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Sex hormone binding globulin in the rat olfactory system

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ABSTRACT

Ovarian steroids are known to act on the olfactory system. Their mode of action, however, is mostly unclear to date since nuclear receptors are lacking in sensory neurons. Here we used immunocytochemistry and RT-PCR to study expression and distribution of sex hormone binding globulin (SHBG) in the rat olfactory system. Single sensory cells in the olfactory mucosa and their projections in the olfactory bulb showed specific SHBG immunostaining as determined by double immunofluorescence with olfactory marker protein OMP. Larger groups of SHBG stained sensory cells occurred in the vomeronasal organ (VNO). A portion of the olfactory glomeruli in the accessory olfactory bulb showed large networks of SHBG positive nerve fibres. Some of the mitral cells showed SHBG immune fluorescence. RT-PCR revealed SHBG encoding mRNA in the olfactory mucosa, in the VNO and in the olfactory bulbs indicating intrinsic expression of the binding globulin. The VNO and its related projections within the limbic system are known to be sensitive to gonadal steroid hormones. We conclude that SHBG may be of functional importance for rapid effects of olfactory steroids on limbic functions including the control of reproductive behaviours through pheromones.

1. Introduction

Olfactory sensory neurons are located in the main olfactory epithelium (MOE), in the vomeronasal organ (VNO) and in the septal organ of Masera (SOM, for review see Zufall and Leinders-Zufall, 2007; Mendoza, 1993; Axel, 1995; Bhatnagar and Meisami, 1998). Areas of sensory cells are surrounded by respiratory epithelium which lines most of the nasal cavities. Sensory cells of the MOE and the SOM extend their axons through the lamina cribrosa to the main olfactory bulb (MOB) to form synapse complexes with mitral cell dendrites, the olfactory glomeruli (Vassar et al., 1994). In rodents, most VNO projections are found in the accessory olfactory bulbs (AOB), which are closely linked to the limbic system (Jia and Halpern, 1996; Witt and Wozniak, 2006). Olfactory neurons seem to be capable of life long regeneration from basal cells (Bedini et al., 1976; Graziadei and Graziadei, 1979; Costanzo, 1991). Mature olfactory sensory cells express a specific olfactory marker protein (OMP) which is lacking in the basal precursor cells (Hartman and Margolis, 1975).

Numerous olfactory receptor proteins have been characterized and localized in membranes of sensory neuron cilia which protrude into the nasal cavity for binding olfactory ligands dissolved in the nasal secretion. Specific olfactory receptors (V1R, V2R, V3R) have been identified in the VNO (Buck and Axel, 1991; Breer, 1993; Mendoza, 1993; Ressler et al., 1993; Vassar et al., 1993; Thürauf et al., 1996; Miyamichi et al., 2005). Gonadal steroid hormones and their derivates are known to act as powerful olfactory ligands (Bensafi et al., 2004; Savic et al., 2005; Berglund et al., 2006; Treyer et al., 2006; Kline et al., 2007; Wyart et al., 2007; Savic and Lindstrom, 2008). Destruction of the VNO results in impairment of sexual behaviour and altered stress response in rodents (Berliner et al., 1996; Keverne, 2004). Interestingly, none of the olfactory receptors described so far seems to have steroid binding properties in spite of their well-established sensitivity to steroids (Robinson et al., 1998; Celsi et al., 2012). Oestrogen function is mediated through well-characterized nuclear receptors (ER-α and ER-β); however, such receptors have not been found in olfactory sensory cells so far (Alekseyenko et al., 2006). Steroid binding globulins and their putative receptors are thought to be involved in rapid steroid effects (for review see Caldwell and Jirikowski, 2014). In the previous study we found the expression of corticosteroid binding globulin (CBG) in the rat olfactory system (Dölz et al., 2013). Sex hormone binding globulin (SHBG) is a homodimeric glycoprotein normally expressed in liver to buffer systemic gonadal steroid levels. We found SHBG and the respective mRNA in the hypothalamic neurosecretory nuclei and in pituitary (Herbert et al., 2006) and it is likely that hypothalamic SHBG is involved in steroid dependent neuroendocrine regulation (Caldwell et al., 2006). The current study examines localization,

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distribution and mRNA expression of SHBG in the rat olfactory system. Immunofluorescence for olfactory marker protein (OMP) was used to identify sensory neurons.

2. Materials and methods

2.1. Tissue preparation

We performed animal experiments in accordance with German law (protocols were approved by Thuringian state government, protocol # 02-040/10). Adult intact male rats ($n = 8$, 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). Two rats received intracerebroventricular injections with colchicine 24 h prior to sacrifice (for details see Möpert et al., 2006). Brains and nasal mucosa were dissected and the MOB, the AOB, the MOE and the VNO were postfixed over night at 4 °C in the same fixative. After washing in PBS, tissue blocks of the MOB and the AOB were cut with a Vibratome (LKB, München, Germany) into 50 μm serial sections. Samples of the MOE and the VNO were immunostained without further sectioning. Another set of tissue samples as described above was dehydrated through ascending ethanol series and embedded in EPON. Acetonitril was used as intermedium, polymerization was performed at 60 °C for three days. Serial semithin sections (1 μm) were cut with a Reichert Ultracut microtome and collected on glass slides (Superfrost, Merck, Darmstadt, Germany). For biochemical experiments formaldehyde fixation was omitted. Tissue samples from the MOB, AOB, MOE, VNO and hypothalamus were dissected and rapidly frozen in liquid nitrogen (LN_2).

2.2. Immunohistochemistry

EPON resin was removed from semithin sections by incubation in 10% sodium methoxide (2 min at RT), followed by rinsing in methanol/benzene 1:1 (2 min at RT), 2× acetone 2 min each. After washing in PBS, consecutive sections were incubated with either rabbit anti-SHBG (Becchis et al., 1996) or with goat anti-OMP, (Chemicon, Temecula, USA). Both antibodies were diluted 1:1000 in PBS, incubation was done overnight at 4 °C in a humid chamber. After washing in PBS, the sections were incubated with anti-rabbit IgG or anti-goat IgG diluted 1:200 in PBS (Sigma–Aldrich, Munich, Germany) for 1 h at RT. Peroxidase–anti-peroxidase complex (rabbit-PAP or goat-PAP, Sigma–Aldrich, Munich, Germany) were 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 3 min. Sections were then dehydrated through ascending ethanol series before mounting them with Entellan® (Merck, Darmstadt, Germany). Sections were evaluated with an Olympus BX 50 photomicroscope with interference contrast illumination.

Immunofluorescence was used for staining vibratome sections and whole mount preparations. Sections were incubated with a mixture of rabbit anti-SHBG and goat anti-OMP as described above, both diluted 1:1000 in PBS, over night in the refrigerator. After washing in PBS, the sections were treated with Alexa Fluor 488 labelled anti rabbit IgG (donkey) and with Alexa Fluor 568 anti-goat IgG (donkey), diluted with 1:200 in PBS, for 1.5 h in the dark. After washing in PBS, the sections and mucosa samples were mounted with Fluoromount (Reagents obtained from Invitrogen/Molecular Probes, Eugen, Oregon, USA) and evaluated with an Olympus BX50 microscope with epifluorescence illumination. Combination of wide band filters (Olympus – U-MWU, excitation wave length 400 nm) was used for simultaneous visualization of both immunoreactivities. Immunohistochemical controls were carried out with the respective normal sera instead of the specific primary antibodies.

2.3. RNA extraction and PCR

RNA was isolated by TRIzol® extraction. 1 ml of TRIzol® (Thermo Fisher Scientific Inc., München, Germany) was added to the samples and homogenized by sonication, followed by incubation for 5 min at RT. The tubes were shaken by hand for 15 s after adding 0.2 ml chloroform (Roth, Karlsruhe, Germany). Samples were again incubated for 3 min at RT and then centrifuged at 12 000 × g for 15 min at 4 °C. The aqueous phase, containing RNA was moved into a new tube. To precipitate RNA 0.5 ml isopropanol was added, followed by 10 min incubation at RT. Thereafter, samples were centrifuged for 10 min at 12 000 × g at 4 °C. After discarding the supernatant 1 ml of 75% ethanol was added and the pellet was resuspended by brief vortexing. After a final centrifugation at 7500 × g for 5 min at 4 °C the supernatant was removed and the pellet air-dried. Total RNA was dissolved in 50 μl of RNase-free water and incubated for 15 min at 55 °C.

For reverse transcription and PCR the QIAGEN OneStep RT-PCR Kit was used. Primer sets for SHBG and β-actin were purchased from Biomol GmbH, Germany. Primer sequences SHBG (rat) were: SHBG – forward primer: 5′-CCA AAC GGT GGT TCT GTC TT-3′ and – reverse primer: 5′-TAA AGC CCC AAG GGA GAG AT-3′ with a amplification product size of 208 bp and β-actin – 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.

Reverse transcription was conducted at 50 °C for 30 min. PCR was run in an T3 Thermocycler (Biometra, Göttingen, Germany) under the following conditions: The initial activation step was 95 °C for 15 min followed by three-step cycling of 94 °C for 30 s, 53 °C for 45 s and 72 °C for 1 min (35 cycles), and a final extension step of 72 °C for 10 min. Final PCR product was separated on an agarose-gel (1.2%, including ethidium bromide) via electrophoresis (110 V, 60 min). Visualization of the amplification product was performed under ultraviolet light. For estimation of PCR product size a Standard DNA size marker was used (Low range DNA ladder, Jena Bioscience, Germany).

3. Results

Immunofluorescence for SHBG was found in scattered cells throughout the main olfactory epithelium. These cells showed in addition immunofluorescence for OMP (Fig. 1). Most of the OMP stained cells did not stain for SHBG. Single OMP- and SHBG-positive axons were found in olfactory glomeruli in the MOB. The majority of the OMP positive neuronal processes within the glomeruli were devoid of SHBG immunostaining (Fig. 2). Thin SHBG positive but OMP negative fibres occurred in MOB glomeruli. Groups of SHBG positive cells and their projections were observed in whole mount preparations of the VNO (Fig. 3). Numerous SHBG positive neuronal processes were found in glomeruli within the AOB. Most of these fibres showed also immunofluorescence for OMP (Fig. 4); however, some of the processes seemed to stain for SHBG only. Semithin sections through the VNO revealed numerous sensory neurons with immunoperoxidase staining for OMP (Fig. 5a). Subsequent semithin sections showed SHBG immunoreactivity mostly confined to apical dendrites (Fig. 5b) and to axons while the perinuclear cytoplasm of sensory cells remained mostly unstained. Other cell types of the MOE and the VNO including goblet cells and basal cells were OMP negative. Some of these cells showed week immunoreactivity for SHBG. Higher magnifications of semithin sections showed concentrations of SHBG immune precipitate in apical dendrites and in olfactory knobs while olfactory cilia remained unstained (Fig. 5c). Single mitral cells and some tufted cells in the MOB showed intense SHBG immunofluorescence in the perinuclear cytoplasm and in processes (Fig. 6). Larger groups of SHBG positive neuronal perikarya were found in the mitral cell layer of the AOB (Fig. 7). After colchicine treatment cytoplasmic SHBG immunostaining of olfactory sensory cells in the MOE, in the VNO and of mitral cells was increased. Sensory neurons contained numerous SHBG positive cytoplasmic inclusion bodies (Fig. 8) while apical dendrites and axons were unstained.

Ethidium bromide staining of agarose gels showed specific amplification product of SHBG encoding transcripts after RT-PCR. Clearly visible bands at the expected size of ca. 200 bp were seen in cDNA preparations derived from RNA extracts from the MOB, the AOB, the VNO and the MOE. Hypothalamus extracts served as positive controls. PCR runs without template showed no reaction product (Fig. 9). Internal controls for PCR with β-actin contained clearly detectable amplification product of approximately 280 bp in all samples.

4. Discussion

The present findings provide evidence for intrinsic expression of SHBG in the rat olfactory system. A small fraction of sensory cells scattered throughout the MOE showed SHBG immunoreactivity as determined by double labelling with OMP. In the VNO SHBG positive sensory neurons were accumulated in groups of cells. Whether non-sensory cells like basal cells or goblet cells express SHBG is the topic of more detailed histochemical studies. In sensory cells SHBG staining was mostly confined to apical dendrites, to olfactory knobs and to axons indicating high turnover of the steroid binding globulin. SHBG seems to be subject to axoplasmic and dendritic transport since colchicine treatment resulted in aggregation of perinuclear immunostaining and a depletion of staining in processes. The appearance of SHBG stained