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Distribution of the vanilloid (capsaicin) receptor type 1 in the rat ovaries on different sexual development period

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SUMMARY

Vanilloid receptor (VR1) is expressed in the central nervous system as well as in various non-neuronal cells (mast cells, keratinocytes and epithelial cells). The VR1 expression in rat ovaries according to the sexual development period was investigated in the present study by immunohistochemistry. Ovaries were removed after euthanasia from pubertal female Sprague Dawley rats (42 day old), from post-pubertal females (56 day old) and from adult females (70 day old) (n = 10 in each group) and tissue samples were processed for conventional histology and for immunohistochemistry using the Streptavidin-Biotin Peroxidase method and a rabbit polyclonal anti-VR1 primary antibody. Serum gonadotropins and sexual steroid concentrations were determined in parallel by commercial ELISA kits. Whatever the sexual development state, the VR1 expression was evidenced in the cytoplasm of various ovarian cell types (granulosa, interstitial, theca and luteal cells) with a moderate intensity albeit the protein accumulation was lower in the theca follicle cells. Furthermore the VR1 ovarian expression seems to be independent from a hormonal control. These results demonstrate the constitutive VR1 expression in rat ovaries.

Keywords: Rat, ovary, vanilloid receptor, immunohistochemistry, sexual development, gonadotropins, sexual steroids.

RÉSUMÉ

Distribution du récepteur de type 1 de la vanilloïde (capsaicine) dans les ovaires de rat en fonction du stade de développement sexuel

Le récepteur de la vanilloïde (VR1) est exprimé dans le système nerveux central ainsi que dans diverses cellules non neuronales (mastocytes, kératinocytes et cellules épithéliales). Dans cette étude, l'expression du VR1 a été recherchée par immunohistochimie dans les ovaires de rattes à plusieurs stades de développement sexuel. Après euthanasie, les ovaires de rattes Sprague Dawley pubères (âgées de 42 jours), post-pubères (âgées de 56 jours) et adultes (âgées de 70 jours) (10 femelles par groupe) ont été prélevés puis fixés en vue d'une analyse histologique conventionnelle et d'une analyse immunohistochimique utilisant une méthode de révélation streptavidine - biotine- péroxydase et un anticorps polyclonal de lapin anti VR1 comme anticorps primaire. Les concentrations sériques en gonadotrophines et en stéroïdes sexuels ont été mesurées en parallèle à l'aide de trousses commerciales ELISA. Quelque soit le stade de développement sexuel, l'expression de VR1 a été observée dans le cytoplasme de différents types de cellules ovariennes (cellules de la granulosa, interstitielles, des thèques et du corps jaune) avec une intensité modérée bien que l'accumulation de cette protéine soit restée plus faible dans les cellules thécales. En outre, l'expression ovarienne du VR1 a semblé indépendante d'un contrôle hormonal. Ces résultats démontrent l'expression constitutive de VR1 dans les ovaires de rat.

Mots clés : Rat, ovaire, récepteur de la vanilloïde, immunohistochimie, développement sexuel, gonadotrophines, stéroïdes sexuels.

Introduction

Ovarian folliculogenesis and atresia is regulated by an interaction among endocrine [27], immune [23], paracrine-autocrine factors [24] and nervous system [28]. The ovaries of the rat receive neural information through sympathetic, cholinergic, peptidergic and sensory nerve fibbers. Sensory innervations play a role in the regulation of ovarian function and the afferent sensory nerves are involved in the regulation of ovarian folliculogenesis or atresia responses to gonadotropins [19]. It has been proposed that ovarian innervations register functional information via receptors localized around the follicles [8]. Several studies have demonstrated SP (substance P)-immunoreactivity, neurokinin A, CGRP (Calcitonin Gene-Related Peptide) in female reproductive organs [6, 26], and with more accuracy, it was shown that sensory nerves innervating the ovary contained SP [5, 22, 25], CGRP and VIP (Vaso-active Intestinal Polypeptide) [3, 13], but the presence of the vanilloid receptor (VR1) has not been demonstrated yet.

The vanilloid receptor, called VR1 is a member of the mammalian transient receptor potential (TRP) channel family [33]. Mammalian TRP channels consist of 6 related protein subfamilies known as TRPV, TRPC, TRPM, TRPP, TRPN and TRPA. The TRPV1 channel, a member of the TRPV subfamily, is referred as the vanilloid receptor 1 (VR1) or the capsaicin receptor [20]. VR1 has been cloned by CATERINA *et al*. [4] from rat dorsal root ganglia and was recognized as a common molecular target for protons and noxious heat (> 43°C) as well as for vanilloid compounds [14]. This receptor is highly expressed in a subset of primary sensory neurons [17] and is responsible for the capsaicin action, but it is also found in diverse areas of the central nervous

system including the limbic system, striatum, hypothalamus, hippocampus, and cerebellum [18]. The role of TRPV1 in the central nervous system is still elusive, although it may mediate endovanilloid signalling promoting the release of excitatory neurotransmitters [15, 16]. There is also evidence that mRNA and protein of VR1 are produced and expressed in non-neuronal cells, including the urinary bladder epithelial cells [2], keratinocytes [9], mast cells [29], human bronchial epithelial cells and rat gastric epithelial cells [11]. Several studies [2, 11, 21] have evidenced VR1 in gastrointestinal, nervous, respiratory and urinary systems but have failed to demonstrate its presence in ovary. The purposes of the present study were to demonstrate the localisation of VR1 in rat ovaries by immunohistochemistry and to identify possible difference in VR1 expression according to the development periods.

Materials and Methods

ANIMALS AND EXPERIMENTAL PROTOCOL

A total of 30 female Sprague Dawley rats (21 days old) obtained from the Experimental Animals Breeding and Research Centre, Uludag University, Turkey, were used for the current study. The animals were handled according to the approved national guidelines for animal care: they were kept under controlled light conditions (Light/Dark 12h: 12h), in temperature (20-24ºC), humidity (60-70%) and fed with an ordinary laboratory diet. Thereafter, they were divided into three equal groups (n = 10) as puberty (42 day old), post puberty (56 day old) and adult (70 day old) rats.

The experimental procedures were approved by the Uludag University Ethical Committee for animal experimentation (Protocol Number: 25.04.2006/1). Animals were deeply anaesthetized with diethyl ether then decapitated by cervical dislocation. Before decapitation, blood was collected from heart into sterile tubes without anticoagulant. After clotting at 4-8°C for 4 hours, blood samples were centrifuged (1 500g, 5 minutes, 4°C) and sera were carefully harvested and stored at -20°C until analysis. Ovaries were surgically removed, dissected from surrounding tissues and fixed in 10% formaldehyde solution. Tissue samples were embedded in paraffin blocks according to routine histological procedures. The 5µm thick sections were stained with Crossman's triple stain for histological investigations and other sections were immunostained for evidencing VR1 in ovaries.

IMMUNOHISTOCHEMISTRY

The standard Streptavidin Biotin Peroxidase complex technique was carried out using the Histostain Plus Kit (Zymed). Briefly, the sections were deparaffinised, hydrated, and all sections were processed with antigen retrieval technique using microwave oven. The sections were placed into buffered citrate (pH 6) for 5x2 minutes. Endogenous peroxidase activity was blocked with 10 minutes incubation in 3% H_2O_2 solution in distilled water. Non-specific antibody binding was reduced by the incubation of the tissues in non-immune serum blocking solution for 1 hour at room temperature before application of the VR1 primary antibody (R-130, Santa Cruz: polyclonal rabbit antibody targeted the N terminal amino-acids 1-130 domain of the rat VR1). The sections were incubated overnight at 4ºC with VR1 antibodies diluted to 1:1000. Thereafter, sections were incubated with biotinylated secondary rabbit antibody for 10 minutes followed by streptavidin conjugated to horseradish peroxidase incubation for 10 minutes at room temperature. Finally, 3.3'-diaminobenzidine (DAB) was used for colour development and haematoxylin was used for counterstaining. Negative control slides processed without primary antibodies were included for each staining. All the slides were coded for asserting blinded investigations and graded according to the following scale: - no staining, + slight, ++ medium, +++ strong [1].

SERUM HORMONE CONCENTRATIONS

Serum LH, FSH, oestrogen, progesterone concentrations were measured by Enzyme-Linked ImmunoSorbent Assays (ELISA) using commercial LH, FSH, oestrogen and progesterone ELISA Kits (Endocrine Technologies, Inc.).

STATISTICAL ANALYSIS

Data were analysed using one-way ANOVA, and post hoc Duncan test (Sigma Stat 3.1). Differences were considered as significant when *P* values were less than 0.05.

Results

The body and ovaries weights according to the different development period are shown in Table I. As expected, the body weight markedly increased according to the age of the female rats, whereas the ovary weights remained stable until the post-puberty period, and moderately increased (by 39.3%) during the adult period. Developing (primordial, primary and secondary) and atretic ovarian follicles were observed during the 3 developmental periods (puberty, post-puberty and adult). Corpus luteum appeared only since the post-puberty period and their number increased with the age of the female rats whereas the number of developing follicles decreased in parallel (figures 1, 2 and 3).

Intra-cytoplasm VR1 accumulation was evidenced by immuno-histochemistry in granulosa and interstitial cells as well as in theca and corpus luteal cells (when present) whatever the development states (figure 4). Negative controls did not give any specific immunostaining for VR1. As shown in Table II, the VR1 expression was weak in the theca follicle cells (figure 5A) and remained low for the 3 developmental periods. The other positive cell types (interstitial cells (figure 5B), granulosa cells (figure 5C) and luteal cells when present (figure 5D)) exhibited a more marked immunolabelling for VR1. Moreover, the immunostaining did not vary according to the sexual development except for the granulosa cells which were strongly labelled for the post-puberty period.

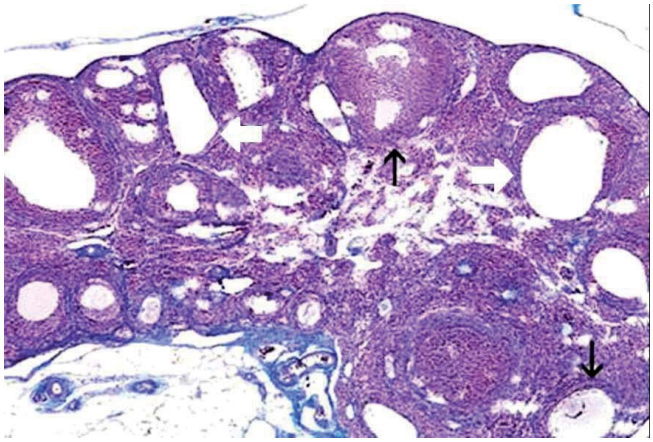

FIGURE 1 : Ovary from a 42 day old female rat (puberty): Developing follicles (black arrows) and atretic follicles (white arrows), Bar: 50 µm.

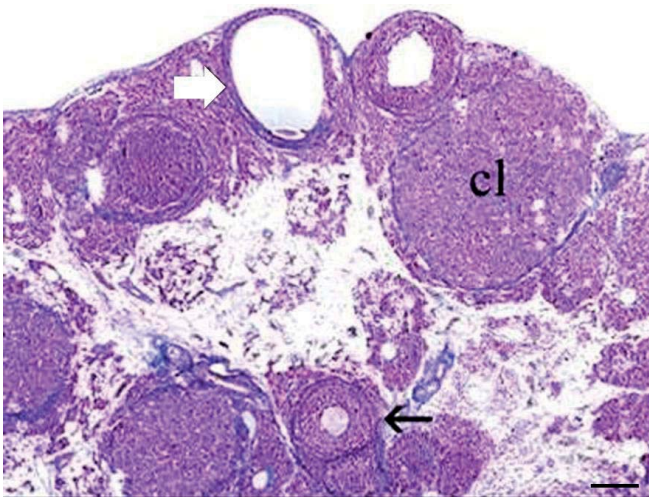

FIGURE 2 : Ovary from a 56 day old female rat (post-puberty): Developing follicles (black arrows), atretic follicles (white arrows) and corpus luteum (cl), Bar: 50 µm.

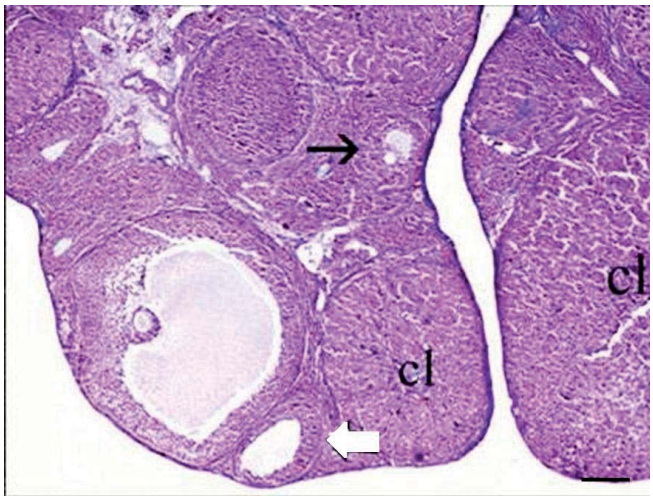

FIGURE 3 : Ovary from a 70 day old female rat (adult): Developing follicles (black arrows), atretic follicles (white arrows) and corpus luteum (cl), Bar: 50 µm.

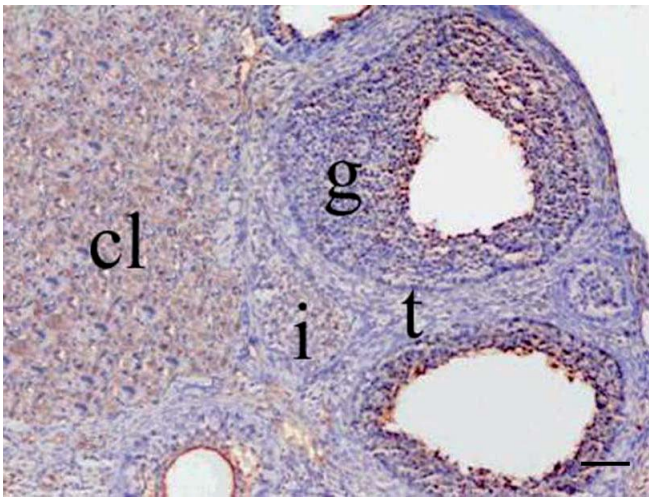

FIGURE 4 : VR1 immunostaining of granulosa cells (g), theca follicle cells (t), interstitial cells (i) and corpus luteal cells (cl) in adult rats (70 day old), Streptavidin Biotin Peroxidase complex technique, Bar: 50 µm.

	Puberty (42 days old)	**Post-puberty (56 days old)**	**Adult (70 days old)**
Body weight (g)	89.25 ± 2.93	132.3 ± 2.48	157.1 ± 2.31
Increase (%)		48.3 ± 0.9	76.0 ± 1.1
Ovary weight (g)	0.028 ± 0.002	0.030 ± 0.002	0.039 ± 0.002
Increase (%)		7.1 ± 0.5	39.3 ± 2.0

TABLE I : Comparison of body and ovaries weights in different development period (puberty, post-puberty and adult, n = 10 in each group). Results are expressed as means ± standard errors.

Ovarian cell types	**Puberty (42 days old)**	**Post-puberty (56 days old)**	**Adult (70 days old)**
Granulosa cells	++	+++	++
Interstitial cells	++	++	++
Theca cells	+	+	+
Luteal cells	Absent	++	++

TABLE II : Intensity of VR1 immunostaining in the different classes of ovarian cells according to the developmental period (puberty, post-puberty and adult). The VR1 accumulation is semi-quantitatively expressed: (-) no staining. (+) weak. (++) moderate. (+++) strong.

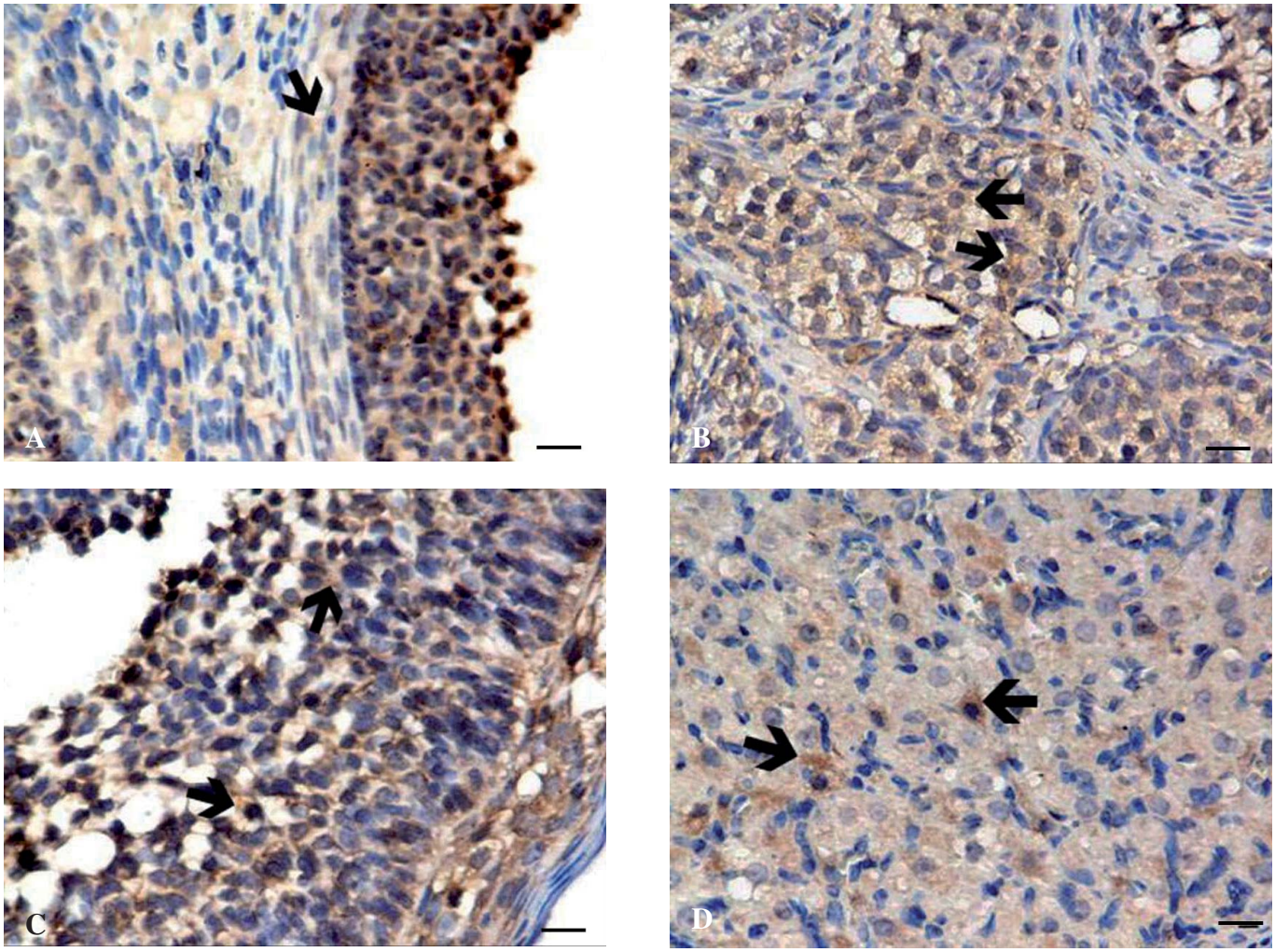

FIGURE 5 : VR1 immunostaining (arrows) of theca follicle cells (**A**), interstitial cells (**B**), granulosa cells (**C**) and luteal cells (**D**) in an adult (70 day old) female rat, Streptavidin Biotin Peroxidase complex technique, Bar: 12.5 µm.

In parallel, serum concentrations of the gonadotropins and sexual steroid hormones were compared between the 3 developmental periods (puberty, post-puberty and adult state) (Table III). It was noted that a marked and significant increase of the serum FSH concentrations characterized the post-pubertal period and that steroid hormones (oestrogens and progesterone) were significantly depressed in adult female rats.

Discussion

In the current study, it was reported for the first time VR1 expression in rat ovaries. Originally, VR1 was thought restricted in dorsal root, trigeminal and noose ganglias and brain [4, 18, 30, 31] but there is growing evidence for its additional expression in non neuronal cells such as bronchial epithelial cells [32], keratinocyte in epidermis [7, 10], urinary bladder epithelial cell [2] and gastric mucosal cell line [12]. These observations suggest that VR1 and its homologues are more broadly distributed than previously thought.

The VR1 protein was accumulated in the cytoplasm of various ovarian cells such as granulosa and interstitial cells, theca follicle and luteal cells albeit minimal expression was recorded in the theca follicle cells. In addition, the protein expression appears independently on the sexual development period. On the other hand, despite some hormonal fluctuations

Hormones	Puberty (42 days old)	Post-puberty (56 days old)	Adult (70 days old)
FSH (µg/L)	0.042 ± 0.300^{a}	1.561 ± 0.412^{c}	0.157 ± 0.060^{b}
LH (µg/L)	0.479 ± 0.133	0.548 ± 0.098	0.447 ± 0.111
Oestrogen (µg/L)	626.72 ± 75.712^{a}	634.21 ± 26.350^{a}	231.88 ± 16.973^{b}
Progesterone (µg/L)	2.181 ± 0.545^{ab}	2.877 ± 1.216^{b}	1.436 ± 0.543^{a}

Different superscipts ^{a,b,c} in the same line indicate significant difference according to the sexual developmental period.

TABLE III : Serum FSH, LH, Oestrogen and Progesterone concentrations in female rats according to the development period (puberty, post-puberty and adult, n = 10 in each group). Results are expressed as means ± standard errors.

recorded according to the development state and as VR1 expression remained roughly constant whatever the rate age, it would be stated that the receptor synthesis appears to be not controlled by gonadotropins or sexual steroids.

As a conclusion, the present study demonstrates a constitutive expression of VR1 in rat ovaries independently of the sexual developmental periods.

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