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Confocal Images of The Colocalization and Upregulation of Vasotocin and Galanin After Water Deprivation in Roosters

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ABSTRACT The aim of this study was to investigate the effects of dehydration on galanin and vasotocin colocalization and galanin expression in the hypothalamo-neurohypophysial system. Adult (age: 22 to 35 weeks) roosters were used and deprived of drinking water for 48 hours in this study. The effects of dehydration were examined by measuring plasma osmolality, plasma levels of potassium, sodium and plasma vasotocin concentration. Double staining immunohistochemistry combined with confocal laser microscopy was utilized to determine the effects of dehydration on hypothalamic magnocellular neurons in the supraoptic and paraventricular nucleus and to verify the extend of colocalization. The results indicate that 48 hours water deprivation caused a moderate dehydration and significantly elevation of plasma osmolality, plasma sodium and vasotocin concentration. Furthermore, the colocalization of galanin and vasotocin was imaged in response to water deprivation only in supraoptic nucleus of hypothalamus. In this study, a close functional link between galanin and vasotocin neurons was found in all dehydrated animals. The simultaneous upregulation and the colocalization of these peptides in response to water deprivation may indicate a site specific control of vasotocin by galanin.

Keywords: *Colocalization, Galanin, Vasotocin, Water Deprivation*

ÖZ

Su Yoksunluğu Sonrası Horozlarda Vasotocin ve Galanin Up-Regülasyonu ve Kolokalizasyonunun Konfokal Görüntüleri

Bu çalışmanın amacı, dehidratasyonun galanin ve vasotocin kolokalizasyonu ve galanin ekspresyonu üzerine etkilerini hipotalamo-nörohipofizeal sistemde incelemektir. Bu çalışmada, yetişkin horozlar (yaş: 22-35 hafta) kullanıldı ve 48 saat içme suyundan mahrum edildiler. Dehidratasyonun etkileri plazma osmolalitesi, plazma potasyum ve potasyum seviyeleri, vasotosin konsantrasyonları ölçülerek incelendi. Supraoptic ve paraventricular nükleuslardaki hipotalamik nöronlarda dehidratasyon etkilerini belirlemek ve kolokalizasyon yayılımını doğrulamak için konfokal lazer mikroskobu ile birlikte çift boyama immunhistokimyası kullanıldı. Sonuçlar, 48 saatlik su yoksunluğunun orta derecede dehidratasyona ve plazma ozmolalitesi, plazma sodyum ve vasotosin konsantrasyonunun önemli ölçüde yükselmesine neden olduğunu göstermektedir. Ayrıca galanin ve vasotosin kolokalizasyonu susuz kalmaya cevaben hipotalamusun sadece supraoptic nükleusunda görüntülendi. Bu çalışmada, tüm dehidre hayvanlarda galanin ve vasotosin nöronları arasında yakın bir fonksiyonel ilişki bulundu. Su yoksunluğuna cevap olarak bu peptitlerin ko-lokalizasyonu ve eş zamanlı olarak up-regülasyonu, galanin tarafından bölgeye özgü bir vasotosin kontrolünü gösterebilir.

Anahtar Kelimeler: *Kolokalizasyon, Galanin, Vasotocin, Su Yoksunluğu*

INTRODUCTION

Vasotocin is an oligopeptitde homologous to vasopressin and oxytocin found in all non-mammalian vertebrates. Vasotocin is synthesized in supraoptic nucleus and paraventricular nucleus of hypothalamus and then transported in neurosecretory granules along the axons to posterior pituitary (Burbach et. al. 2001). Vasotocin is known to be involved in the regulation of water balance and also regulation of uterine contractions for oviposition in avian species. Galanin is a 29 amino acid peptide and found to be a potent modulator of neuroendocrine regulation. Galanin is distributed throughout the central nervous system and particularly concentrated within the basal forebrain, hypothalamus, septal region and amygdala in the rat and monkey (Merchenthaler et al. 1993). Dehydration is inducible in terrestrial animals by water

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deprivation and also has been shown to be an effective stimulus for vasotocin in avian species. One of the main action of vasotocin is the stimulation of renal water reabsorbtion at the distal convolute tubues and the collecting ducts in the kidney (Stallone and Braun 1986a). There are several reports demonstrating that either water deprivation (Mühlbauer et al. 1992; Chaturvedi et al. 2001) or administration of hypertonic saline (Koike et al. 1986; Stallone and Braun 1986a) cause to elevate the plasma osmolality and the plasma vasotocin levels in chickens.

The aim of this study was to investigate effects of dehydration on galanin and vasotocin colocalization. Besides the control of water balance, vasotocin is strongly involved in the mechanisms leading to egg laying in female birds. Thus, to avoid any influence with egg laying, only rooster were used. To this end the expression pattern and signal intensities of vasotocin and galanin in supraoptic and in paraventricular nucleus of hypothalamus were evaluated.

MATERIALS and METHODS

Experimental design and tissue preparation

A total of adult 16 roosters (Lohman- Selected Leghorn) were used at the age of 22-35 weeks. Animal maintenance and the experiments were conducted in accordance with the relevant laws and regulations that govern the treatment of experimental animals (AZ: 5096-4250213). All studies were performed at the Institüt für Tierzucht Mariensee der Bundesforshungsanstalt für Landwirtscahaft. One group of rooster (n=8) was deprived from water for 48 hours. At the end of dehydration period, blood sample (1 ml) from each animal (8 normohydrated and 8 dehydrated) was withdrawn from wing vein into the heparinize tube. Plasma was separated by centrifugation (3000 rpm, 15min, 4 °C) for measurement of AVT, plasma osmolality, plasma sodium and potassium concentration. Plasma osmolality was determined by vapor pressure osmometry (Wescor 5500, Wescor Inc, Logan, USA). Plasma levels of sodium and potassium were measured by using gas analysing system (Rapid Lab 865, Bayer Healthcare, Fernwald, Germany). Immediately after the withdrawal of the blood samples, roosters were euthanasied and perfused. Perfusion was performed via carotid arteris with 150 ml 0.1M phosphate buffer saline (PBS pH 7.4) contaning 0.2% heparin followed by 360ml Zamboni's fixative (4% paraformaldehyd and 150 ml saturated picric acid in 0.1 M PBS, pH 7.4). The brains were removed and postfixed for 6-8 hours in the same fixative. They placed into 25% sucrose solution in 0,1 phosphate buffer saline (PBS) for cryoprotection. The brains were then stored at -80 °C until cut into transverse serial sections of 40 µm using a cryostat (Reicher-Jung, 2800, Frigocut-E). Fifty sections from each brain, corresponding to the levels of brain chicken atlas (Kuenzel and Masson 1988) were collected in 0.02 M PBS and processed for immunohistochemistry.

Procedure of the immunohistochemistry and laser scanning confocal microscopy

In this study the indirect method of immunohistochemistry with use of a fluorescent label was utilized for laser scanning confocal microscopy. Immunohistochemical procedures were performed essentially as described by Jurkevich et al. 1999**.** The sections were washed six times in 0.02 M PBS at room temperature. After that the sections were incubated for 30 min in 0.02 M PBS containing 10% normal goat serum (Dako, Hamburg) and 0.2% Triton X-100. The sections were then incubated with rabbit polyclonal antibody against vasotocin diluted 1:30.000 and guinea pig antibody against galanin (Peninsula laboratories, California) diluted 1:600 in 0.02 M PBS with 1% normal goat serum, 0.2% Triton X-100 and 0.1% sodium azide for 22 hours at 4 °C. After that, the sections were rinsed in 0.02 M PBS and incubated with goat anti-rabbit IgG coupled with ALEXA 555 (Vector lab, Burlingame, TI-1000) diluted 1: 400 and goat anti-guinea pig IgG coupled with fluorescein (FITCH, Vector, FI-7000) diluted 1:400 in 0.02 M PBS with 0.2 % Triton X-100, 1% normal goat serum and 0.1% sodium azide in the dark at room temperature for 90 min. After washing, the sections were rinsed again in 0.02 M PBS with 0.2% Triton X-100 and mounted on gelatine coated slides with vectashield mounting medium (Vector, H-1000) and then sealed with coverslips using colorless nail polish. The slides were stored at 4 °C and protected from the light. Thus, the tissues were prepared for examination under laser scanning confocal microscopy.

Processing and analysis of data

The student's t-test was employed to determine the differences in plasma vasotocin, osmolality, sodium, and potassium between the control and dehydration group. Pearson correlation test was used to verify correlation between plasma vasotocin and osmolality levels in dehydrated and normohydrated animals. Statistical analysis was performed using a software package (Sigma Stat, Version 2.0). No attempt was undertaken to statistically analyse the neuroanatomical data.

RESULTS

Effects of dehydration on blood parameters

As a results of 48 hours water deprivation, the levels of plasma vasotocin ($p \leq 0.001$), plasma sodium ($p \leq 0.001$) and plasma osmolality ($p \leq 0.001$) were significantly increased in dehydrated animals. On the other hand, there were no significant differences in plasma potassium levels between normohyrated and dehydrated groups. Neither in normohydrated ($r=0.41$; $p>0.05$) nor in dehydrated ($r=0.009$; $p>0.05$) animals was a significant correlation detected between plasma vasotocin and plasma osmolality levels (Table 1).

Table 1. Effects of 48 hours water deprivation on plasma osmolality and plasma concentration of vasotocin (AVT), sodium (Na) and potassium (K) in adult roosters

Treatment	Osmolality (Mmol/l) mean±SEM	Sodium (Na) (Mmol/l) mean±SEM	Potassium (K) (Mmol/l) mean±SEM	(AVT) (Mmol/l) mean±SEM
Dehydrated	312.25±2.65* (n=8)	166.56±1.84* (n=8)	3.65±0.20 (n=8)	44.19±5.23* (n=6)
Control	295.00±3.12 (n=8)	155.05±1.29 (n=8)	3.57±0.15 (n=8)	15.39±2.91 (n=6)

*Values indicate mean ±S.E.M; n=8 (for AVT n=6) ; *p=<0.001 (Student ' s t-test)

Vasotocin and galanin colocalization in supraoptic nucleus

The intensity of immunolabeling in supraoptic nucleus was weaker for galanin than for vasotocin (Figure 1) in all brain sections of the 8 normohydrated animals.

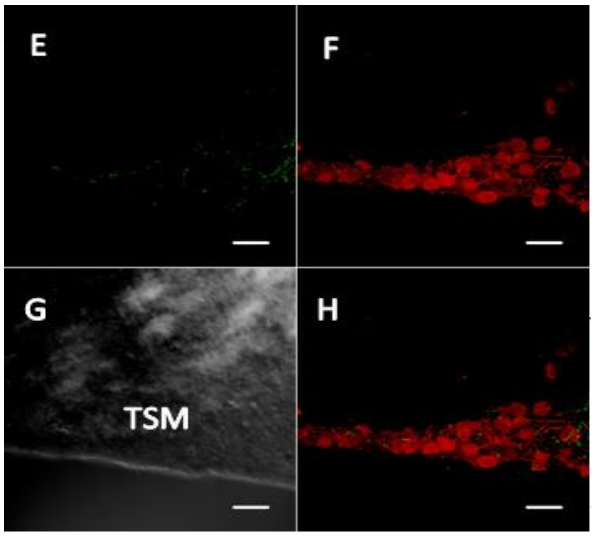

Figure 1. Demonstration of galanin (green) and vasotocin (red) immunoreactivity in supraoptic nucleus in normohydrated adult roosters. Galanin-containing neurons were not found but a few of neuronal fibers of galanin (E) were stained in this region. Vasotocin-containing neurons showed strong labeling in both cell bodies (F) and nerve fibers. Tractus septomesencephalicus (TSM) shown in diffential interference contrast (G) for better orientation. The colocalization of galanin and vasotocin was not found (H) in normohydrated animals. Scale bar indicates 50 µm

The cell bodies of galanin neurons were not found but interestingly, a few neuronal fibers of galanin neurons were detected in supraoptic nucleus in all normohydrated animals (Figure 1E). Vasotocin-containing neurons showed strong labeling in both cell bodies and nerve fibers in supraoptic nucleus in all normohayrated animals (Figure 1F). In normohydrated animals, the colocalization of galanin and vasotocin was not detected in supraoptic nucleus (Figure 1H).

The pattern of distribution and the intensity of the labelling were different in dehydrated animals. In all 8 dehydrated animals, in contrast to the normohydrated animals, galanin immunorecative neurons were observed in sections (Figure 2A). A clear upregulation of vasotocin was found in supraoptic nucleus in all dehydrated animals (Figure 2B). The colocalization of galanin and vasotocin was also detected in dehydrated animals (Figure 2D). The signals of colocalization appeared in yellow flourescence in all sections (Figure 2D).

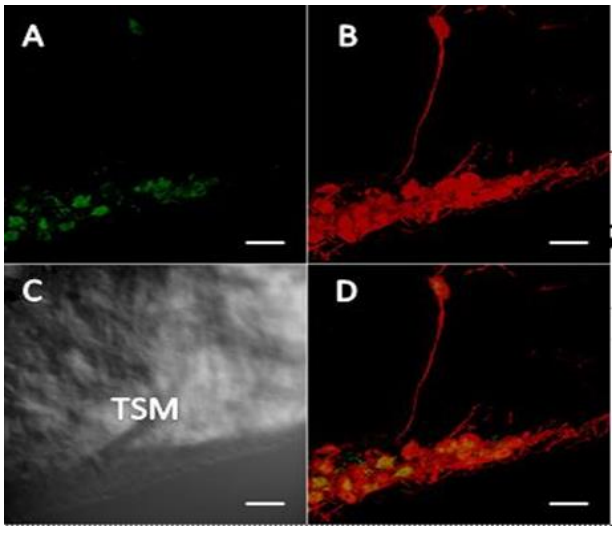

Figure 2. Demonstration of galanin (green) and vasotocin (red) immunoreactivity in supraoptic nucleus in dehydrated adult roosters. Galanin (A) and vasotocin (B) immunoreactive neurons were detected in this region. Tractus septomesencephalicus (TSM) shown in diffential interference contrast (C) for better orientation. The colocalizations of galanin and vasotocin were found in yellow fluorescence (D) in dehydrated animals. Scale bar indicates 50 µm

Lack of vasotocin and galanin colocalization in paraventricular nucleus

Magnocellular neurons of paraventricular nucleus were also heavily stained for vasotocin (Figure 3F and Figure 4B) and dendritic fibers of galanin were intensely detected in both normohydrated (Figure 3E) and dehydrated (Figure 4A) animals. The colocalization of galanin and vasotocin was not detected in normohydrated (Figure 3H) and dehydrated animals (Figure 4D).

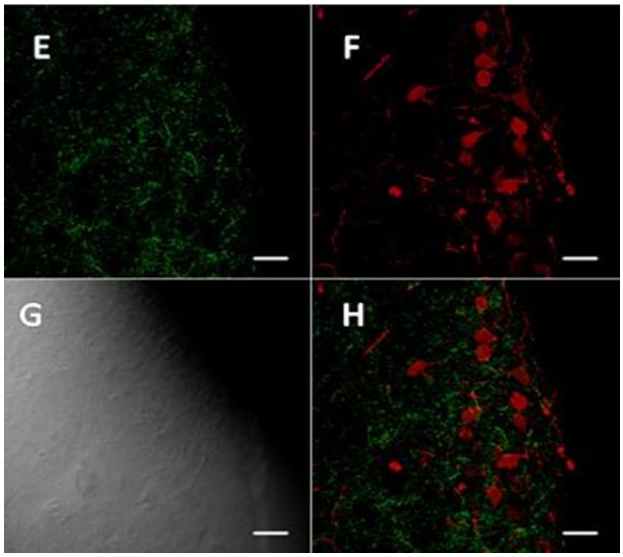

Figure 3. Demonstration of galanin (green) and vasotocin (red) immunoreactivity in paraventricular nucleus in normohydrated adult roosters. Neuronal fibers of galanin cells (E) were detected and vasotocin (F) immunoreactive neurons were intensively stained in this region. Diffential interference contrast (G) shown for better orientation. The colocalization of galanin and vasotocin was not found (H) in paraventricular nucleus. Scale bar indicates 50 µm

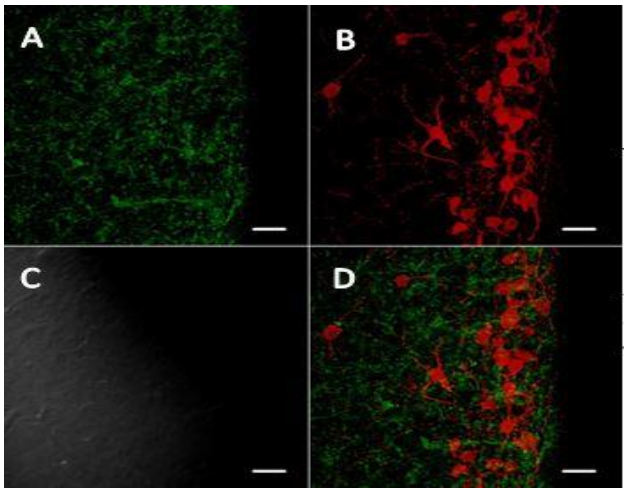

Figure 4. Demonstration of galanin (green) and vasotocin (red) immunoreactivity in paraventricular nucleus in dehydrated adult roosters. Neuronal fibers of galanin cells (A) were detected and vasotocin (B) immunoreactive

neurons were intensively stained in this region. Diffential interference contrast (C) shown for better orientation. The colocalization of galanin and vasotocin was not found (D) in paraventricular nucleus. Scale bar indicates 50 µm

DISCUSSION and CONCLUSION

The osmoregulatory system within the hypothalamo-nuerohypophysial axis was stimulated in this study and the results showed that water deprivation for 48 hours as an osmotic stimulus caused an increase in the plasma vasotocin levels concomitant with enhancement of plasma osmolality. This is in agreement with data published by Nouwen et al. (1984) and Arad et al. (1985). On the other hand, a correlation was not found between vasotocin and osmolality levels in this study; neither in normohydrated nor in dehydrated animals. This is also in agreement with data published by Nouwen et. al. (1984). But, some other researches found a correlation between plasma vasotocin and plasma osmolality during water deprivation (Arad et. al. 1985; Stallone and Braun 1986). The discrepancies could be due to the differences in the breeds, sex, age and housing of the animals used in different experiments. The results of immunohistochemistry combined with confocal laser microscopy study confirm the intensifying of vasotocin in supraoptic as well as paraventricular nucleus in response to the 48 hours of water deprivation. This increment indicates that osmotic challenge has resulted in activation of vasotocin expressing in these nuclei and the dehydration has been moderate in all dehydrated animals. Magnocellular hypothalamo-neurohypophysial system as well as the parvocellular hypothalamo-anterior pituitary system contain large number of galanin-containing neurons (Palkovits et al. 1987). In the rat, cell bodies of galanin neurons were detected in supraoptic and paraventricular nucleus and terminal of their axons were found in the posterior lobe of the pituitary (Palkovits et al. 1987; Arad et al. 1990; Gundlach and Burazin 1998). GAL has been assumed to have an inhibitory action on the biosynthesis and axoal transport of oxytocin but these effects of galanin has not been assumed on vasopressin in normohydrated rats (Ciosek 2000). But, interestingly, above authors indicated that in salt-loaded rats GAL attenuates the secretion of oxytocin and vasopessin. This is in good agreement with present data showing an upregulation of galanin and vastocin only in dehydrated animals. A work of laboratory (Klein et al. 2006) confirm the presence of galanin-containing and vasotocin-containing neurons in paraventricular nucleus of chickens and roosters. They also could not observe any colocalization of galanin and vasotocin in these nuclei. However, in the supraoptic nucleus they could determine significantly higher degree of colocalization. This is in good agreement with the present finding, which indicate a colocalization of galanin and vasotocin only in the supraoptic nucleus. The present data shows for the first time the enhanced expression of galanin and vasotocin in the hypothalamo-neurohypophysial system due to osmotic stress. This could explain that vasotocin is controlled by paracrine/autocrine secretion and activity of galanin in supraoptic nucleus. Interestingly, a subpopulation of magnocellular neurosecretory neurons in supraoptic nucleus expressed vasotocin only after water deprivation. It is possible that these cells are the sites of colocalization. It is also interesting to note that a dense dendritic fibers of galanin and vasotocin neurons investigated in supraoptic and paraventricular nucleus. This result confirms previous research that a dendritic release of vasopressin has been shown in the hypothalamic magnocellular nuclei (Morris et al. 1998; Pow and Morris 1989; Wang et al. 1995; Ludwig 1998).

In conclusion, the results of the present study show a close functional link between galanin and vasotocin neurons. The colocalization and simultaneous upregulation of these peptides in response to water deprivation may indicate a site specific control of vasotocin by galanin.

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