Int J Basic Sci Med. 2019;4(1):4-9 Original Article

http://ijbsm.zbmu.ac.ir/ doi 10.15171/ijbsm.2019.02

Central Substance P Attenuates RF-amid-related Peptide-3 Impacts on the Serum Level of Luteinizing Hormone in Wistar Rats

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Received May 21, 2018
Accepted June 1, 2019
Published online March 31, 2019

Abstract

Introduction: It is well-established that gonadotropin-inhibitory hormone (GnIH) and its mammalian orthologues (RFRP: RF-amid related peptides) can decrease gonadotrophin secretion. Moreover, substance P (SP) is another modulator of the secretion of gonadotropins in a species-dependent manner. This study aimed to find out the impacts of concomitant infusion of RFRP-3 and SP on luteinizing hormone (LH) concentration.

Methods: Forty-two rats were arbitrarily assigned to 7 groups (n=6 per group). Animals in the experimental groups were intracerebroventricularly injected with saline +DMSO, SP, RFRP-3, SP + RFRP-3, SP + RF9 (RFRP-3 receptor antagonist), SP + P234 (kisspeptin receptor antagonist) + RFRP-3 and SP + CP-96,345 (SP receptor antagonist) + RFRP-3 in a final volume of 3 µL. Blood samples were collected at 30-minute intervals after injections, and serum was used to measure the LH concentration by radioimmunoassay.

Results: According to the results, injections led to the elevation of LH serum concentration at 30-minute post injection ($P<0.05$) in the SP and SP+RF9 groups. Moreover, administration of either RFRP-3 or SP + RFRP-3 + SP receptor antagonist strikingly decreased the LH mean serum concentration at 30-minute after injections ($P<0.05$). On the contrary, the infusion of SP+RFRP-3 and SP+RFRP-3+P234 caused no dramatic changes in the LH mean serum concentration.

Conclusion: In general, the data showed that SP suppresses the impacts of RFRP-3 on the serum levels of LH.

Keywords: Substance P, RF-amid-related peptid-3, GnRH, Luteinizing hormone, Kisspeptin, Rat

Please cite this article as follows: Rahdar P, Khazali H. Central substance P Attenuates RF-amid-related peptide-3 impacts on the serum level of luteinizing hormone in Wister rats. Int J Basic Sci Med. 2019;4(1):4-9. doi:10.15171/ijbms.2019.02.

Introduction

Reproductive axis across the majority of vertebrates is controlled by the integration of environmental and internal signals. These inputs are processed and integrated at the hypothalamic level for releasing stimulatory or inhibitory neuropeptides into the hypophyseal-portal bloodstream.[1] Gonadotropin-releasing hormone (GnRH), the hypothalamic decapeptide, is the final integrator that regulates the secretion of pituitary gonadotropins. This neuropeptide, which is synthesized in the hypothalamus and is released in median eminence (ME), plays a serious role in promoting steroidogenesis and gametogenesis in both sexes. Most participating neuropeptides in the regulation of reproduction exert their effects via up-regulation or down-regulation of GnRH or inducing its upstream signaling pathways.[2]

Gonadotropin-inhibitory hormone (GnIH) is a hypothalamic dodecapeptide (SIKPSAYLPLRF amide) that was first identified in quail. GnIH is capable of regulating the secretion of gonadotropins at distinct levels from hypothalamus, pituitary gland, and gonads.[3] GnIH belongs to RF-amid peptide family, and fulfils its functions via 2 G-protein-coupled receptors: GPR 147 (with higher affinity for GnIH) and GPR74.[4,5] One GnIH and 2 GnIH-related peptides are processed from precursor mRNA of GnIH that contain

LPXRF-amid (X=L or Q) at their C terminus. The motif plays a critical role in the attachment of GnIH to specific receptors.[6]

Some studies conducted on the brains of mammals have reported that three orthologues of RFRPs are encoded: RFRP-1, -2 and -3. In rodents, two GnIH orthologues including RFRP-3 and RFRP-1 were detected,[4,7,8] from which RFRP-3, akin to avian GnIH, could reduce gonadotropin's secretion in mammals.

GnIH neural cell bodies are majorly concentrated in dorsomedial hypothalamic area (DMH) in mammals. The fibers of GnIH-secreting neurons have been reported to be found in different areas, some of which participate in the regulation of reproduction.[9] Altogether, the spread of GnIH immunoreactive fibers and their contact with GnRH neurons is almost similar among vertebrates.

In the brains of rodents, approximately one-third of GnRH-secreting neurons and lower percentage of kisspeptin-secreting neurons in rostral periventricular nucleus produce GnIH receptor mRNA. These reports revealed that the regulatory impacts of GnIH on GnRH-stimulated gonadotropin secretion can be directly or indirectly mediated by kisspeptin pathway.[10]

In addition, tachykinin peptides including substance P (SP), and neurokinin A and B exert leading roles in the control of reproduction at hypothalamic, hypophyseal, and gonadal levels, via binding to three G-protein-coupled receptors viz neurokinin-1 receptor (NK1R), neurokinin-2 receptor (NK2R), and neurokinin-3 receptor (NK3R). SP, the neurotransmitter and neuromodulator composed of 11 amino acid residues, is encoded by tachykinin precursor 1 (*TAC1*) gene[11], and is associated with the perception of pain and inflammatory processes in the brain by acting through NK1R.[12] SP has been reported to represent significant effects in the incidents that lead to the promotion of gonadotrophin secretion in studied subjects.[13] Accordingly, the results of a microscopic observation in mammals revealed that SP neurons provide axosomatic and axodendritic inputs to GnRH neurons.[14]

Compelling evidence suggests that SP and specific agonists of NK1R elevate the activity rate of kisspeptin-secreting neurons of ARC, which act as the main upstream stimulator of GnRH neurons, and consequently increase the secretion of GnRH-stimulated gonadotropin in rats.[15] in addition, SP-immunoreactive cells are present in various regions of hypothalamus including PVN, ARC, DMH, and amygdala, and with a lower number in ME.[16] In rats, hypothalamic neural systems that contain SP neurons are located in the ARC, and projection to ME and SP-immunoreactive ends provide contact with the capillaries of the hypophyseal portal system.[17]

However, it appears that the precise interaction of GnIH and SP, as the afferents responsible for regulating GnRH secretion and its critical upstream pulse generator, namely kisspeptin neurons, is currently unknown. This experimental study aimed to find out the impacts of the third ventricle interaction of SP and RFRP-3 on the luteinizing hormone (LH) serum levels among female rats.

Materials and Methods

Animals

Adult ovariectomized estrogen-primed female Wistar rats (220-250 g) were kept in the sterile cages in the animal center with controlled environmental conditions, as the temperature was 22±2°C, with 12:12 hour light–dark cycle, and the approximate humidity of 46%. Rats were given ad libitum access to food and water.

Stereotactic Surgery and Injections

All procedures of animal surgery and handling were performed as previously described.[18] Subjects were deeply anesthetized following intraperitoneal injection of a mixture of ketamine (80 mg/kg BW) and xylazine (10 mg/kg BW). Central injections were administered with one 22-gauge guide cannula, which was inserted into the third cerebral ventricle, based on the instructions of the atlas.[19] Using the stainless steel screws and dental cement, the guide cannula was fixed on the skull bone. After recovery, subjects were haphazardly assigned to 7 groups (n=6 per group). Subjects in the experimental groups were intracerebroventricularly injected with saline+DMSO (3 µL), SP (2.5 nmol), RFRP-3 (5 nmol), SP (2.5 nmol) + RFRP-3 (5 nmol), SP (2.5 nmol) + RF9 (RFRP-3 receptor antagonist, 20 nmol), SP (2.5 nmol) + P234 (kisspeptin receptor antagonist, 1 nmol) + RFRP-3 (5 nmol) or SP (2.5 nmol) + CP-96,345 (SP receptor antagonist, 5 nmol) + RFRP-3 (5 nmol).

All materials, SP (Ana Spec Co, USA), P234 (Phoenix Pharmaceutical Inc, USA), RFRP-3 (Tocris Co, USA), CP96-345 (Tocris Co, USA), and RF9 (Tocris Co, USA) were dissolved in 50% normal saline and 50% DMSO. Solutions were fresh and were prepared in a volume of 3 µL and administrated with a 27-gauge injector with the help of a Hamilton microliter syringe. The doses of drugs were selected based on the reports which determined their effective dosage in ICV injection.[20-23]

Radioimmunoassay for Measuring LH

A volume of 0.5 mL blood samples were collected at 0, 30, and 60 minutes following the injections via the tail vein.[24] After centrifugation for 15 minutes at 3000 rpm, the resultant serum was kept at –20°C for further assessment. Mean serum LH concentration was measured using rat LH [I125] RIA kit (Izotop, Hungary) according to the manufacturers' protocol, and the sensitivity of the kit was 0.09 ng/mL.

Statistical Analysis

The normalcy of the data was determined using the one-sample Kolmogorov-Smirnov test. Results were shown

as the mean ± SEM. The analysis of data was carried out utilizing two way-ANOVA followed by post hoc Tukey test. SPSS software (version 25.0) was used for data analysis and *P* value <0.05 was considered statistically significant.

Results

The statistical analysis in each group showed that SP promoted the mean serum concentration of LH at 30-minute time point post-injection compared to the first time point (0 minute, $P < 0.05$). While, injection of RFRP-3 resulted in the decrease of LH mean serum concentration at 30-minute time point post-injection compared to the 0-minute time point ($P > 0.05$, Figure 1).

Co-injections of SP + RFRP-3 had no dramatic impact on the LH mean serum concentration; while, that of SP + RF9 considerably promoted LH mean serum concentration at 30-minute time point post-injection compared to the baseline ($P < 0.05$, Figure 2).

Administration of SP + RFRP-3 + P234 led to the non-significant elevation of LH mean level at 30 minutes after injection, as well as a non-significant decrease at 60 minutes following the injection. On the contrary, by the infusion of SP + RFRP-3 or SP antagonist, the LH mean serum concentration showed a considerable decrement at 30 minutes after injection compared to the baseline ($P < 0.05$, Figure 3).

No significant difference was observed between the groups at baseline considering the LH mean serum concentration. At 30 minutes after injection, the LH mean serum concentration was significantly higher in the SP-injected subjects compared to the rats in the RFRP-3 and control groups and lower in the RFRP-3-injected group compared to either SP, SP + RF9, SP + RFRP-3 or control groups. The LH serum concentration was significantly higher in the SP + RF9 group compared to the RFRP-3, SP + RFRP-3 + P234, SP + RFRP-3 + SP antagonist, and control groups. Moreover, the decrease in LH concentration following the administration of SP + RFRP-3 + SP antagonist was significant compared to the groups administered with SP, and SP + RF9 ($P < 0.05$, Figure 4).

At 60 minutes after injection, our data indicated that the differences in the LH serum concentration following the injection of SP, RFRP-3, SP+RFRP-3, and SP+RFRP-3+P234 were not significant. Whereas, the increase in the mean serum concentration of LH in the SP + RF9 group was significant compared to the RFRP-3-administrated and control groups ($P < 0.05$, Figure 5).

Discussion

The effects of central interaction of SP and RFRP-3 on the serum level of LH were investigated in the present study. Based on the results, the central administration of SP led to significant elevation of the LH serum levels and the central infusion of RFRP-3 led to the decrease of LH serum levels. The results of our study are in line with the results of other experiments which demonstrated that the ICV injection of SP could increase the secretion of LH in female rats.[25] Both GnRH and kisspeptin neurons express RFRP-3 receptor and GPR147, and previous investigations have confirmed that the activity of kisspeptin neurons decrease following the ICV injection of RFRP-3; so RFRP-3 can inhibit GnRH signaling pathway either directly or indirectly by the involvement of kisspeptin.[10]

Furthermore, our results revealed that SP and RFRP-3 neutralize the effects of one another on the serum level of LH. This study was the first one to determine the

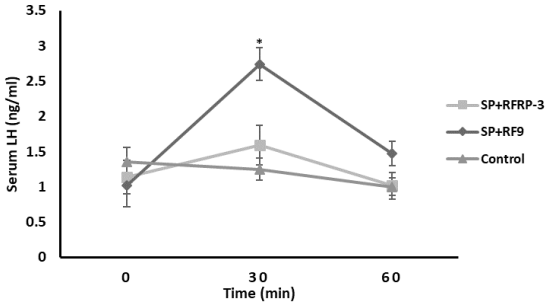

Figure 2. Impacts of ICV Infusion of Saline, SP+RFRP-3, and SP+RF9 at 0, 30, and 60 Minutes Following the Injection on the LH Mean Serum Concentration in Wistar Rats (n = 6 per group)
Note: Results are illustrated as mean ± SEM, *$P < 0.05$.

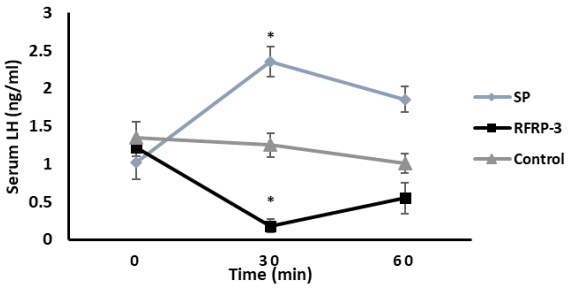

Figure 1. Impacts of the ICV Infusion of Saline, SP, and RFRP-3 at 0, 30, and 60 Minutes Following the Injection on the LH Mean Serum Concentration in Wistar Rats (n = 6 per group).
Note: Results are illustrated as mean ± SEM, *$P < 0.05$.

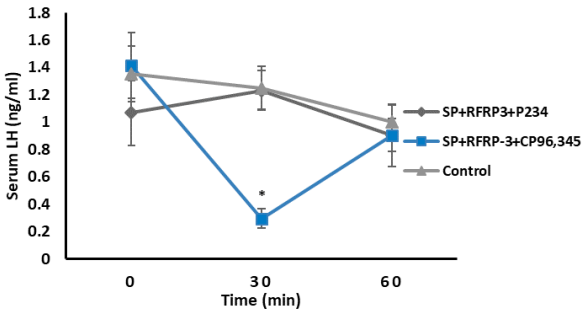

Figure 3. Impacts of ICV Infusion of Saline, SP+RFRP-3+P234, and SP+RFRP-3+CP96,345 at 0, 30, and 60 minutes Following the Injection on the LH Mean Serum Concentration in Wistar Rats (n = 6 per group).
Note: Results are illustrated as mean ± SEM, *$P < 0.05$.

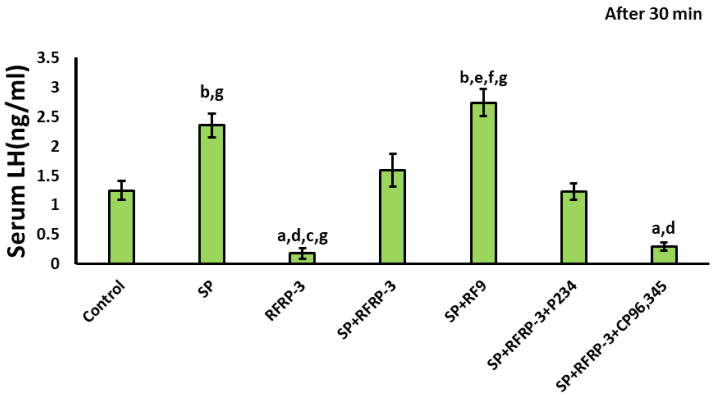

Figure 4. The Comparison of LH Serum Levels at 30 Minutes Following the ICV Injection of Saline, SP (2.5 nmol), RFRP-3 (5 nmol), RF9 (20 nmol), Co-infusion of SP+RFRP-3 and SP + RF9, SP+ RFRP-3+ P234 (1nmol) and SP+ RFRP-3+ CP96,345(5 nmol) in Wistar Rats.
Note: Lowercase letters indicate significant differences; a) Compared to SP group, b) Compared to RFRP-3 group, c) Compared to SP+RFRP-3 group, d) Compared to SP + RF9 group, e) Compared to SP+ RFRP-3+ P234 group, f) Compared to SP+ RFRP-3+ CP96,345 group, and g) Compared to control group. Results are illustrated as mean ± SEM, $P<0.05$, n=6 per group.

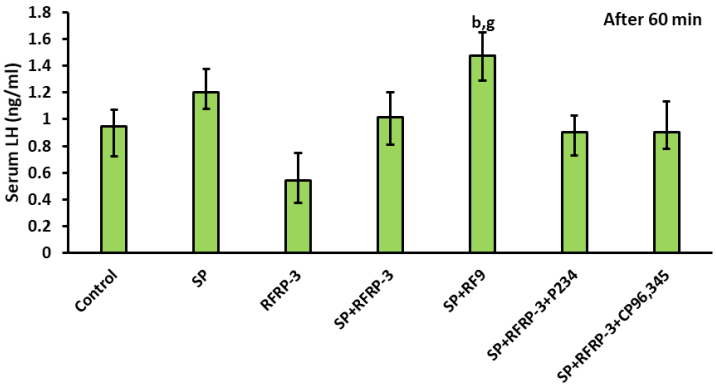

Figure 5. The Comparison of LH Serum Levels at 60 Minutes Following the ICV Injection of Saline, SP (2.5 nmol), RFRP-3 (5 nmol), RF9 (20 nmol), Co-infusion of SP+RFRP-3 and SP + RF9, SP+ RFRP-3+ P234 (1nmol) and SP+ RFRP-3+ CP96,345 (5 nmol) in Wistar Rats
Note: Lowercase letters indicate significant differences; a) Compared to SP group, b) Compared to RFRP-3 group, c) Compared to SP+RFRP-3 group, d) Compared to SP + RF9 group, e) Compared to SP+ RFRP-3+ P234 group, f) Compared to SP+ RFRP-3+ CP96,345 group, and g) Compared to control group. Results are illustrated as mean ± SEM, $P<0.05$, n=6 per group.

interactions of SP and RFRP-3 as influencers of GnRH/kisspeptin system. It also indicated that co-injection of these materials culminated in the neutralization of their effects on the serum levels of LH.

RF9 is an antagonist of neuropeptide FF receptors, namely GPR147 and GPR74. It has been demonstrated that RF9 can also act as kisspeptin agonist and elevate GnRH secretion by the activation of kisspeptin/GPR54 pathway in addition to the suppression of RFRP-3 function.[26] As reported in the relevant literature, the injection of RF9 can increase gonadotropins secretion mediated either by binding to GPR 54 on GnRH neurons or by binding to GPR147 and GPR74 on kisspeptin/GnRH secreting neurons.

It has been proven that ICV injection of kisspeptin can promote the LH levels in vivo, and several studies have divulged that ICV injection of P234 (peptide 234) blocks the kisspeptin signaling pathway and its stimulatory effect on HPG axis.[27] Kisspeptin neurons are the main upstream regulator of GnRH neurons and accumulating data demonstrated that these neurons mediate the impacts of many influencers of GnRH neurons such as SP and RFRP-3.[28]

P234 is the selective antagonist of kisspeptin receptor, which opposes the effects of kisspeptin pathway in mice, ewes, and monkeys.[27] A previous investigation indicated that P234 administration resulted in a dramatic suppression of LH secretion via binding to G-protein-coupled receptor 54 (GPR54) on the target neurons.[29]

The results of our study indicated that co-infusion of SP+ RFRP-3+ P234 did not exert considerable impact on the LH concentration. In fact, in the case that P234 abolishes the binding of kisspeptin to its receptors, SP continues to exhibit its impact probably without the involvement of kisspeptin and successfully inhibits RFRP-3 impacts. Therefore, it seems there are other pathways

involved in the function of SP on GnRH neurons and consequently LH. Our findings agreed with the investigations that unveiled SP neurons send projections to GnRH neurons.[14]

CP96,345 was introduced as the potent antagonist which binds to NK1R. In this context, previous studies showed that the effects of SP were significantly suppressed in the presence of CP96,345. In this manner, our results demonstrated that by the ICV administration of CP96,345, the suppressive effects of SP on the function of RFRP-3 diminished and RFPR-3 could reduce the LH level. Nonetheless, the effect of RFRP-3 on HPG axis was not significantly reinforced in co-administration with SP receptor antagonist (CP96,345). In this line, some studies suggested that the ICV administration of NK1R antagonist failed to alter the serum concentration of LH.[15] Further investigations are propounded to determine the interactive effects of other neuropeptides and neuromodulators which are secreted by upstream neurons of GnRH neural populations on the hormonal aspects of the function of reproductive axis.

Conclusion

To sum up, in this study, the interactive effect of central infusion of SP and RFRP-3 and their antagonists on the serum level of LH in female rats was investigated. We showed that SP and RFRP-3 modulated the effect of each other on the serum concentration of LH. In addition to kisspeptin neurons, there are other pathways that participate in modulating the impact of SP on reproductive axis.

Ethical Approval

All steps of this study were taken in the Laboratory Animal Center, according to the protocols of the Institutional Animal Care and Usage Committee, Shahid Beheshti University, Tehran, Iran.

Conflicts of Interest

None.

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