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Localization of sex hormone binding globulin in the rat vomeronasal organ

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ABSTRACT

Volatile and non-volatile derivates of gonadal steroids are known to act as pheromones in many mammalian species. Pheromones have multiple effects on the brain via the olfactory system. Their primary port of entry seems to be the vomeronasal organ (VNO) but the underlying cellular and molecular mechanisms are unclear so far. Recently we localized sex hormone binding globulin (SHBG) in both the main and the accessory olfactory system of rat with immunocytochemistry and RT-PCR. The accessory olfactory system consisting of VNO and accessory olfactory bulb showed high expression of SHBG.

In the present paper we studied SHBG expression in the VNO in greater detail. In semithin sections we found SHBG immunostaining in the perinuclear cytoplasm of some of the sensory neurons, in sensory cilia and in their axons. A portion of the basal cells and some of the goblet cells in the non-sensory epithelium showed intense SHBG staining. SHBG was abundant in exocrine cells of the vomeronasal glands, perhaps compartimentalized in secretory vesicles. In situ hybridization revealed specific signals in sensory and non-sensory cells of the VNO. Our findings indicate that SHBG expressed in the VNO may be liberated into nasal secretions to bind aerosolic steroids. SHBG in sensory cells may be involved in signaling actions of pheromones.

Introduction

In most mammals species-specific and gender-specific olfactory stimuli, often referred to as pheromones, are known to act powerfully on social and sexual behaviours. Aerosolic gonadal steroid hormones and their derivates excreted in urine, sweat or other discharges play a decisive role in triggering behavioural and endocrine changes when detected by the main and the accessory olfactory system (Achiraman et al., 2010; Beckman, 2002; Bensafi et al., 2003, 2004; Brennan and Kendrick, 2006; Brennan and Keverne, 2004; Jacob and McClintock, 2000; Jacob et al., 2001, 2002; Keverne, 2005).

Olfactory sensory cells are located in the main olfactory epithelium (MOE) within the nasal mucosa and in the vomeronasal organ (VNO) also termed Jacobson's organ, described first in domestic animals in the 1810s (Jacobson et al., 1998). In most mammalian species the VNO is found at the medial base of the nasal cavity forming the vomeronasal duct (VND), a blind tube that is surrounded by an aggregation of seromucous glands and a vein plexus. The muscular layer of these blood vessels is rather thick, thought to support a pump mechanism for transport of fluids within the VND (Meredith et al., 1980). The VND is lined with two different epithelia: The sensory epithelium (SE) containing sensory neurons, basal cells and support cells and the non-sensory epithelium (NSE) consisting of ciliated cells, goblet cells and basal cells. Sensory cells extend their axons through the lamina cribrosa of the ethmoidal bone to the accessory olfactory bulb (AOB) to form synapses with dendrites of mitral cells in olfactory glomeruli (Halpern and Martínez-Marcos, 2007; for review see Mucignat-Caretta, 2010).

Effects of gonadal steroids on behaviour and on endocrine regulation via the olfactory system are well known for a long time: "Vandenberg effect" describes the acceleration of sexual maturation in female mice upon sensing androgens (Vandenbergh, 1973). Smelling androgens from a foreign male interrupts pregnancy in certain rodents (Bruce and Parrott, 1960). "Whitten effect", the blockade of estrus cycle in mice held in groups seems to be triggered by oestrogen effects on the VNO (Whitten, 1959). Olfactory sensory cells are devoid of nuclear receptors for gonadal

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steroid hormones. Effects of olfactory steroids are usually fast, indicating non genomic effects perhaps mediated through membrane receptors. Known olfactory receptors do not seem to have binding properties for steroids (Alekseyenko et al., 2006; for review see Zufall and Leinders-Zufall, 2007).

In a previous study we described the expression of corticosteroid binding globulin (CBG) in rat olfactory system (Dölz et al., 2013). Recently we observed localization and production of sex hormone binding globulin (SHBG) in MOE, main olfactory bulb (MOB), VNO and in AOB (Ploß et al., 2014). SHBG is a serum glycoprotein mostly expressed in liver with high affinity for gonadal steroids. Systemic SHBG is thought to buffer systemic estrogens and androgens (Caldwell et al., 2006). We observed the intrinsic expression of SHBG in other organs including brain and pituitary indicating its involvement in rapid "non-genomic effects" of steroids (Caldwell and Jirikowski, 2014). Given the functional importance of the VNO for central responses to olfactory steroids (for review see Kelliher, 2007) we now took a closer look at the distribution of SHBG via immunostaining and the localization of the hybridization product of SHBG mRNA in VNO structures of male and female rats.

Materials and methods

Tissue preparation

Animal experiments were performed in accordance with German law (protocol # 02-040/10, approved by Thuringian state government). Adult male ($n = 3$) and female ($n = 3$) Wistar rats (bw ca. 250 g) were sacrificed by prolonged ether anaesthesia followed by cardiac perfusion with 4% paraformaldehyde in PBS (0.1 M sodium phosphate buffer pH 7.2, containing 0.9% NaCl). We dissected the medial portion of the nasal mucosa and postfixed it at 4° C overnight in the same fixative. After fixation, tissue samples were rinsed in PBS. Tissue blocks containing the VNO were dissected with the aid of a stereo microscope (Leitz, Wetzlar, Germany). Samples were then dehydrated through ascending ethanol series and embedded in Epon. Acetonitrile was used as intermedium. Polymerization was performed at 60° C for three days. Serial semithin (1 μm) sections were cut with a Reichert Ultracut microtome and mounted onto glass slides (Superfrost, Merck, Darmstadt, Germany).

Immunohistochemistry

Epon resin was removed by rinsing semithin sections in 10% sodium methoxide (2 min at RT), followed by incubating in methanol/benzene 1:1 (2 min at RT), 2x acetone 2 min each. Thereafter we washed consecutive sections in PBS and incubated them with rabbit anti-SHBG (Becchis et al., 1996) diluted 1:500 in PBS at 4 °C in a humid chamber overnight. After washing sections in PBS for 30 min they were incubated with anti-rabbit IgG diluted 1:200 in PBS (Sigma–Aldrich, Munich, Germany) for 1 h at RT followed by washing in PBS. Peroxidase-anti-peroxidase complex (rabbit-PAP, Sigma–Aldrich, Munich, Germany) was used at a dilution of 1:200 in PBS, 1 h at RT. Immunoprecipitates were stained with DAB and H_2O_2 (Sigma FAST kit) for 5 min. Incubations with rabbit normal serum instead of the primary antibody were performed as immunocytochemical controls. Stained slides were dehydrated through ascending ethanol series and mounted with Entellan® (Merck, Darmstadt, Germany).

In situ hybridization

Synthetic oligonucleotide probes (Department of Virology, FSU, Jena, Germany) complementary to a fragment of mRNA encoding rat SHBG were labelled by 3′ tailing with 5′-bromo-2-deoxyuridine (BrdU) as described earlier (Jirikowski et al., 1989). The sequence of the antisense probe (design of probe according to EMBL Database, Heidelberg, Germany) was ACG TGT AGG AAT TTA GGG CCT CT, taken from the SHBG cDNA sequence (Reventos et al., 1988). The complementary BrdU labelled sense probe was used for control hybridizations. Epoxy resin was removed from the semithin sections as described above but omitting the rehydration step. Labelled probes were applied in hybridization buffer (Omnibuff, Wack Chemie, Germany), 10 pM/ml. Incubation was performed at 38 °C for 2 h. Then sections were incubated with a mouse monoclonal antibody to BrdU (Progen, Heidelberg, Germany) diluted 1:400 in PBS overnight at 4 °C. Immunoprecipitates were stained with anti-mouse IgG diluted 1:100 in PBS followed by incubation with mouse PAP complex (both for 90 min at RT). Sections were again stained with DAB and H_2O_2. Hybridized sections were dehydrated, cover-slipped with Entellan® as described above.

Microscopic evaluation was performed with an Olympus BX 50 photomicroscope equipped with phase contrast illumination and a DP10 camera.

Results

Epon embedding and light microscopic histochemistry of semithin sections allows for optimal preservation of cellular and subcellular structures and high resolution localization of cytoplasmic reaction product. We observed specific immunoreactivity for SHBG in a variety of cells throughout the VNO. Within the sensory epithelium SHBG immunostaining was most pronounced in apical dendrites, some of them in close apposition (Fig. 1, black arrow) to capillaries (Fig. 1, black asterisk). Olfactory cilia also showed immunostaining for SHBG (Fig. 1, white arrow). The perinuclear cytoplasm of some of the sensory neurons contained SHBG staining (Fig. 1, white asterisk). Other cell types within the sensory epithelium including basal cells and supporting cells were SHBG negative.

The vomeronasal glands in the periphery of the VND contained abundant SHBG immunoreactivity. This staining was confined to large cytoplasmic structures within exocrine cells suggesting localization in secretory vesicles (Fig. 2, black arrows). The upper portion of cilia of the respiratory epithelium of the nasal mucosa showed SHBG immunoreactivity (Fig. 3, black arrow), whereas the cytoplasm of these cells was SHBG negative.

Short cilia of NSE cells lining the VND on the opposite side of the sensory epithelium were stained for SHBG (Fig. 4). Granulated SHBG positive precipitates were found in some of the goblet cells and in basal cells of the NSE (arrow).

Immunostaining for BrdU revealed hybridization signal of SHBG encoding transcripts in the perinuclear cytoplasm or in nuclei of some of the sensory cells. Some of these showed SHBG hybridization signal in nuclei and in cytoplasm (Fig. 5). BrdU staining was also found in goblet cells, in basal cells and in exocrine cells of the VNO. Control sections hybridized with the sense probe were devoid of BrdU immunoreactivity.

There were no apparent gender differences in the distribution of SHBG expression in the samples studied.

Discussion

Steroid binding globulins SHBG and CBG seem to be expressed in the olfactory system (Dölz et al., 2013; Ploß et al., 2014). The VNO is likely a prominent source of steroid binding globulins in the olfactory system as indicated by our immunohistochemical observations. Transcription and translation of SHBG probably occurs in both the main and the accessory olfactory system as indicated by the presence of SHBG encoding mRNA as shown by in situ hybridization and by RT-PCR (Ploß et al., 2014). This intrinsic expression may be independent from systemic SHBG known to originate from liver.

We could not observe clear gender differences in the distribution of SHBG in the VNO although gonadal steroids were shown to influence the development of a sexual dimorphic VNO neonatally and maintain its anatomical structure during adulthood (Alekseyenko et al., 2006; Guillamón and Segovia, 1997). Since the current study did not employ quantitative methods, possible sex differences may have escaped our observation. On the other hand it is known that SHBG binds both estrogens and androgens (Caldwell et al., 2006) and the cellular and molecular mechanisms of steroid targeting in the VNO may be similar in both sexes. The possibility of triggering alternating SHBG levels in the VNO by steroid hormones and their functional importance has yet to be determined.

There may be different functional properties of SHBG in sensory and in non-sensory cells of the VNO. SHBG expressed in goblet cells and in exocrine cells of the vomeronasal glands is most likely subject to secretion into the VND. SHBG staining patterns in these cells suggests confinement to secretory granules. This assumption