ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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THE EXPRESSION OF VANILLOID RECEPTOR SUBTYPE-1 (VR1) IN GONADOTROPIN-RELEASING HORMONE IN RAT BRAIN

SIÇAN BEYNİNDE VANİLLOİD RESPTÖR ALTGRUP TİP-1'İN GONODOTROPİN SALGILAYAN HORMON NÖRONLARINDA İFADE EDİLMESİ

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Öz

Amaç
Kapsikum biberlerinde bulunan ve ısı hissini üreten lipofilik bir vanilloid madde olan Kapsaisin (KAP), birincil afferent nöronlar üzerinde belirgin bir uyarıcı etkiye sahiptir ve uzun zamandan beri hassas nöronları tanımlamak için nöroparmakolojik bir araç olarak kullanılmaktadır. Kapsaisin etkisini Vanilloid Reseptör Alt Türü-1 (VR1) ile gösterir. Kapsaisin, çeşitli beyin bölgelerini etkiler, beyinde vanillod duyarlı nöronların var olduğu çeşitli çalışmalarda bildirilmiştir. Hipotalamusta bulunan VR1 reseptörleri büyük olasılıkla ağrı vücut ısısının düzenlenmesi, besin alımı, metabolik denge vb. diğer önemli fonksiyonlara sahiptir. Bu çalışmada, VR1 reseptörlerinin üreme üzerindeki potansiyel fizyolojik rolünü araştırmayı amaçladık.

Gereç ve Yöntem
Bu amaç ile sıçan beyninde VR1 ifade eden Gonodotropin Salgılayan Hormonu (GnRH) nöronlarının dağılımını araştırmak için ikili immünoflöresan tekniğini kullandık.

Bulgular
Çoğu VR1 pozitif GnRH nöronları ağırlıklı olarak organum vaskülozum lamina terminalis ve preoptik alanda, daha az olarak ise medial septum diyagonal bandında gözlendi.

Sonuç
VR1'in GnRH nöronlarında ifade edilmesi önemlidir, bu bulgu VR1'in üreme aksında rol oynayabileceğini gösterebilir.

Anahtar Kelimeler: GnRH, VR-1, Ko-lokalizyon

Abstract

Objective
Capsaicin (CAP), a lipophilic vanilloid substance found in Capsicum peppers which produces the sensation of heat, has a prominent excitatory action on primary afferent neurons, and has long been used as a neuropharmacological tool to identify sensitive neurons. Capsaicin shows its effect via Vanilloid Receptor Subtype-1 (VR1). Capsaicin affects a variety of brain regions, the presence of vanillod sensitive neurons in the brain has been reported in several studies. The VR1 receptors present in the hypothalamus most likely have other important functions including regulation of body temperature, food intake, metabo-

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lic balance etc. In this study we aimed to investigate potential physiological role of VR1 receptors in reproduction.

Material and Methods
Fort his purpose we performed dual immunofluorescence staining to explore the distribution of Gonodotropin Releasing Hormone (GnRH) neurons that express VR1 in rat brain.

Results
Most VR1 positive GnRH neurons found predominantly in organum vasculosum lamina terminalis and preoptic area lesser extend in medial septum diagonal band.

Discussion
The findings that VR1 is expressed in GnRH neurons is of great importance, it can be speculated that VR1 may have a role in reproductive axis.

Keywords: GnRH, VR-1, co-localization

Introduction

Capsaicin (CAP), the main active ingredient of hot chili peppers, has long been used as a neuropharmacological vehicle to localize sensitive neurons and their role in pathophysiological mechanisms (1). From the periphery into the central nervous system, Nociceptive information is transmitted by CAP-sensitive primary sensory neurons. CAP exerts its effects by binding and activating to the vanilloid receptors (VRs), the cloned CAP receptor, vanilloid receptor 1 (VR1) is also called transient receptor potential vanilloid subtype 1 (TRPV1) a non-selective cation channel with six transmembrane domains Which are structurally related to a member of the TRP (transient receptor potential) channel family (2). VR1 is expressed in numerous neurons throughout the brain activates specific vanilloid receptors at the sensory nerve endings and causes burning pain. VR1; its highest density was detected in the locus coeruleus (LC), mediobasal hypothalamus and preoptic area (PA), and, to a lesser extent in the olfactory bulb, which are regions known to be involved in various sensory, autonomic and neuroendocrine functions, but much less is known about the specific cell types in which TRPV1 is located and the intracellular localization in these cells (3,4).

In addition, local application of CAP acting via VR1 receptors can directly excite and/or inhibit hypothalamic-preoptic neurons in vivo and evokes glutamate output from hypothalamic slices, suggesting that certain glutamatergic terminals and/or neurons are sensitive to CAP in the hypothalamus (5). There also is a complete overlap between tyrosine hydroxylase and VR1 (3). Several lines of evidence suggest that CAP may participate in the control of neuroendocrine mechanisms, particularly the hypothalamus-pituitary-gonadal axis. Thus, uterine and cervical afferent nerves are predominantly C-type primary afferents conveying nociceptive, pain and probably non-nociceptive sensations and are more sensitive in an estrogen-dominant milieu. Other examples of reproductive responses and behaviors dependent on afferent innervation of proximal vagina and cervix include facilitation of female rat mating posture, myometrial contractions facilitating passage of sperm through the cervix and uterus to the oviduct, and activation of a neuroendocrine reflex essential to augment the corpus luteal progesterone secretion required to maintain pregnancy (6-9). More recently, in female rats the role of sensory feedback in neuroendocrine regulation were assessed with intrathecal injection of the CAP into the lumbosacral region of the spinal cord on reproductive function. In this study it has been shown that estrous cycle or female sexual behavior were not effected by intrathecal injection of CAP. Although CAP treated animals showed a dramatic reduce in fertility, according to the vehicle treated control, later on this is related with the attenuated capacity of vaginal/cervical excitation to produce pseudopregnancy (10). In addition it has been shown that functional non-neuronal TRPV1 receptor proteins are expressed in the rat endometrium, and this expression is regulated by estrogen (11).

Another study reported a significant improvement in ovarian tissue damage and hormonal levels in Curcumin and CAP treated rats with cyclophosphamide-induced Premature Ovarian Failure (12). In contrast of these findings CAP, which adversely affects spermatogonial survival, has been reported to induce apoptosis in spermatogonial cell lines (13). As relevant to the ratio of CAP exposed to cells, another publication reported that VR1 is important in defense of testis against heat stress as VR-1 is a thermo regulated cation channel (14).

VR1 has been shown to be present in hypothalamic regulatory regions (3,4), but to this date it is not known whether it is related to the female reproductive function (15). As far as it is known GnRH is a neurohormone secreted from the hypothalamus as a result of the estradiol feedback effect, and its frequency, am-

plitude and onset are critical for reproductive function (16,17). In order to examine if these effects of CAP are mediated by actions on gonadotropin-releasing hormone (GnRH) neurons, dual immunofluorescence labeling was performed to identify VR1 expressing GnRH neurons.

Material and Methods

All experimental procedures carried out in this study were approved by the Bursa Uludağ University Experimental Animal Care and Usage Committee.

Tissue:
Brain tissues were obtained from 50 days old female Sprague-Dawley rats (n=6) following the perfusion fixation with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4; 400 ml/animal). Forty-micrometer thick vibratome sections were taken throughout the rostro-caudal extend of the hypothalamus and collected in Tris-HCl buffer (0.05 M, pH 7.6).

Antigen Retrieval (AR): To test the working dilution of the antibody without using AR procedure resulted in very weak to none immunoreaction in preliminary immunofluorescence staining studies. In order to get a specific staining AR method was performed: 1.5% hydrogen peroxide was applied to the sections for blocking the endogenous peroxidase activity, subsequently sections were washed then processed for microwave-aided AR., a Bosch model 882 G microwave oven with an operating frequency of 2450 MHz and 900 W power output was used. The sections were immersed in a glass beaker with 80 ml Citro Plus (Bio Genex). The minimum and maximum temperature was set to 38 and 42 0C, respectively, and the alarm was set to 45 0C. Then the sections were heated at 900 W for 30 minutes. The final temperature of the buffer solution was 40-45 0C. Sections were kept in the buffer solution until the temperature has dropped to the room temperature. Then the sections were washed and processed for immunofluorescence staining.

Immunohistochemistry:
Sections were incubated for 96 hours in goat anti-VR1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100 in the above blocking buffer at 4^0C, following the blocking incubation in normal horse serum (% 10 in Tris-HCl buffer containing 0.1% Na-azide and 0.2% Triton X-100). After the termination of the incubation by three washes in Tris-HCl buffer, the sections were then incubated for 60 minutes in the affinity purified and cross-absorbed biotin-conjugated donkey anti-goat (1:300, Jackson Immunoresearch Labs, West Grove, PA). Subsequently the sections were treated with the streptavidin conjugated Cy5 (1:100, Jackson Immunoresearch Labs, West Grove) for fluorescence immunohistochemistry. After three final washes of 10 minutes each, the sections were incubated with the normal horse serum to block the non specific bindings. Then sections were incubated for 72 hours in guinea pig anti-GnRH (a gift from L. Jennes) at a dilution of 1:500. After three washes in Tris-HCL buffer, sections were then incubated for 60 minutes in the fluorescein-conjugated affinity pure donkey anti-guinea pig IgG (1:100, Jackson Immunoresearch Labs, West Grove, PA). Subsequently three final washes of 10 minutes each, the sections were mounted and analyzed with a- Zeiss LSM-510 Meta confocal laser scanning microscope (Germany) equipped with 63x plan-apo objective for detection of VR1 and GnRH. The 488 nm Argon ion and He-Ne, 633 nm red He-Ne laser lines were used to excite GnRH and VR1 respectively. Single optical sections (5 µm in thickness) and serial images on z-axis at 2µm intervals were collected by the LSM-510 Software (Germany) running on a Siemens-Nixdorf workstation. The final 3-D reconstructions and direct measurements on 3-D images were performed using Zeiss LSM 510 v3.0 software (Germany).

Spinal ganglion was used as a positive control for VR1 staining (Figure 1). Specificity controls included omission of the primary antibody; substitution of the primary antibody by normal serum. All negative control experiments resulted in the absence of staining.
Image Analysis Each GnRH -immunoreactive neuron in areas Organum Vasculosum of the Lateralis Terminalis (OVLT), Preoptic Area (POA) and Medial Septum of Diagonal Band was examined for the presence of VR1 protein. The anatomical identification of these regions was based on the brain map (18).

Sections were analyzed at x200 magnification for cell counting. All GnRH-positive cells, with and without VR1-positive neurons in the OVLT, POA and Medial Septum Diagonal Band were counted bilaterally and blindedly. The percentage of VR1-positive GnRH neurons was calculated within each animal relative to all GnRH-positive cells in the same animal.

Results

Immunohistochemistry revealed that the overall distribution of VR1-expressing neurons and GnRH neurons in the hypothalamus was consistent with previous data. Our results also showed that in order to achieve VR1 labeling with this particular antibody, usage of antigen retrieval method is necessary, since

few labeling was seen when the sections had no pre-treatment.

In addition, neurons which were double-labeled for GnRH and VR1 were detected in the Organum Vasculosum of the Lateralis Terminalis (OVLT), Preoptic Area (POA) and Medial Septum of Diagonal Band. Most GnRH neurons surrounding the OVLT co-expressed VR1 (Figure 2), lesser extent in POA (Figure 3) and only some GnRH neurons in the medial septum-diagonal band contained VR1 (Figure 4).

Both of the antibody exhibited cytoplasmic staining in the neuron, GnRH immunostaining was homogenous pattern in the cytoplasm in contrast of that VR1 immunostaining was more predominantly punctate pattern. In Anteroventral Periventrivular nucleus VR1 expression was seen mostly in glial cells (Figure 5).

OVLT

Many immunolabeled GnRH and VR1neurons were detected within the OVLT. About 50 % GnRH neurons expressed VR1 protein in that area (Table I).

POA

Similarly, about 40 % GnRH neurons expressed VR1 protein in the preoptic area (Table I).

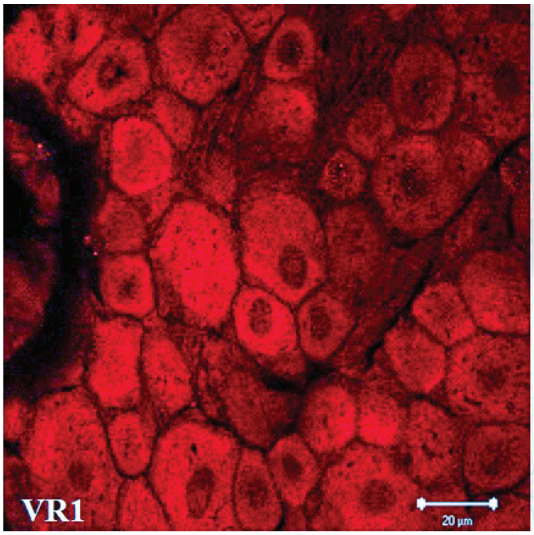

Figure 1
Positive control of VR1 immunofluorescence staining in Spinal Ganglion neurons. The VR1 staining was evident in the cytoplasm

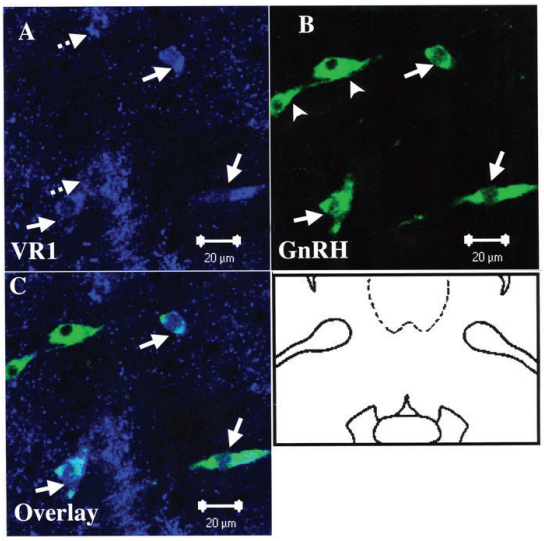

Figure 2
VR1 and GnRH immunofluorescence in Organum Vasculosum of the Lateralis Terminalis (OVLT). Dual immunofluorescence staining of A; VR1 (blue) and B; GnRH (green), C; Overlay of VR1 and GnRH stainings in the OVLT. Overlay of VR1 and GnRh stainings reveals that the neurons indicated with arrows in figures A and B are VR1 expressing GnRH neurons (bluish green) whereas the ones indicated with dashed arrows in figure A express only VR1 and with arrowheads in figure B express only GnRH. Both of the antibodies exhibited a cytoplasmic staining in the neurons.

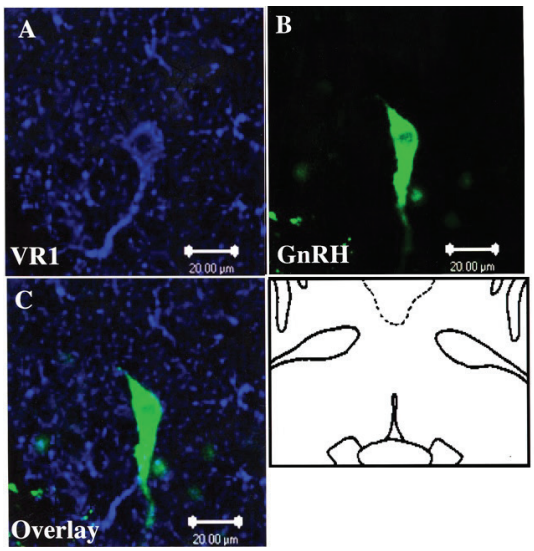

Figure 3
VR1 and GnRH immunofluorescence in the anteroventral periventricular nucleus. Immunofluorescence staining of A; VR1 (blue) and B; GnRH (green), C; Overlay of VR1 and GnRH stainings in the anteroventral periventricular nucleus. Overlay shows co-localization of VR1 and GnRH in anteroventral periventricular nucleus. Both of the antibodies exhibited a cytoplasmic staining in the neurons and glial cells.

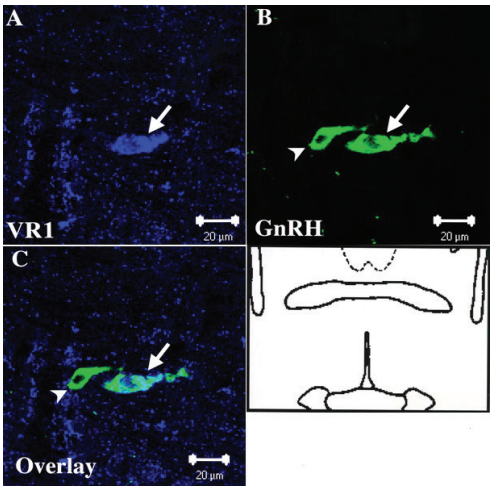

Figure 4
VR1 and GnRH immunofluorescence in the rostral medial preoptic nucleus. Immunofluorescence staining of A; VR1 (blue) and B; GnRH (green), C; Overlay of VR1 and GnRH stainings in the rostral medial preoptic nucleus. Overlay of VR1 and GnRH stainings reveals that the neurons indicated with arrows in figures A and B are VR1 expressing GnRH neurons (arrow, bluish green) whereas the one indicated with the arrowhead expressed only GnRH. Both of the antibodies exhibited a cytoplasmic staining in the neurons.

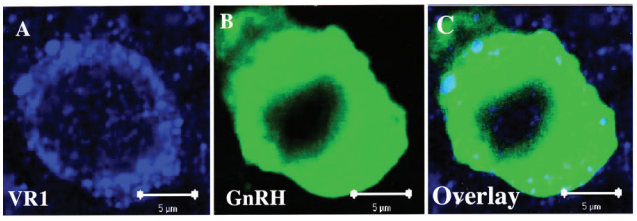

Figure 5
VR1 and GnRH immunofluorescence in the soma of neuron. Immunofluorescence staining of A; VR1 (blue), B; GnRH (green), and C; Overlay of VR1 and GnRH stainings in the The aquamarine dots in the overlay of VR1 and GnRH stainings in figure C correspond to synaptic inputs of VR1 in GnRH soma.

Table 1 The mean number of GnRH neurons and VR1 expressing GnRH neurons counted

Area	Total number of animals	Mean number of GnRH neurons counted	Mean number of GnRH neurons expressing VR1
OVLT	6	70.33 ±7.05	34.66±3.58
POA	6	36.66±3.02	15.33±2.13

•Values are the mean and SEM

Conclusion

Vanilloid receptor 1, known as the capsaicin receptor in primary sensory neurons, is known as a molecular sensor for nociception [19]. VR1 protein and mRNA expression is reported in various regions of the central nervous system (3,4,5, 20, 23,24), and also in non-neural cells such as in premeiotic male germ cells, ovarian granulosa and interstitial cells, mast cells, and urinary bladder epithelial cell (13,14, 21, 22).

Studies showed that capsaicin enhances glutamatergic synaptic transmission in locus coeruleus and substantia nigra by activation of VR1 receptors [5,23].

Mezey et al., (3) reported immunopositive cells in the suprachiasmatic nucleus, numerous weak immunopositive cells in the anterior hypothalamic nuclei, the paraventricular nuclei among the magnocellular hypothalamic neurons, the dorsomedial hypothalamic nucleus, and the arcuate nuclei. In contrast to the strength of evidence that VR1 is widely distributed in the brain, much less is known about the specific cell types in which VR1 is located and the intracellular localization in these cells. Toth et al., reported that VR1expression was seen in hippocampus, cortex, cerebellum, olfactory bulb, mesencephalon and hindbrain, high expression was seen in the cell bodies and dendrites of neurons in the hippocampus and in the cortex (4).

In the view of the literature, VR1 expression was reported in various regions of the brain of the rat, being found in astrocytes and pericytes as well as in neurons (3,4,5, 20, 23,24). Its localization is consistent

with multiple functions within the central nervous system.

In our study though we did not stain all rostro-caudal serial sections, we stained the sections of our focus of interest brain regions where GnRH neurons located. In contrast to the findings of Mezey and Toth et al., we observed that most VR1 expression is predominantly seen in OVLT and POA regions of the brain in our study. This is possibly due to the in sensitivity difference of the methods like antigen retrieval and the use of different antibodies.

These results suggest that vanilloid receptors could participate in the action of various neuronal systems, in addition to their known role in the modulation of pain perception.

One such system is the GnRH neuronal system that expresses VR1 receptors. The presence of VR1 receptor in cytoplasm of GnRH neurons, VR1 synaptic inputs in GnRH soma suggests that capsaicin could participate in the control of reproductive processes by affecting the secretory activity of GnRH neurons by direct receptor-mediated processes.

As far as we know this is the first study exploring the potential physiological function of VR1 receptors that co-localise with GnRH neurons in OVLT and POA. Further studies should be done whether these co-localization percentage changes during the steroid induced LH surge between young rat and middle-aged rats.

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