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Original Communication

Rat RFamide-related peptide-3 suppresses sex behavior and luteinizing hormone levels in the adult female rat

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ABSTRACT

Much is known about neuropeptides that stimulate the hypothalamic-pituitary-gonadal axis, however, less is known about peptides that may inhibit this pathway. Recently, a peptide (RFRP-3) was shown to act as a luteinizing hormone inhibitor in males. The purpose of this study was to investigate the effects of RFRP-3 on sex behavior and the estrus-related increase in luteinizing hormone levels in female rats. We hypothesized that central ICV administration of RFRP-3 (500 ng) would suppress both female sex behavior and LH levels in ovariectomized, hormone-primed female rats. Each animal received both RFRP-3 and vehicle, counterbalanced for order, with injections separated by at least one week. For the sex behavior study, after RFRP-3 or vehicle infusion the females were placed in a cage with an experienced male and sex behavior was recorded for one hour (i.e., lordosis, mounts, intromissions and ejaculations). For the measurement of LH levels, blood was collected by tail nick 30 minutes and 90 minutes after the RFRP-3 or vehicle infusion. LH levels were measured in the same animals that had been previously tested in the sex behavior study. Results indicate that RFRP-3 suppressed sexual receptivity in the female rat by significantly decreasing lordosis quotient between 45 and 90 minutes post-RFRP-3 injection, $p<0.05$. In addition, the LH surge was suppressed in RFRP-3 injected animals compared with vehicle-injected controls ($p = 0.004$). These results suggest that RFRP-3 may act as an important regulator of LH levels and sex behavior during estrus.

KEYWORDS: rat, lordosis, sex behavior, rat RFamide-related peptide

INTRODUCTION

In mammals, reproduction is regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Secretion of gonadotropin-releasing hormone (GnRH) regulates this axis and controls the synthesis and secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which then stimulate the production of gonadal steroids. Both negative and positive feedback by gonadal steroids regulate GnRH neuron firing and this regulation is crucial for optimization of fertility and successful reproduction. Much is known about neuropeptides that stimulate the HPG axis (e.g. GnRH, galanin like peptide [GALP]), however less is known about peptides that may inhibit this pathway. Recently, an RFamide peptide was shown to act as an inhibitor of this axis by decreasing both LH levels and reproductive behavior [1, 2, 3, 4, 5, 6, 7, 8].

This peptide, a 12-amino acid sequence (Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2) which inhibits gonadotropin secretion was first discovered in birds [2, 3, 9, 10, 11, 12, 13]. This novel neuropeptide was shown to directly inhibit LH release from cultured quail anterior pituitaries

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and thus was given the name gonadotropin-inhibitory hormone [GnIH; 12]. High concentrations of GnIH-immunoreactive (-ir) neurons were found in the paraventricular nucleus (PVN) of the hypothalamus of the quail [13]. GnIH-ir fibers were found scattered among the ventral paleostriatum, septal area, preoptic area, and optic tectum, [7] as well as in the median eminence, further supporting the role of GnIH as a regulator of pituitary gonadotropins [7]. GnIH receptors were found in the pituitary and hypothalamus of the quail, which suggested the probable direct action of GnIH in these structures [7, 14]. Importantly, GnRH neurons also express GnIH receptors, indicating the possibility that GnIH may directly regulate GnRH secretion [15].

More recently, mammalian homologs of GnIH (RFRP-1 and RFRP-3) were identified in rats, mice and hamsters [4, 5, 16, 17, 18]. In all three species, GnIH (RFRP-3)-ir cells were concentrated in the dorsomedial nucleus of the hypothalamus (DMH), a region important in reproductive regulation [4, 5]. While these GnIH (RFRP-3) neurons are localized to a specific region of the brain, GnIH (RFRP-3) nerve fibers are widely distributed. Kriegsfeld and colleagues [5] identified RFRP-3 fibers in male and female rats, hamsters and mice: they found fibers in all areas that contain GnRH neurons and fibers (medial septum, diagonal band of Broca, preoptic area, and anterior hypothalamus) and in regions that do not contain GnRH cells or fibers (septal and preoptic areas, amygdala, bed nucleus of the stria terminalis). In male rats, RFRP-3 fibers have been found distributed in areas of the brain known to regulate both reproductive hormones and behavior: the bed nucleus of the stria terminalis (BnST), the medial preoptic area (MPOA), the medial and lateral septal areas (MS and LS), and the paraventricular thalamic nucleus (PVT) [4]. In the preoptic area of male rats, GnIH (RFRP-3) fibers lie in close proximity to approximately 75% of GnRH cell bodies [5]. A study by Johnson and colleagues [4] demonstrated that in male rats intracerebroventricular (ICV) GnIH (RFRP-3) administration decreased tonic plasma LH levels and reduced the number of mounts, intromissions and ejaculations.

Control of LH, particularly the surge, is critical for female ovulation and estrous behavior. In the hypothalamus of the female Syrian hamster, over 40% of GnRH neurons receive GnIH (RFRP-3) projections [5]. In females, GnRH cell bodies are believed to be responsible for initiating GnRH and LH surges, suggesting the possibility of direct inhibitory action on GnRH neurons [4]. Indeed, recent studies suggest that RFRP-3 can act directly on GnRH neurons and the anterior pituitary [5, 9, 10, 12, 19] in birds and mammals. For example, in response to surge induction by estrogen and progesterone in female ovariectomized (OVX) rats, chronic RFRP-3 treatment caused a reduction in c-FOS activation of GnRH neurons when compared to controls, suggesting the modulation of GnRH neurons by RFRP-3 [20].

The effect of GnIH (RFRP-3) on hormone levels and behavior has recently been investigated. This peptide inhibits sex behaviors and decreases LH secretion in songbirds [2]. Both systemic and local administration of GnIH (RFRP-3) reduced tonic LH levels in OVX female hamsters [5]. In a similar experiment, however, OVX female rats exposed to low-level estradiol administration (insufficient to cause a GnRH/LH surge) and RFRP-3 did not demonstrate changes in LH pulse frequency, amplitude or concentration [20]. These studies demonstrate that with some treatments of GnIH (RFRP-3) tonic circulating LH levels in a female can be reduced. However, the effects of GnIH (RFRP-3) on sex behavior and LH surge necessary for ovulation have not been simultaneously investigated in intact female rodents or ovariectomized animals hormone-primed to induce estrus. We hypothesized that inhibition by RFRP-3 will directly suppress lordosis and the LH surge. Alternatively, GnIH may decrease GnRH or LH directly, and thereby decrease estrogen at the time of estrus and indirectly decrease sex behavior.

The goal of the present study was to determine whether GnIH (RFRP-3) would reduce female rat sexual behavior and surge-associated increase in LH in animals ovariectomized (OVX) and hormone-primed for estrus. The experiment used acute ICV administration of RFRP-3 and recorded lordosis quotient and LH concentrations at the time of day that the surge and sex behavior occur. We also tested the disruption in female behavior by examining disruption in male behavior.

MATERIALS AND METHODS

Animals

Female rats (180-200 g) were obtained from Charles River Laboratories (Wilmington, Virginia). The animals were housed in pairs until surgery, and following surgery they were housed individually. Both food (Purina Rodent Chow 5001) and charcoal filtered (estrogen free) water were available *ad libitum*. The lights were on a 12:12 light/dark cycle, with lights-on at 0500 h. The University Committee for the Care and Use of Animals at the University of Michigan approved all methods, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Ovariectomies (OVX)

Approximately ten days after arrival, all animals were ovariectomized under isoflurane anesthesia (4-5% for induction, 1-2% for maintenance). A small bilateral incision was made below the bottom rib on the dorsolateral surface to allow exposure of the ovary. Sterile chromic gut suture was used to tie off the ovarian artery and the ovary was removed. After the ovaries were removed, dissolvable suture was used to close the muscle wall and wound clips to close the skin. After two weeks of recovery, vaginal smears were taken from each rat for a minimum of five days to confirm complete cessation of cycling indicating complete ovarian removal.

Lateral ventricle cannulation

Following recovery from OVX and confirmed cessation of cycling, the rats were anesthetized using isoflourane (4-5% for induction, 1-2% for maintenance), given pre-operative analgesic and positioned in a stereotaxic instrument. A single incision was made on the midline of the scalp. A stainless steel 15 mm 30 gauge (G) cannula (Small Parts Inc., Miami Lakes, FL) was placed in the lateral ventricle at 1.5 mm lateral to the midline, 1.0 mm posterior to Bregma, and 3.8 mm inferior of the dura mater. The cannula was anchored to the skull using dental cement and bone screws. A steel stylet (Plastics One, Roanoke, VA, USA) was placed in the cannula to prevent blockage and exposure to outside contaminants. The animals were given a week to recover before ICV injections. The injections were given using a 28 gauge injector (Plastics One, Roanoke, VA) attached to polyethylene tubing and a 5 ul Hamilton Syringe.

Hormone treatment

After at least one week of recovery from cannula implantation, animals were primed with estradiol and progesterone. On day one, 10 ug of b-estradiol benzoate (Sigma Chemical, St. Louis, MO) in 0.1 ml of peanut oil was injected subcutaneously (s.c.) at ZT 8 (1300 h; ZT8 = zeitgeber time, 8 hours after lights on). Forty-eight hours later 500 ug of progesterone (Sigma Chemical, St. Louis, MO) in 0.1 ml of peanut oil was injected s.c. This treatment reliably induces estrous behavior 4 hours later [21] (ZT12 in our studies).

Intracerebroventricular (ICV) injections

To determine the effects of RFRP-3 on female typical sex behavior and LH levels, female rats were given ICV injections of 500 ng of RFRP-3 (made by Genosys, Inc., gift to us by Gregory Fraley, see [4] for more detailed description) or CSF vehicle (Harvard Apparatus, Holliston, Massachusetts) in 3 ul total volume 30 minutes prior to lights off (3.5 hours after the progesterone injection). This dose was chosen based on previous studies in males and females [4, 5]. After injection, the injector tip was left in the cannula for two additional minutes to ensure no backflow. A cross-over design was used for both experiments and all animals received both CSF injection and RFRP-3 injection, separated by at least one week, with half of the animals receiving RFRP-3 first and the other half receiving vehicle first.

Experiment 1: Effects of ICV RFRP-3 on sex behavior

30 minutes after ICV injection of either RFRP-3 or vehicle the female rats (n = 6) were placed in a clear housing cage with a sexually experienced male rat (at the time of lights off, ZT12). As described previously, a cross-over design was used such that each female was given both RFRP-3 and vehicle one week apart. Sex behavior was video recorded for one hour and the number of lordosis responses by females and the total number of mounts alone, mounts with intromission, and ejaculations by males was determined from

the video record, this data was divided into four 15 minute bins. A mount alone was recorded if the male approached the female from behind and placed both forelegs on her flanks. A mount with intromission was recorded if the mount was followed by the insertion of the penis into the vagina and was followed by male self-genital grooming. An ejaculation was recorded if the male rat showed an intromission with a longer-lasting thrust followed by self-genital grooming and a period of disinterest in the female. Lordosis behavior was recorded when the female arched her back and lifted her tail to one side in response to male behavior. Lordosis quotient (LQ) was scored as the frequency of lordosis divided by total number of mounts (mount alone, mount to intromission, and mount to ejaculation). Videos were scored by two individuals blind to the treatment condition of the female.

Experiment 2: Effects of RFRP-3 on LH levels

After ICV injections of either CSF or RFRP-3 in a cross-over design described above, tail nicks were performed on female rats (n = 6) to collect blood samples to measure LH concentration at the same times as the sex behavior recordings were previously collected. These time points were chosen so LH levels could be determined at the same times behavioral changes were observed, and because LH is elevated during the time of behavioral estrus, near the time of the LH surge. One blood sample (200 ul) was collected at the time of lights off (ZT 12, 1700 h, 30 min after RFRP-3 or vehicle injection) and a second sample was collected at ZT 13 (1800 h, 90 min after RFRP-3 or vehicle injection). All blood samples were placed on ice, and then centrifuged. Plasma was stored in a -20^{o}C freezer until LH concentrations were measured with a radioimmunoassay.

Radioimmunoassay

LH concentrations of samples run in duplicate were measured at Northwestern University (Evanston, IL, Brigitte Mann) with reagents from NIH. The anti-serum was anti-rLH-S11, the standard was rLH-RP3, the assay sensitivity was 0.1 ng/ml and the intra-assay coefficient of variation was 3.25%. The interassay variation was 4.1%. For more detailed methods see Johnson *et al*. [4].

Perfusions

Cannula placement was confirmed by cresyl violet staining of brains from perfused animals. All animals used in the study had cannulae successfully placed in the lateral ventricle.

Statistical design

All data are expressed as a mean ± SEM for each treatment. Sex behaviors were analyzed using repeated measure ANOVA and post-hoc analysis. Hormone data was analyzed using repeated measures 2-way ANOVA with RFRP-3/vehicle as one variable and time (ZT12/ZT13) as the other. Differences with significance level of $p<0.05$ were considered meaningful.

RESULTS AND DISCUSSION

Similar to studies with RFRP-3 in male rodents [4, 5] and the avian homolog, GnIH, in birds [2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 22], we found that ICV administration of RFRP-3 decreased both sex behavior and LH release in estrous female rats. We also observed that males paired with RFRP-3 treated females exhibited fewer intromissions compared to males paired with vehicle treated females, most likely because of the poor lordosis of the females.

Experiment 1: Effects of ICV RFRP-3 on sex behavior

There was an overall treatment effect such that RFRP-3 administration caused a significant decrease in lordosis quotient (LQ) compared to vehicle treatment ($F(1,10) = 5.214$, $p = 0.046$) and an overall change in LQ over time ($F(3,30) = 3.620$, $p = 0.024$). Post-hoc analysis revealed a significant treatment effect (decrease in LQ of RFRP-3 treated animals) at 45-60 minutes, 60-75 minutes, and 75-90 minutes post-RFRP-3 injection ($F(1,10) = 5.034$, $p = 0.049$; $F(1,10) = 5.384$, $p=0.043$; $F(1,10) = 6.781$, $p = 0.026$, respectively, Figure 1). Male sex behavior in response to females given RFRP-3 or vehicle was also altered. The total number of mounts (mounts alone and mounts with intromissions) did not differ significantly between treatment groups (Figure 2). Overall, the effect of treatment on the number of intromissions approached significance ($F(1,10) = 3.969$, 0.074). Post-hoc analysis revealed that male rats paired

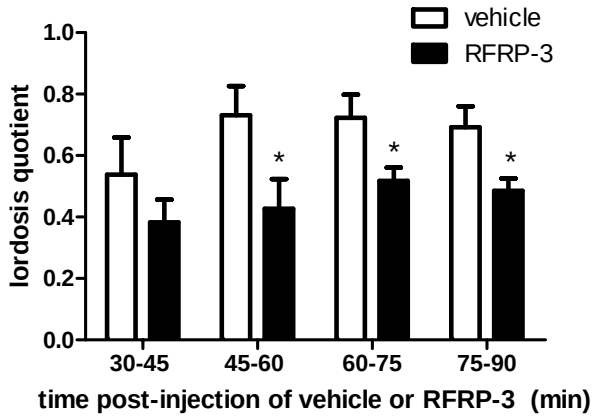

Figure 1. Lordosis quotient was significantly reduced in females administered RFRP-3 compared to vehicle from 45 to 90 minutes post RFRP-3 infusion. Lordosis quotient was calculated by dividing the total lordosis events by total male sex behaviors. * = $p<0.05$.

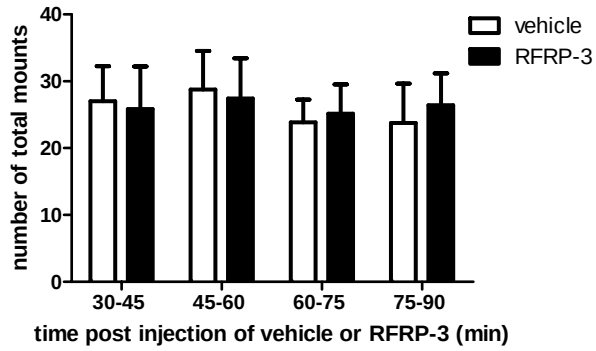

Figure 2. The total number of mounts (mounts alone and mounts with intromissions) in response to females treated with RFRP-3 did not differ from the response to vehicle treated controls.

with RFRP-3 treated females exhibited significantly fewer intromissions compared to males paired with vehicle injected females from 30-45 minutes post-RFRP-3 injection ($F(1,10) = 6.824$, $p = 0.026$, Figure 3). The number of ejaculations did not differ between the two groups (data not shown).

Over 40% of GnRH neurons in the hypothalamus receive RFRP projections (Kriegsfeld *et al.*, 2006). Additionally, Anderson and colleagues (2009) have demonstrated that chronic RFRP-3 treatment resulted in a reduction in c-FOS activation of GnRH neurons in hormone-primed, OVX rats compared to vehicle-treated controls at the time of the expected LH surge. Not only does

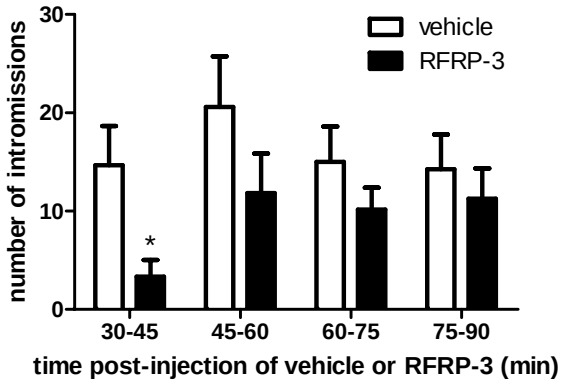

Figure 3. The number of intromissions was significantly decreased in response to females treated with RFRP-3 compared to vehicle treated females from 30 to 60 minutes post RFRP-3 infusion. * = $p<0.05$.

GnRH modulate the HPG axis release of LH and FSH, but it also impacts sexual receptivity in the rat [23]. GnRH injections in hormone-primed rats have been shown to enhance lordosis behavior [24]. The results from the current study combined with the work of Anderson and colleagues [20] suggest that GnRH neurons could be directly inhibited by RFRP-3, thereby reducing sexual receptivity. In the first 15 minutes that the animals were paired there was not a significant decrease in lordosis quotient in the RFRP-3 treated animals, however after 15 minutes the females treated with RFRP-3 demonstrated a reduced lordosis quotient for the remainder of the test (45-90 minutes post injection), suggesting that RFRP-3 can suppress female sexual behavior for at least 90 minutes after exposure.

The difference in sex behavior elicited from the males in response to female treatment further supports the conclusion of a change in female receptivity. Males paired with less receptive RFRP-3 treated females exhibited the same number of total mounts (mounts alone and mounts with intromissions) but fewer successful intromissions compared to pairing with control females, suggesting that the hormone-primed females maintained their attractiveness although their receptivity declined.

Experiment 2: Effects of RFRP-3 on LH levels

OVX rats were hormone-primed with estradiol and progesterone to stimulate the near estrus-timed

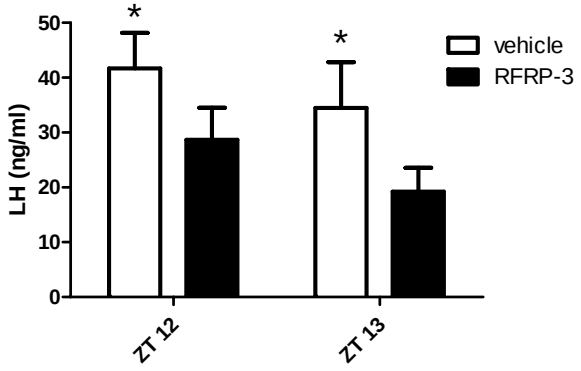

Figure 4. RFRP-3 treatment caused a significant decrease in luteinizing hormone levels at ZT12 (30 min after injections) and ZT13 (90 min after injection) compared to vehicle treatment. * = $p<0.05$.

surge in LH. RFRP-3 treatment caused a significant decrease in LH levels compared to vehicle treatment at ZT12 and ZT13 ($F(1,20) = 4.856$, $p = 0.039$; Figure 4). These times were chosen so LH levels could be determined at the same time as behavioral changes were observed, and because LH is elevated during the time of behavioral estrus, near the time of the LH surge.

Although both vehicle and RFRP-3 treated animals exhibit an increase in LH, the amplitude of this increase is suppressed in the females treated with RFRP-3 at the times studied. RFRP-3 did not completely ablate the increase in LH, perhaps because it was administered after the estradiol, and therefore likely after the LH had already started to increase. A RFRP-3 mediated decrease in tonic LH levels has previously been observed in males and non-hormone-primed OVX females [4, 5]. In hormone primed females, Anderson and colleagues (2009) report that chronic ICV infusion of RFRP-3 appeared to decrease LH levels, but the result was not significant, additionally, there was no effect of RFRP-3 on tonic pulsatile LH secretion. More recently it has been shown that ICV RFRP-3 administration can inhibit LH secretion in gonadectomized (non-hormone primed) male and female rats [25]. In addition, a study by Gibson and colleagues [26] in female Syrian hamsters showed that RFRP-immunoreactive cell numbers are reduced during the LH surge and that estrogen is required for daily changes in RFRP levels. A decrease in the LH amplitude following the estrogen rise reduces the likelihood of ovulation and successful fertilization [27]. The current data adds to previous work by demonstrating significant decreases in surging LH levels after acute administration of RFRP-3, and the effect is observed in the same paradigm that demonstrates a decrease in female sexual behavior.

CONCLUSION

Overall, we demonstrated that RFRP-3 treatment in an ovariectomized, hormone-primed female rat will reduce female sex behavior and simultaneously dampen the increase in LH, both measures are controlled by GnRH and estrogen. To our knowledge, this is the first study of acute RFRP-3 effects on sex behavior and LH levels in estrous female rodents. Interestingly, Kirby and colleagues [28] demonstrated that both acute and chronic restraint stress in male rats leads to an upregulation of RFRP expression in the hypothalamus, and that RFRP mRNA levels were negatively correlated with circulating LH levels. This suggests that RFRP neuron activity can be influenced by the stress axis, and if the same is true in females, RFRP could provide a rapid method for controlling fertility in response to sudden stressful changes in the environment. This study, as well as others, has shown that RFRP-3 appears to be important in the regulation of the neuroendocrine system of both male and female rodents. Future studies should examine the direct action of this peptide by injecting directly into areas of the brain known to regulate reproduction, as well as exploring its role in other mammalian species.

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REFERENCES

1. Anderson, G. M., Relf, H-L., Rizman, M. Z. and Evans, J. J. 2009, Endocrinol., 150, 1834-1840.
2. Bentley, G. E., Jensen, J. P., Kaur, G. J., Wacker, D. W., Tsutsui, K. and Wingfield, J. C. 2006a, Horm. Behav., 49, 550-555.
3. Bentley, G. E., Kriegsfeld, L. J., Osugi, T., Ukena, K., O'Brien, S., Perfito, N., Moor, I. T., Tsutsui, K. and Wingfield, J. C. 2006b, J. Exp. Zool., 305A, 807-814.
4. Johnson, M. A., Tsutsui, K. and Fraley, G. S. 2007, Horm. Behav., 51, 171-180.
5. Kriegsfeld, L. J., Mei, D. F., Bentley, G. E., Ubuka, T., Mason, A. O., Inoue, K., Ukena, K., Tsutsu, K. and Silver, R. 2006, PNAS, 103, 2410-2415.
6. Kriegsfeld, L. J., Gibson, E. M., Williams III, W. P., Zhao, S., Mason, A. O., Bentley, G. E. and Tsutsui, K. 2010 J. Neuroendocrinol., 22, 692-700.
7. Tsutsui, K., Bentley, G. E., Ubuka, T., Saigoh, E., Yin, H., Osugi, T., Inoue,K., Chowdhury, V. S., Ukena, K., Ciccone, N., Sharp, P. J. and Wingfield, J. C. 2007, Gen. Comp. Endocrinol., 153, 365-370.
8. Ubuka, T., Ukena, K., Sharp, P. J., Bentley, G. E. and Tsutsui, K. 2005, Endocrinol., 147, 1187-1194.
9. Bentley, G. E., Perfito, N., Ukena, K., Tsutsui, K. and Wingfield, J. C. 2003, J. Endocrinol., 15, 794-802.
10. Osugi, T., Ukena, K., Bentley, G. E., O'Brien, S., Moore, I. T., Wingfield, J. C. and Tsutsui, K. 2004, J. Endocrinol., 182, 33-42.
11. Satake, H., Hisada, M., Kawada, T., Minakata, H., Ukena, K. and Tsutsui, K. 2001, Biochem. J., 354, 379-385.
12. Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fijisawa, Y., Kikuchi, M., Ishii, S. and Sharp, P. J. 2000, Biochem. Biophys. Res. Commun., 275, 661-667.
13. Ukena, K., Ubuka, T. and Tsutsui, K. 2003, Cell Tissue Res., 312, 73-79.
14. Yin, H., Ukena, K., Ubuka, T. and Tsutsui, K. 2005, J. Endocrinol., 184, 257-266.
15. Ubuka, T., Kim, S., Huang, Y., Reid, J., Jiang, J., Osugi, T., Chowdhury, V. S., Tsutsui, K. and Bentley, G. E. 2008, Endocrinol., 149, 268-278.
16. Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe T., Kikuchi, K., Terao, Y., Yano, T., Yamamoto, T., Kawamata, Y., Habata, Y., Asada, M., Kitada, C., Kurokawa, T., Onda, H., Nishimura, O., Tanaka, M., Ibata, Y. and Fujino, M. 2000, Nat. Cell Biol., 2,703-708.
17. Yano, T., Iijima, N., Kakikara, K., Hinuma, S., Tanaka, M. and Ibata, Y. 2003, Brain Res., 982, 156-167.
18. Yano, T., Iijima, N., Hinuma, S., Tanaka, M. and Ibata, Y. 2004, Brain Res. Dev. Brain Res., 152, 109-20.
19. Ciccone, N. A., Dunn, I. C., Boswell, T., Tsutsui, K., Ubuka, T., Ukena, K. and Sharp, P. J. 2004, J. Neuroendocrinol., 16, 999-1006.
20. Anderson, G. M., Relf, H-L., Rizman, M. Z. and Evans, J. J. 2009, Endocrinol., 150 1834-1840.
21. Jenkins, W. J. and Becker, J. B. 2001, Behav. Brain Res., 121, 119-128
22. Ubuka, T., Ueno, M., Ukena, K. and Tsutsui, K. 2003, J. Endocrinol., 178, 311-318.
23. Rothfeld, J. M., Carstens, E. and Gross, D. S. 1985, Peptides, 6, 603-608.
24. Moss, R. L. and McCann, S. M. 1973, Science, 181, 177-179.
25. Pineda, R., Garcia-Galiano, D., Sanchez-Garrido, M. A., Romero, M., Ruiz-Pino, F., Aguilar, E., Dijicks, F. A., Blomenrohr, M., Pinilla, L., van Noort, P. I. and Tena-Sempere, M. 2010, Am J. Physiol. Endocrinol. Metab., 299, E39-E46..
26. Gibson, E. M., Humber, S. A., Jain, S., Williams III, W. P., Zhao, S., Bentley, G. E., Tsutsui, K. and Kriegsfeld, L. J. 2008, Endocrinol., 149, 4958-4969.
27. Meek, L. R. and Lee, T. M. 1994, Biol. Reprod., 51, 725-730.
28. Kirby, E. D., Geraghty A. C., Ubuka, T., Bentley, G. E. and Kaufer, D. 2009, PNAS., 106, 11324-11329.