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ORIGINAL ARTICLE

Expression and co-localization of RFRP-3 and kisspeptin during breeding and non-breeding season in the hypothalamus of male rhesus monkey (*Macaca mulatta*)

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Abstract

Propose: The mechanism that underpins how RFRP-3 and kisspeptin interacts are not fully understood in higher primates. This study therefore set out to assess RFRP-3 and kisspeptin expression and their morphological interactions in the breeding, and in the non-breeding period in monkey hypothalamus.

Methods: Eight mature male macaques (*Macaca mulatta*) in the breeding season (February; $n = 4$) and non-breeding season (June; $n = 4$) were used. To reveal the expression and co-localization of RFRP-3 and kisspeptin, double-labeled immunohistochemistry was performed. Testicular volume, sperm count, and plasma testosterone level were also measured to validate the breeding and non-breeding paradigms.

Results: Testicular volume, plasma testosterone level, and sperm count showed a significant reduction during non-breeding season. The number of kisspeptin-positive cells was significantly increased during the breeding season ($p < 0.05$), whereas more RFRP-3-positive cell bodies were seen in the non-breeding season ($p < 0.01$). Close contacts of RFRP-3 fibers with kisspeptin cells showed no significant difference ($p > 0.05$) across seasons. However, co-localization of RFRP-3-ir cell bodies onto kisspeptin IR cell bodies showed a statistical increase ($p < 0.01$) in non-breeding season.

Conclusion: In higher primates, RFRP-3 decreases kisspeptin drives from the same cells to GnRH neurons in an autocrine manner causing suppression of the reproductive axis during the non-breeding period.

KEYWORDS
kisspeptin, reproduction, RFRP-3, rhesus monkey, seasons

1 | INTRODUCTION

The neuropeptides Kp (kisspeptin) and RFRP-3 (RFamide-related peptide-3) have a pivotal role in regulating reproduction. In addition, kisspeptin is fundamental to GnRH neuronal activity, leading to increased gonadotrophin secretion and triggering the HPG-axis across mammals, including humans.[1,2] KISS1 gene codes kisspeptin, a hypothalamic peptide with an Arg-Phe-NH2 C-terminal sequence that belongs to the RFamide family.[3–5] Kisspeptin is the ligand of GPR54 (commonly known as KISS1 receptor), to initiate the GnRH pulsatile secretion.[6] Gonadotropin-inhibitory hormone (GnIH) was first discovered to show a potent inhibitory role in the function of GnRH

neurons in the hypothalamus of Japanese quail.[7,8] In the literature, RFRP-3 has been recognized as the homologue of GnIH among mammals.[9] Similarly, RFRP-1 and RFRP-3 both are the product of the same gene in mammals.[10] RFRP-3 halts gonadotropin secretion in various species via binding to GPR147, signifying its reproductive axis inhibitory action.[11–13]

Reproductive activity in many mammalian species coordinates with a specific time of year, therefore, known as seasonal breeders. Seasonal species are either long-day (LD) breeders or short-day (SD) breeders. Long-day breeders (rodents) produce offspring during the spring and summer with a few weeks of gestation. Whereas, short-day breeders, such as sheep, goats, deer, or rhesus monkeys, breed during the fall with a few months of gestation.[14] In the context of seasonal reproduction, the promising role of kisspeptin and RFRP-3 have been investigated in several studies, providing evidence that they are excellent candidates for regulating the reproductive axis in various seasons.

During the long-day period, Syrian hamsters had a higher level of Kiss1 mRNA in the arcuate nucleus, and greater testicular weight and plasma testosterone level. However, when the animals were housed in short day for 8–10 weeks, the level of Kiss1 mRNA, testis size, and testosterone level decreased.[15] Similarly, administering kisspeptin-10 under photoinhibitory conditions restores sexual function in Syrian hamsters.[16] These studies indicate that kisspeptin tightly regulates the reproductive status in Syrian hamster and a low level of kisspeptin in short-day cause the inhibition of reproduction. In addition, the influence of season on the expression of Kiss1 has also been studied in sheep. Notably, the kisspeptin mRNA was higher during the onset of the breeding season (a short-day period) in the arcuate nucleus.[17] Furthermore, 12.4 nmol/h kisspeptin delivery in seasonally acyclic ewes triggers ovulation.[18] On the other hand, in the non-breeding period (long day) kisspeptin expression in the sheep was markedly declined.[19] This shows that the arcuate of sheep undergoes seasonal regulation of Kiss1 expression to drive seasonal reproduction.

Subsequently, the role of RFRP-3 in seasonal reproduction has also been renowned in many studies. In male hamsters (responders to short-day length), eight weeks' constant short-day exposure significantly decreased RFRP-3 cell bodies and mRNA as compared to long-day animals. Whereas, nonresponders to short-day length showed RFRP-3 expression significantly when compared to long-day animals.[20]

In the sheep hypothalamus, RFRP-3 protein expression was elevated in the non-breeding period.[21] Similarly, RFRP-3 terminal connection opposing GnRH neuron increase in the ovine non-breeding period.[21] Ewes maintained in a long-day photoperiod (non-breeding season) showed a higher RFRP-3 mRNA expression as compared to ewes maintained in a short-day photoperiod.[22] During the short-day photoperiod decrease in RFRP-3 peptide synthesis, the decline in fibers toward GnRH neurons and RFRP-3 release into hypophysial blood circulation were seen in sheep.[21,23]

Further research found that RFRP-3's role in seasonal reproduction is complex, with varied expression patterns in rodents and mammals.[24] These findings suggest that RFRP-3 has either a species-specific role or when it binds to its receptor, it interacts with numerous G proteins, resulting in a variety of seasonal reproduction effects in different species.

RFRP-3 receptors (GPR147 and GPR74) expression was investigated on the kisspeptin neuron in male and in female mice. Along with RFRP-3 receptors, 35 percent of kisspeptin neurons received RFRP-3 fibers in the arcuate nucleus.[25] Similarly, RFRP-3 intracerebroventricular injection substantially decreased kisspeptin mRNA in both female rats and mice.[26,27] Altogether, these findings reveal that kisspeptin and RFRP-3 are the key processers involved in seasonal breeding in communication with GnRH neurons. Likely, the kisspeptin neuronal population is modulated by the direct action of RFRP-3. However, there are few studies that have investigated the morphological interaction between RFRP-3 and the kisspeptin system in higher primates controlling seasonal reproduction. Thus, the aim of our study was to find out the expression of RFRP-3 and kisspeptin and their morphological interaction: co-localization in the breeding and non-breeding season using monkey as a model animal.

2 | MATERIALS AND METHODS

2.1 | Animals

A total of 8 mature male rhesus monkeys (*Macaca mulatta*) four in the breeding season (February; day length 11.16 h; temperature 15°C), with a body weight of 9–15 kg (mean SEM: 11.62 1.24 kg) and a testicular volume of 30–54 ml (mean SEM: 42.48 4.93 ml) and the remaining four in the non-breeding season (June; day length 14.41 h; temperature 33°C), with a body weight of 5–12 kg (mean SEM: 7.67 1.41 kg) and a testicular volume of 12–18 ml (mean SEM: 14.83 1.18 ml) were purchased and used in our experiment. Monkeys were kept and cared for in isolated cages of the Department Primate Facility Centre. The monkeys were fed with fresh fruits and bread on a daily basis and also provided water with free access. The entire experimental process and protocol were conducted under the guideline and approval of the ethical committee of Zoology department at Quaid-i-azam University (Islamabad, Pakistan).

2.2 | Blood sampling and hypothalamic tissues collection

Four animals were euthanized on two consecutive days (two animals per day) in the breeding period (February), whereas non-breeding monkeys were also scarified using the same scheme (June). The monkeys were first anesthetized by injecting 10–20 mg/kg body weight of Ketamine hydrochloride intramuscularly (Ketamax, Rotex Medica,). Each animal's body weight and testicular volume were noted before euthanization. A single blood sample from each animal was taken from the saphenous vein to measure testosterone levels. A blood sample (2.5 ml) was taken in a heparinized syringe and

immediately shifted into a chilled glass tube. In order to obtain blood plasma, the samples were processed for centrifugation at 3500 rpm for 15 minutes (Kokusan-H-103RS centrifuge,). The separated plasma was kept at −20°C for hormone analysis. After sedation, a high dose of ketamine hydrochloride was injected intravenously to euthanize the animals. After that, the hair from the skull were shaved off, and removed skin and muscle with the help of a scalpel. Later on, the skull bone was cut out with the help of a sharp bone cutter. Brains were removed carefully and immediately placed on a cold glass plate. Hypothalamic blocks were taken out corresponding to the procedure as available in the earlier study.[28] Centrally median eminence, posteriorly mammillary bodies and anteriorly optic chiasm, a coronal cut was made rostral to the anterior commissure and next to the mammillary bodies. Furthermore, 5 mm of two parasagittal were made on both sides of the midline. At the caudal boundary of the optic chiasm, a cut was made and a square of hypothalamic tissue was obtained. Finally, the hypothalamic blocks were separated by making a cut at the midline, and normal saline was used to wash out blocks of hypothalamus.

2.3 | Hypothalamic tissue fixation and processing

Hypothalamic block fixation was carried out for 24 h in 4% paraformaldehyde and phosphate buffer saline (PBS) at 4°C. Following fixation, the tissue blocks were dehydrated in a 20% and then 30% sucrose solution (Fischer Chemical, hire,). The fixed tissues were then cut into 20 μm thick sections at −25°C using cryostat (Bright OTF 5000, A-M Systems, Sequim,). After sectioning, the sections were recovered and preserved at −20°C in cryoprotectant solution: 30 percent ethylene glycol, 1 percent polyvinylpyrrolidone, and 30 percent sucrose, till further analysis.

2.4 | Semen analysis and histological study of testicular tissues

For the purpose of semen analysis, the epididymis was extracted from both breeding and non-breeding animals and washed with normal saline. Random cuts were made in the epididymis at different points and semen was collected in a phosphate buffer saline (PBS) solution to prepare a mixture. A volume of 20 microliters of the mixture containing spermatozoa was taken and filled the hemocytometer chamber. After filling, the hemocytometer was put in a covered petri dish with moistened filter paper for 10 min to allow the spermatozoa to settle down. The hemocytometer was divided into five large squares. All sperm heads were counted in the large centre square (1 mm X 1 mm X 0.1 mm). Those spermatozoa on the left and top lines of each square were counted, and those on the right and bottom lines were not included. A general formula for sperm count was used as: No. of sperm/mL = dilution factor x count in 5 chambers x 0.05 x 106. For testicular histology, both groups' testicular tissues were collected and fixed in Bouin's solution (25 ml formalin, 75 ml picric acid solution, and 5 ml acetic acid). After fixation, the tissues were processed using the paraffin wax embedding method. Tissue blocks were sectioned at a 20 μm thickness on the microtome. The sections were stained with hematoxylin and eosin for observing general testicular histology and assessing the spermatogenic status of animals. On the ocular micrometer, the testicular diameter of seminiferous tubules and epithelial height were measured at 20x and 40x using a compound microscope (Nikon SE, Shinagawa,).

2.5 | Immunohistochemistry on hypothalamic tissues

Kisspeptin and RFRP-3 expression and their co-localization were assessed in the arcuate area (ARC) of monkey hypothalamus through a standard double-labeled immunohistochemistry. Guinea pig-anti-RFRP-3 (cat.no: GPARFRP3.1) was used as a primary antibody for RFRP-3, while the primary antibody for kisspeptin was sheep anti-human (kindly provided by Prof. Dr. Stephen Bloom). Alexa Flour 488 labeled goat anti-guinea pig (cat. no: A-11073) and Texas red rabbit anti-sheep (cat.no: ab6745) were used as secondary antibodies. The specificity of primary antibodies was confirmed by previous immunocytochemical studies.[29,30] Each animal had four sections stained in total. Three sections were processed for dual labeled immunostaining and one section remained as control. Primary antibodies' specificity was determined by omitting them from the control sections. To remove the cryoprotectant, the sections were washed eight times with PBS (0.01 percent) at 25°C for 15 min each time. Following washing, the sections were shaken for 2 h in incubation solution (10% of each: goat and rabbit serum., 0.05% Triton-X100, and 0.1% bovine serum albumin in PBS) to prevent nonspecific secondary antibody binding. Afterward, sections were washed and incubated for 48 h at −4°C in primary antibodies anti-RFRP-3 (1:1000) and anti-kisspeptin (1: 120000) dilutions. Control sections were incubated for 48 h only in the incubation solution without primary antibodies.

After incubation sections were again washed three times in PBS at room temperature for 15 min each time. After washing, a cocktail of secondary antibodies was used for incubation in the dark for 2 h (Alexa Flour 488 labeled goat anti-guinea pig at 1:500 and Texas red conjugated rabbit anti-sheep diluted at 1:500). Finally, sections were washed with PBS 3 times after secondary bodies incubation. Sections were mounted on super frosted glass slides (SC-24976, Statlab,) and coverslipped with Gelvatol. Slides were covered by aluminium foil and placed in the dark at 4°C until fluorescent microscopy was carried out.

2.6 | Fluorescent microscopy

Immunostained sections of ARC were studied (40x resolution) under a fluorescent microscope (AMEP-4615, EVOS, Bothel,) to determine immunoreactive-like cell bodies (IR) for RFRP-3,

kisspeptin, and their co-localization. Photographs of the interesting area (ARC) were captured with a digital camera connected to the fluorescent microscope for all animals. Immunoreactivities for RFRP-3 and kisspeptin were observed as green and red fluorescence, respectively. Co-localisation of RFRP-3 and kisspeptin was examined by superimposing images of RFRP-3 and kisspeptin IR cell bodies taken from the same area. The expression of RFRP-3 and kisspeptin, and their co-localization, were measured using Image J software. Total immunoreactive cells for RFRP-3 and kisspeptin were counted for each animal in a defined arcuate area. The mean ± SEM number of immunoreactive RFRP-3 and kisspeptin cells was then estimated for both breeding and non-breeding groups. Double immunostaining was evaluated by counting the superimposed cell bodies of RFRP-3 and kisspeptin.

2.7 | Elisa

A commercially available ELISA kit for human testosterone was used to measure monkey testosterone levels in blood plasma (Astra; Biotech; GmbH,). The assay was carried out according to the described protocol available in the kit. The assay's sensitivity was 0.05 ng/mL, with 9% interassay and 10% intra-assay coefficients of variation.

2.8 | Measurement of testicular volume

$V = (\pi w^2 l)/6$ was used to measure the testicular volume (ml) in which "l" shows the length and "w" describes the width of each testicle. Each animal's total testicular volume was calculated by combining the volumes of both the right and left testis.

2.9 | Statistical analyses

RFRP-3 and kisspeptin positive cells and their co-localisation in the ARC were compared using a simple unpaired *t*-test in both seasons. The difference was supposed to be statistically significant at $p \leq 0.05$. Graphs and data analyses were carried out using Graph Pad Prism version 7.04 (Graph Pad Software,).

3 | RESULTS

3.1 | Body weight, testicular volume, and plasma testosterone level

Body weight of monkeys in the breeding (mean ± SEM: 11.62 ± 1.24 kg) and non-breeding seasons (mean ± SEM: 7.67 ± 1.41 kg) was not statistically different. Testicular volume of monkeys in the non-breeding period was significantly ($p < 0.01$) reduced (mean ± SEM: 14.83 ± 1.18 ml vs 42.48 ± 4.93 ml). Similarly, a significant decline ($p < 0.05$) in circulating testosterone levels was seen in the non-breeding season (mean ± SEM: 0.55 ± 0.15 ng/mL vs 2.03 ± 0.46 ng/mL).

3.2 | Sperm count, epithelial height, and tubular diameter

Photomicrographs of hematoxylin and eosin–stained testicular sections for animals in breeding and non-breeding seasons are presented in Figure 1A, showing a low spermatogenic status and regression in tubular diameter in non-breeding animals. Data on mean epithelial height and tubular diameter of seminiferous tubules are shown in Figure 1B. A marked increase ($p < 0.001$) was seen in the tubular diameter during breeding season (189.56 ± 8.97 vs 98.32 ± 9.29. While no significant difference (57.14 ± 5.04 vs 35.45 ± 2.52) in epithelial height was observed in the two seasons. Data on the mean total number of sperm count/mL of each group are presented in Figure 2. A comparison of the mean total sperm count showed a significant decrease ($p < 0.05$) during non-breeding season.

3.3 | Expression profile of RFRP-3 and Kisspeptin

RFRP-3 and kisspeptin immunofluorescence positive cells in ARC of the monkey hypothalamus in both seasons are presented in Figure 3. The mean total number of RFRP-3 positive cells was significantly greater (at $p < 0.01$ NB; 423.75 ± 34.30 vs B; 189.75 ± 50.63) in the non-breeding season. Whereas, the total number of kisspeptin-positive cells (mean ± SEM: B; 360.25 ± 67.85 vs NB; 160.50 ± 14.56) were significant at $p < 0.05$ in the breeding season with respect to the non-breeding season (Figure 4).

3.4 | Close contact and co-localization of RFRP-3 and kisspeptin

Photomicrographic evidence of morphological interaction between RFRP-3 IR fibers onto kisspeptin IR cell bodies in the ARC of monkey hypothalamus is shown in Figure 5. Photomicrographs of co-localization of RFRP-3 with kisspeptin IR cell bodies are shown in Figure 6. No significant difference ($p > 0.05$) between the two groups was evident in close contacts of RFRP-3 IR fibers and kisspeptin IR cell bodies. Furthermore, there was a significant difference ($p < 0.01$) between the two groups in the mean number of RFRP-3 and kisspeptin co-localisation, showing more co-localization in the non-breeding season (Figure 7).

4 | DISCUSSION

The neuronal population in the hypothalamus produces RFRP-3 and kisspeptin, which appear to be crucial in the regulation of seasonal

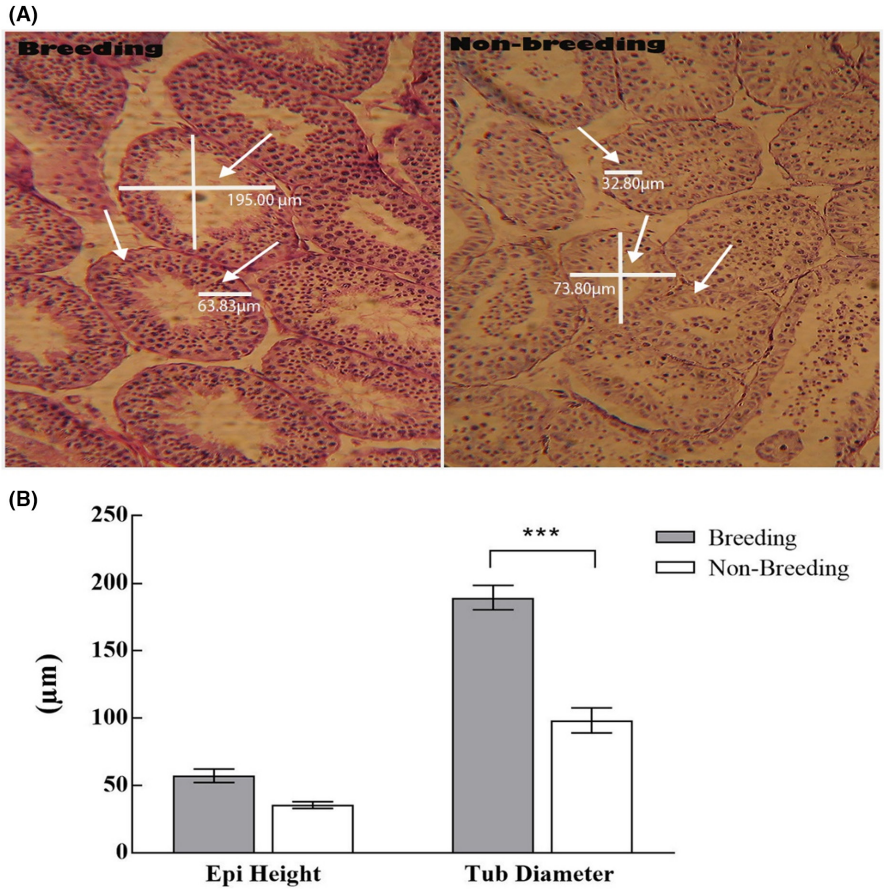

FIGURE 1 (A) Represent stained testicular section of male monkey (*Macaca mulatta*) at breeding and non-breeding period. Photomicrograph shows normal spermatogenesis in breeding season along with greater tubular diameter and epithelial height. While the non-breeding testicular section show regression in tubular diameter and low spermatogonic status (H&E staining 20x, magnification). (B) Comparison of mean ± SEM epithelial height and tubular diameter of seminiferous tubule in breeding and non-breeding season male monkeys. A significant reduction (***$p < 0.001$) in tubular diameter was noted during non-breeding season in comparison with breeding period

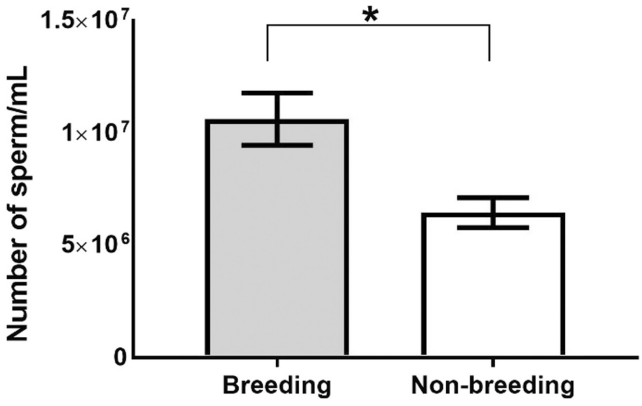

FIGURE 2 Shows the comparison of mean ± SEM sperm count per mL in mature monkey testis in the breeding and non-breeding period. Number of sperm count decline (*$p < 0.05$) in non-breeding season with respect to breeding season

reproduction. The current study was designed to determine RFRP-3 and kisspeptin expression and their morphological interaction: close contacts and co-localization in the monkey hypothalamus during the breeding and non-breeding period. This is a novel study to our knowledge, which evidences morphological interaction between these two neuropeptides in male monkeys across the breeding and non-breeding season. Similarly, our results from the current study in the hypothalamic tissues (ARC) of male monkeys revealed that seasonal variation in kisspeptin expression is influenced by the RFRP-3 drives. The first question was to confirm breeding period and non-breeding period paradigms. Measuring serum gonadotrophins is not feasible and reliable in rhesus monkeys because of lesser specificity of the kits available. Thus we assessed plasma testosterone concentration, believing that serum gonadotrophin levels ultimately translate to synthesis and release of sex steriods. We also measured testicular volume, and sperm count to further confirm the animal breeding and non-breeding state. We found that the HPG-axis activity is suppressed in the non-breeding period resulting in a significant decrease in testicular volume, plasma testosterone concentration, and declining sperm count. Our results are in line with previous studies that found a substantial decrease in testicular volume and plasma testosterone levels in non-breeding rhesus monkeys.[31,32]

Kisspeptin and RFRP-3 own pivotal regulatory functions in seasonal reproduction across mammalian species. Thus, with respect to the breeding and non-breeding seasons, we assessed both RFRP-3 and kisspeptin expression patterns in the monkey hypothalamus. Our findings showed, that there were more kisspeptin-positive cells in the breeding period than non-breeding. Our finding seems to be consistent with earlier studies, which found more kisspeptin in Syrian hamsters and sheep during breeding period.[15,17] Another study also supports our result, where kisspeptin expression and kiss1 mRNA were observed to be higher in ewes' ARC during the breeding season.[21] Furthermore, our finding of decreased kisspeptin expression in the non-breeding period was in accord with lower levels of Kiss1

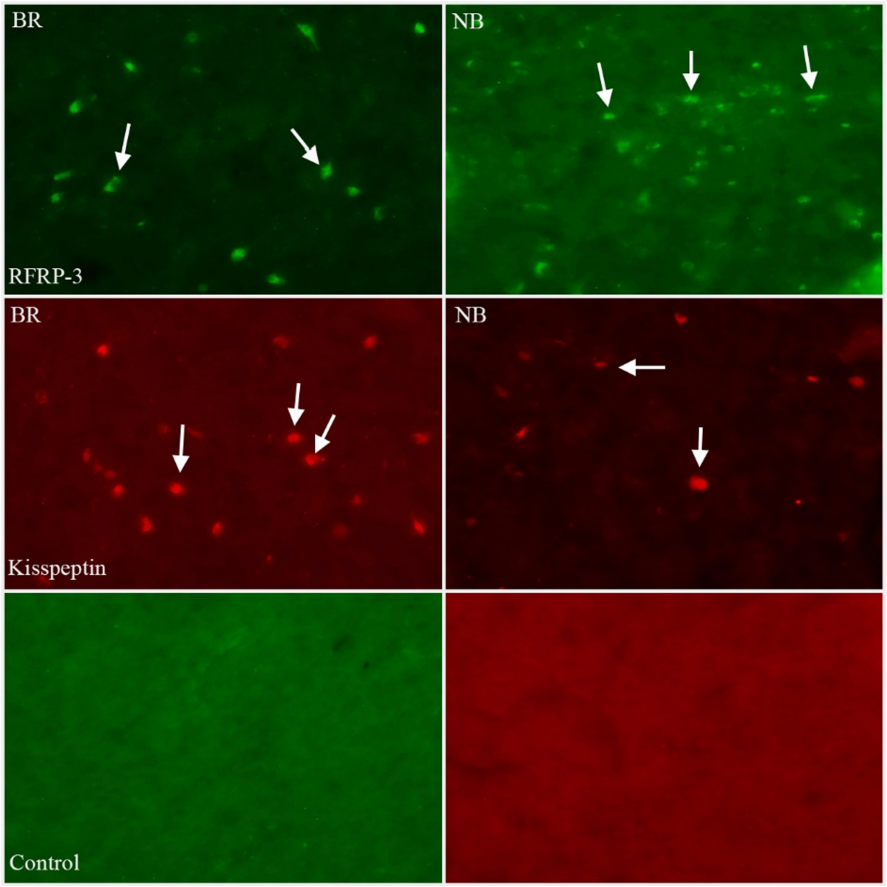

FIGURE 3 Fluorescent photomicrograph showing RFRP-3 and kisspeptin-like immunoreactive cell bodies in the hypothalamic sections (arcuate area) of male monkey in the breeding (BR) and non-breeding (NB) period. During the non-breeding season, more RFRP-3-positive cells were observed using goat anti-guinea pig conjugated with Alexa fluor 488 for RFRP-3. While more kisspeptin positive cells were seen during the breeding season using Texas red-conjugated rabbit anti-sheep for kisspeptin. The primary antibody-omitted control section shows no fluorescence

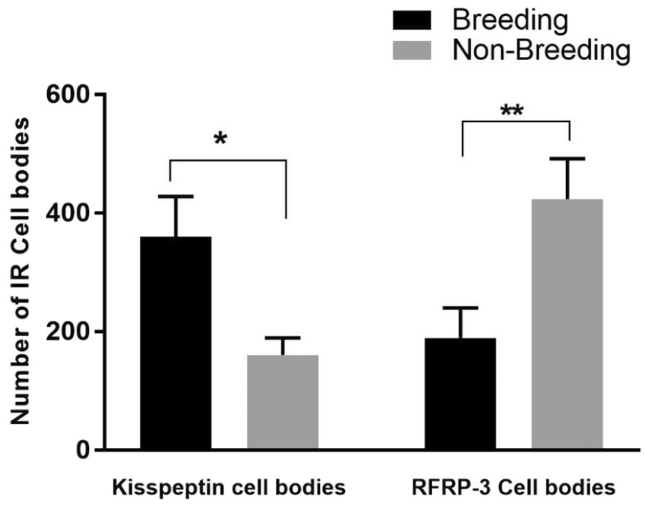

FIGURE 4 Presents comparison of total number (mean ± SEM) of RFRP-3 and kisspeptin-positive cells observed in three random hypothalamic section in arcuate area of (*Macaca mulatta*) in breeding and non-breeding period. Non-breeding period showed more RFRP-3-positive cells (**$p < 0.01$) in comparison with breeding season. Whereas, kisspeptin-positive cells were more (*$p < 0.05$) in breeding in relation to non-breeding period

expression and kisspeptin activity in the ewe hypothalamus in the non-breeding period.[17] Our results confirm that kisspeptin expression increases during the breeding season, triggering HPG-axis activity and vice versa. Moreover, we found more RFRP-3 positive cells in the monkey hypothalamus (ARC) during the non-breeding season. Our findings seem to be in line with those of previous research on seasonal breeders. In the non-breeding period, RFRP-3 IR and its neuroanatomical connection to GnRH neurons were significantly increased in the DMH and ARC of Syrian hamsters.[20] RFRP-3 expression was also higher in the hypothalamus of sheep during the non-breeding period.[21] We also saw fewer RFRP-3-positive cells during the breeding season. The previous study also supports our observation, where RFRP-3 cells were not obvious in the breeding period.[21] Likely, RFRP-3 and GnRH neuronal interactions in the POA region of sheep also decrease during the breeding season.[21] A similar study on sheep found that a short stimulatory day duration resulted in a significant decrease in RFRP-3.[22] Altogether, these findings show that both RFRP-3 and kisspeptin act in harmony to regulate GnRH neuronal activity in different seasons, resulting in annual fluctuation in fertility.

Despite kisspeptin and RFRP-3's effective and opposite roles on the HPG-axis, their putative interaction in higher primates has not been thoroughly investigated. RFRP-3 and kisspeptin co-localisation, and RFRP-3 fiber to kisspeptin cell body contact, is the major finding of our study. Interestingly the co-localisation was seen greater in the non-breeding period, which reveals that a subset of ARC cells could express both RFRP-3 and kisspeptin in higher primates to regulate seasonal reproduction. Our result of co-localization is consistent with a recent study, that found

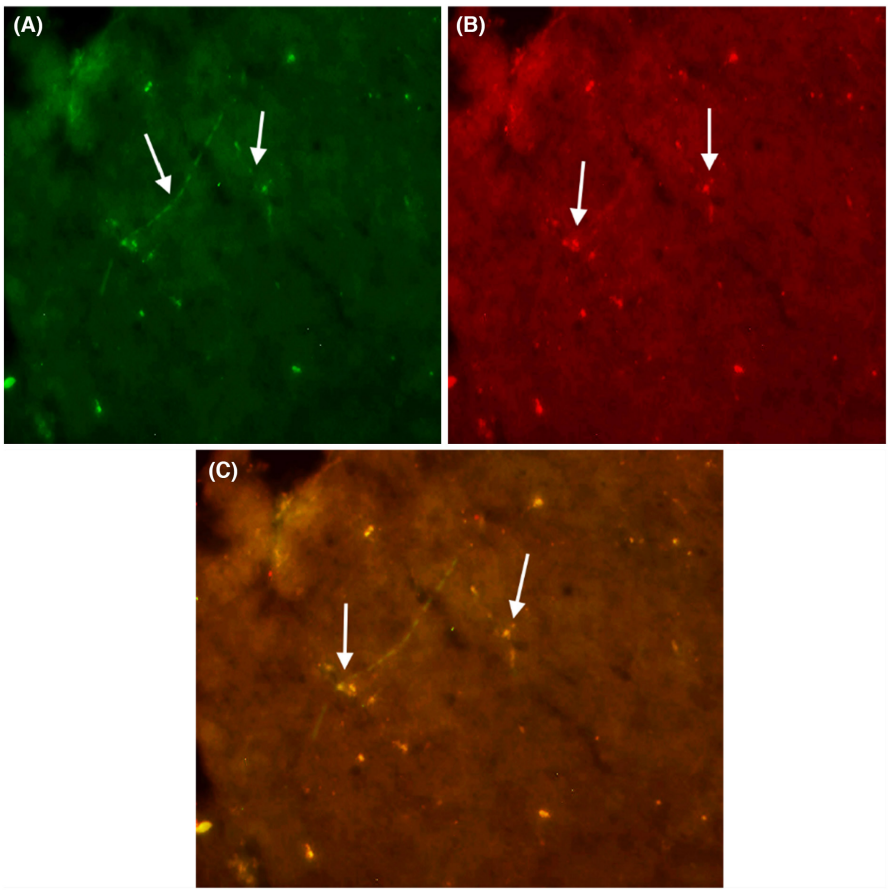

FIGURE 5 Fluorescent photomicrograph showing close contacts of RFRP-3 IR fibers onto kisspeptin immunoreactive cell bodies in the hypothalamic section (arcuate area) of male monkey. (A) Shows RFRP-3 fiber, (B) shows kisspeptin immunoreactive cell bodies, (C) shows the overlap of RFRP-3 IR fibers on kisspeptin cell bodies

co-expression of kisspeptin and RFRP-3 in the same neuronal population of the hypothalamus in ovariectomized rats suggesting a direct interaction of RFRP-3 and kisspeptin protein.[33] Thus, it can be assumed that RFRP-3 decrease kisspeptin drives from the same cells to GnRH neuron in autocrine manner during non-breeding period. Likewise, such RFRP-3 basal inhibition of kisspeptin neurons is reduced, leading to a stimulatory kisspeptin influence on GnRH neurons in the breeding season.

Furthermore, previous research showed that the hypothalamic ARC cell model (mHypoA-55) expressed kisspeptin, neurotensin (NT), corticotrophins-releasing hormone (CRH), and RFRP-3.[34] NT, a neuropeptide, notably express in AVPV and ARC area of mammals and has been shown to involved in feeding and reproductive functions. Similarly, CRH is largely synthesized in paraventricular nucleus and involved as a key processor for stress responses. Findings of this study showed that under the influence of estradiol along with RFRP-3 expression, both NT and CRH had inhibitory effects on Kiss-1 gene expression in the ARC kisspeptin neurons.[34] Thus we can assume that during non-breeding season greater RFRP-3 expression might enhance the NT and CRH expression both of which may inhibit kiss-1 gene function reducing kisspeptin to GnRH, resulting in reproductive axis suppression.

In our study, RFRP-3 IR fibers toward kisspeptin cell bodies were also seen. However, this interaction showed no significant difference in both seasons. We may speculate that RFRP-3 and kisspeptin neuronal contacts might be equal in both seasons; however, RFRP-3 expression is increased during the non-breeding season enhancing the effect of RFRP-3, which, in turn, halts the kisspeptin expression supported by more immunoreactive cell bodies co-localization. In this regard, previous studies supported our findings that RFRP-3 fibers appose to kisspeptin in ARC.[35,36] Similarly, intra-cerebroventricular injection of RFRP-3, significantly reduced kisspeptin mRNA level in the female rat and mice hypothalamus.[26,27] It has also been shown that a plausible proportion of kisspeptin neurons in the anteroventral-periventricular-nucleus and the ARC area express GPR147, but only the ARC kisspeptin neurons receive RFRP-3 fiber input, suggesting the kisspeptin neuronal population is modulated via direct interaction of RFRP-3 in the ARC.[25] The present study report evidence supporting the morphological interaction of RFRP-3 and kisspeptin in the monkey hypothalamus. However, the neuroanatomical relationship between the RFRP-3 and kisspeptin systems is still unclear. Therefore, further research is warranted to investigate this co-localization, using co-immunoprecipitation to substantiate the claim that both peptides are either produced in the same cells or in adjacent cells with respect to the breeding and non-breeding seasons.

5 | CONCLUSION

In conclusion, RFRP-3 expression prolongs during the non-breeding period possibly making direct contact with kisspeptin neurons in the

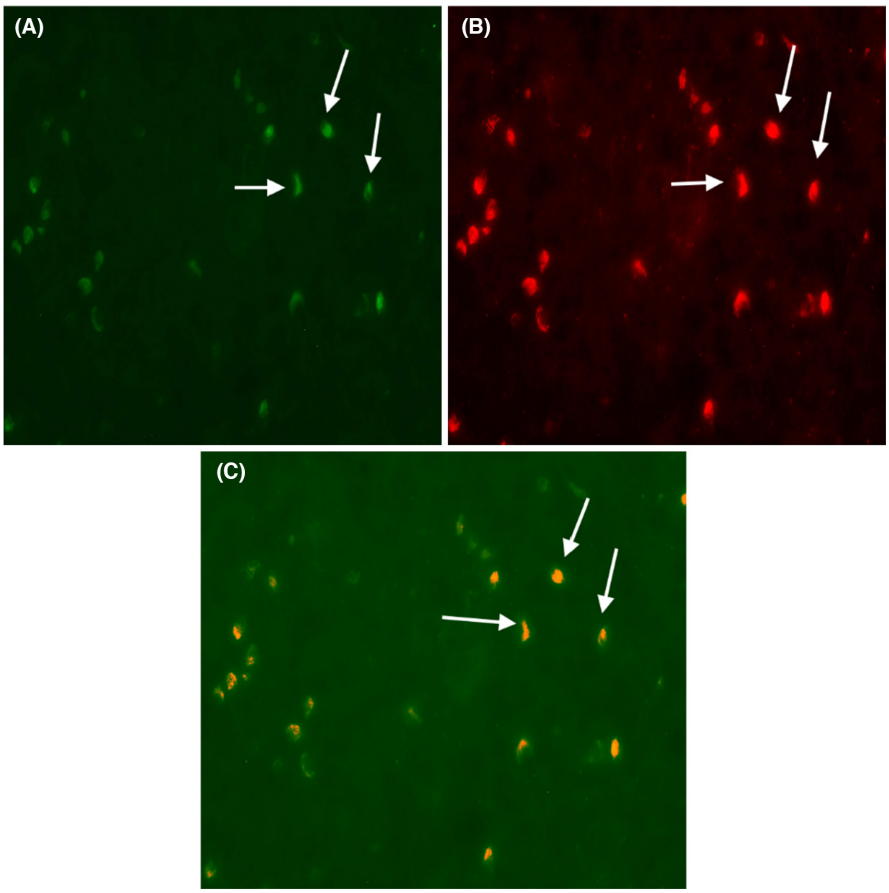

FIGURE 6 Fluorescent photomicrograph showing co-localization of RFRP-3 and kisspeptin immunoreactive cell bodies in the hypothalamic section (arcuate area) of male monkey. (A) Shows RFRP-3-positive cells, (B) indicates kisspeptin-positive cells, and (C) reveals merged RFRP-3 and kisspeptin cell bodies

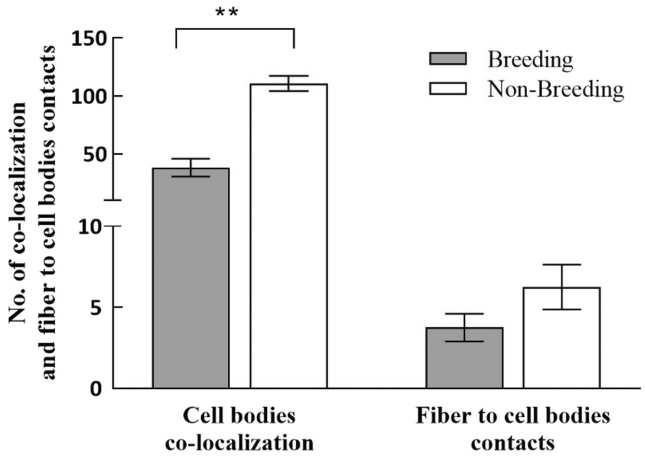

FIGURE 7 Comparison of mean ± SEM total number of close contacts of RFRP-3 fibers onto kisspeptin cell bodies and co-localizations of RFRP-3 and kisspeptin in three random section of hypothalamus (arcuate area) of male monkey during breeding and non-breeding season. No statistical difference was observed in number of close contacts across two seasons. Whereas, co-localization between RFRP-3 and kisspeptin IR cell bodies were significantly increased (**$p < 0.01$) in non-breeding season

ARC of higher primates. Similarly, intra-neuronal co-localization of RFRP-3 and kisspeptin suggests a direct intracellular link between RFRP-3 and kisspeptinergic system in the suppression of GnRH activity during the non-breeding season.

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CONFLICT OF INTEREST

Safdar khan, Bakhtawar Batool, Hira zubair, Riffat Bano, Shakil ahmad, and Muhammad Shahab declare that they have no conflict of interest.

ETHICAL STATEMENT

The experimental work was approved by the ethical committee of Zoology department at Quaid-i-azam University (Islamabad, Pakistan).

ANIMALS STUDIES

All institutional and national guidelines for the care and use of laboratory animals were followed.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients being included in the study.

CLINICAL TRIALS

The current study does not include any human clinical trials.

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