation (d)	Cause of death
1	98-090	M	58	41:00	72	Sudden death, cardiac failure
2	98-028	F	91	NA	49	Lung embolism with deep venous thrombosis; extensive metastasized adeno-carcinoma of the pancreas
3	96-085	M	84	9:00	30	Heart failure by uremia
4	99-116	M	78	4:20	43	Pancreatic cancer
5	01-011	F	46	10:25	35	Multi organ failure (toxic cause not excluded), pneumonia

NBB number, Netherlands Brain Bank number; PMD, postmortem dalay; Fixation, fixation time; M, male; F, female; NA, not available.

material and clinical information for research purposes. Clinico-pathological data are presented in Table 1.

Histology

The hypothalamus was fixed in formalin and embedded in paraffin. Serial 6 μm coronal sections were made from the hypothalamus from the level of the lamina terminalis to the mamillary bodies. For anatomical orientation, every 100th hypothalamic section was collected and mounted on Superfrost plus slides (Menzel, Germany) and subsequently dried for at least 2 days at 37 °C, followed by a Nissl staining (0.5% thionine in distilled water).

Western blot

The protein samples of human hypothalamus and BE(2)-C cell (a human neuroblastoma cell line) lysate were run on a 10% SDS polyacrylamide gel and then were transferred onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA). Membranes were blocked for 1 h at 37 °C in PBS-Tween buffer (0.01 M PBS and 0.05% Tween-20) containing 5% non-fat milk and incubated for 2 h at room temperature with the primary antibody diluted in PBS-Tween buffer with 0.5% non-fat milk. Immunoblotting was performed using an anti-RARα antibody (SC-551, Santa Cruz, CA, USA). As a negative control, another blot from the same run containing the same human hypothalamus sample also was blocked but incubated in PBS-Tween buffer without primary antibody. After washing, membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega, Madison, WI, USA). After rinsing, subsequent detection was performed using the ECL Western blot system (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) according to the manufacturer's instruction.

RAR-α immunohistochemistry

The paraffin sections were dewaxed, hydrated, rinsed in TBS (Tris buffer saline: 0.05 M Tris, 0.9% NaCl, pH 7.6) for 10 min and treated with 0.3% hydrogen peroxide in TBS for 30 min to quench endogenous peroxidase activity. After being washed in TBS (3×10 min), the sections were treated with microwaves (700W) in 0.05 M citrate buffered saline (pH 6.0) for 2×10 min for antigen retrieval. The sections were then blocked with 5% goat serum in TBS with 0.3% triton x-100 (TBST) for 30 min at 37 °C. Tissues were incubated with a polyclonal rabbit anti-RAR-α (raised against a peptide mapping at the C-terminus of RARα1 and RARα2 of human origin, dilution, 1:100; SC-551, Santa Cruz Biotechnology, Santa Cruz, CA, USA; for specificity tests see the Discussion section) in TBST with 5% goat serum for 1 h at 37 °C and then overnight at 4 °C. The next day all sections were washed in TBS and exposed to a biotinylated secondary goat anti-rabbit antibody (dilution, 1:200; Vector Laboratories, Burlingame, CA, USA) in TBST with 5% goat serum for 1 h at 37 °C. Following washes in TBS, sections were incubated in the avidin-biotin complex (dilution, 1:200; Vector Laboratories, Burlingame, CA, USA) in TBST for 1 h. Finally the sections were exposed to the substrate, 0.05% 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) in TBS with 0.03% H_2O_2 for 5 min. After washing with TBS, sections were dehydrated and cleared in increasing concentrations of ethanol and finally xylene, coverslipped with neutral balsam (Sinopharm Chemical Reagent, Shanghai, China).

Immunofluorescence double staining and confocal laser scanning microscopy

To further characterize the RARα-immunoreactive (ir) cells in the suprachiasmatic nucleus (SCN), PVN and supraoptic nucleus (SON), we performed immunofluorescence double staining of RARα-vasopressin (AVP) and RARα-corticotropin releasing hormone (CRH), respectively. Hypothalamic sections were pretreated as described above in the RARα immunocytochemical staining (rehydrated and antigen retrieved). After washing in TBS, sections were incubated for 30 min in 0.5% triton X-100 to permeabilize the tissue. Sections were then blocked with 5% goat serum in TBST for 30 min at 37 °C to reduce nonspecific binding. For double staining of RARα-AVP, sections were incubated for 1 h at 37 °C with polyclonal rabbit anti-RARα (1:100) and monoclonal mouse anti-AVP [(VP III-D-7) at 1:100 dilution, which recognizes Phe in position 3 as the most important determinant in the AVP ring (a generous gift from Dr. A. Silverman to Dr. F. W. Van Leeuwen, Netherlands Institute for Brain Research, Amsterdam, The Netherlands; for details, see Hou-Yu et al. (1982) and Wu et al. (2006); only processed AVP can be recognized by this antibody; de Bree and Burbach, 1994] diluted in TBST with 5% goat serum and then incubated overnight at 4 °C. The next day the sections were washed and incubated with FITC-labeled goat anti-rabbit antibody (1:200, Southern Biotech, Birmingham, AL, USA) and biotinylated anti-mouse antibody (1:200, Vector) diluted in TBST with 5% goat serum for 1 h at 37 °C. For double staining of RARα-CRH, sections were incubated for 1 h at 37 °C with polyclonal rabbit anti-RARα (1:100) and monoclonal rat anti-CRH "PFU83" [(IgG2a subclass) at dilution of 1:1000, which is directed at the C-terminal part (amino acids 38-39) of rat/human CRH protein (kindly donated by Prof. FJ H Tilders, Vrije Universiteit, Amsterdam, The Netherlands); for details see Bao et al. (2005); a radioimmunoassay has proved it to be highly specific, with no significant binding of pituitary hormones and hypothalamic peptides other than CRH (van Oers et al., 1989); moreover, the specificity of this antibody has been confirmed by our previous studies by a spot blot test and solid phase pre-adsorption (Raadsheer et al., 1993)] diluted in TBST with 5% goat serum and then incubated overnight at 4 °C. The next day the sections were washed and incubated with FITC-labeled goat anti-rabbit antibody (1:200, Southern Biotech, Birmingham, AL, USA) and biotinylated anti-rat antibody (1:200, Vector) diluted in TBST with 5% goat serum for 1 h at 37 °C. After washing, the sections were incubated with Cy3-labeled streptavidin (1:500, Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) for 30 min at 37 °C. Finally, sections were rinsed in TBS, coverslipped with glycerin and analyzed on a Zeiss 510 confocal laser scanning microscope (CLSM)