Androgen Receptor Distribution, PAS and Alcyan Blue Reaction in the Vomeronasal Organ and the Nasal Septum Mucosa of the Developing Male Rat

Distribución del Receptor de Andrógeno, Positividad a PAS y Alcyan Blue en el Órgano Vomeronasal y la Mucosa del Tabique Nasal Durante el Desarrollo de la Rata Macho

Núñez Chichet, M E.; Genovese, P. & Bielli, A.

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SUMMARY: The vomeronasal organ (VNO) is a tubular chemoreceptive organ which detects environmental pheromones and is essential for mammalian normal reproductive activity. A sensitive chemoreceptive neuroepithelium lines the concave VNO wall and the opposite, convex wall is lined by a pseudostratified, receptor-free epithelium. The secretion of the Jacobson glands (JG) -tubuloacinary glands lying in the lamina propria of the VNO - is essential for pheromones contacting vomeronasal organ chemoreceptors. Tubuloacinary glands lying in the connective tissue of the nasal septum mucosa (Bowman glands), can be classified as ventral and dorsal, according to their location. Positivity to PAS and Alcyan blue reactions and androgen receptor immunolocalization was evaluated with a semiquantitative system (0, 1, 2 or 3 crosses) in JG, ventral Bowman glands (VBG), dorsal Bowman glands (DBG), sensitive chemoreceptive neuroepithelium (Ne), receptor-free epithelium (RFE) and nasal septum respiratory epithelium (RE) histological slides of male rats at 5, 15, 25 and 35 days of age. The VBG and JG were intensely positive to PAS reaction since 5 days of age, and weakly positive to Alcyan blue reaction (AB) since 15 days of age, but not at 5 days of age. Both JG and VBG were strongly positive to androgen receptor immunolocalization at all ages studied (15, 25 and 35 days of age). DBG were always negative to both PAS and AB reactions. The NE and the RFE and nasal mucosa luminal epithelium were negative. To our knowledge, this is the first report of androgen receptor (AR) immunolocalization in the vomeronasal organ and the nasal septum mucosa. The present results also suggest an influence of androgens on the mechanism of pheromones contacting with the Ne of the VNO through regulation of the Jacobson glands secretion.

KEY WORDS: Vomeronasal organ; Chemoreception; Development; Histochemistry; Immunohistochemistry.

INTRODUCTION

The vomeronasal organ (VNO) is a chemoreceptive organ which detects environmental pheromones and is essential for mammalian normal reproductive activity (Keverne, 2004). It is tube-shaped, lies at both sides of the ventral nasal septum within a hard bony and cartilaginous capsule that lies at the base of the nasal septum (Wysocki, 1979) and its lumen is crescent-shaped (Vacarezza *et al.*, 1981). A sensitive chemoreceptive microvillar neuroepithelium (Ne) lines the concave VNO wall and the opposite, convex wall is lined by a ciliated pseudostratified, receptor-free epithelium (RFE). The VNO varies in structure lengthwise (Vacarezza *et al.*) and can be divided along its longitudinal axis into four regions. Jacobson glands (JG) are located in the connective tissue underlying the RFE (Taniguchi

et al., 1996). The secretion of the JG is essential for pheromones contacting VNO chemoreceptors (Eccles, 1982; Mendoza, 1986; Taniguchi *et al.*, 1993). Bowman glands (BG) are similar tubuloacinary glands lying in the connective tissue of the nasal septum mucosa (Taniguchi *et al.*, 1996).

Schiff's periodic acid (PAS) stain and Alcyan Blue (AB) stain are histochemical methods widely used to demonstrate mucous secretions. The PAS technique oxidizes 1,2-glycols into aldehyde groups, which then react with the Schiff reagent producing a magenta stain. Neutral polysaccharides are positive to PAS stain. On the other hand, AB technique evidences weakly acid sulfated mucinous substances producing a cyan stain (Merck KGaA, 2004).

Oikawa *et al.* (1994) found chinchilla's (*Chinchilla laniger*) JG are positive to PAS reaction and negative to AB reaction. The morphology and histochemistry of the septal glands that empty into the nasal cavity has been described as either serous, mucous, or seromucous in nature (Roslinski *et al.*, 2000). Alcyan blue and PAS reaction positivity varies between species: all JG are positive to PAS reaction in rats older than 7 days (Garrosa, 1986) whereas JG are negative to AB stain in pre and postnatal rats. However, Bowman glands are positive to AB reaction from 7 days of age on (Garrosa). Human JG but neither *Microcebus murinus* nor *Microtus* spp. are positive to AB reaction (Roslinski *et al.*). However, JG are positive to PAS reaction in all three species. In humans, the dorsal acini of the BG (DBG) are negative to PAS reaction (Katz & Merzel, 1977), but most acini are PAS positive in the ventral BG (VBG) and in all JG. Consequently, there seems to be some degree of regional specialization in the secretion of JG and BG, with differences between species.

On the other hand, although the ontogeny of PAS and AB reaction has been described for rat JG, no reports exist regarding rat BG PAS or AB reaction. Furthermore, none of the previous papers described what region was sampled in spite that it is well established that the VNO's four anatomical regions differ widely in structure.

Androgens are essential for normal male reproduction and development. Androgen receptor (AR) is a member of the steroid and nuclear receptor protein superfamily (Freedman, 1998). Androgen receptor is a soluble protein that is present in the cytoplasm linked to heat shock proteins and upon agonist binding, it translocates to the nucleus (Gao *et al.*, 2005). Thus, AR location indicates sites of androgen action. No description exists regarding AR localization in the VNO and the nasal septum mucosa.

The objective of the present work was to study PAS positivity, AB positivity and AR immunolocalization in the VNO most caudal region and the neighboring nasal septum mucosa during postnatal life and puberty in the male rat.

MATERIAL AND METHOD

Animals and sampling procedures. All procedures described below were carried out following rules described by the Committee of Animal Ethical Management of the Veterinary Faculty of Uruguay. Five Sprague-Dawley virgin 2-month-old female rats were mated by the same male. After parturition, pups were interchanged to arrange five 8-pups litters, where at least 7 pups were males. Pups (total n=56)

were weaned at 20 days of age and fed thereafter with standard rat chow. Pups were weighed every other day and slaughtered (n= 7) at 5, 15, 25 and 35 days of age. Rats were decapitated, their snouts cut out, skin and superficial soft tissues trimmed away, and the remaining sample was immersion-fixed in 4% buffered formaline, pH = 7.2. After 7 days fixation, samples were decalcified in 14% ethilen diamino tetra acetic acid solution until snout bones became flexible. Samples were then sectioned so that the VNO was divided into four pieces of similar length. The caudalmost piece was dehydrated in increasingly concentrated ethanol solutions and embedded into paraffin wax. Samples were sectioned serially from the caudal end of the VNO. When VNO lumina became patent in the resulting sections, fifteen 7 µm-thick histological sections per animal were mounted on sylanised glass slides (5 sections/slide). The first 5 sections per animal were used to study AR localization, the next 5 sections to study PAS reaction localization and the last 5 sections to study AB localization.

To study PAS positivity, sections were oxidized in 1% periodic acid solution (1g sodium periodate, distilled water up to 100 mL), rinsed in distilled water, immersed in Schiff's stain for 10 min., rinsed again and stained with Meyer's haematoxylin, dehydrated, cleared and mounted.

To study Alcyan Blue positivity, sections were stained with Alcyan Blue solution (1.0 g Alcyan Blue 8GX stain in 100 mL, 0.1N chlorhydric acid) for 30 min., dried with filter paper, dehydrated, cleared and mounted. In order to study AR localization, antigens in sylanised sections were exposed by humid heat incubation in 0,3% buffer citrate solution (pH=7.5), treated with a mouse primary antibody (AR441, Dako Cytomation, Carpinteria, CA, USA) anti human AR diluted 1:15, and developed with a streptavidin-biotin commercial kit (LSAB+, Dako Cytomation, Carpinteria, CA, USA). Positive substances were evidenced with 5 min DAB stain (diamino bencidine) incubation. A slide which was not treated with the primary antibody was used as a negative control.

Histological slides were evaluated blindly by two experienced observers with a semiquantitative system (0, 1, 2 or 3 crosses) in a light microscope (Olympus BX 50, Tokyo, Japan) at 400x. Staining positivity was evaluated in 6 histological structures: JG, VBG, DBG, VNO neuroepithelium (Ne), VNO receptor-free epithelium (RE) and nasal septum respiratory epithelium (NRE) for all three techniques used.

Body weights were expressed as means $\pm$ SD. Body weight differences between ages were studied by repeated measures Anova with differences at $P \leq 0.05$.

RESULTS

Body weights at slaughter day increased along the experiment (5 days of age: 16.49 ± 0.84 ; 15 days of age: 29.24 ± 1.98 ; 25 days of age: 674.29 ± 7.45 ; 35 days of age: 104.33 ± 6.56). Staining results are shown in Table I.

PAS reaction (Figs. 2. A, D, G, J, M).

Both JGs and VBGs were positive to PAS reaction but staining was more intense in VBGs. PAS reaction intensity increased slightly from 5 to 15 days of age but did not change later. Dorsal Bowman glands were negative. The NRE was positive (goblet cells). The VNO's Ne showed positive material only in its luminal surface. The VNO's RFE was negative except for a few positive epithelial cells which looked similar to goblet cells.

Alcyan Blue reaction. (Figs. 2. B, E, H, K, N).

Both JGs and VBGs were mildly positive to AB technique but DBGs were negative. Alcian blue reaction intensity increased slightly from 5 to 15 days of age both in VBG and JG but did not change later. The NRE was intensely positive to AB reaction at all ages studied (in goblet cells) although it was slightly weaker at 5 days of age than later. The VNO's Ne and RFE showed material positive to AB reaction only on their luminal surfaces. Such material showed stronger positivity on the RFE surface.

Androgen receptor immunolocalization (Figs. 2. C, F, I, L, O).

Several slides belonging to the 5 days of age group were wiped off. Consequently, the entire group was eliminated for AR detection. Both JGs and VBGs were strongly positive to AR immunolocalization at all ages studied (15, 25 and 35 days of age). Dorsal Bowman glands were weakly positive. The VNO's Ne and RFE and the NRE were negative.

Fig. 1. The nasal septum of the rat at the VNO's caudalmost region. Location of the VNO and its luminal epithelia, neighboring glands and NRE.

Fig. 2. Ventral Bowman's glands and nasal respiratory epithelium in rats: Fig. A.- 15 days, PAS-Haematoxylin technique (arrow head) = VBG, (*) = NRE, n = nasal cavity; Fig. B.- 5 days, AB technique; Fig. C.- 25 days, AR immunopositivity. Dorsal Bowman's glands and nasal respiratory epithelium in rats: Fig. D.- 35 days, PAS-Haematoxylin technique, (arrow head) = DBG, (*) = nasal respiratory epithelium, c = cartilage; Fig. E.- 15 days, AR immunopositivity; Fig. F.- 25 days, AB technique. Jacobson's glands in rats: Fig. G.- 5 days, PAS-Haematoxylin technique, (arrow head) = JG, (b)= blood vessels; Fig. H.- 25 days, AR immunopositivity; Fig. I.- 15 days, AB technique. Vomeronasal neuroepithelium of the vomeronasal organ in rats: Fig. J.- 25 days, Ne, PAS-Haematoxylin technique, L = lumen of the VNO; Fig. K.- 5 days, AB technique; Fig. L.- 35 days, AR immunopositivity. Receptor-free epithelium of the vomeronasal organ in rats: Fig. M.- 5 days, PAS-Haematoxylin technique, (**) = RFE; Fig. N.- 25 days, AB technique; Fig. O.- 35 days, with AR immunopositivity. Bar = 10 µm.

Table I. Distribution of PAS and AB reaction positivity and immunolocalization (AR) of androgen receptor in VNO's Ne, surface of Ne, RFE, surface of RFE, JG, DBG and VBG and in the NRE of the septum in rats of 5, 15, 25 and 35 days of age. IHC = immunohistochemistry.

AGE (days)	Technique	DBG	VBG	JG	NRE	VNO			
						Ne		RFE	
						Epithelium	Surface	Epithelium	Surface
5 (n= 6)	PAS	0	3	1	2	0	1	T	1
15 (n= 6)	PAS	0	2	2	2	0	2	1	1
25 (n= 6)	PAS	0	3	2	2	0	2	2	1
35 (n= 6)	PAS	0	3	2	2	0	2	3	1
5 (n= 6)	AB	0	0	1	2	0	1	0	2
15 (n= 6)	AB	0	1	2	3	0	1	0	2
25 (n= 6)	AB	0	2	2	3	0	1	0	2
35 (n= 6)	AB	0	1	2	3	0	1	0	2
15 (n= 6)	AR IHC	1	2.5	2	0	0	0	0	0
25 (n= 6)	AR IHC	1	2.5	3	0	0	0	0	0
35 (n= 6)	AR IHC	1	2	2	0	0	0	0	0

DISCUSSION

PAS reaction. The pattern we found for PAS reaction positivity in VBG and JG has already been reported for rats (Garrosa *et al.*) and humans (Roslinski *et al.*). The pattern we found in DBG is similar to what was found in humans (Roslinski *et al.*) but has not been reported for rats previously. Furthermore, the slight layer of AB- and PAS- positive material we found coating the VNO's Ne and the RFE has already been reported for *Microtus* and *Microcebus* (and suggested as a characteristic of the luminal environment) and for humans, where secretions were most copious (Roslinski *et al.*).

Alcian Blue reaction. Our results show weak AB reaction in VBG and JG at 15, 25 and 35 days of age, and not at 5 days of age, whereas Garrosa *et al.* found no AB stain positivity in pre and postnatal rats. Roslinski *et al.* found AB reaction in human JG but not in *Microcebus murinus* and *Microtus* spp. The AB stain positivity we found in the VNO epithelial apical surfaces (both Ne and RFE) is probably caused by dispersion of JG's secretion. The VBG acini showed heterogeneous PAS and AB positivities indicating that at least in developing rats, most acini are seromucous in nature. We did not find any purely mucous acini. The increase with age in glands positivity to PAS and AB reactions is probably due to increases in volume or changes in the composition of their secretion. Both possibilities suggest changes in VNO function as rats grow. The PAS and AB reactions positivity in the surface of both VNO's epithelia seems to be caused by a thin layer of JG's secretion, since no positive structures were seen deeper within the epithelia.

Roslinski *et al.* observed that the RFE had unicellular mucous glands and cilia (both AB and PAS-positive), similar to respiratory epithelia, but did not find such cells in *Microcebus murinus* nor in *Microtus* spp. We have found PAS positive cylindrical cells in the rat RFE. This positivity increases as rats grow, at least until 35 days of age. Although such PAS positive cells have not been found either in *Microcebus murinus* or in *Microtus* spp they might be similar to the PAS positive cells found by Roslinski in humans. The differences in AB and PAS reaction between VNO's RFE and NRE suggest differences in secretion composition between such epithelia. Similar differences in secretion composition can be inferred between dorsal and ventral BG considering AB and PAS reactions, although such differences appear later in life (15 days of age) for AB stain. Androgen receptor immunolocalization To our knowledge, this is the first report of AR immunolocalization in the VNO and the nasal septum mucosa. The VNO is important for pheromone detection and thus for reproductive activity. We would expect some influence of androgens on VNO activity. Androgen exposure history determines reactivity to pheromone exposure (Dominguez-Salazar *et al.*, 2002). Muroi *et al.* (2006) have recently demonstrated a competitive effect of androgen on pheromone detection in mice. The fact that AR has not been detected in the VNO Ne suggests that in case androgen influences pheromone detection in rats it exerts its action at some other site. Meredith had suggested one of such sites is probably the central nervous system. Another site where such influences might act are the JG, since their secretion is necessary for pheromones reaching VNO Ne

and the positivity of JG epithelial cells to AR indicates their activity is androgen dependent. The uneven distribution of AR in BGs suggests regional specialization, with VBGs situated closer to the VNO being similar to JG and different in AR sensitivity to DBG, whose sensitivity to androgens seems to be very slight if any at all. In conclusion, we have described the postnatal ontology of PAS and AB reaction in the caudal VNO and nasal septum mucosa and have reported for the first time AR immunolocalization in these organs. The present results suggest differences in secretion composition between VNO's RFE and NRE, and also suggest

an influence of androgens on the mechanism of pheromones contacting with the VNO Ne through regulation of the JG secretion.

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NÚÑEZ CHICHET, M.E.; GENOVESE, P. & BIELLI, A. Distribución del receptor de andrógeno, positividad a PAS y Alcian Blue en el órgano vomeronasal y la mucosa del tabique nasal durante el desarrollo de la rata macho. *Int. J. Morphol.*, 25(3):579-585, 2007.

RESUMEN: El órgano vomeronasal (VNO) es un órgano quimiorreceptor, tubular, que detecta feromonas ambientales. Es esencial para la actividad reproductiva normal de los mamíferos. El VNO está formado por un neuroepitelio quimiosensible (pared cóncava, Ne) y un epitelio pseudoestratificado (epitelio libre de receptores: RFE) en la pared opuesta, convexa. La secreción de las glándulas tubuloacinares de Jacobson (JG), ubicadas en la lámina propia del VNO, son esenciales para que las feromonas entren en contacto con el Ne. Las glándulas tubuloacinares ubicadas en la mucosa del tabique nasal (glándulas del Bowman), se pueden clasificar como ventrales (VBG) y dorsales (DBG), según su localización. La positividad a las técnicas de PAS, Azul de Alcian (AB) e inmunolocalización del receptor de andrógeno (AR), fueron evaluadas con un sistema semicuantitativo (0, 1, 2 o 3 cruces) en láminas histológicas de JG, VBG, DBG, Ne, RFE y del epitelio del tabique nasal (RE) en ratas macho de 5, 15, 25 y 35 días de edad. Las VBG y JG fueron intensamente positivas a la reacción de PAS desde los 5 días de edad y débilmente positivas a la reacción de AB desde los 15 días de edad, pero no a los 5 días de la edad. Las JG y VBG fueron fuertemente positivas a la inmunolocalización del AR en las edades estudiadas (15, 25 y 35 días de edad). Las DBG fueron siempre negativas a las reacciones de PAS y AB. El NE, el RFE y el RE fueron negativos. Según nuestro conocimiento, éste es el primer informe de inmunolocalización del AR en el VNO y la mucosa del tabique nasal. Los actuales resultados sugieren una influencia de los andrógenos en el mecanismo por el cual entran en contacto las feromonas y el Ne, a través de la regulación por andrógenos de la secreción de las JG.

PALABRAS CLAVE: Órgano vomeronasal; Quimiorrepción; Desarrollo; Histoquímica; Inmunohistoquímica.

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Correspondence to:
Dra. María Eugenia Núñez Chichet
Area of Histology and Embryology
Dept. of Morphology and Development
Veterinary Faculty
Lasplaces 1550
Montevideo, - URUGUAY

Tel.: +598 2 622 29 33, Fax.: +598 2 622 64 13.

Email: maeugenu@gmail.com

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