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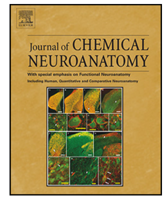

The rat vomeronasal organ is a vitamin D target

A. Rodewald*, V.M. Gebhart, H. Oehring, G.F. Jirikowski

Institute of Anatomy II, University Hospital, Friedrich Schiller University, Jena, Germany

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ABSTRACT

We studied the expression of vitamin D receptor and of vitamin D binding protein in the rat vomeronasal organ. With immunofluorescence, in situ hybridization and with reverse transcriptase PCR we found both proteins in sensory as well as in non-sensory cells. Sensory neurons contained immunoreactivity for vitamin D3 receptor in nuclei, in portions of the cytoplasm, and in apical dendrites and their microvilli. Vitamin D binding protein was observed in sensory neuron axons and cytoplasm, mostly confined to dendrites. Colocalization appeared in the contact zone of supporting cells and sensory dendrites. Both proteins were also found in single ciliated cells within the non-sensory epithelium. Vitamin D binding protein was also localized in secretory vesicles in a portion of the vomeronasal glands. Our findings suggest that the rat vomeronasal organ is a vitamin D target.

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1. Introduction

The vitamin D (1α,25-dihydroxycholecalciferol) receptor (VDR) belongs to the family of nuclear steroid receptors (Mangelsdorf et al., 1995). VDR was found in a large variety of tissues with immunocytochemistry, in situ hybridization and with autoradiography (Wang et al., 2012; Yang et al., 2012). The biologically active form of vitamin D, calcitriol (VD3), performs its transcriptional functions through VDR. Several studies documented that VD3 may be a neurosteroid (Harms et al., 2011) important for brain development (Harms et al., 2011; McGrath et al., 2004). VDR was found in the rat olfactory mucosa and olfactory bulb (Glaser et al., 1999). Parts of the hypothalamus and of the limbic system express VDR, in part colocalized with oxytocin (Prufer and Jirikowski, 1997; Prufer et al., 1999) and it has been proposed that VD3 is involved in the control of limbic functions and in human affective disorders (Armstrong et al., 2006; Ganji et al., 2010; Jorde et al., 2006). Almost 90% of serum vitamin D is bound to Vitamin D binding protein (DBP) (Bikle et al., 1986; Speeckaert et al., 2006), a 55 kDa protein that is mostly synthetized and liberated in liver (Speeckaert et al., 2006; Cooke and David, 1985). DBP expression has been shown to occur also in specific regions of the rat brain (Jirikowski et al., 2009).

In mammals the vomeronasal organ (VNO) has been suspected to be the main target of steroid pheromones thus triggering social behaviors (Clancy et al., 1984; Holy et al., 2000; Leypold et al., 2002; Luo and Katz, 2004). The VNO is located bilaterally at the lower portion of the nasal septum. It hosts the vomeronasal duct (VND) which is medially lined by the sensory epithelium (SE) and laterally by the non-sensory epithelium (NSE). The SE contains sensory neurons, basal cells, supporting cells and intraepithelial capillaries (Barrios et al., 2014; Breipohl et al., 1979; Dennis et al., 2003; Hofer et al., 2000). Sensory neurons extend their dendrites towards the lumen of the VND. They form protrusions that extend microvilli which have been termed knoblike dendritic endings (Hofer et al., 2000) since they share some similarities with the olfactory knobs which host cilia with membrane associated olfactory receptors. The NSE contains several cell types: Dark, light and pale non-ciliated cells, and ciliated columnar cells (Elgayar et al., 2014). Vomeronasal glands are located in the submucosal layer of the NSE, their excretory ducts feed into the VND (Adams and Wiekamp, 1984; Doving and Trotier, 1998). Sensory cells in olfactory mucosa and in the VNO express the olfactory marker protein (OMP) (Bock et al., 2009; Farbman and Margolis, 1980; Fleischer et al., 2006). The functional properties of this 19 kDa protein are still mostly unknown. It is used as a molecular marker for olfactory neurons and their projections in different species (Barrios et al., 2014; Berghard et al., 1996; Smith et al., 2011).

In previous studies we observed the expression of sex hormone binding globulin and of corticosteroid binding globulin in the rat VNO (Dolz et al., 2013; Ploss et al., 2014a, 2014b). We also demonstrated a small OMP-positive group of cells in the NSE (Rodewald et al., 2016). Experiments in lizards indicated that

* Corresponding author at: Institut für Anatomie II, Universitätsklinikum Jena, Teichgraben 7, 07743 Jena, Germany.
E-mail address: Andrea.Rodewald@med.uni-jena.de (A. Rodewald).

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provitamin D_3 (7-Dehydrocholesterol) has pheromone like properties (Martin and Lopez, 2006).

The current study seeks to examine the distribution of VDR and DBP in the rat VNO. In situ hybridization with bromodeoxyurdine (BrdU) labelled synthetic oligo nucleotide probes complementary to DBP encoding transcripts and RT–PCR of RNA extracts from tissue homogenates were performed to assess the intrinsic expression of DBP and VDR.

2. Materials and methods

2.1. Tissue preparation

Adult Wistar rats (male n = 4, females n = 4) were killed by prolonged ether anesthesia followed by cardiac perfusion with 4% formaldehyde in PBS (0,1 M sodium phosphate buffer pH 7.2, containing 0.9% NaCl). Tissue samples containing the VNO were rapidly dissected and postfixed in the same fixative. Samples were dehydrated through ascending ethanol series and embedded in EPON 812 (Sigma-Aldrich, München, Germany). Serial semithin sections (1 μm) were cut on a Reichert Ultracut microtome, mounted onto APES-coated glass slides (Thermo Scientific, Braunschweig, Germany) and air dried.

2.2. Electron microscopy

EPON embedded tissue block containing the VNO were brought to size (approximately 1 × 1 mm) and cut with low angle diamond knives (DIATOME, Biel, Switzerland) into serial ultrathin sections (70 nm, Reichert Ultracut). Sections were collected onto Formvar coated nickel grids (H9 SPEC, Science Services, Munich, Germany) and contrasted with 1% uranyl acetate in H2O (10 min at RT) followed by freshly prepared 2% aqueous lead citrate solution. Sections were examined in an EM 902 (ZEISS, Oberkochen, Germany) at 80 kV.

2.3. Immunohistochemistry

Epoxy resin was removed by incubation in 10% sodium methoxide, methanol/benzene 1:1 and two times in acetone for two minutes each and afterwards rinsed in Tris buffered saline (TBS). The sections were preincubated in 2% normal donkey serum in TBS at room temperature (RT) for 30 min to block non-specific reactions. Concentrated bovine serum albumin BSA-c™ 10% (Aurion, Wageningen, Netherlands) was diluted to 0,1% and added to all antibodies to block further non-specific reactions. After rinsing in TBS sections were incubated for a specific double immunostaining with goat anti-VDR (Chemicon Temecula, USA) diluted 1:500 in TBS and rabbit anti-DBP (Atlas Antibodies, Stockholm, Sweden) diluted 1:200 in TBS in a humid chamber at 4 °C overnight. After washing in TBS sections were incubated in the dark with donkey Alexa Fluor 568 labelled anti-goat IgG (Invitrogen, Karlsruhe, Germany) diluted 1:200 in TBS and Alexa Fluor 488 labelled anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) at RT for 60 min. Sections were mounted with Mowiol containing 2% DAPI (AppliChem, Darmstadt, Germany) and examined with an Olympus BX 50 microscope equipped with epifluorescence illumination (filter combinations WU and WIBA). An Olympus DP10 camera was used for microphotography. Immunohistochemical negative controls were performed with normal goat or normal rabbit serum instead of the specific primary antibodies. All controls were unstained. Positive controls for DBP were exemplarily performed with rat liver sections to verify specific staining (results not shown). For a specific double staining with rabbit anti-DBP diluted 1:200 in PBS and goat anti-OMP (Wako chemicals, USA) diluted 1:500 in PBS the sections were incubated in a humid chamber at 4 °C overnight. After rinsing in PBS sections were incubated in the dark with Fab Cy3 labelled anti-goat IgG (Molecular Probes, Eugene, USA) and Alexa Fluor 488 labelled anti-rabbit IgG diluted 1:200 in PBS at RT for 60 min. Controls were performed with the respective normal serum instead of the specific antibody and with nuclear stain (Hoechst) diluted 1:1000 in PBS at RT for 15 min.

2.4. In situ hybridization

A synthetic 25 mer oligonucleotide probe complementary to DBP encoding mRNA was designed as previously described (Jirikowski et al., 2009) (Bioscientia, Jena). The probe was labelled by 3′ tailing with 5′bromo-’2-deoxyuridine (BrdU) (Sigma) with terminal transferase (TTT Kit, Boehringer Mannheim). For details of this method see (Jirikowski et al., 1989). Epoxy resin was removed from semithin sections as described above prior to hybridization with the BrdU labelled probe (10pM in hybridization buffer, (Omnibuff, Wack Chemie) 1 h at 37 °C in a humid chamber). For details on the sequence of the DBP probe and the hybridization procedure see reference (Jirikowski et al., 2009). The respective sense probe was used for control purposes. After hybridization sections were washed in PBS and incubated with a mouse monoclonal antibody to BrdU, diluted 1:200 in PBS (Progen, Heidelberg, Germany) over night at 4 °C. After washing in PBS, sections were incubated with a goat anti-mouse IgG, labelled with Alexa Fluor 488 (Molecular Probes), diluted in PBS 1:200. After another washing step, sections were subjected to fluorescence microscopy as described above.

2.5. Reverse transcriptase PCR

Rats were killed by prolonged ether anesthesia and decapitation. VNOs were rapidly dissected. Total RNA was isolated by TRIzol® extraction. Samples were collected in reaction tubes with 1 ml TRIzol® and homogenized mechanically (Ultraturrax T3) followed by sonication. Homogenized samples were incubated for 5 min at RT. 0.2 ml chloroform was added and the tubes were shaken by hand for 15 s. After an incubation for 3 min at RT samples were centrifuged at 12000 x g for 15 min at 4 °C. The aqueous phase, containing the isolated RNA, was placed into a new tube. For RNA precipitation samples were mixed with 0.5 ml of 100% isopropanol, incubated for 10 min at RT and then again centrifuged at 12000 x g for 10 min at 4 °C. Supernatant was removed from the tube. The pellet was resuspended by briefly vortexing in 1 ml 75% ethanol. After centrifugation (7500 x g, 5 min, 4 °C) the supernatant was discarded. The air-dried pellet was resuspended in 50 μl of RNase-free water and incubated in a heat block at 55 °C for 15 min.

Reverse transcription and PCR were conducted with QIAGEN OneStep RT-PCR Kit. The used primer sets for VDR, DBP as well as β-actin, are available from Biomol GmbH, Germany. The following primer sequences for VDR (rat) were used: forward primer: 5′- TGA AGG CTG CAA AGG TTT CT-3′ and reverse primer: 5′-TAG CTT GGG CCT CAG ACT GT-3′ with a product size of 250 bp. For DBP (rat) we used the following primers: forward primer: 5′-TAC TTC ATG CCA ACT GCT GA-3′ and reverse primer: 5′-AGG GTT TTC AGG GTC GTA TC-3′ with a product size of 179 bp. The primers for the housekeeping gene β-actin (rat) were: forward primer: 5′- CAC ACT GTG CCC ATC TAT GA-3′ and reverse primer: 5′- CCG ATA GTG ATG ACC TGA CC-3′ with a product size of 272 bp.

The cycling conditions (Biometra T3 Thermocycler) included 50 °C for 30 min for the reverse transcription and 95 °C for 15 min

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