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Gonadotropin inhibitory hormone and RF9 stimulate hypothalamic-pituitary-adrenal axis in adult male rhesus monkeys

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ABSTRACT

Stress activates gonadotropin inhibitory hormone (GnIH), hypothalamic-pituitary-adrenal axis (HPA-axis) and represses hypothalamic-pituitary-gonadal axis (HPG-axis) but RF9 administration relieves stress-induced repression of the HPG-axis. Importantly, it was not known whether GnIH signaling and RF9 synthetic peptide modulate the HPA axis. To assess this, mammalian orthologs of GnIH (RFRP-1 and RFRP-3) and RF9 were administered to intact adult male rhesus monkeys. RFRP-1 (125 μg/animal), RFRP-3 (250 μg/animal) and RF9 (0.1 mg/kg BW) were intravenously (*iv*) injected into normal fed ($n = 4$) monkeys. Additionally, a single bolus *iv* injection of RF9 (0.1 mg/kg BW) was also administered to 48 h fasted monkeys ($n = 4$) to check the effects of RF9 signaling on an activated HPA-axis. Serial blood samples were collected, centrifuged and the obtained plasma was used for the analysis of cortisol by specific enzyme immunoassay. RFRP-1 treatment significantly increased cortisol levels while RFRP-3 increased the plasma cortisol, but the effect was non-significant. RF9 treatment significantly increased cortisol levels in normal fed animals. In contrast, RF9 injection did not significantly alter circulating cortisol in fasted monkeys. In conclusion, our results suggest stimulatory action of RFRPs and RF9 on the HPA axis in the adult male monkeys. However, the mechanism and site of action of RFRP-1 and RF9 along the HPA-axis is still unknown. Therefore, further studies are needed to decipher the mechanism and site of action of RFRPs and RF9 on the HPA axis in primates.

1. Introduction

Various factors regulate reproductive functions (Ullah et al., 2017; Ullah et al., 2016; Wahab et al., 2011; Wahab et al., 2015). These cascades exist mainly in three components, the hypothalamus (Castillo et al., 1992; Döcke, 1979), the pituitary (HORI et al., 1969), and the gonads (Bolt et al., 1971), which collectively form the hypothalamic-pituitary-gonadal axis (HPG-axis). Gametogenesis and steroidogenesis in gonads are mainly regulated by pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) [for a review, see (Themmen and Huhtaniemi, 2000)]. LH and FSH are regulated by hypothalamic gonadotropin releasing hormone (GnRH) (Martynska et al., 2014; Schally et al., 1971). In turn, GnRH secretion is regulated by various stimulatory and inhibitory signaling peptides such as ghrelin, leptin, neuropeptide Y (NPY), agouti-related protein (AgRP), amphetamine-regulated transcript (CART), alpha-melanocyte-stimulating hormone (α-MSH), orexin A(Martynska et al., 2014), corticotrophin-releasing hormone (CRH) (Lebrethon et al., 2007), kisspeptin (Gottsch et al., 2004), gamma-aminobutyric acid (GABA) (Zhu et al., 2015), etc.

Tsutsui et al. (2000) originally discovered GnIH in the quail brain as an inhibitor of the HPG-axis. Subsequently, GnIH orthologs were identified in vertebrates from fish to mammals (Jadhao et al., 2017; Tsutsui et al., 2010; Tsutsui et al., 2007; Tsutsui et al., 2009; Tsutsui et al., 2012; Tsutsui et al., 2006; Tsutsui and Ukena, 2006; Ukena et al., 2016; Ullah et al., 2016). Similar to avian GnIH and GnIH-related peptides (GnIH-RPs), all the recognized mammalian and primate GnIH peptides possess a common LPXRFamide (X = L or Q) sequence at C-terminal [for reviews, see (Tsutsui et al., 2010; Tsutsui et al., 2009; Tsutsui et al., 2012; Tsutsui et al., 2015)] and are thus termed as LPXRFamide peptides. Mammalian orthologs of GnIH are termed as RFamide-related peptide 1 and 3 (RFRP-1 and -3). RFRPs not only inhibit reproduction (Anjum et al., 2016; Calisi et al., 2016; Ullah et al.,

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2016) but have many other functions too (Ferris et al., 2015; Kim, 2016; Kovács et al., 2017; McConn et al., 2014).

It is a well-established fact that stress disrupts various reproductive parameters in avian and mammalian species (Cyr and Romero, 2007; Deviche et al., 2014; Eskiocak et al., 2005; Lerman et al., 1997; Sheiner et al., 2002; Wada et al., 1996). Similarly, various hormones, including RFRPs, CRH, ACTH, oxytocin, vasopressin, cortisol, etc., are involved in the regulation of stress (Bao et al., 2014; Li et al., 2016; Toonen et al., 2016; Ullah et al., 2016; Yan et al., 2014). It is also well-known that different stress conditions increase hypothalamic GnIH/RFRPs expression (Johnson et al., 2007; Kirby et al., 2009; Retana-Marquez et al., 2003). Importantly, GnRH neurons in the hypothalamus (Ubuka et al., 2008; Ubuka et al., 2009b), gonadotrophs in the pituitary (Gibson et al., 2008; Rizwan et al., 2012; Smith et al., 2012; Ubuka et al., 2009b), and interstitial cells in the gonads (Bentley et al., 2008; McGuire and Bentley, 2010) express GPR147 (GnIH receptor). Based on the above evidence, it is reasonable to speculate that GnIH/RFRPs can directly inhibit reproduction at all the three components of the HPG-axis (Ubuka et al., 2014). It is also well-estiblished that an activated HPA-axis during stress conditions can down-regulate the GnRH system (Geraghty and Kaufer, 2015; Ralph et al., 2016; Rivier and Rivest, 1991; Rivier et al., 1986). The repressed GnRH system reduces LH secretion (Duruisseau et al., 1979; Gonzalez-Quijano et al., 1991).

Likewise, the HPA-axis regulates many functions including stress (Filaferro et al., 2014; Liew et al., 2015; Ullah et al., 2016). Activation of the HPA-axis has been reported during stress conditions [for a review, see (Ullah et al., 2016)]. Additionally, the localization of CRH secreting neurons near RFRP fibers in the hypothalamus (Qi et al., 2009) suggests the possibility of a direct stimulatory effect of RFRPs on CRH expression in the hypothalamic paraventricular nucleus (PVN). Likewise, RFRP-3 stimulates parvocellular paraventricular perikarya by disinhibiting GABAergic afferent signals (Jhamandas et al., 2007). This evidence suggests the indirect role of RFRPs in the activation of CRH secreting neurons. Additionally, neurons of supraoptic and PVN areas also express GPR147. Most importantly, RFRP-1 administration induces anxiety and changes behavior in rats (Kaewwongse et al., 2011). These data collectively suggest that RFRPs induce stress related changes by activating the HPA-axis.

Similarly, our previous studies have reported that RF9 administration completely rescues fasting (Batool et al., 2014) and cortisol (Naz et al., unpublished data) induced repression of the HPG-axis. Importantly, an activated HPA-axis during stress conditions represses the HPG-axis but RF9 administration relieves such repression [for a review, see (Ullah et al., 2016)]. These findings suggest that RF9 may directly affect the HPA-axis. Therefore, the current study has been conducted to explore whether GnIH/RFRPs signaling and RF9 have any role in regulating the HPA-axis in normal fed and 48 h fasted adult male rhesus monkeys.

2. Materials and methods

2.1. Animals

A total of 16 (n = 4/experiment) adult intact male rhesus monkeys (*Macaca mulatta*) were used in the present study. The animals were kept in individual cages under standard laboratory conditions (photoperiod 12 h dark and 12 h light, temperature 25 °C) in the Primate Facility of the Department of Animal Sciences at the Quaid-i-Azam University, Islamabad, Pakistan. Housing, feeding, chair-restraint-training, and sedation of animals have been described in our previous studies (Wahab et al., 2008; Wahab et al., 2010; Wahab et al., 2012). All animal experiments were approved by the Departmental Committee for Care and Use of Animals.

2.2. Pharmacological agents

Human RFRP-1 and RFRP-3 were purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, Ca, USA). RF9 was prepared by China peptides (Shanghai, China) and normal saline was purchased locally. Working solutions of RFRP-1, RFRP-3 and RF9 were prepared in normal saline (0.9%). Heparin and ketamine (Rotex medica, Trittau, Germany) were purchased locally.

2.3. Venous catheterization

For non-interrupted withdrawal of sequential blood samples and administration of RFRP-1, RFRP-3, and RF9, it was necessary to insert a cannula into the saphenous vein of the animals. The animals were sedated by intra-muscular (*im*) injection of ketamine (5 mg/kg BW) and a cannula (Cathy; 0.8 × 25 mm/22G; HMD Healthcare Ltd., Horsham, UK) was inserted into the saphenous vein. A butterfly tube (length 300 mm, volume 0.29 ml, 20 GX3/4; JMS, Singapore) was used to connect the inserted cannula with a syringe. Blood samples and drug injections were only done after the complete recovery of the animals from sedation.

2.4. Experimental design

Four experiments were conducted in the present study and 4 animals were used in each experiment.

2.4.1. Experiment 1

A group of 4 normal fed monkeys, ranging in body weight from 8.7 to 12 kg was used for the assessment of the role of RFRP-1 on the HPA-axis. In order to perform the experiment, RFRP-1 (125 µg or 0.076 µmol/animal, *iv* bolus) was injected into the saphenous vein immediately after collecting the 0 time point blood sample. Serial blood samples (1.5 ml) were collected with 15 min intervals for 15 min before and 120 min after the injection. Heparinized syringes were used for blood collection.

2.4.2. Experiment 2

A group of 4 normal fed monkeys, ranging in body weight from 9 to 12.5 kg was used for the assessment of the role of RFRP-3 on the HPA-axis. In order to perform the experiment, RFRP-3 (250 µg or 0.257 µmol/animal, *iv* bolus) was injected into the saphenous vein immediately after collecting the 0 time point blood sample. Serial blood samples (1.5 ml) were collected with 15 min intervals for 15 min before and 150 min after the injection. Heparinized syringes were used for blood collection.

2.4.3. Experiment 3

A group of 4 normal fed monkeys with an average body weight of 11.1 ± 00.60 kg was used for the evaluation of the effects of RF9 on the HPA-axis. In order to perform the experiment, RF9 was administered as an *iv* bolus in the saphenous vein. Two iv bolus injections of RF9 (0.1 mg or 0.2 µmol/kg BW) were injected at 0 and 120 time points and serial blood samples (1.5 ml) were collected with 20 min intervals for 240 min after drug administration. Heparinized syringes were used for blood collection.

2.4.4. Experiment 4

A group of 4 monkeys with an average body weight of 10.10 ± 0.46 kg was used for the evaluation of the effects of RF9 on the HPA-axis after a 48 h fast. Serial blood samples were obtained before and after drug administration at 30 min intervals. The RF9 (0.1 mg or 0.2 µmol/kg BW) was injected as iv bolus at 0 and 120 time points and serial blood samples (1.5 ml) were collected for 240 min after drug administration in heparinized syringes.

Blood collection for cortisol measurement was strictly started at

11:30 am in all the experiments. During sampling, as to avoid hypovolemic stress, an equal amount (1.5 ml) of heparinized saline (5 IU/ml) was administered after each blood sample. The blood samples were centrifuged at 3500 rpm and the plasma obtained was stored at –20 °C until hormonal assay. The dose (125 μg/animal, *iv* bolus) of RFRP-1 was in equimolar with the effective doses of kisspeptin (Shahab et al., 2005), another neuropeptide. The dose (250 μg/animal, *iv* bolus) of RFRP-3 was estimated according to the previous study in sheep (Clarke et al., 2008). RF9 was administered intravenously at a dose of 0.1 mg/Kg BW, based on the previous studies (Batool et al., 2014; Caraty et al., 2012).

To investigate the effects of RFRP-1 (Experiment 1) and RFRP-3 (Experiment 2) on plasma cortisol in normal fed conditions, we collected blood samples at 15 mints intervals for 2.5 h (0–150 mints) but the last two samples in Experiment 1 suffered from a technical problem so we excluded those samples. Similarly, to investigate the effects of RF-9 on plasma cortisol in normal fed (Experiment 3) and fasting conditions (Experiment 4), we collected blood samples for 4 h (0–240 min) but to avoid the large numbers of samples, we collected blood samples at 20 min intervals in experiment 3 and at 30 min intervals in experiment 4.

2.5. Enzyme immunoassay (EIA) of cortisol

The plasma cortisol levels were measured by using a commercial ELISA kit for human cortisol (Monobind Inc. Lake Forest, CA, USA). The sensitivity of this assay was 3.66 ng/ml and the coefficient of variation was < 8%. The assay was performed according to the manufacturer's protocol.

2.6. Statistical analyses

One way ANOVA with repeated measures was used to analyze the time related variations in cortisol levels after the peptides administration while a *t*-test was used to compare overall mean cortisol levels observed in pre- and post-peptides administration periods. For the identification of significant difference, $P < 0.05$ was taken and all data were expressed as mean ± standard error of mean (SEM). GraphPad Prism version 5 (Software Inc., San Diego, CA, USA) was used for data analysis.

3. Results

3.1. Experiment 1: effects of RFRP-1 administration on plasma cortisol levels in normal fed monkeys

Time related changes in mean plasma cortisol levels before and after RFRP-1 administration are shown in Fig. 1. A significant ($P < 0.05$) time related increase in cortisol levels after RFRP-1 administration was observed with peak cortisol levels prevalent between 60 and 120 min. Comparison of overall mean plasma concentrations of cortisol before and after RFRP-1 administration is shown in Fig. 2. Mean cortisol levels were significantly ($P < 0.05$) increased after RFRP-1 administration.

3.2. Experiment 2: effects of RFRP-3 administration on plasma cortisol levels in normal fed monkeys

Time related variations in the mean concentration of cortisol after RFRP-3 administration in normal fed adult male monkeys are shown in Fig. 3. There were no significant time related alterations in cortisol level. The comparison of overall mean plasma cortisol levels before and after RFRP-3 administration is shown in Fig. 4. Again there was no significant difference between pre- and post-peptide mean plasma cortisol levels.

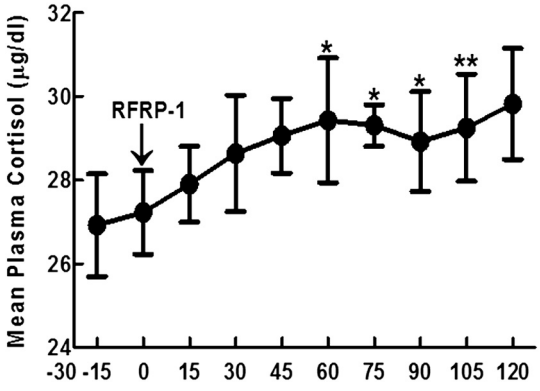

Fig. 1. Changes in mean ± SEM plasma cortisol concentration before and after *iv* administration of RFRP-1 (125 μg/animal; arrow) in normal fed adult male rhesus monkeys ($n = 4$). One way ANOVA with repeated measures identified significant time related variations in cortisol level. Further, Dunett's post hoc test indicated that mean plasma cortisol levels were significantly elevated during the period 60–120 min after RFRP-1 administration (*$P < 0.05$, **$P < 0.01$).

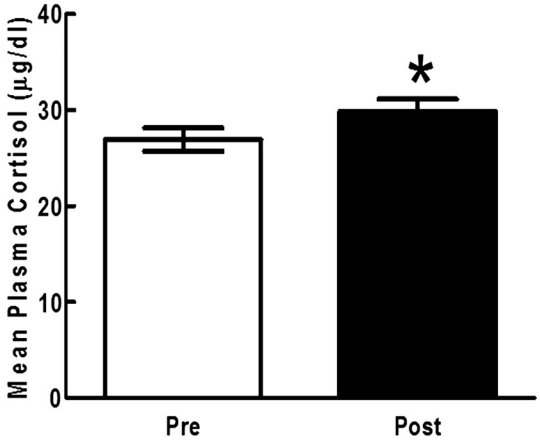

Fig. 2. Comparison of overall mean ± SEM plasma cortisol levels observed in pre- (− 15, 0 min) and post- (15–120 min) RFRP-1 administration periods in adult male rhesus monkeys ($n = 4$). RFRP-1 administration significantly (*$P < 0.05$) increased mean cortisol levels in normal fed monkeys.

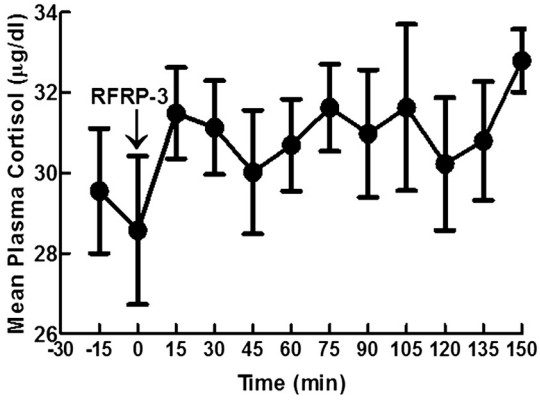

Fig. 3. Changes in mean ± SEM plasma cortisol levels before and after *iv* RFRP-3 administration (250 μg/animal; arrow) in normal fed adult male rhesus monkeys ($n = 4$). One way ANOVA with repeated measures identified non-significant time related variations in cortisol level.

3.3. Experiment 3: effects of RF9 administration on plasma cortisol levels in normal fed monkeys

The time related variations in cortisol concentration after RF9

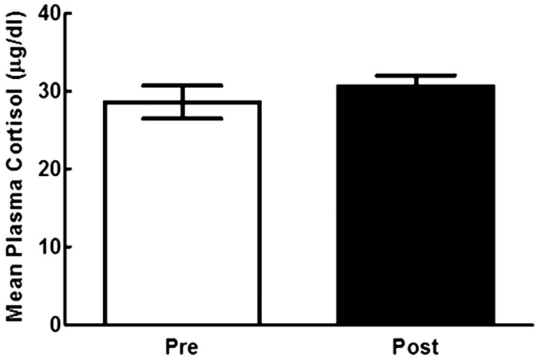

Fig. 4. Comparison of overall mean ± SEM plasma cortisol levels observed in the pre- (− 15, 0 min) and post- (15–150 min) RFRP-3 administration periods in normal fed adult male rhesus monkeys ($n = 4$). RFRP-3 administration did not change mean cortisol levels.

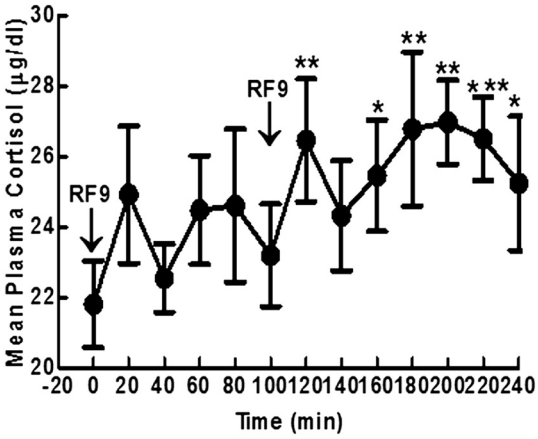

Fig. 5. Changes in mean ± SEM plasma cortisol concentration before and after *iv* administration of RF9 (0.1 mg/kg BW; *iv* bolus; arrow) in normal fed adult male rhesus monkeys ($n = 4$). One way ANOVA with repeated measures identified highly significant time related variations in cortisol level. Further, Dunett's post hoc test indicated that mean plasma cortisol levels were significantly elevated during the period 120–240 min after RF9 administration ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$).

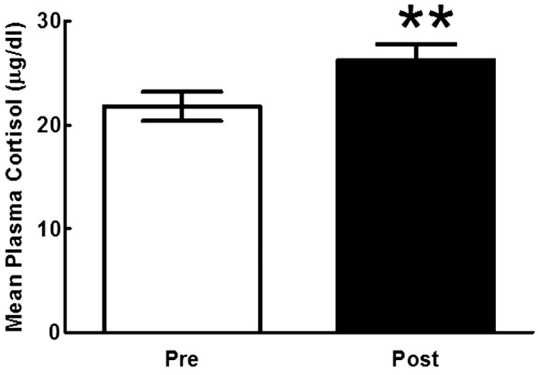

Fig. 6. Comparison of overall mean ± SEM plasma cortisol levels observed in the pre- (0 min) and post- (180–240 min) RF9 administration periods in normal fed adult male rhesus monkeys ($n = 4$). RF9 administration significantly ($^{**}P < 0.01$) increased overall mean plasma cortisol levels in normal fed monkeys.

administration in normal fed adult monkeys are shown in Fig. 5. A robust ($P < 0.0001$) time related increase in cortisol levels after RF9 administration was observed with peak cortisol levels occurring between 120 and 240 min. Comparison of overall mean plasma cortisol levels observed during pre- and post-RF9 administration periods is shown in Fig. 6. Mean cortisol levels were markedly ($P < 0.01$) raised during the last hour (180–240 min) after RF9 injection as compared to

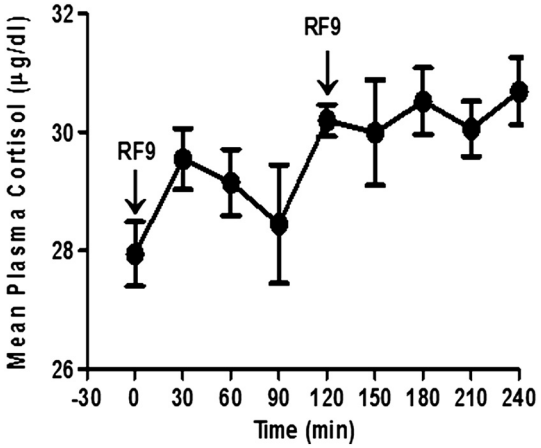

Fig. 7. Changes in mean ± SEM plasma cortisol concentration before and after *iv* administration of RF9 (0.1 mg/kg BW; *iv* bolus; arrow) in 48 h fasted adult male rhesus monkeys ($n = 4$). One way ANOVA with repeated measures identified only non-significant time related variations in cortisol levels.

pre-RF9 (0 min) levels.

3.4. Experiment 4: effects of RF9 administration on plasma cortisol levels in 48 h fasted monkeys

Pattern of minute-to-minute variations in cortisol level after the administration of RF9 in 48 h fasted adult monkeys is shown in Fig. 7. There were no time related significant variations in cortisol level after RF9 administration. The comparison of overall mean plasma concentration of cortisol before and after RF9 administration is shown in Fig. 8. A non-significant difference in pre- and post-cortisol concentrations was observed after RF9 administration.

4. Discussion

In the current study we analyzed the effects of mammalian GnIH (RFRP-1 and RFRP-3) signaling and RF9, a synthetic peptide in the regulation of the HPA-axis in adult male rhesus monkeys. RFRP-1, RFRP-3 and RF9 were administered in normal fed conditions (also in the 48 h fast condition at least for RF9). Importantly, GnIH and the HPA-axis are activated during stress conditions (Ullah et al., 2016), therefore we hypothesized that GnIH signaling may contribute to stimulating the HPA-axis. As during stress conditions, RFRPs are already elevated and, therefore, we thought that administration of RFRPs to fasted animals was not very relevant. Accordingly we measured

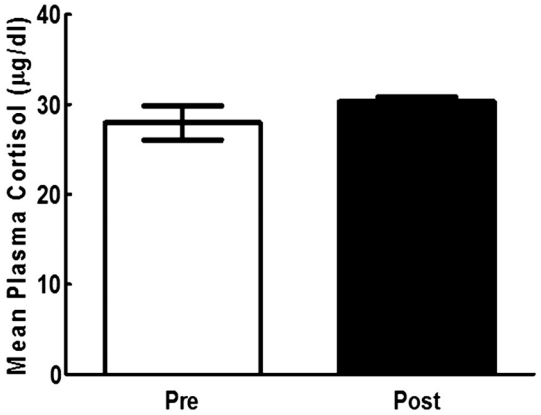

Fig. 8. Comparison of overall mean ± SEM plasma cortisol levels observed in the pre (0 min) and post (30–240 min) RF9 administration periods in 48 h fasted adult male rhesus monkeys ($n = 4$). RF9 administration did not affect mean cortisol levels.

circulating levels of cortisol, the end product of the HPA-axis, in response to GnIH administration. It is well-known that the hypothalamus secretes CRH that reaches the pituitary through the hypophyseal portal system and stimulates ACTH cells in the anterior pituitary to release ACTH that, in turn, stimulates adrenal glucocorticoid, cortisol, secretion in systemic blood (Alldredge, 2010; Kalra et al., 1999; Kormos and Gaszner, 2013). In the present study, a significant stimulatory effect of RFRP-1 administration was observed on circulating cortisol levels in adult male monkeys. Similarly, RFRP-3 administration was shown to increase cortisol levels, albeit marginally. These results suggest that RFRP-1 is more potent with regard to stimulating the HPA-axis in higher primates. It is likely that the observed stimulatory effect of RFRP-1 in monkeys is mediated centrally. CRH expressing neurons are present very close to RFRP fibers in the hypothalamus (Qi et al., 2009). This evidence suggests the possibility that RFRPs may have a direct role in the stimulation of CRH expressing neurons in PVN. Parenthetically, RFRP-3 has been shown to cause stimulation of PVN cell bodies by disinhibiting GABAergic afferent signals (Jhamandas et al., 2007). Based on the latter finding, it can be speculated that in the activation of CRH, RFRPs may also have an indirect role. Neurons of PVN express GPR147 and RFRP-1 administration induces anxiety and changes in behaviors (Kaewwongse et al., 2011). RFRP-1 has been shown to cause no effect on the basal or CRH induced ACTH secretion from the cultured pituitary cells but its central administration in rats has been observed to elevate cortisol secretion (Samson et al., 2003). Similarly, higher expression of ACTH has been documented after the central administration of RFRPs (Kaewwongse et al., 2011; Ullah et al., 2016). It is interesting to mention here that stress stimulates RFRPs (GnIH) expressing neurons (Clarke et al., 2008). This evidence suggests that RFRPs regulate the HPA-axis centrally at the hypothalamic level and as cortisol is the end parameter of the HPA-axis, we therefore measured cortisol as a marker of an activated HPA-axis. Additionally, increased concentrations of ACTH and cortisol in various animal models after RFRP-1 and 3 administration and close localization of CRH neurons in PVN near to RFRP fibers, suggest the presence of RFRP receptors on CRH neurons. However, there is no concrete evidence available yet for the presence of RFRPs receptors on CRH cells; it is therefore an important point for future research.

Similarly, a stimulated HPA-axis and a repressed HPG-axis have been reported during stress conditions [for a review, see (Ullah et al., 2016)]. In our previous studies, RF9 administration relieved a fasting (Batool et al., 2014) and cortisol (Naz et al. un-published data) induced repressed HPG-axis in monkeys. These findings suggest that RF9 may regulate the HPA-axis. To explore this phenomenon, we injected RF9 into normal fed and 48 h fasted adult male monkeys and plasma cortisol levels were examined.

Interestingly and paradoxically, RF9 administration was observed to stimulate cortisol levels in normal fed animals. In 48 h fasted monkeys, RF9 treatment was noted to cause no effect on cortisol secretion. Importantly, RF9 was developed as an antagonist of the neuropeptide FF receptors (NPFFRs) [NPFF1R or GPR147 and NPFF2R or GPR74], activated by a wide range of neuropeptides including RFRP-3 (Simonin et al., 2006). NPFF1R or GPR147 is considered the receptor for RFRPs (Hinuma et al., 2000; Ubuka et al., 2009a). However, recent studies have shown that RF9 not only antagonizes NPFF driven hyperalgesia (Simonin et al., 2006) but paradoxically increases food intake similar to NPFF (Maletínská et al., 2013). Additionally, it has been reported that RF9 acts as an agonist of kisspeptin receptor (GPR54) on GnRH neurons to stimulate GnRH firing rate independent of RFRP-3 (Liu and Herbison, 2014; Min et al., 2015). Therefore, our results also raise the possibility that RF9 may act as an agonist in monkeys to stimulate the HPA-axis. This notion is also supported by an analogous earlier observation that an NPY antagonist 1229 acted as an NPY agonist on NPY4 receptor in adult male monkeys (Shahab et al., 2003). Our results and the above mentioned studies (Liu and Herbison, 2014; Maletínská et al., 2013; Min et al., 2015; Shahab et al., 2003) collectively suggest that RF9 has multiple and off-target actions and, therefore, at some receptors it acts as an antagonist while on others it acts as an agonist. Based on the above studies, we tend to speculate that RF9 acts as an agonist of the HPA-axis and hence its administration in monkeys occasions stimulation of cortisol secretion. Although we are the first to report that RF9 acts as an agonist of the HPA-axis, we do not know the mechanism and site of action of RF9 along the HPA-axis and further studies are required. Paradoxically, RF9 administration to fasted monkeys caused no effect on cortisol levels. RF9 acts as an agonist of kisspeptin receptor (GPR54) on GnRH neurons to stimulate GnRH firing rate independent of RFRP-3 (Liu and Herbison, 2014; Min et al., 2015). Similarly, our own studies reported that RF9 administration has stimulated a fasting induced repressed reproductive axis by stimulating testosterone levels (Batool et al., 2014) but didn't effect cortisol levels (the current study) when injected into 48 h fasted monkeys. This evidence collectively suggests that RF9 directly stimulates kisspeptin-GPR54 signaling to activate the reproductive axis during fasting induced stress independent of RFRPs-GPR147 and cortisol signaling. Importantly, RF9 cannot cross the blood brain barrier and, therefore, the effect of RF9 on kisspeptin-GPR54 system seems peripheral, not central. However, to verify the aforementioned, careful assessment of RF9 interaction with GPR54 and GPR147 using neuropharmacology tools would be necessary. Additionally, as it is well-established that fasting situations stimulate the HPA-axis and cortisol levels in various species including monkeys (Rivier and Rivest, 1991; Rivier et al., 1986; Roky et al., 2004; Wahab et al., 2008), we therefore intuitively speculate RF9 may be ineffective when the HPA-axis is already stimulated.

In summary, our findings support a stimulatory role of RFRPs signaling on the HPA-axis in adult male monkeys. Additionally, RF9 likely acts as an agonist of the HPA-axis but RF9 signaling is ineffective when the HPA-axis is already stimulated. Importantly, the mechanism and site of action of RF9 along the HPA-axis is not known and further studies are required.

Author contribution statement

RU, MS, JF and FW designed the current study and wrote the manuscript. RU performed the experiments while AB, MW, RN and TR provided help during experimentations.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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References

Alldredge, B., 2010. Pathogenic involvement of neuropeptides in anxiety and depression. Neuropeptides 44, 215–224.
Anjum, S., Krishna, A., Tsutsui, K., 2016. Possible role of GnIH as a mediator between adiposity and impaired testicular function. Front. Endocrinol. 7, 6.
Bao, L.-L., Jiang, W.-Q., Sun, F.-J., Wang, D.-X., Pan, Y.-J., Song, Z.-X., Wang, C.-H., Yang, J., 2014. The influence of psychological stress on arginine vasopressin concentration in the human plasma and cerebrospinal fluid. Neuropeptides 48, 361–369.
Batool, A., Naz, R., Wazir, M., Azam, A., Ullah, R., Wahab, F., Shahab, M., 2014. Acute fasting-induced repression of the hypothalamic-pituitary-gonadal axis is reversed by RF-9 administration in the adult male macaque. Horm. Metab. Res.(Hormon-und Stoffwechselforschung Hormones et metabolisme). 46 927-832.
Bentley, G.E., Ubuka, T., McGuire, N.L., Chowdhury, V.S., Morita, Y., Yano, T., Hasunuma, I., Binns, M., Wingfield, J.C., Tsutsui, K., 2008. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. Gen. Comp. Endocrinol. 156, 34–43.
Bolt, D., Kelley, H., Hawk, H., 1971. Release of LH by estradiol in cycling ewes. Biol.

Reprod. 4, 35–40.
Calisi, R.M., Geraghty, A.C., Avila, A., Kaufer, D., Bentley, G.E., Wingfield, J.C., 2016. Patterns of hypothalamic GnIH change over the reproductive period in starlings and rats. Gen. Comp. Endocrinol. 237, 140–146.
Caraty, A., Blomenröhr, M., Vogel, G., Lomet, D., Briant, C., Beltramo, M., 2012. RF9 powerfully stimulates gonadotrophin secretion in the ewe: evidence for a seasonal threshold of sensitivity. J. Neuroendocrinol. 24, 725–736.
Castillo, R.H., Matteri, R.L., Dumesic, D.A., 1992. Luteinizing hormone synthesis in cultured fetal human pituitary cells exposed to gonadotropin-releasing hormone. J. Clin. Endocrinol. Metab. 75, 318–322.
Clarke, I.J., Sari, I.P., Qi, Y., Smith, J.T., Parkington, H.C., Ubuka, T., Iqbal, J., Li, Q., Tilbrook, A., Morgan, K., 2008. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. Endocrinology 149, 5811–5821.
Cyr, N.E., Romero, L.M., 2007. Chronic stress in free-living European starlings reduces corticosterone concentrations and reproductive success. Gen. Comp. Endocrinol. 151, 82–89.
Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., Valle, S., 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. Gen. Comp. Endocrinol. 203, 120–131.
Döcke, F., 1979. Neurohormonal regulation of male gonad function. Zeitschrift fur die gesamte innere Medizin und ihre Grenzgebiete 34, 589–592.
Duruisseau, P., Tache, Y., Brazeau, P., Collu, R., 1979. Effects of chronic immobilization stress on pituitary-hormone secretion, on hypothalamic factor levels, and on pituitary-responsiveness to LHRH and TRH in female rats. Neuroendocrinology 29, 90–99.
Eskiocak, S., Gozen, A.S., Yapar, S.B., Tavas, F., Kilic, A.S., Eskiocak, M., 2005. Glutathione and free sulphydryl content of seminal plasma in healthy medical students during and after exam stress. Hum. Reprod. 20, 2595–2600.
Ferris, J.K., Tse, M.T., Hamson, D.K., Taves, M.D., Ma, C., McGuire, N., Arckens, L., Bentley, G.E., Galea, L.A.M., Floresco, S.B., Soma, K.K., 2015. Neuronal gonadotrophin-releasing hormone (GnRH) and astrocytic gonadotrophin inhibitory hormone (GnIH) immunoreactivity in the adult rat hippocampus. J. Neuroendocrinol. 27, 772–786.
Filaferro, M., Ruggieri, V., Novi, C., Calò, G., Cifani, C., Micioni Di Bonaventura, M.V., Sandrini, M., Vitale, G., 2014. Functional antagonism between nociceptin/orphanin FQ and corticotropin-releasing factor in rat anxiety-related behaviors: Involvement of the serotonergic system. Neuropeptides 48, 189–197.
Geraghty, A.C., Kaufer, D., 2015. Glucocorticoid regulation of reproduction. In: Wang, J.-C., Harris, C. (Eds.), Glucocorticoid Signaling: From Molecules to Mice to Man. Springer New York, New York, NY, pp. 253–278.
Gibson, E.M., Humber, S.A., Jain, S., Williams 3rd, W.P., Zhao, S., Bentley, G.E., Tsutsui, K., Kriegsfeld, L.J., 2008. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. Endocrinology 149, 4958–4969.
Gonzalez-Quijano, M., Ariznavarreta, C., Martin, A., Treguerres, J., Lopez-Calderon, A., 1991. Naltrexone does not reverse the inhibitory effect of chronic restraint on gonadotropin secretion in the intact male rat. Neuroendocrinology 54, 447–453.
Gottsch, M., Cunningham, M., Smith, J., Popa, S., Acohido, B., Crowley, W., Seminara, S., Clifton, D., Steiner, R., 2004. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145, 4073–4077.
Hinuma, S., Shintani, Y., Fukusumi, S., Iijima, N., Matsumoto, Y., Hosoya, M., Fujii, R., Watanabe, T., Kikuchi, K., Terao, Y., 2000. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. Nat. Cell Biol. 2, 703–708.
HORI, T., IDE, M., MIYAKE, T., 1969. Pituitary regulation of preovulatory estrogen secretion in the rat. Endocrinol. Jpn. 16, 351–360.
Jadhao, A.G., Pinelli, C., D'Aniello, B., Tsutsui, K., 2017. Gonadotropin-inhibitory hormone (GnIH) in the amphibian brain and its relationship with the gonadotropin releasing hormone (GnRH) system: an overview. Gen. Comp. Endocrinol. 240, 69–76.
Jhamandas, J.H., Simonin, F., Bourguignon, J.-J., Harris, K.H., 2007. Neuropeptide FF and neuropeptide VF inhibit GABAergic neurotransmission in parvocellular neurons of the rat hypothalamic paraventricular nucleus. Am. J. Phys. Regul. Integr. Comp. Phys. 292, R1872–R1880.
Johnson, M.A., Tsutsui, K., 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. Horm. Behav. 51, 171–180.
Kaewwongse, M., Takayanagi, Y., Onaka, T., 2011. Effects of RFamide-related peptide (RFRP)-1 and RFRP-3 on oxytocin release and anxiety-related behaviour in rats. J. Neuroendocrinol. 23, 20–27.
Kalra, S.P., Dube, M.G., Pu, S., Xu, B., Horvath, T.L., Kalra, P.S., 1999. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr. Rev. 20, 68–100.
Kim, J.S., 2016. What's in a name? Roles of RFamide-related peptides beyond gonadotrophin inhibition. J. Neuroendocrinol. 28 n/a-n/a.
Kirby, E.D., Geraghty, A.C., Ubuka, T., Bentley, G.E., Kaufer, D., 2009. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. Proc. Natl. Acad. Sci. 106, 11324–11329.
Kormos, V., Gaszner, B., 2013. Role of neuropeptides in anxiety, stress, and depression: from animals to humans. Neuropeptides 47, 401–419.
Kovács, A., László, K., Zagoracz, O., Ollmann, T., Péczely, L., Gálosi, R., Lénárd, L., 2017. Effects of RFamide-related peptide-1 (RFRP-1) microinjections into the central nucleus of amygdala on passive avoidance learning in rats. Neuropeptides 62, 81–86.
Lebrethon, M.-C., Aganina, A., Fournier, M., Gerard, A., Parent, A.-S., Bourguignon, J.-P., 2007. Effects of in vivo and in vitro administration of ghrelin, leptin and neuropeptide mediators on pulsatile gonadotrophin-releasing hormone secretion from male rat hypothalamus before and after puberty. J. Neuroendocrinol. 19, 181–188.
Lerman, S.A., Miller, G.K., Bohlman, K., Albaladejo, V., Léonard, J.-F., Devas, V., Clark, R.L., 1997. Effects of corticosterone on reproduction in male sprague-dawley rats. Reprod. Toxicol. 11, 799–805.
Li, J., Li, H.-X., Shou, X.-J., Xu, X.-J., Song, T.-J., Han, S.-P., Zhang, R., Han, J.-S., 2016. Effects of chronic restraint stress on social behaviors and the number of hypothalamic oxytocin neurons in male rats. Neuropeptides 60, 21–28.
Liew, H.-K., Huang, L.-C., Yang, H.-I., Peng, H.-F., Li, K.-W., Tsai, A.P.-Y., Chen, S.-Y., Kuo, J.-S., Pang, C.-Y., 2015. Therapeutic effects of human urocortin-1, -2 and -3 in intracerebral hemorrhage of rats. Neuropeptides 52, 89–96.
Liu, X., Herbison, A.E., 2014. RF9 excitation of GnRH neurons is dependent upon Kiss1r in the adult male and female mouse. Endocrinology 155, 4915–4924.
Maletínská, L., Tichá, A., Nagelová, V., Špolcová, A., Blechová, M., Elbert, T., Železná, B., 2013. Neuropeptide FF analog RF9 is not an antagonist of NPFF receptor and decreases food intake in mice after its central and peripheral administration. Brain Res. 1498, 33–40.
Martynska, L., Wolinska-Witort, E., Chmielowska, M., Kalisz, M., Baranowska, B., Bik, W., 2014. Effect of orexin A on the release of GnRH-stimulated gonadotrophins from cultured pituitary cells of immature and mature female rats. Neuropeptides 48, 199–205.
McConn, B., Wang, G., Yi, J., Gilbert, E.R., Osugi, T., Ubuka, T., Tsutsui, K., Chowdhury, V.S., Furuse, M., Cline, M.A., 2014. Gonadotropin-inhibitory hormone-stimulation of food intake is mediated by hypothalamic effects in chicks. Neuropeptides 48, 327–334.
McGuire, N.L., Bentley, G.E., 2010. Neuropeptides in the gonads: From evolution to pharmacology. Front. Pharmacol. 1, 114.
Min, L., Leon, S., Li, H., Pinilla, L., Carroll, R.S., Tena-Sempere, M., Kaiser, U.B., 2015. RF9 acts as a KISS1R agonist in vivo and in vitro. Endocrinology 156, 4639–4648.
Qi, Y., Oldfield, B., Clarke, I., 2009. Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. J. Neuroendocrinol. 21, 690–697.
Ralph, C.R., Lehman, M.N., Goodman, R.L., Tilbrook, A.J., 2016. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? Reproduction 152, R1–R14.
Retana-Marquez, S., Bonilla-Jaime, H., Vazquez-Palacios, G., Martinez-Garcia, R., Velazquez-Moctezuma, J., 2003. Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. Horm. Behav. 44, 327–337.
Rivier, C., Rivest, S., 1991. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: Peripheral and central mechanisms. Biol. Reprod. 45, 523–532.
Rivier, C., Rivier, J., Vale, W., 1986. Stress-induced inhibition of reproductive functions: Role of endogenous corticotropin-releasing factor. Science 231, 607–609.
Rizwan, M.Z., Poling, M.C., Corr, M., Cornes, P.A., Augustine, R.A., Quennell, J.H., Kauffman, A.S., Anderson, G.M., 2012. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology 153, 3770–3779.
Roky, R., Houti, I., Moussamih, S., Qotbi, S., Aadil, N., 2004. Physiological and chronobiological changes during Ramadan intermittent fasting. Ann. Nutr. Metab. 48, 296–303.
Samson, W.K., Keown, C., Samson, C.K., Samson, H.W., Lane, B., Baker, J.R., Taylor, M.M., 2003. Prolactin-releasing peptide and its homolog RFRP-1 act in hypothalamus but not in anterior pituitary gland to stimulate stress hormone secretion. Endocrine 20, 59–66.
Schally, A., Arimura, A., Kastin, A., Matsuo, H., Baba, Y., Redding, T., Nair, R., Debeljuk, L., White, W., 1971. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. Science 173, 1036–1038.
Shahab, M., Balasubramaniam, A., Sahu, A., Plant, T., 2003. Central nervous system receptors involved in mediating the inhibitory action of neuropeptide Y on luteinizing hormone secretion in the male rhesus monkey (*Macaca mulatta*). J. Neuroendocrinol. 15, 965–970.
Shahab, M., Mastronardi, C., Seminara, S.B., Crowley, W.F., Ojeda, S.R., Plant, T.M., 2005. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc. Natl. Acad. Sci. U. S. A. 102, 2129–2134.
Sheiner, E.K., Sheiner, E., Carel, R., Potashnik, G., Shoham-Vardi, L., 2002. Potential association between male infertility and occupational psychological stress. J. Occup. Environ. Med. 44, 1093–1099.
Simonin, F., Schmitt, M., Laulin, J.-P., Laboureyras, E., Jhamandas, J.H., MacTavish, D., Matifas, A., Mollereau, C., Laurent, P., Parmentier, M., 2006. RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. Proc. Natl. Acad. Sci. U. S. A. 103, 466–471.
Smith, J.T., Young, I.R., Veldhuis, J.D., Clarke, I.J., 2012. Gonadotropin-inhibitory hormone (GnIH) secretion into the ovine hypophyseal portal system. Endocrinology 153, 3368–3375.
Themmen, A.P., Huhtaniemi, I.T., 2000. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. Endocr. Rev. 21, 551–583.
Toonen, R.B., Wardenaar, K.J., van Ockenburg, S.L., Bos, E.H., de Jonge, P., 2016. Using state space methods to reveal dynamical associations between cortisol and depression. Nonlinear Dynamics Psychol. Life Sci. 20, 1–21.
Tsutsui, K., Saigoh, E., Ukena, K., Teranishi, H., Fujisawa, Y., Kikuchi, M., Ishii, S., Sharp, P.J., 2000. A novel avian hypothalamic peptide inhibiting gonadotropin release. Biochem. Biophys. Res. Commun. 275, 661–667.
Tsutsui, K., Bentley, G.E., Bedecarrats, G., Osugi, T., Ubuka, T., Kriegsfeld, L.J., 2010. Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. Front. Neuroendocrinol. 31, 284–295.
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., Wingfield, J.C., 2007. The general and

comparative biology of gonadotropin-inhibitory hormone (GnIH). Gen. Comp. Endocrinol. 153, 365–370.
Tsutsui, K., Saigoh, E., Yin, H., Ubuka, T., Chowdhury, V., Osugi, T., Ukena, K., Sharp, P., Wingfield, J., Bentley, G., 2009. A new key neurohormone controlling reproduction, gonadotrophin-inhibitory hormone in birds: discovery, progress and prospects. J. Neuroendocrinol. 21, 271–275.
Tsutsui, K., Ubuka, T., Bentley, G.E., Kriegsfeld, L.J., 2012. Gonadotropin-inhibitory hormone (GnIH): discovery, progress and prospect. Gen. Comp. Endocrinol. 177, 305–314.
Tsutsui, K., Ubuka, T., Son, Y.L., Bentley, G.E., Kriegsfeld, L.J., 2015. Contribution of GnIH research to the progress of reproductive neuroendocrinology. Front. Endocrinol. 6, 179.
Tsutsui, K., Ubuka, T., Yin, H., Osugi, T., Ukena, K., Bentley, G.E., Ciccone, N., Inoue, K., Chowdhury, V.S., Sharp, P.J., Wingfield, J.C., 2006. Mode of action and functional significance of avian gonadotropin-inhibitory hormone (GnIH): a review. J. Exp. Zool. A Comp. Exp. Biol. 305, 801–806.
Tsutsui, K., Ukena, K., 2006. Hypothalamic LPXRF-amide peptides in vertebrates: identification, localization and hypophysiotropic activity. Peptides 27, 1121–1129.
Ubuka, T., Kim, S., Huang, Y.C., Reid, J., Jiang, J., Osugi, T., Chowdhury, V.S., Tsutsui, K., Bentley, G.E., 2008. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. Endocrinology 149, 268–278.
Ubuka, T., Lai, H., Kitani, M., Suzuuchi, A., Pham, V., Cadigan, P.A., Wang, A., Chowdhury, V.S., Tsutsui, K., Bentley, G.E., 2009a. Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. J. Comp. Neurol. 517, 841–855.
Ubuka, T., Morgan, K., Pawson, A.J., Osugi, T., Chowdhury, V.S., Minakata, H., Tsutsui, K., Millar, R.P., Bentley, G.E., 2009b. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. PLoS One 4, e8400.
Ubuka, T., Son, Y.L., Tobari, Y., Narihiro, M., Bentley, G.E., Kriegsfeld, L.J., Tsutsui, K., 2014. Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor. Front. Endocrinol. 5, 8.
Ukena, K., Iwakoshi-Ukena, E., Osugi, T., Tsutsui, K., 2016. Identification and localization of gonadotropin-inhibitory hormone (GnIH) orthologs in the hypothalamus of the red-eared slider turtle, *Trachemys scripta* elegans. Gen. Comp. Endocrinol. 227, 69–76.
Ullah, R., Shen, Y., Zhou, Y.-D., Huang, K., Fu, J.-F., Wahab, F., Shahab, M., 2016. Expression and actions of GnIH and its orthologs in vertebrates: current status and advanced knowledge. Neuropeptides 59, 9–20.
Wada, H., Shibuya, A., Adachi, H., 1996. Effects of long-term psychological stress on sexual behavior and brain catecholamine levels. J. Androl. 17.
Wahab, F., Aziz, F., Irfan, S., Zaman, W.U., Shahab, M., 2008. Short-term fasting attenuates the response of the HPG axis to kisspeptin challenge in the adult male rhesus monkey (*Macaca mulatta*). Life Sci. 83, 633–637.
Wahab, F., Bano, R., Jabeen, S., Irfan, S., Shahab, M., 2010. Effect of peripheral kisspeptin administration on adiponectin, leptin, and resistin secretion under fed and fasting conditions in the adult male rhesus monkey (*Macaca mulatta*). Horm. Metab. Res. 42, 570–574.
Wahab, F., Quinton, R., Seminara, S.B., 2011. The kisspeptin signaling pathway and its role in human isolated GnRH deficiency. Mol. Cell. Endocrinol. 346, 29–36.
Wahab, F., Shahab, M., Behr, R., 2015. The involvement of gonadotropin inhibitory hormone and kisspeptin in the metabolic regulation of reproduction. J. Endocrinol. 225, R49–R66.
Wahab, F., Zaman, W.-U., Shahab, M., 2012. Differential response of the primate HPG axis to N-methyl-D, L-aspartate, but not to Kisspeptin challenge under euglycemic and hypoglycemic conditions. Horm. Metab. Res. 44, 451.
Yan, Y., Wang, Y.-L., Su, Z., Zhang, Y., Guo, S.-X., Liu, A.-J., Wang, C.-H., Sun, F.-J., Yang, J., 2014. Effect of oxytocin on the behavioral activity in the behavioral despair depression rat model. Neuropeptides 48, 83–89.
Zhu, J., Xu, X.h., Knight, G.E., He, C., Burnstock, G., Xiang, Z., 2015. A subpopulation of gonadotropin-releasing hormone neurons in the adult mouse forebrain is γ-aminobutyric acidergic. J. Neurosci. Res. 93, 1611–1621.
Ullah, R., Su, Y., Shen, Li C., Xu, X., Zhang, J., Huang, K., Rauf, N., He, Y., Cheng, J., Qin, H., Zhou, Y., Fu, Junfen, 2017. Postnatal feeding with high-fat diet induces obesity and precocious puberty in C57BL/6J mouse pups: a novel model of obesity and puberty. Front. Med. http://dx.doi.org/10.1007/s11684-017-0530-y.