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The Localization of R-Spondin1 and R-Spondin 3 Peptides in Rat Hypothalamus: An Immunohistochemical Study

Localización de los Péptidos R-Spondin1 y R-Spondin3 en el Hipotálamo de Rata: Un Estudio Inmunohistoquímico

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SUMMARY: The R-spondin protein family is a group of proteins that enhance Wnt/b-catenin signaling and have pleiotropic functions in stem cell growth and development. In the literature reviews, there is no histomorphological study showing the localization and distribution of R-spondins in different hypothalamic nuclei. For this reason, the purpose of this study was to determine the localization, distribution characteristics, and densities in the hypothalamic nuclei of neurons expressing Rspo1 and Rspo3 proteins. The free-floating brain sections of the male rats who were not exposed to any treatment were stained with the indirect immunoperoxidase method using the relevant antibodies. As a result of the immunohistochemical studies, it was determined that neurons expressing the Rspo1 protein were found in large numbers in the supraoptic nucleus (SON), the suprachiasmatic nucleus (SCh), anterior paraventricular nucleus, periventricular hypothalamic nucleus (PeV), anterior hypothalamic area, magnocellular preoptic nucleus (MCPO) and the lateral hypothalamic area (LH) from the hypothalamic nuclei, while they were localized in fewer numbers in the arcuate nucleus (ARC). Rspo3 protein expression was found in neurons localized in the hypothalamic nuclei SON, paraventricular nucleus (PVN), PeV, ARC, ventromedial nucleus (VMH), LH, anterior parvicellular nucleus, and zona inserta (ZI). In addition, neurons synthesizing both peptides were found in the cortex and hippocampus regions (H). Rspo1 and 3 proteins are expressed in hypothalamic energy homeostatic areas, thus these proteins may be involved in the regulation of food intake.

KEY WORDS: R-spondin 1; R-spondin 3; Hypothalamus; Immunohistochemistry; Rat.

INTRODUCTION

Food intake is a complex physiological behavior that is controlled by central and peripheral signaling mechanisms, which is transformed into behavior mainly by neuropeptides released from the central nervous system into circulation (Berthoud & Morrison, 2008). In the last decade, many neuropeptides have been identified that alter food intake and energy expenditure, particularly in the neuronal pathway. R-spondin (roof plate-specific spondin; RSPO) family proteins have been identified as potential ligands for Leucine-rich repeats (LGR) containing G protein-coupled receptors (GPCRs) (Carmon *et al*., 2011). These proteins regulate the Wnt/b-catenin signaling pathway by binding to LGR4, LGR5, and LGR6 receptors (Gong *et al*., 2012). The RSPO protein family consists of four independent members, RSPO1, RSPO2, RSPO3, and RSPO4 (Nam *et al*., 2006). Although the R-spondin protein family has pleiotropic functions in stem cell growth and development (Gong *et al*., 2012), a recent study has focused on the fact that this peptide may play a role in the anorexigenic effect by suppressing food intake in the brain (Li *et al*., 2014). Subsequent studies have confirmed these findings (Li *et al*., 2019; Otsuka *et al*., 2019) and RSPOs/LGR4 signaling has been proposed as a novel mechanism to regulate food intake. There are limited studies on the physiological functions and signaling pathways of LGR4 in the literature. In addition, no histomorphological study shows the localization and distribution of R-spondin-expressing neurons in the hypothalamic nuclei in detail. Our main objective is to determine the localization, distribution characteristics, and densities of neurons expressing Rspo1 and Rspo3 proteins in the hypothalamus using immunohistochemistry technique.

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MATERIAL AND METHOD

Animals. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Experimental Ethical Committee of Bursa Uludag University (Approval Number: 2021-14/09, Project Name: THIZ-2021-645). Sixty-day-old male Sprague-Dawley rats weighing 250 to 300 g were obtained from the Bursa Uludag University Laboratory Animal Breeding and Experimental Center at which the animals were kept in a light- and temperature-controlled facility (a 12:12 h light-dark cycle with lights on at 7:00 am at 21 ºC) with food and water freely available (*ad libitum*). The animals were deeply anesthetized with xylazine and ketamine and were sacrificed by trans-cardiac perfusion fixation with 4 % paraformaldehyde (PFA) in 0.13 M Sorenson's phosphate buffer, pH 7.4 (300 ml/animal).

Tissue Preparation. Brains were carefully removed and post-fixed overnight in the 4 % PFA fixative at +4°C. Microwave ovens (MWO) induced antigen retrieval (AR) procedure was applied to all of the brain tissue obtained after perfusion fixation in order to regain the antigenicity in the tissue. For this purpose, a 10 mM pH 8.5 ethylene diamine tetraacetic acid disodium salt 2-hydrate (EDTA) solution prepared was used during the AR process. MWO was used at a reduced power of 600 W, 3 min and the final temperature of the solution was 84 °C. The brain tissues which were left to cool, were taken to distilled water 1.5 h later and then to Tris-HCl buffer (0.05 M, pH 7.6). Serial 40 µm-thick coronal sections from the brain were cut with a vibratome and collected in scintillation vials containing Tris-HCl buffer. After the brain sections were washed 3 times with Tris-HCl buffer they were kept in cryoprotectant at -20 °C until use. Antigen retrieval procedure was performed with 1 mM EDTA solution in some sections where Rspo3 mouse monoclonal antibody was used. Sections were incubated with antigen retrieval solution at a final temperature of 80-85 °C in an incubator set at 110 °C for 30 min. Afterward, the sections were kept in the same solution for 15 min at room temperature (20-22 °C) without opening the lid of the vials. Antigen retrieval protocols used in the experiments are presented in Table I.

Immunohistochemistry Procedures. Tris-HCl buffer (0.05 M, pH 7.6) was used for all washing steps with the exception of blocking and primer antibody incubation. Before the immunohistochemical applications, the sections were removed from -20 °C to room temperature, and the solution temperature was expected to reach ambient temperature. An orbital shaker with appropriate agitation was used for all incubation and washing steps. Free-floating sections were washed in Tris-HCl buffer to remove cryoprotectant and then the antigen retrieval procedure was applied to restore antigenicity only to the sections to be incubated with Rspo-3 primary antibody (Table I). Sections were incubated in blocking buffer including 10 % normal horse serum, 0.1 % sodium azide, and 0.2 % Triton X-100 in Tris-HCl for 2 hours in order to prevent non-specific binding. This buffer was also used for the dilution of primary and secondary antibodies. After the blocking serum step, sections were incubated with mouse Rspo-1 and Rspo-3 primary antibodies, with varying incubation times and temperatures (Table I). Sections were washed 3 times for 10 min with Tris-HCl buffer. Sections were exposed to biotin-SP-conjugated donkey anti-mouse IgG (1:300, Jackson Immunoresearch Labs, West Grove, PA) for 2 h followed by primary antibody incubation. Sections were processed with ABC Elite Standard Kit (Vector Labs, Burlingame, CA, USA) for 60 min and then stained with diaminobenzidine (DAB 12,5 mg and 1,3 µl hydrogen peroxide in 25 ml Tris-HCl buffer) substrate chromogen solution. Sections were washed, collected onto glass slides, dried, and mounted with DPX. Negative control experiments included the omission of primary or secondary antibodies, which revealed no specific staining.

Cell Counting and Statistical Analysis. Stained sections were analyzed and photographed with an Olympus BX-50 photomicroscope attached to a CCD color camera (Olympus DP71, CCD, 1.5 million pixels, Olympus Corporation, Tokyo, Japan). The coordinates of the sections prepared as a result of single labeled were determined according to the stereotaxic rat brain atlas. Sections including SON (between bregma -0.48 mm and -1.44 mm), PeV (between bregma -0.24 mm and -3.60 mm), PVN (between bregma -1.32 mm and -1.92 mm), ARC (between bregma -2.12 mm and -3.80 mm) and LH (between bregma -2.04 mm and -3.60 mm) were analyzed for the presence of Rspo immunoreactivity (Paxinos & Watson, 2009). In these intervals, sections taken from 5 different levels of the hypothalamus in the rostrocaudal plane were examined. Care was taken to ensure that the sections were equidistant from each other and at the same coordinate for each subject. Data were collected by two observers blind to the experiment, and sections were semi-quantitatively evaluated for the presenceand density of R-spondin 1 and R-spondin 3 peptides in hypothalamic neurons. Thus, if most of the neurons in the particular nucleus express the respective subunit, a scoring of "+++" was used. While "++" was used for a moderate number of neurons, "+" was used if a few neurons, and "-" was used to mean no immunopositivity.

Table I. Properties of the primary antibodies.

Antibodies	Supplier and Catalog Number	Dilution, Incubation Time and Temperature	Fixative	Antigen Retrieval (AR)			
				Buffer	pH	Temperature and Time	MWO induced AR
Rspo1 mouse monoclonal antibody	OriGene, TA804626S	1:3000, Overnight, RT	% 4 PFA + % 7.5 picric acid	–	–	–	+
Rspo3 mouse monoclonal antibody	OriGene, TA804633	1:3000, Overnight RT and 48 h, +4 ˚C	% 4 PFA + % 7.5 picric acid	1 mM EDTA	8.5	85 ˚C 5 min	+

RT: room temperature, MWO: Microwave ovens.

RESULTS AND DISCUSSION

In this study, the localization and distribution characteristics of neurons expressing R-spondin 1 and R-spondin 3 proteins from RSPO family members in the hypothalamus and their densities in the hypothalamic nuclei were determined by immunohistochemical approach. Since the level of Rspo2 mRNA was not detected in the rat brain and Rspo4 mRNA was expressed only in the habenular nucleus of the epithalamus, only the expression of the proteins of Rspo1 and Rspo3 was examined in this study. Intense immunoreaction involving the Rspo1 peptide was observed in a large number of neurons, particularly localized in the SON, the SCh, PVN, medial septal nucleus (MS), and in the horizontal (HDB), and vertical (VDB) nuclei of the diagonal band of Broca (Fig. 1). Moderate expression of Rspo1 was found in neurons located in the anterior parvicellular nucleus (PaAP), LH, magnocellular preoptic

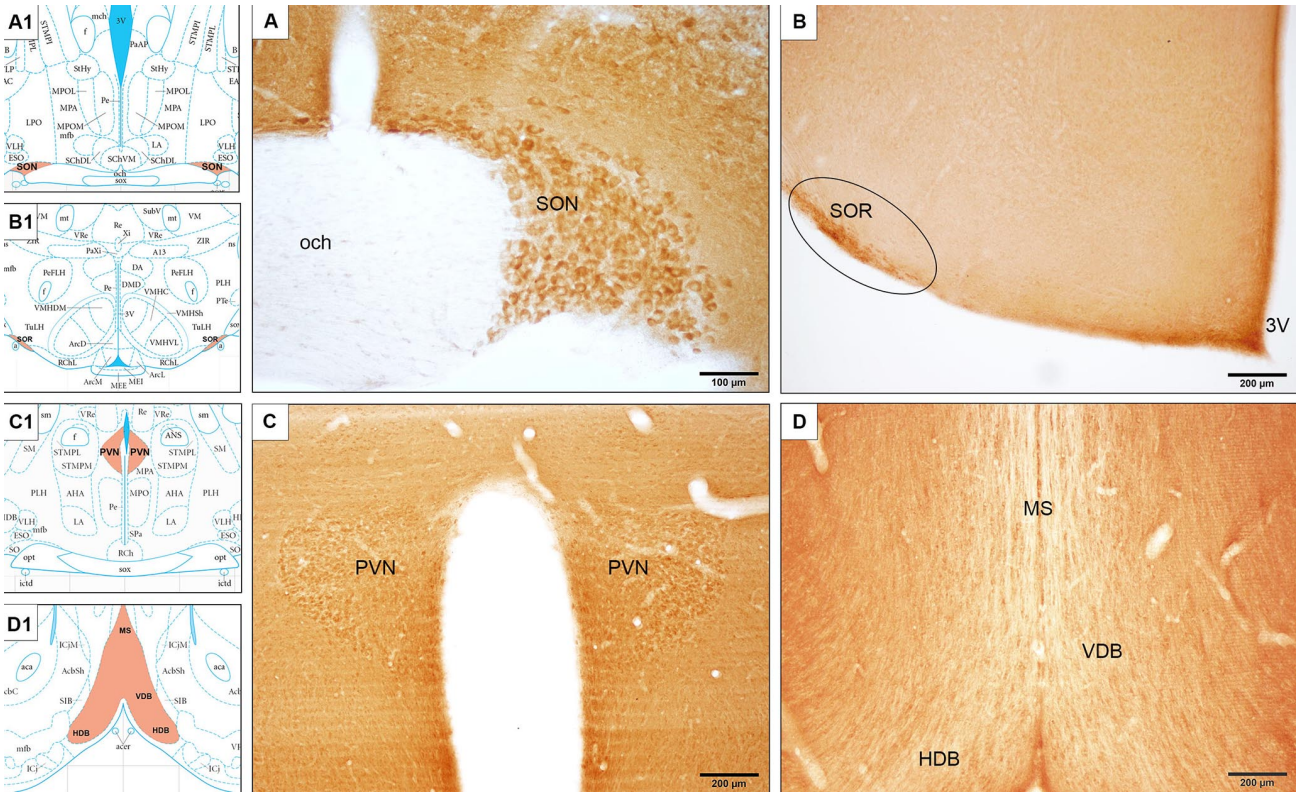

Fig. 1. High levels of Rspo 1 immunoreactivity are distinguished neurons localized in (A) supraoptic nucleus (SON), (B) suprachiasmatic nucleus (SCh), (C) paraventricular nucleus (PVN), (D) medial septal nucleus (MS), vertical (VDB) and horizontal (HDB) nuclei of Broca's diagonal band. (A1-D1) According to the Paxinos & Watson (2009) rat atlas, localizations of the hypothalamic nuclei are seen. 3V: Third ventricle, och: optic chiasm

nucleus (MCPO), ventrolateral preoptic nucleus (VLPO), and red nucleus (RN) (Fig. 2). In addition, a small number of neurons expressing Rspo1 were detected in the PeV, ARC, and VMH (Fig. 3). In the sections immunostained with the Rspo3 antibody, it was determined that this antibody also revealed immune-positive neurons located in different levels of the hypothalamus with a widespread distribution. Neurons expressing-Rspo 3 were found extensively in the diagonal band (DB) at the preoptic level of the hypothalamus, SON, SCh, and both parvocellular and magnocellular parts of the paraventricular nucleus, VMH, and LH (Fig. 4). Intense Rspo 3 positivity was also observed in the cortex areas of the brain. Moderately dense and randomly dispersed localized cells showing Rspo 3 expression were observed in the PeV around the third ventricle, ZI, and H region. Fewer and weaker immune reactions were observed in neurons located in the ARC (Fig. 5). Rspo 3 immune positivity was not found in any of the neurons localized in the MS, HBD, VDB, PaVP,

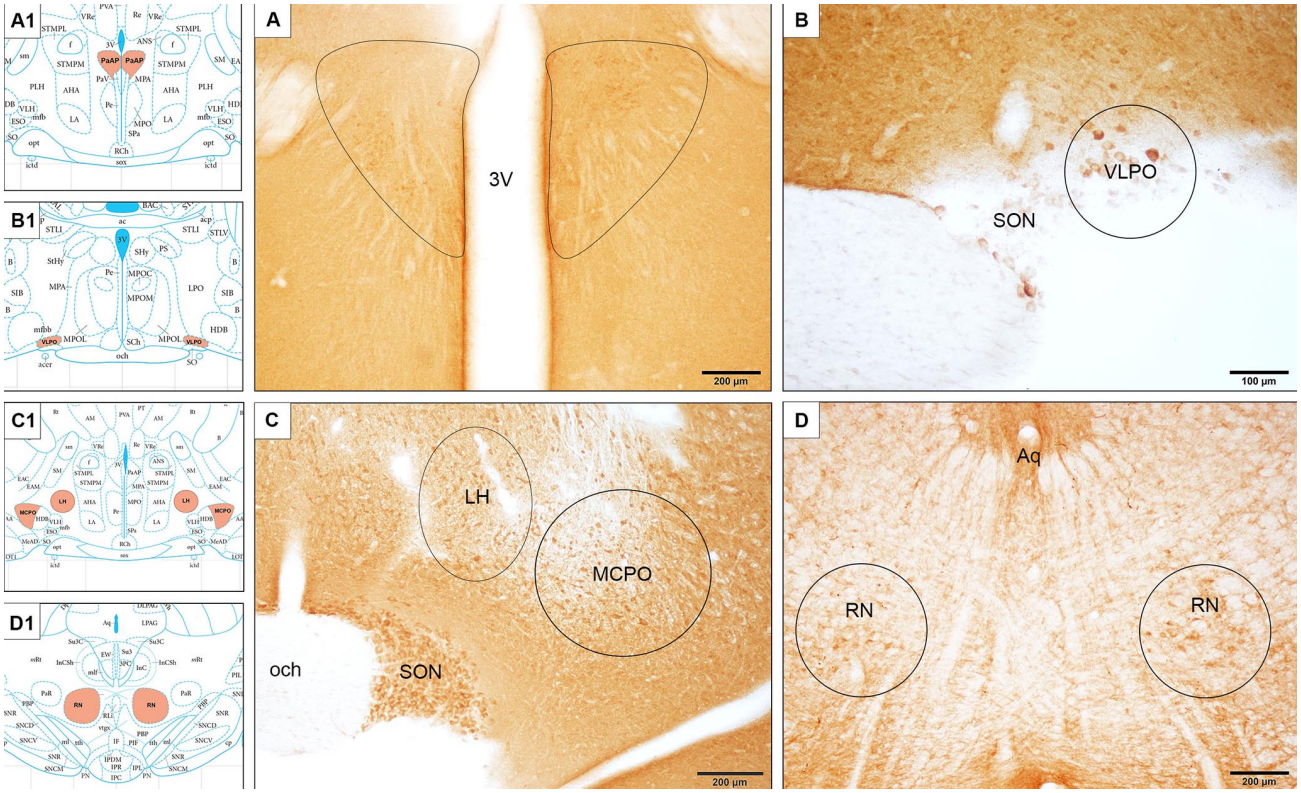

Fig. 2. Neurons that moderately express Rspo 1 are seen in (A) anterior parvicellular nucleus (PaAP), (B) ventrolateral preoptic nucleus (VLPO), (C) magnocellular preoptic nucleus (MCPO), lateral hypothalamic area (LH), and (D) red nucleus (RN). (A1-D1) According to the Paxinos & Watson (2009) rat atlas, localizations of the hypothalamic nuclei are seen. 3V: Third ventricle, och: optic chiasm, Aq: aqueductus cerebri, SON: supraoptic nucleus.

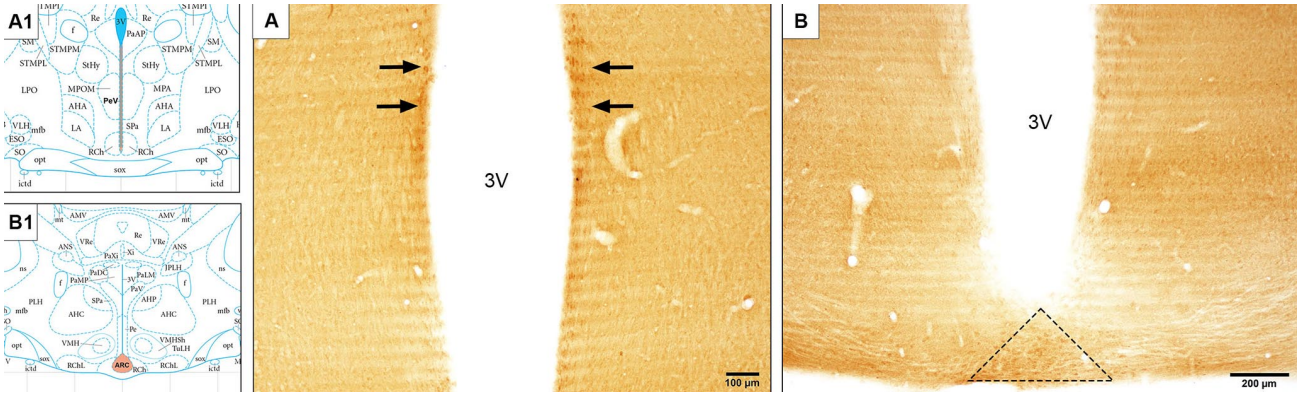

Fig. 3. Few Rspo 1-immunopositive neurons were observed in (A) periventricular nucleus (PeV) and (B) arcuate nucleus (ARC). (A1-B1) According to the Paxinos & Watson (2009) rat atlas, localizations of the hypothalamic nuclei are seen. 3V: third ventricle, the triangular area with a dashed arrow: arcuate nucleus, arrows (→): Rspo-1 positive neurons in the periventricular nucleus.

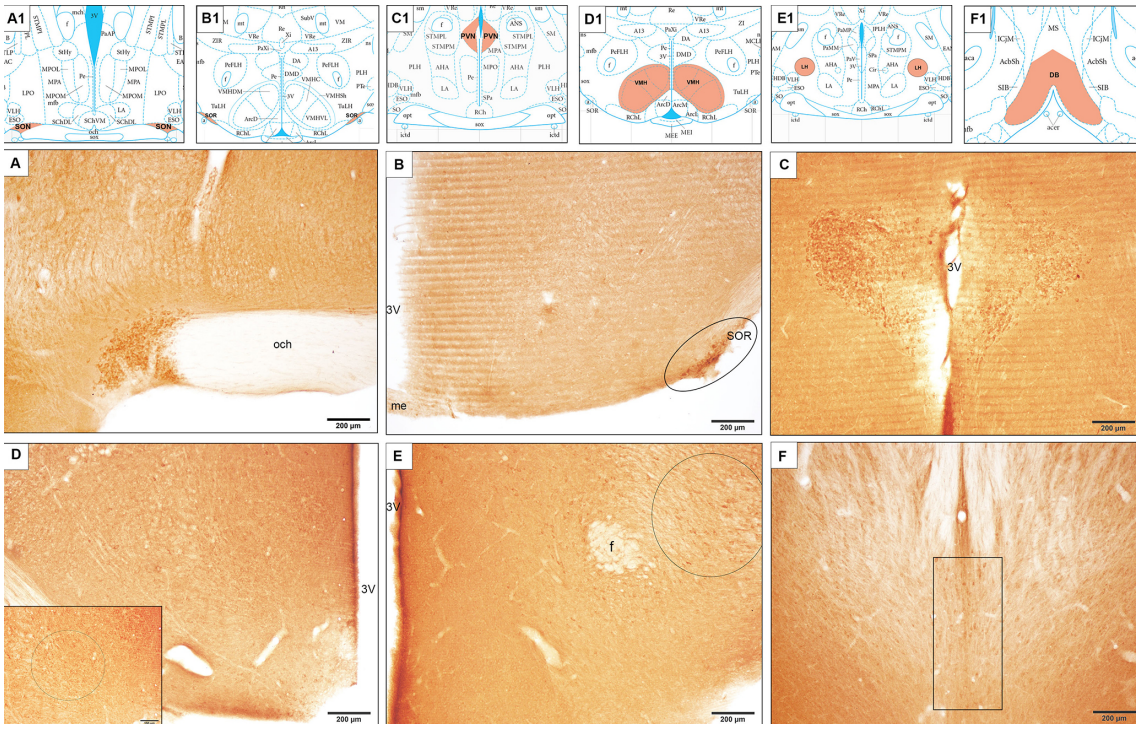

Fig. 4. High-intensity Rspo 3 immunoreactivity is distinguished in neurons (A) supraoptic nucleus (SON), (B) suprachiasmatic nucleus (SCh), (C) paraventricular nucleus (PVN), (D) ventromedial hypothalamic nucleus (VMH), (E) ventromedial nucleus in the area indicated by the arrows (20X magnification), (F) the lateral hypothalamus (LH), (G) diagonal band (DB). (A1-F1) According to the Paxinos & Watson (2009) rat atlas, localizations of the hypothalamic nuclei are seen. 3V: Third ventricle, och: optic chiasm, f: fornix, me: median eminence.

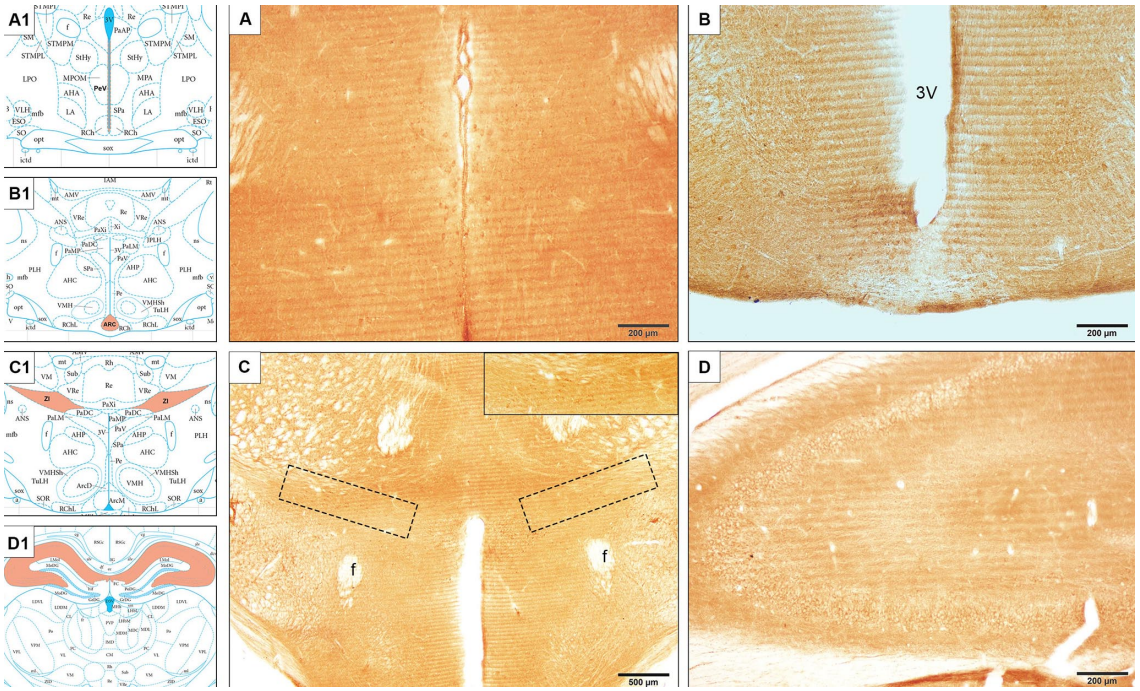

Fig. 5. Moderate expression of Rspo 3 is observed in neurons located in (A) periventricular nucleus (PeV), (B) arcuate nucleus (ARC), (C) zona inserta (ZI), and photographs of zona inserta at 20X magnification is selected in the figure and (D) hippocampus. (A1-D1) According to the Paxinos & Watson (2009) rat atlas, localizations of the hypothalamic nuclei and hippocampus are seen. 3V: Third ventricle, f: fornix.

MCPO, VLPO, and RN areas (Table II). In recent years, several groups of researchers have identified R-spondin (Rspo) family proteins as potential endogenous ligands for LGRs (Glinka *et al*., 2011; Ruffner *et al*., 2012). These proteins function via Wnt/b-catenin signaling to regulate cell proliferation, differentiation and adult stem cell homeostasis by binding to LGR4, LGR5 and LGR6 receptors (de Lau *et al*., 2011). LGR4 contributes to the regulation of energy metabolism, including food intake, energy expenditure and lipid metabolism, as well as pancreatic b-cell proliferation and insulin secretion (Li *et al*., 2015). Although LGR4 expression has been demonstrated in cerebellar Purkinje cells, researchers have reported that LGR4 can modulate motor activity by regulating the development and differentiation of neural cells in the cerebellum (Guan *et al*., 2014). High expression of LGR4 has been demonstrated in the hypothalamic ARC (Li *et al*., 2005). LGR5 is the receptor expressed in ventral midbrain (VM) dopaminergic (DA) neurons of developing mice. Researchers have demonstrated that RSPO2 promotes both mDA differentiation and neurogenesis in human ESCs (Gyllborg *et al*., 2018). Recent studies have revealed a relationship between LGR4 and energy metabolism in areas ranging from food intake and obesity to lipid metabolism. In situ hybridization studies revealed that LGR4 mRNA and ligands were highly expressed in the ARC, VMH and median eminence, which are areas of the hypothalamus related to energy homeostasis and feeding behavior, and it has been reported that this molecular relationship plays a role in the regulation of food intake (Li *et al*., 2019). In addition, LGR4 mRNA was expressed in layers II and III in the cortex, in CA1, CA2, and CA3 regions in the hippocampus, in the dentate gyrus (DG), in the amygdala nucleus, and in the habenular nucleus of the epithalamus (Hbs) (Li *et al*., 2014). While the expression of LGR5 mRNA was undetectable, LGR6 was found to be expressed only in the median eminence and epithelial cells of the inferior portion of the third ventricle (Li *et al*., 2014). Another study revealed that LGR4 mRNA is highly expressed in the medulla oblongata and pons in E14 embryos and expressed in the spinal cord and H region in the adult mouse (Schoore *et al*., 2005). Guan *et al*. (2014) reported that expression of LGR4 is highly detected in cerebellar Purkinje cells (PCs), while the role of LGR4 in the central nervous system has not been studied.

The researchers showed that Rspo1 mRNA was highly expressed in the VMH, while Rspo3 mRNA was extensively expressed in the PVN (Li *et al*., 2014). In our studies, we found that Rspo1 and Rspo3 peptides are expressed in many nuclei of the hypothalamus, which are responsible for the regulation of food intake. When all regions of the hypothalamus from the preoptic to the tuberal level were examined, it was found that the expression of Rspo 1 and Rspo 3, in which the number of neurons is high and cytoplasmic involvement is observed intensely. On the other hand, in the nuclei at the mammary level, scattered and less number of intermediate immune positive cells were found only for the Rspo 1 peptide. Intense immunoreaction involving the Rspo 1 peptide was observed in a large number of neurons, especially localized in the SON, SCh, PVN, the MS of the hypothalamus, and horizontal and vertical arm nuclei of Broca's diagonal band. Moderate Rspo 1 positivity is observed in neurons located in the anterior PaAP, LH, MCPO, VLPO, and RN. In addition, neurons expressing a small number of Rspo 1 peptides were also detected in the PeV, ARC, and VMH. Immune-reactive neurons belonging to the Rspo 3 subunit were intensely detected in most neurons in the diagonal band at the preoptic level of the hypothalamus, in the SON at the anterior level, in the SCh and in both the parvocellular and magnocellular parts of the PVN, the VMH at the tuberal level, and the LH. Intense Rspo 3 positivity was also observed in the cortex areas of the brain. Moderate density and randomly distributed localized neurons showing Rspo 3 expression were observed in the PeV around the third ventricle, ZI, and H region; however, light immunopositivity and a small number of cells were detected for the ARC. Rspo 3 immune positivity was not found in any neurons localized in MS, HBD, VDB, PaVP, MCPO, VLPO, and RN areas.

Table II. Semi-Quantitative Expression Levels of Rspo1 and Rspo3 Peptides in the Hypothalamus

LOCALIZATION	AntiBODIES	
Preoptic Level	**Rspo 1**	**Rspo 3**
Medial Septal Nucleus (MS)	+++	–
Broca's Diagonal Band (BDB)	+++	+++
Magnocellular Preoptic Nucleus (MCPO)	++	–
Ventrolateral Preoptic Nucleus (VLPO)	++	–
Anterior Level		
Periventricular Nucleus (PeV)	+	++
Suprachiasmatic Nucleus (SCh)	+++	+++
Paraventricular Nucleus (PVN)	+++	+++
Anterior Parvisellüler Nucleus (PaAP)	++	+
Supraoptic Nucleus (SON)	+++	+++
Tuberal Level		
Arcuate Nucleus (ARC)	+++	++
Ventromedial Hypothalamic Nucleus (VMH)	+	+++
Lateral Hypothalamus (LH)	++	+++
Mamillare Level		
Red Nucleus (RN)	++	–
Hippocampus	++	+++

"+++" is used for high number of neurons, "++" is used for a moderate number of neurons, "+" is used if a few numbers of neurons or "–" is used to mean no immunopositivity.

It has been reported that after icv injection of R-spondins, the mRNA expression level of the anorexigenic

peptide pro-opiomelanocortin (POMC) increased and the mRNA expression of the orexigenic peptide Neuropeptide-Y (NPY) decreased in the rat arcuate nucleus (Li *et al*., 2014). In another study using the Lgr4 gene knockout mouse model, it was reported that POMC expression was decreased while NPY expression was not unaffected. After the icv injection of Rspo1 into wild-type mice, anorexigenic POMC neurons localized in the ARC have shown c-Fos activation (Otsuka *et al*., 2019). Another study found that neurons containing cocaine- and amphetamine-regulated transcript (CART) localized in the arcuate nucleus expressed both LGR4 and the insulin receptor, c-Fos expression was induced in these neurons after icv injection of Rspo1, and anorexigenic effects of the RSPO peptide were reduced after silencing of CART (Li *et al*., 2019). It has been shown that Rspo1 and Rspo 3 peptide levels are down-regulated by fasting, while these levels are up-regulated by the satiety factor insulin. The inhibition of food intake observed after icv injection of Rspo1 or Rspo3 supports this concept (Li *et al*., 2014). The Rspo1 peptide is more potent than Rspo3 in inhibiting food intake and binds to LGR4 with higher affinity (Carmon *et al*., 2011).

Although these studies in the literature have determined that Rspo1 and Rspo3 peptides play an important role in regulating food intake, no histomorphological studies have been found showing the localization of neurons synthesizing these peptides in the hypothalamic nuclei. For the first time in the literature, to our knowledge, we showed the expression of Rspo 1 and Rspo 3 in the hypothalamic neurons in an abstract form (Coskan *et al*., 2022). The findings of the present study detailed the localization and distribution of these neurons in the hypothalamus.

In this study, the immunohistochemistry technique, which is a frequently used histological method for cellular protein localization in tissues, was used. Although immunohistochemistry is a technique that gives visual results and has high evidence power, it is limited to only detectable levels of R spondin protein expressed by neurons, and only these brain neurons were defined as R spondin positive neurons. There may also be other R spondin-positive neurons in the central nervous system that we could not identify using this technique. Therefore, further studies using other methods such as in situ hybridization are needed to better understand the expression pattern of R spondins. In conclusion, the fact that Rspo1 and Rspo3 peptides, which are ligands of LGR4, are synthesized in many nuclei of the hypothalamus that play a role in the control of food intake suggests that these peptides may play a role in the regulation of energy homeostasis as anorexigenic factors. The findings obtained from our study may lead to the planning of further studies on the functions and signaling mechanisms of LGR4 and Rspo proteins related to food intake in the hypothalamus.

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RESUMEN: La familia de proteínas R-espondina es un grupo de proteínas que mejoran la señalización de Wnt/b-catenina y tienen funciones pleiotrópicas en el crecimiento y desarrollo de las células madre. En las revisiones de la literatura no existen estudios histomorfológicos que muestren la localización y distribución de las R-espondinas en diferentes núcleos hipotalámicos. Por esta razón, el propósito de este estudio fue determinar la localización, características de distribución y densidades en los núcleos hipotalámicos de neuronas que expresan las proteínas Rspo1 y Rspo3. Secciones de cerebro flotantes de ratas macho que no fueron expuestas a ningún tratamiento se tiñeron con el método de inmunoperoxidasa indirecta utilizando los anticuerpos pertinentes. Como resultado de los estudios inmunohistoquímicos, se determinó que las neuronas que expresan la proteína Rspo1 se encontraron en gran número en el núcleo supraóptico (SON), el núcleo supraquiasmático (SCh), el núcleo paraventricular anterior, el núcleo hipotalámico periventricular (PeV), el núcleo hipotalámico anterior área, núcleo preóptico magnocelular (MCPO) y el área hipotalámica lateral (LH) de los núcleos hipotalámicos, mientras que se localizaron en menor número en el núcleo arqueado (ARC). La expresión de la proteína Rspo3 se encontró en neuronas localizadas en los núcleos hipotalámicos SON, núcleo paraventricular (PVN), PeV, ARC, núcleo ventromedial (VMH), LH, núcleo parvicelular anterior y zona inserta (ZI). Además, se encontraron neuronas que sintetizan ambos péptidos en las regiones de la corteza y el hipocampo (H). Las proteínas Rspo1 y 3 se expresan en áreas homeostáticas de energía hipotalámicas, por lo que estas proteínas pueden estar involucradas en la regulación de la ingesta de alimentos.

PALABRAS CLAVE: R-espondina 1; R-espondina 3; Hipotálamo; Inmunohistoquímica; Rata.

REFERENCES

Berthoud, H. R. & Morrison, C. The brain, appetite, and obesity. *Annu. Rev. Psychol., 59*:55-92, 2008.

Carmon, K. S.; Gong, X.; Lin, Q.; Thomas, A. & Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/-catenin signaling. *Proc. Natl. Acad. Sci., 108(28)*:11452-7, 2011.

Coskan, N.; Gok-Yurtseven, D. & Eyigor, O. *Immunohistochemical Localization of R-Spondin Expressing Neurons in the Rat Hypothalamus*. Ankara, NICHE2022, 15th National and 1st International Congress of Histology and Embryology, 2022. pp.134.

de Lau, W.; Barker, N.; Low, T. Y.; Koo, B. K.; Li, V. S. W.; Teunissen, H.; Kujala, P.; Haegebarth, A.; Peters, P. J.; van de Wetering, M.; *et al*. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature, 476(7360)*:293-7, 2011.

Glinka, A.; Dolde, C.; Kirsch, N.; Huang, Y. L.; Kazanskaya, O.; Ingelfinger, D.; Boutros, M.; Cruciat, C. M. & Niehrs, C. LGR4 and LGR5 are R-spondin receptors mediating Wnt/-catenin and Wnt/PCP signalling. *EMBO Rep., 12(10)*:1055-61, 2011.

Gong, X.; Carmon, K. S.; Lin, Q.; Thomas, A.; Yi, J. & Liu, Q. LGR6 is a high affinity receptor of R-spondins and potentially functions as a tumor suppressor. *PLoS One, 7(5)*:e37137, 2012.

Guan, X.; Duan. Y.; Zeng, Q.; Pan, H.; Qian, Y.; Li, D.; Cao, X. & Liu, M. Lgr4 protein deficiency induces ataxia-like phenotype in mice and impairs long term depression at cerebellar parallel fiber-purkinje cell synapses. *J. Biol. Chem., 289(38)*:26492-504, 2014.

Gyllborg, D.; Ahmed, M.; Toledo, EM.; Theofilopoulos, S.; Yang, S.; Ffrench-Constant, C. & Arenas, E. The matricellular protein R-spondin 2 promotes midbrain dopaminergic neurogenesis and differentiation. *Stem Cell Rep., 11(3)*:651-64, 2018.

Li, J. Y.; Chai, B.; Zhang, W.; Fritze, D. M.; Zhang, C. & Mulholland, M. W. LGR4 and ıts ligands, R-spondin 1 and R-spondin 3, regulate food ıntake in the hypothalamus of male rats. *Endocrinology, 155(2)*:429-40, 2014.

Li, J. Y.; Kuick, R.; Thompson, R. C.; Misek, D. E.; Lai, Y. M.; Liu, Y. Q.; Chai, B. X.; Hanash, S. M. & Gantz, I. Arcuate nucleus transcriptome profiling identifies ankyrin repeat and suppressor of cytokine signalling box-containing protein 4 as a gene regulated by fasting in central nervous system feeding circuits. *J. Neuroendocrinol., 17(6)*:394-404, 2005.

Li, J. Y.; Wu, X.; Lee, A.; Zhou, S. Y. & Owyang, C. Altered R-spondin 1/CART neurocircuit in the hypothalamus contributes to hyperphagia in diabetes. *J. Neurophysiol., 121(3)*:928-39, 2019.

Li, Z.; Zhang, W. & Mulholland, M. W. LGR4 and its role in intestinal protection and energy metabolism. *Front. Endocrinol. (Lausanne), 6*:131, 2015.

Nam, J. S.; Turcotte, T. J.; Smith, P. F.; Choi, S. & Yoon, J. K. Mouse cristin/R-spondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate beta-catenin-dependent gene expression. *J. Biol. Chem., 281(19)*:13247-57, 2006.

Otsuka, A.; Jinguji, A.; Maejima, Y.; Kasahara, Y.; Shimomura, K.; Hidema, S. & Nishimori, K. LGR4 is essential for R-spondin1-mediated suppression of food intake via pro-opiomelanocortin. *Biosci. Biotechnol. Biochem., 83(7)*:1336-42, 2019.

Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates*. 6th ed. Amsterdam, Elsevier, Academic Press, 2009.

Ruffner, H.; Sprunger, J.; Charlat, O.; Leighton-Davies, J.; Grosshans, B.; Salathe, A.; Zietzling, S.; Beck, V.; Therier, M.; Isken, A.; *et al*. R-spondin potentiates Wnt/-catenin signaling through orphan receptors LGR4 and LGR5. *PLoS One, 7(7)*:e40976, 2012.

Schoore, G. V.; Mendive, F.; Pochet, R. & Vassart, G. Expression pattern of the orphan receptor LGR4/GPR48 gene in the mouse. *Histochem. Cell Biol., 124(1)*:35-50, 2005.

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