RESEARCH ARTICLE Indian Journal of Animal Research, Volume 57 Issue 6: 708-713 (June 2023)

Comparative Expression Profile of RFRP-3 in Hypothalamus and Testis of Adult Rats

Asim Chaudhuri[1], Pradip Kumar Das[1], Probal Ranjan Ghosh[1], Joydip Mukherjee[1], Kinsuk Das[1], Partha Das[2], Dipak Banerjee[1]

10.18805/IJAR.B-4713

ABSTRACT

Background: The present study focused on the localization and comparative expression analysis of RFRP-3 gene in the hypothalamus and testis of adult male rats.

Methods: Brain and testis tissue samples were collected from adult Wister rats and used for immunohistochemistry for identification and localization RFRP-3 producing neurons. The expression level of the RFRP-3 gene was assessed by Real-time PCR and compared with muscle tissue.

Result: The results confirmed the presence of RFRP-3 secreting neurons in the hypothalamus and in the testes. Scattered RFRP-3 in cell bodies was identified primarily in the VMH, DMH and surrounding the third ventricle of the brain. In testis, RFRP-3 was detected in Leydig cells, Sertoli cells and germ cells. The expression level of RFRP-3 gene in the hypothalamus was observed to be significantly higher in hypothalamus than in testicular tissue ($p<0.01$) of adult rats.

Key words: Hypothalamus, Immunohistochemistry, Rat, RFRP-3, Testis.

INTRODUCTION

Hypothalamic-pituitary-gonadal axis plays a pivotal role in regulating mammalian reproduction (Harris, 1964). Kisspeptins and RFamide-related peptides (RFRPs) are two crucial neuropeptides that regulate GnRH secretion from the hypothalamus. Kisspeptins stimulate GnRH secretion, whearse RFRP-3 inhibits GnRH secretion in birds and mammals. RFRP-3 regulates reproduction and influences food intake, stress response and sexual behavior (Tsutsui and Ubuka, 2020). RFRPs (RFRP-1 and RFRP-3) are RFamide peptide, which contains arginine-phenylalanine-amide at C-terminal end.

The RFamide-related peptide-3 (RFRP-3), also known as gonadotropin inhibiting hormone (GnIH) in mammals. Different studies have shown that GnIH (RFRP-3) acts directly in the hypothalamic hypophysial system, offers direct actions on the gonads and prevents gonadotropin release (Tsutsui and Ubuka, 2020). RFRP-3 and its receptor have been detected in testicular interstitial cells and germ cells and pseudostratified columnar epithelial cells in the epididymis. GnIH (RFRP-3) and its receptors are also expressed in the gonads and reproductive organs of birds (Bentley *et al*., 2008; McGuire and Bentley, 2010) and mammals (Zhao *et al*., 2010). The sites of secretion and expression patterns of GnIH/RFRP-3 and its receptor suggest a role in steroid synthesis, gamete maturation and apoptosis. Recent studies indicate that GnIH/RFRPs can have an autocrine or paracrine effect on a wide range of gonadal functions in birds and mammals.

In mice, the expression of RFRP-3 and its effect on steroidogenesis on GnRH receptor (GnRHR) and LH-receptor (LHR) in the testis has been tested (Anjum *et al*., 2012; Anjum *et al*., 2014). The presence of RFRP-3 and its receptors in the Syrian hamster (Zhao *et al*., 2010) suggested a role in spermatogenesis and androgens synthesis (Zhao *et al*., 2010). The effects of RFRP-3 depend on gonadotropins in primates and rats (Anjum *et al*., 2014). The expression of RFRP-3 is affected by photoperiod and reproductive conditions (Zhao *et al*., 2010).

Several studies were conducted on different avian and mammalian species for molecular characterization of RFRP-3 and its physiological distribution and function; however, there are very few studies on the expression analysis of RFRP-3 gene in the mammalian testis. Therefore, it is the need of the hour to comprehensively analyze the expression level of the RFRP-3 gene in the hypothalamic region of the brain and testis in rats. Present study was designed to localize RFRP-3 secreting cells and comparative expression analysis of RFRP-3 gene in hypothalamus and testis of the adult male rats.

[1]Department of Veterinary Physiology, West Bengal University of Animal and Fishery Sciences, Kolkata-700 037, West Bengal, India. [2]Department of Veterinary Anatomy and Histology, West Bengal University of Animal and Fishery Sciences, Kolkata-700 037, West Bengal, India.

Corresponding Author: Dipak Banerjee, Department of Veterinary Physiology, West Bengal University of Animal and Fishery Sciences, Kolkata-700 037, West Bengal, India. Email: dipakndri@gmail.com

How to cite this article: Chaudhuri, A., Das, P.K., Ghosh, P.R., Mukherjee, J., Das, K., Das, P. and Banerjee, D. (2023). Comparative Expression Profile of RFRP-3 in Hypothalamus and Testis of Adult Rats. Indian Journal of Animal Research. 57(6): 708-713. doi: 10.18805/IJAR.B-4713.

Submitted: 09-07-2021 **Accepted:** 07-03-2022 **Online:** 29-04-2022

MATERIALS AND METHODS

The experiments were performed on adult male Wister rats free from physical and anatomical abnormalities. The animals were maintained under 12/12-h light-dark cycle and constant temperature (24°C) and given ad-libitum access to pelleted food and fresh drinking water. All the experiments were performed following the guidelines of the Regional Animal Ethics Committee and the Rules for Experimental Animals of the University.

Brain (hypothalamus) and testiscular tissue samples were collected from the rats after lightly anesthetized with isoflurane in 10% neutral buffered formalin (NBF) for histological and immunohistochemical studies.

The samples for immunochemistry were fixed in NBF for 48 h at room temperature. They were dehydrated through a graded series of ethyl alcohol and frozen before being cut into 5-7 µm sections. For RNA extraction, brain (hypothalamus), pituritary, testis and muscle tissue samples were removed and immersed in RNA-later solution (Thermo Fisher Scientific, Santa Clara, CA, USA) and frozen at -80°C until used.

The sections of the samples of 5 µm thickness were prepared with the help of Leica 2125 DM rotary microtome after preparing a stanadred paraffin blocks and fixed for histological studies in 10% NBF solution. The slides were treated with Mayer's Haematoxylin followed by 2% Eosin and rinsed in 90% ethyl alcohol. Finally, mounted with DPX.

The sections were treated with phosphate buffered saline PBS and 0.3% H_2O_2 in methanol for quenching endogenous peroxidase. Blocking of the background was done by 10% normal horse serum. Tissue sections were then incubated for 4 hours at 37°C with RFRP-3 antibody (RFRP-3 antibody was kindly given by Kazuyoshi Tsutsui, Professor, Center for Medical Life Science Waseda University, Tokyo, Japan) diluted in PBS. The slides were then incubated with the secondary antibody using the ABC universal staining kit (Wuhan Boster Biological Technology Co., Ltd., China). The peroxidise activity was assessed using 0.03% 3,3′-diaminobenzidine tetrahydrochloride (Wuhan Boster Biological Technology Co, Ltd., China) in 0.05 M Tris, pH 7.6 and 0.1% H_2O_2 for 5 min. For testing immunoreaction specificity, the primary antiserum was replaced in the control phase with 1% normal horse serum; the pre-adsorption of the RFRP-3 antiserum with the RFRP-3 antigen (10 µg/ml). The slides of both histology and immunohistochemistry were observed under Leica DM 2000 Microscope in different magnifications and localizing RFRP-3-immunoreactive (ir) cells were done.

Tissue samples stored in RNA-later solution were homogenized in a Polytron homogenizer and total RNA was extracted using Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instruction. After checking the integrity of RNA (1.5% agarose gel) and determining the concentration of RNA (NanoDrop ND-1000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA) total RNA was treated with RNase-free DNAseI (Fermentas Life Sciences, Canada) to exclude genomic DNA contamination. The absence of contaminant genomic DNA in RNA preparations was tested using RNA as a template in real-time PCR assays (RNA not reverse-transcribed to cDNA). Total RNA from each sample was reverse transcribed into cDNA in a 20 µl reaction mixture using High Capacity RNA-to-cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

The primers for RFRP-3 were designed from the available sequences in NCBI GenBank (Table 1), optimized synthesized and standardized (Fig 1) along with the housekeeping gene (RPS18) for amplification by quantitative real-time polymerase chain reaction (qPCR). The relative expression level of mRNA transcripts of the RFRP-3 gene was measured by qPCR (Roche LC480) using the SYBR Green I (Roche Diagnostics, Switzerland). Relative quantification of a target gene was done by comparing the expression of RPS18 as per Pfaffl (2001) method. The specificity of each primer pair was confirmed using melt curve analysis and samples run on a 2% agarose gel with a 100-bp DNA ladder (Invitrogen) to verify the generation of a single product of the correct size (Fig 1A).

Data were analyzed by one-way analysis of variance (ANOVA) considering significant level at $p<0.05$. All statistical analyses of the qPCR data used CT values, whereas graph (Fig 4) indicate the fold changes ($2^{-\Delta\Delta CT}$).

RESULTS AND DISCUSSION

Localization of RFRP-3 secreting cells in the hypothalamus and testis

Different nucleus were identified by histological study with HE staining (Fig 2). RFRP-3 labeled cells were mainly identified (by immunochemistry) in the dorsomedial nucleus of the hypothalamus (DMH) and ventromedial nucleus of the hypothalamus (VMH) with some labeled cells along the sides of the third ventricle (3V) in the rostral-caudal extent of the hypothalamus (Fig 2). RFRP-3-ir fibers are also detected along the sides of the and above the dorsal edge of the 3V. A higher density of fibers was found in the caudal extent of the hypothalamus.

Table 1: Real-time primers for RFRP-3 and RPS18 genes (housekeeping gene).

Gene	F/R	Primer sequence	Melting point (Tm in °C)	Product size (bp)
RFRP-3	F	GCGATTCATTTTATTGACTTTAGCAACTTCAAGC	60	143 bp
	R	TTACCCCTTTTGGGATTCCTCTCAGCTG	61	
RPS18	F	TGCGAGTACTCAACACCAACATCGATGG	61	184 bp
	R	GGATTCTGCATAATGGTGATCACACGTTCC	62	

The results of this study confirmed the presence of scattered RFRP-3-ir cell bodies in the VMH, DMH and surroundings the third ventricles in male rats. Similar findings, *viz.* RFRP3-secreting cells in DMN and fibers of neurons extend to the hippocampus, preoptic area (POA), paraventricular nucleus (PVN), anterior hypothalamus and the lateral hypothalamus' rostral aspects were recorded in rats by Rizwan *et al*. (2009); in mouse DMH (Kriegsfeld *et al*., 2006), surrounding the VMH in the rat (Johnson *et al*., 2007) and in PVN and DMH in Sheep (Smith *et al*., 2008). The expression of GnIH mRNA in the neurons of DMH was observed in mice by Ukena and Tsutsui (2001), in hamster

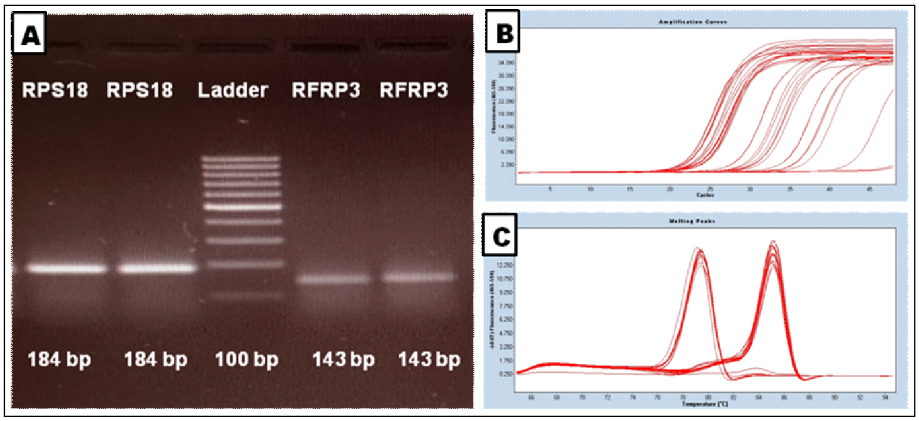

Fig 1: (A) Real-time PCR amplified products of RFRP-3 and RPS18 genes on agarose gel 3%; (B) Amplification Curves of RFRP3 and RPS18 genes; (C) Melting peaks of RFRP3 and RPS18 genes.

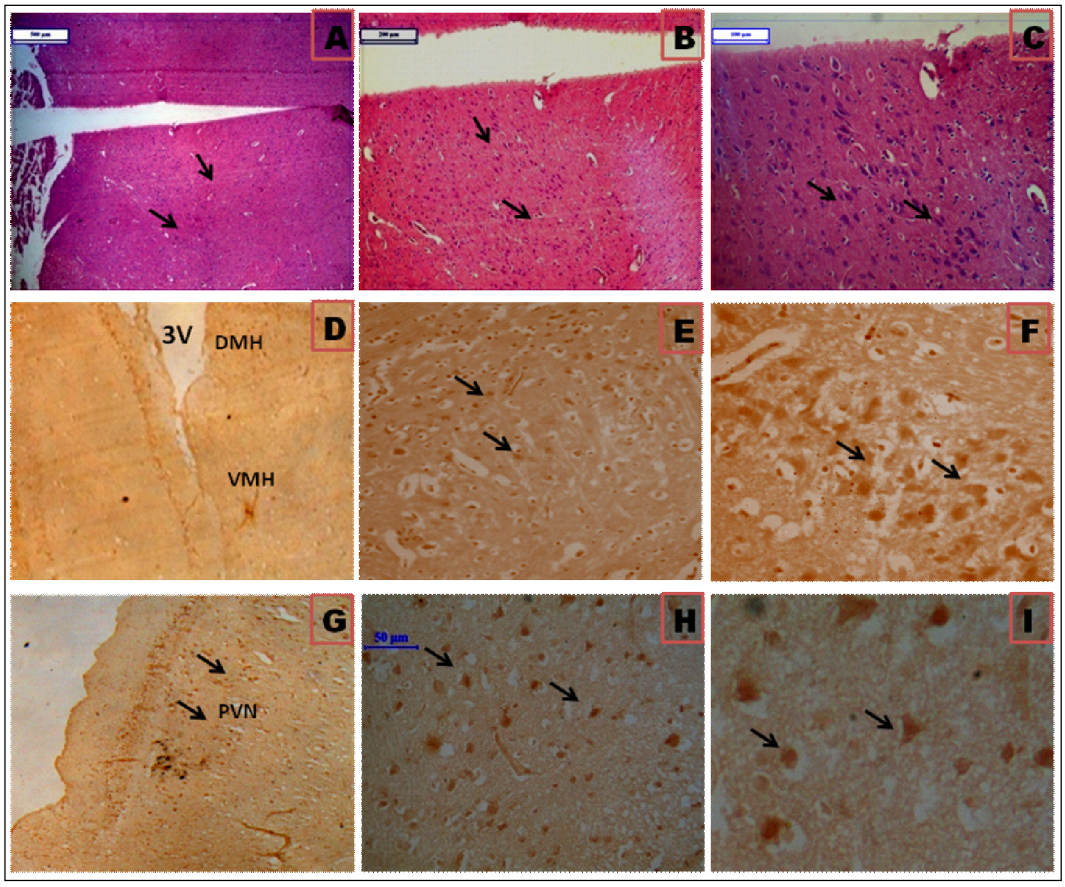

Fig 2: Hypothalamic sections showing the distribution of RFRP-3-immunoreactive neurons;
(A) HE staining of PVN; Panels B and C are enlargements of panels A.
(D) RFRP 3-immunoreactive cell bodies within the DMH and VMH. Panels E and F are enlargements of panels B.
(G) RFRP3-immunoreactive neurons within the PVN. Panels H and I are enlargements of panels G.
(Scale bar - A: 500 µm, B: 200 µm, C: 100 µm, D: 500 µm, E: 100 µm, F: 50 µm, G: 500 µm, H: 50 µm, I: 50 µm).

(Ubuka *et al*., 2012) and in DMH and PVN of the sheep (Qi *et al*., 2009) and in mare (Thorson *et al*., 2014).

Thompson *et al*. (1996) reported the projections of DMH axons in the intrahypothalamic, *i.e*., neuroendocrine and pre-autonomic hypothalamic regions and to some extent in the extrahypothalamic sites, namely, brainstem and the telencephalon. Thompson *et al*. (2003) explained the DMH, with five other preoptic nuclei, *i.e*., anteroventral and anterodorsal preoptic, parastrial, median preoptic and anteroventral periventricular, form a complex interconnected network that plays a key role in coordinating neuroendocrine, autonomic and somatic responses to external stimuli, sensory feedback and cognitive/motivational input. Any lesions in DMH prevent the seasonal onset of reproductive quiescence which is mediated by increased gonadal steroid negative feedback in the Syrian hamster (Lewis *et al*., 2002).

In our study, the RFRP-3 was observed in both Leydig cells and the seminiferous tubules at the level of germ and Sertoli cells of rats (Fig 3). Similar findings were obtained in different species, *viz*. in avian species (McGuire and Bentley, 2010), pigs (Zheng *et al*., 2015), rodents (Zhao *et al*., 2010; Anjum *et al*., 2012). Therefore, our study revealed the testes as a site of RFRP-3 biosynthesis in rats, according to the earlier work in birds (Bentley *et al*., 2008) and mammals (Iwasa *et al*., 2017). It was reported that Leydig cells is the main site of RFRP-3 synthesis and have a local effect on Leydig cells to inhibit secretion of testosterone in birds (McGuire and Bentley, 2010) as well as in mammals (Anjum *et al*., 2014; Lents *et al*., 2017) *via* the repression of LHR, steroidogenic acute regulatory (StAR) protein, cytochrome P450 side-chain cleavage and 3β-hydroxysteroid dehydrogenase expression (Anjum *et al*., 2014; Zheng *et al*., 2015) and probably preventing protein kinase A (PKA) activation (Lents *et al*., 2017).

Relative expression profile of RFRP-3 gene

Melting curve analysis did not yield any non-specific peak from each primer set tested. Additionally, every PCR product generated a prominent band with an expected size in the gel electrophoresis analysis (Fig 1). These indicated that non-specific amplification with the primer sets tested was not detected in the real-time PCR analysis. Present study also indicated that the mRNA of RFRP-3 was significantly highly expressed ($p<0.01$) in the hypothalamus than the pituitary and testis; pituitary had a higher ($p<0.05$) expression than the testis and no expression was observed in muscle tissue (Fig 4).

Our results support previous studies on other species (Legagneux *et al*., 2009; Tan *et al*., 2021). Ubuka *et al*. (2014) observed the expression of GnIH precursor mRNA in the interstitium. Rizwan (2012) observed that RFRP mRNA is mainly expressed at high levels in the hypothalamus and eye and a very low level in the testis. The presence of RFRP

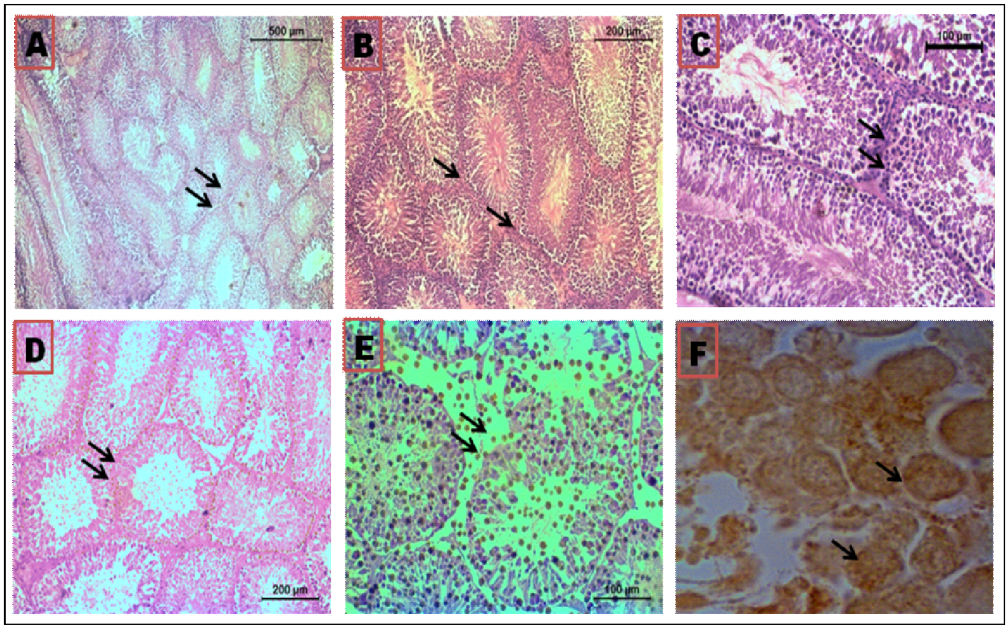

Fig 3: Testicular sectionsshowing the distribution of RFRP-3-immunoreactive cells.

H&E staining of testis; seminiferous tubule pointed by arrow A (4X) and Interstitial cells of Leydig, pointed by arrows B (10X) and C (20X). H&E.

Photomicrograph showing spermatogonium, pointed by arrows D (10X) IHC, Interstitial cell of Leydig pointed by arrows E (20X), Panels F is enlargements of panels E.

IHC Positive immunostaining in brown color, counterstaining with hematoxylin; RFRP-3 peptide is located in different stages of spermatogenesis. The signal is also present in Sertoli cells. Strong RFRP-3 signal is observed in Leydig cells.

(Scale bar- A: 500 µm, B: 200 µm, C: 100 µm, D: 200 µm, E: 100 µm, F: 50 µm).

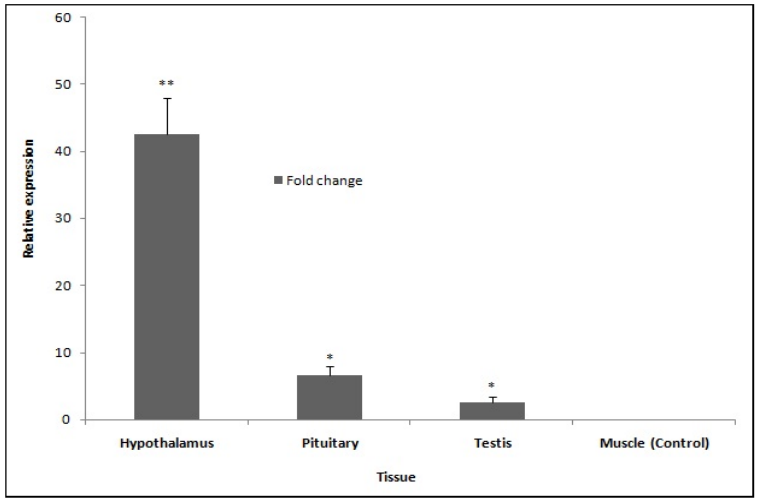

Fig 4: Relative expression profile of RFRP-3 genes in hypothalamus, testis and pituitary gland in comparison to muscle tissue as control (Relative mRNA expression levels were normalized with RPS18 mRNA. Data represent mean±SD. "∗∗" between bars indicated difference was extremely significant ($p<0.01$); "∗" between bars was significant ($p<0.05$).

and its receptors in the seminiferous tubules and developing spermatids have confirmed in the Syrian hamster and suggested the inhibitory role of RFRP in the local regulation of testicular functions during the regressive phase of the reproductive cycle (Zhao *et al*., 2010). Expression of RFRP and its receptor (GPR147) has been demonstrated in spermatocytes and suggests a possible role of RFRP in the final maturation of spermatids (Zhao *et al*., 2010). McGuir and Bentley *et al*. (2010) confirmed the expression of GnIH and its receptor, GPR147, in Leydig cells of the testis and GnIH reduced the effect of LH on testosterone secretion. Apoptotic cell death in the testis may result from a GnIH-induced decrease in testicular testosterone (Tsutsui *et al*., 2010). Based on current understanding, it appears that GnIH may have the potential for healing of the disease that requires down-regulation of reproductive function.

CONCLUSION

This study confirmed the presence of RFRP-3 secreted neurons in the hypothalamus in male rats. Scattered RFRP-3-ir cell bodies were identified to be mainly surrounding the VMH, DMH and third ventricles. RFRP-3-ir cells are clustered in DMH. DMH projects extensively to neuroendocrine and preautonomic hypothalamic regions. Immunohistochemical studies revealed the presence of RFRP-3 peptide in rat testes. This finding is consistent with the results obtained in different species. RFRP -3 was mainly expressed at high levels in the hypothalamus and very low levels in the testes.

ACKNOWLEDGEMENT

The authors are thankful to Kazuyoshi Tsutsui, Professor, Laboratory of Integrative Brain Sciences, Department of Biology, Waseda University, Tokyo, Japan for providing the RFRP-3 antibody for this study.

Conflict of interet: None.

REFERENCES

Anjum, S., Krishna, A. and Tsutsui, K. (2014). Inhibitory roles of the mammalian GnIH ortholog RFRP3 in testicular activities in adult mice. Journal of Endocrinology. 223: 79-91.

Anjum, S., Krishna, A., Sridaran, R. and Tsutsui K. (2012). Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH-receptor and their possible roles in testicular activities from birth to senescence in mice. Journal of Experimental Zoology. 317A: 630-644.

Bentley, G.E., Ubuka, T., McGuire, N.L., Chowdhury, V.S., Morita, Y., Yano, T., Hasunuma, I., Binns, M., Wingfield, J.C. and Tsutsui, K. (2008). Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. General and Comparative Endocrinology. 156(1): 34-43.

Harris, G.W. (1964). Sex hormones, brain development and brain function. Endocrinology. 75: 627-648.

Iwasa, T., Matsuzaki, T., Yano, K. and Irahara, M. (2017). Gonadotropin-inhibitory hormone plays roles in stress-induced reproductive dysfunction. Frontiers in Endocrinology (Lausanne). 8: 62. doi: 10.3389/fendo.2017.00062.

Johnson, M.A., Tsutsui, K. and Fraley, G.S. (2007). Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion and has variable effects on sex behavior in the adult male rat. Hormones and Behavior. 51: 171-180.

Kriegsfeld, L.J., Mei, D.F., Bentley, G.E., Ubuka, T., Mason, A.O., Inoue, K., Ukena, K., Tsutsui, K. and Silver, R. (2006). Identification and characterization of a gonadotropininhibitory system in the brains of mammals. Proceedings of the National Academy of Sciences. 103: 2410-2415.

Legagneux, K., Bernard-Franchi, G., Poncet, F., La Roche, A., Colard, C., Fellmann, D., Pralong, F. and Risold, P.Y. (2009). Distribution and genesis of the RFRP-producing neurons in the rat brain: Comparison with melanin-concentrating hormone and hypocretin-containing neurons. Neuropeptides. 43: 13-19.

Lents, C.A., Thorson, J.F., Desaulniers, A.T. and White, B.R. (2017). RFamide-related peptide 3 and gonadotropin-releasing hormone-II are autocrine-paracrine regulators of testicular function in the boar. Molecular Reproduction and Development. 84: 994-1003.

Lewis, D., Freeman, D.A., Dark, J., Wynne-Edwards, K.E. and Zucker, I. (2002). Journal of Neuroendocrinology. 14: 294-299.

McGuire, N.L. and Bentley, G.E. (2010). A functional neuropeptide system in vertebrate gonads: Gonadotropin-inhibitory hormone and its receptor in testes of field-caught house sparrow (*Passer domesticus*). General and Comparative Endocrinology. 166: 565-572.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research. 29: 2002-2007.

Qi, Y., Oldfield, B.J. and Clarke, I.J. (2009). Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. Journal of Neuroendocrinology. 21: 690-697.

Rizwan, M.Z., Poling, M.C., Corr, M., Cornes, P.A., Augustine, R.A., Quennell, J.H., Kauffman, A.S. and Anderson, G.M. (2012). RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology. 153(8): 3770-3779.

Rizwan, M.Z., Porteous, R., Herbison, A.E. and Anderson, G.M. (2009). Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. Endocrinology. 150: 1413-1420.

Smith, J.T., Coolen, L.M., Kriegsfeld, L.J., Sari, I.P., Jaafarzadehshirazi, M.R., Maltby, M., Bateman, K., Goodman, R.L., Tilbrook, A.J. and Ubuka, T. (2008). Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: A novel medium for seasonal breeding in the sheep. Endocrinology. 149: 5770-5782.

Tan, Y.G., Xu, X.L., Cao, H.Y., Mao, H.G. and Yin, Z.Z. (2021). RFamide-related peptides' gene expression, polymorphism and their association with reproductive traits in chickens. Poultry Science. 100: 488-495.

Thompson, R.H. and Swanson, L.W. (2003). Structural characterization of a hypothalamic visceromotor pattern generator network. Brain Research. Brain Research. Review. 41: 153-202.

Thompson, R.H., Canteras, N.S. and Swanson, L.W. (1996). Organization of projections from the dorsomedial nucleus of the hypothalamus: A PHA-L study in the rat. Journal of Comparative Neurology. 376: 143-173.

Thorson, J.F., Prezotto, L.D., Cardoso, R.C., Sharpton, S.M., Edwards, J.F., Welsh Jr., T.H., Riggs, P.K., Caraty, A., Amstalden, M. and Williams, G.L. (2014). Hypothalamic distribution, adenohypophyseal receptor expression and ligand functionality of RFamide-related peptide 3 in the mare during the breeding and nonbreeding seasons. Biology of Reproduction. 90(2): 28: 1-9.

Tsutsui, K. and Ubuka, T. (2020). Discovery of gonadotropin-inhibitory hormone (GnIH), progress in GnIH research on reproductive physiology and behavior and perspective of GnIH research on neuroendocrine regulation of reproduction. Molecular and Cellular Endocrinology. 514: 110914. doi: 10.1016/j.mce.2020.110914.

Tsutsui, K., Bentley, G.E., Bedecarrats, G., Osugi, T., Ubuka, T. and Kriegsfeld, L.J. (2010). Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. Frontiers in Neuroendocrinology. 31(3): 284-295.

Ubuka, T., Inoue, K., Fukuda, Y., Mizuno, T., Ukena, K., Kriegsfeld, L.J. and Tsutsui, K. (2012). Identification, expression and physiological functions of Siberian hamster gonadotropin inhibitory hormone. Endocrinology. 153: 373-385.

Ubuka, T., Son, Y.L., Tobari, Y., Narihiro, M., Bentley, G.E., Kriegsfeld, L. and Tsutsui, K. (2014). Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor. Frontiers in Eendocrinology. 5: 8. https://doi.org/10.3389/fendo.2014.00008.

Ukena, K. and Tsutsui, K. (2001). Distribution of novel RFamide-related peptide-like immuno reactivity in the mouse central nervous system. Neuroscience Letters. 300: 153-156.

Zhao, S., Zhu, E., Yang, C., Bentley, G.E., Tsutsui, K. and Kriegsfeld, L.J. (2010). RF amide related peptide and messenger ribonucleic acid expression in mammalian testis: Association with the spermatogenic cycle. Endocrinology. 151: 617-627.

Zheng, L., Su, J., Fang, R., Jin, M., Lei, Z., Hou, Y., Ma, Z. and Guo, T. (2015). Developmental changes in the role of gonadotropin-inhibitory hormone (GnIH) and its receptors in the reproductive axis of male Xiaomeishan pigs. Animal Reproduction Science. 154: 113-120.