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The actions of neuropeptide SF on the hypothalamic–pituitary–adrenal axis and behavior in rats

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ABSTRACT

Present experiments focused on measuring the effect of neuropeptide SF (NPSF) on the hypothalamus–pituitary–adrenal (HPA) axis and behavior. The peptide was administered in different doses (0.25, 0.5, 1, 2 μg) intracerebroventricularly to rats, and the behavior of which was then observed by telemetry and open-field test. Effect of NPSF on core temperature was also measured *via* telemetry. Plasma ACTH and corticosterone concentrations were measured to assess the influence of NPSF on the HPA activation. In addition, the changes in corticotrophin-releasing hormone (CRH) level in the hypothalamic paraventricular nucleus were continuously monitored by means of intracerebral microdialysis. Our results showed that NPSF augmented paraventricular CRH release and increased ACTH and corticosterone levels in the plasma. The release of corticosterone was successfully blocked by the pre-treatment of the CRH antagonist α-helical $CRH_{9–41}$. Spontaneous and exploratory locomotor activity was also stimulated according to the telemetric and open-field studies. However, NPSF only tended to alter stereotyped behavior in the open-field experiments. These results demonstrate that NPSF may play a physiologic role in the regulation of such circadian functions as the activity of motor centers and the HPA axis, through the release of CRH.

1. Introduction

RFamide neuropeptides play a diverse role in several processes in the nervous system [1], and especially their role in pain modulation [1] has been verified by several papers, since their discovery [2]. The most intensely investigated members of the family are neuropeptide FF (NPFF), prolactin-releasing peptide (PrRP), and the kisspeptins, which are produced by five different genes (*farp-1* to *5*) [1]. NPFF, NPAF and NPSFs (or PQRFamides) from different species share the N-terminal sequence homology (Pro-Gln-Arg-Phe: PQRF), but they differ considerably in structure containing 10, 18 and 8 amino acids, respectively (NPFF: FLFQPQRF-NH_2, NPAF: AGEGLNSQFWSLAAPQRF-NH_2, porcine NPSF: SLAAPQRF-NH_2, human NPSF: SQAFLFQPQRF) [3–5]. They derive from a common precursor through alternative processing [5,6].

In the present study we focused on the physiological activities of NPSF, which was first isolated from the rodent brain and spinal cord [7] but its human expression was already predicted by genetic studies [5]. NPSF, as other PQRFamides, is mainly expressed in the CNS especially in the dorsal horn of the spinal cord, the hypothalamus, the medulla and the limbic system [4,8]. PQRFamides bind to a separate family of G protein-coupled receptor [9] processed from the same receptor gene *rfr-3* [1].

A great deal of information is available on the physiologic and pathophysiologic function of PrRP, kisspeptins and NPFF. PrRP stimulates the release of prolactin [10], activates the hypothalamic–pituitary–adrenal (HPA) axis [11], and facilitates motivated behavioral paradigms such as grooming [12]. It also regulates autonomic processes, such as the pressor response [11], feeding [13] and thermoregulation [14]. The first biological action associated with kisspeptin was the suppression of metastasis in melanoma [15], but since then plethora of publications [16–20] has demonstrated the pivotal role of the kisspeptin system in the regulation of the reproductive axis. Further, recent data has supported that kisspeptin positive neurons may regulate other physiological functions of behavior, the HPA axis and thermoregulation [21,22]. The role of NPFF and NPAF has also been thoroughly investigated in pain modulation [23,24]. Moreover, other publications pointed out that they may play an equally important role in the regulation of endocrine, autonomic and behavioral processes. They inhibit feeding [25,26] and stimulate locomotion [27,28] and NPAF activates the HPA axis [28]. Since little information is available in the literature on the functions of NPSF, and one can observe apparent differences in the activity profile of RFamides, a systematic evaluation of the endocrine, autonomic and behavioral activities of NPSF appeared necessary. Only two aspects of its physiological functions have been clarified. NPSF, like NPFF, has marked antianalgesic properties [1,3,29,30] and it exerts anorectic, antidipsogenic and antilipolytic effects [4,31].

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Therefore, in the present experiments, after intracerebroventricular (icv.) administration of different doses of NPSF, we recorded behavioral and endocrine parameters of rats. Spontaneous locomotion (continuous monitoring with telemetry), exploratory and stereotyped behaviors (in an open-field system) were observed in the behavioral experiments. As regards the HPA response, the ACTH and corticosterone levels were measured in the trunk blood, while CRH release was measured with the help of microdialysis from the paraventricular nucleus (PVN). Since CRH is the central regulator of the HPA axis [32], and it appears to mediate the HPA activation elicited by other RFamide neuropeptides [11], the CRH receptor antagonist α-helical $CRH_{9–41}$ was used to antagonize the NPSF-evoked endocrine response. Core temperature was also recorded continuously by telemetry.

2. Materials and methods

2.1. Animals

The animals were kept and handled during the experiments in accordance with the instructions of the Ethical Committee for the Protection of Animals in Research of the University of Szeged. Male Wistar rats weighing 150–250 g upon arrival were used and they were between 2.5 and 3 months old at the time of testing. The rats were kept in their home cages at a constant room temperature on a standard illumination schedule, with 12-h light and 12-h dark periods (lights on from 6.00 a.m.). Commercial food and tap water were available *ad libitum*. The rats were allowed a minimum of 1 week to acclimatize before surgery. To minimize the effects of nonspecific stress, the rats were handled daily. Every experiment was carried out separately and one animal was used only for open-field test, hormonal assays or telemetry. Only were the animals in the telemetric studies treated more than once and treatment days were separated by recovery days. Therefore, 6 circadian registrations were gained from one animal since icv. cannulas usually remain permeable for 2 weeks before formation of glial scars.

2.2. Surgery

For icv. peptide administration, the rats were implanted with a stainless steel Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle under pentobarbital (Nembutal, Phylaxia-Sanofi, Budapest; 35 mg/kg, ip.) anesthesia. The stereotaxic coordinates were 0.2 mm posterior, 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface, according to the atlas of [53]. Cannulas were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of at least 5 days.

For implantation of an internal radio transmitter (E-Mitter: temperature-activity transponder), the rats were anesthetized with pentobarbital (35 mg/kg, ip.). The abdomen was opened by making a 2-cm midline incision along the linea alba. The E-Mitter was placed in the abdominal cavity along the sagittal plane in front of the caudal arteries and veins, but dorsal to the digestive organs. The abdominal opening was then closed with absorbable suture material, while the skin was closed with stainless steel suture material. After a recovery of 5 days the rats were implanted with the stainless steel Luer cannula for icv. administration.

After all experiments, the animals (apart from the hormone assays and superfusion, in which they were sacrificed by decapitation) were euthanized with pentobarbital, and decapitated. To verify the permeability of the cannulas, methylene blue was injected into each decapitated head and the brains were dissected. Only animals with correctly located cannulas were used for statistical evaluation.

2.3. Open-field experiments

All the open-field experiments were carried out between 8:00 a.m. and 10:00 a.m., and the behavioral parameters were recorded visually from approximately 1.5 m by trained assistants who were blind to the experimental groups. To establish a dose–response curve different doses of NPSF (human NPSF, 0.25, 0.5, 1, 2 μg, Bachem, Switzerland) dissolved in saline, or saline alone (control animals), in a volume of 2 μl, were injected icv. into conscious rats. Thirty minutes after NPSF administration, the rats were removed from their home cages and placed in the center of a square, wooden, white open-field box (60 × 60 cm) consisting of 36 squares (10 × 10 cm each). The standard source of illumination was a 60 W bulb situated at a distance of 80 cm. The open-field box was cleaned between sessions with 96% ethyl-alcohol. The horizontal locomotor activity was characterized by the total number of squares crossed during a 5-min test session (square crossing). Vertical locomotor activity was characterized by the number of rearings (standing on the hind legs, leaning on open-field walls included). Groomings (face washing, forepaw licking and head stroking), other stereotyped behavioral paradigms (hunching and stretched attending) and the number of defecations were also observed. Every episode of face washing, forepaw licking and head stroking was counted as a separate grooming session, independently from how long they actually lasted.

2.4. Telemetry

Different doses of NPSF (0.5, 1 or 2 μg) or saline alone (control animals), in a volume of 2 μl, were injected icv. into conscious rats, between 8:20 and 8:30 a.m. The spontaneous motor activity and core temperature were recorded continuously in the animals' home cages. The system uses the E-Mitter, an implanted radiotelemetry device (Mini Mitter, USA), to determine the temperature, motor activity data and ECG. The E-Mitter obtains power from a radiofrequency field produced by an energizer/receiver placed below the cage of the animals. The counts were recorded continuously and the output from the receivers was managed by VitalView, a Windows-based data acquisition system.

2.5. Determination of plasma corticosterone

All experiments were carried out between 8:00 a.m. and 10:00 a.m. The animals were either treated with increasing concentrations of NPSF or received combined treatment (first the antagonist and 30 min later the most effective dose of the peptide). To establish a dose–response curve different doses of NPSF (human NPSF, 0.25, 0.5, 1 μg, Bachem, Switzerland) dissolved in saline, or saline alone (control animals), in a volume of 2 μl, were injected icv. into conscious rats. The dose range was set up in accordance with the literature data [28,33]. In the antagonist study animals were subjected to combined treatment with α-helical $CRH_{9–41}$ (Bachem, Switzerland) and NPSF. The dose of $CRH_{9–41}$ has been tested in several previous studies [34,35] and *per se* did not affect the HPA axis. The dose of NPSF was that which had proved most effective while establishing the dose–response curve. All the peptides were injected icv., in a volume of 2 μl, while the control animals received saline alone. The antagonists were applied 30 min before the NPSF treatment, since previous studies had demonstrated that they were most effective at this time [54]. In both experimental settings 30 min after NPSF treatment, the rats were sacrificed by decapitation, and approximately 3 ml of blood was collected in heparinized tubes for corticosterone assay. The plasma corticosterone level was determined by fluorescence assay [55].

2.6. Determination of plasma ACTH

All experiments were carried out between 8:00 a.m. and 9:00 a.m. The most effective concentration of NPSF (0.5 μg), that was used in the corticosterone assay or saline alone (control animals), in a volume of 2 μl, was injected icv. into conscious rats. Ten minutes after the treatment with NPSF, the animals were sacrificed and 2 ml of blood was

collected in EDTA-containing tubes. The ACTH concentrations of the samples were determined by a solid-phase two-site sequential chemiluminescent immunometric assay (Immulite 2000, Diagnostics Products Corporation, Los Angeles, USA). The analytical sensitivity of the assay is 5 pg/ml, the intraassay precision is 6.8% and the interassay precision is 8.2%.

2.7. Determination of hypothalamic CRH release

To investigate the highest level of the HPA axis microdialysis was used according to our previous publications [36] with the following modifications. One week before the microdialysis experiments the animals received icv. cannulas aimed at the right lateral cerebral ventricle. The microdialysis probe was implanted into the left paraventricular nucleus (PVN); stereotaxic coordinates: 1.5 mm caudal to the bregma, 0.5 mm lateral to the midline, and 7.5 mm ventral to the surface of the brain. After the 2-h equilibrium period, the most effective concentration of NPSF (0.5 μg), that was used in the corticosterone assay or saline alone (control animals), in a volume of 2 μl, was injected icv. Then perfusates were collected through the probes implanted into the PVN, in one fraction, at a flow rate of 4 μl/min, for 30 min, yielding samples of 120 μl. For the determination of the CRH content 50 μl samples were used in duplicates with a mouse/rat CRH-high sensitivity ELISA kit (CosmoBio Company, Ltd., Japan). This kit is based on a sandwich ELISA and practically shows no cross-reactivity to urocortins or other related peptides. The recommended detection range of the assay is 0.078–2.5 ng. The intraassay CV in rat plasma is 3.47–10.53% and the interassay CV is 2.01–5.19%.

2.8. Statistical analysis

Values are presented as means ± S.E.M. Statistical analysis of the results was performed by different forms of general linear model (GLM) or *t*-test for independent samples. While for the telemetric observations repeated measure GLM was applied, for endocrine expreiments one-way GLM. The differences between groups were examined by Tukey's *post hoc* comparison test, and a probability level of 0.05 or less was accepted as indicating a statistically significant difference. Data reductions and statistical analyses were performed by SigmaPlot 12.0 (Systat Software, Inc., Chicago, IL) and IBM SPSS Statistics 20.0 (IBM Corporation, Armonk, NY).

3. Results

3.1. Effects of NPSF on open-field behavior

NPSF activated both horizontal (GLM, $F_{4,51} = 2.39$, $p < 0.05$; Fisher's *post hoc* test: $p < 0.05$ for 0.5 μg NPSF *vs.* control; Fig. 1) and vertical locomotion ($F_{4,51} = 3.75$, $p < 0.05$; Tukey's *post hoc* test: $p < 0.05$ for 0.5 μg NPSF *vs.* control and $p < 0.05$ for 1 μg NPSF *vs.* control; Fig. 1) in a statistically significant manner. Although NPSF also showed a tendency to increase the number of groomings and the number of defecations these actions did not prove to be statistically significant (Fig. 1).

3.2. Effects of NPSF on spontaneous locomotion, and thermoregulation

Although NPSF activated spontaneous locomotion (Fig. 2a), it revealed only a tendency to increase the core temperature (Fig. 2b). Analysis of locomotor data between 8.00 a.m. and 12.00 a.m., revealed significant effect of NPSF treatment (repeated measure GLM, Between-Subject, $F_{3,39} = 17.99$; $p < 0.01$; Tukey's *post hoc* test: $p < 0.01$ for both 1 μg NPSF *vs.* the control and 2 μg NPSF *vs.* the control).

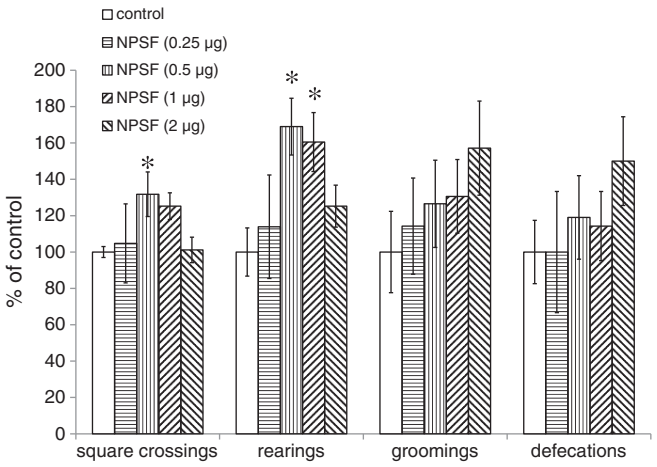

Fig. 1. Effects of NPSF on square crossing, rearing and grooming responses and the number of defecations. Data are expressed as means ± S.E.M. Numbers in brackets are the numbers of animals used. Abbreviation: NPAF = neuropeptide AF. Symbols: * = $p < 0.01$ *vs.* the control.

3.3. Effects of NPSF on HPA response

NPSF increased the adrenal corticosterone secretion (a more than twofold increase at 0.5 μg; Fig. 3) in a dose-dependent manner. Both the 0.5 and the 1 μg NPSF-treated groups proved statistically different from the control (GLM, $F_{3,46} = 6.69$; $p < 0.01$; Tukey's *post hoc* test: $p < 0.01$ *vs.* control in both cases). NPSF also increased ACTH level in the plasma ($t_{(9)} = -3.59$ and $p < 0.01$ *vs.* control) and enhanced CRH ($t_{(9)} = -2.51$ and $p < 0.05$ *vs.* control) release from the PVN at 0.5 μg (Fig. 4). The CRH receptor antagonist α-helical $CRH_{9–41}$ pretreatment inhibited the NPSF-evoked corticosterone release to a statistically significant extent ($F_{3,40} = 8.41$, $p < 0.05$; Tukey's *post hoc* test: $p < 0.05$ for $CRH_{9–41}$ + NPSF *vs.* NPSF; Fig. 5).

4. Discussion

In the open-field studies, NPSF stimulated exploratory behavior (horizontal and vertical locomotion) in a dose-dependent manner (Fig. 1). This PQRFamide also showed a tendency to increase the number of grooming and defecations. Therefore, NPSF, like NPAF [28] and PrRP [12] appears to activate exploratory behavior, while NPFF seems to inhibit it [27]. These differences should be attributed to the differences in the activity and distribution of their preferred receptors [1,9,37]. In the telemetric experiments NPSF also stimulated spontaneous locomotion in a dose-dependent manner, which confirms our findings with NPAF (Fig. 2) [28].

In our experiments, NPSF activated the HPA axis dose-dependently and this effect was exerted, with certainty, at a central level (Figs. 3–5). The paraventricular site of action is suggested by the route of administration and confirmed by both indirect and direct lines of evidence. First, the pre-administration of the CRH antagonist almost completely abolished the NPSF evoked response (Fig. 5.), and second, NPSF also brought about significant CRH release from the PVN (Fig. 4). The endocrine results are in harmony with the action of PrRP and NPAF on the HPA system [11,28] and suggest that further studies are necessary to investigate the action of other PQRFamides, such as NPFF and NPVF on the HPA axis. According to the literature, NPFF receptors can activate the parvocellular neurons in the PVN through the disinhibition of converging GABAergic inputs [38]. Similar mechanism can explain the remarkable effect of NPSF on spontaneous, exploratory and stereotyped behaviors, which are also under the control of CRH release [39–41].

Nevertheless, our findings reflect that NPSF has a distinct activity profile, since, unlike NPAF [28], it does not seem to have impact on the thermoregulatory and cardiac centers. Neither did it show anxiogenic

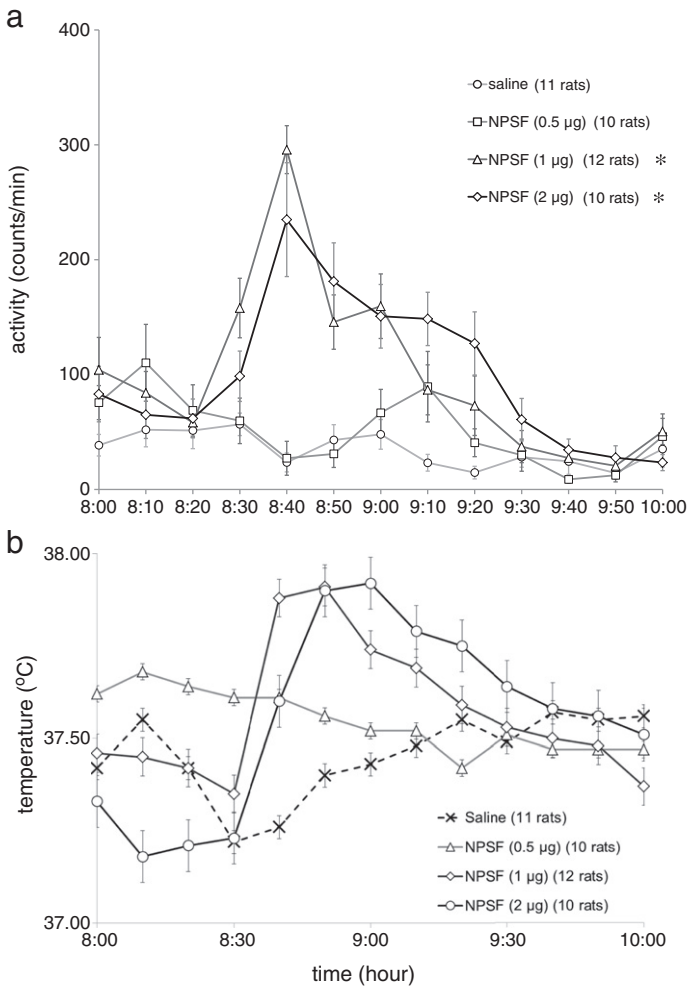

Fig. 2. a. Effect of NPAF on the spontaneous motor activity recorded by telemetry. Data are expressed as means ± S.E.M. Numbers in brackets are the numbers of circadian registrations. Abbreviation: NPSF = neuropeptide SF. Symbol: *p < 0.05 *vs.* control. b. Effect of NPSF on core temperature. Data are expressed as means ± S.E.M. Numbers in parentheses are the numbers of animals used. Abbreviation: NPSF = neuropeptide SF.

properties. These differences could be explained by the receptor affinity of the peptides. NPFF binds to NPFF-1 (GPR147), while NPAF to NPFF-2 (GPR74), while NPSF shows equal but weaker affinity to both NPFF-1 and NPFF-2. Further, the effects of NPSF can also be mediated by the activation of the acid sensing ion channels (ASICs) [1]. These differences are supported by literature data, too. NPAF and NPFF, unlike PrRP [42] elicit hyperthermia [28,43], but regarding the pressor response even NPFF and NPAF exert opposite actions [28,44], *via* the diverse arsenal

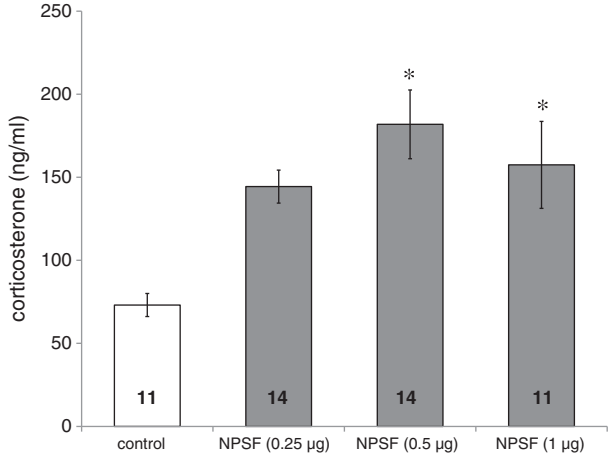

Fig. 3. Effect of NPSF on corticosterone release. Data are expressed as means ± S.E.M. Numbers within bars are the numbers of animals used. Symbols: *p < 0.05 *vs.* the control. Abbreviation: NPSF = neuropeptide SF.

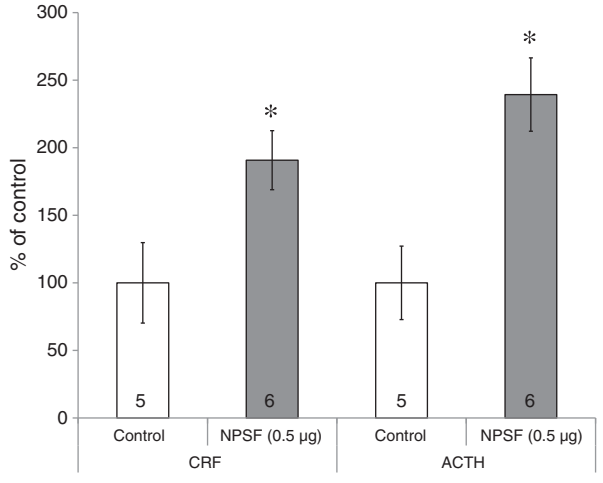

Fig. 4. Effects of NPSF on CRH and ACTH release. Data are expressed as means ± S.E.M. Numbers within bars are the numbers of animals used. Symbols: *p < 0.05 *vs.* the control. Abbreviation: NPSF = neuropeptide SF.

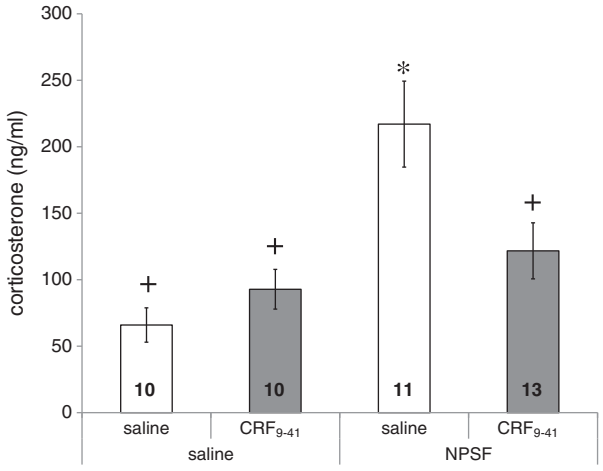

Fig. 5. Effect of α-helical $CRH_{9–41}$ pretreatment on NPAF evoked corticosterone release. Data are expressed as means ± S.E.M. Numbers within bars are the numbers of animals used. Symbols: *p < 0.05 *vs.* the control. Abbreviation: NPSF = neuropeptide SF.

of signal transduction processes. RFamides predominantly inhibit the cAMP–protein kinase A (PKA) system [4,45], but activation of Phospholipase C (PLC), mitogen activated protein kinase (MAPK), calcineurin and NFκB can also influence hormone secretion, chemotaxis and the organization of the cytoskeleton [37]. Further, the NPSF positive neurons in the hypothalamus, the medulla and the limbic system [1,8], interact with catecholaminergic [4] and neuropeptide Y positive (NPY) centers [46], in the regulation of endocrine, autonomic and behavioral processes [4,47] and, like neuromedin S [48], take part in the implementation of the suprachiasmatic control of these circadian processes [16,28,33,49,50]. They apparently harmonize the activity of the HPA axis, behavior and food intake [4,31]. Indirect mechanism, like the release of endogenous opiates or other neuropeptides can also mediate their effects in nociception, motor activities and thermoregulation [23,24,34,35,51,52].

In conclusion, our data suggest that centrally released NPSF, acting at the paraventricular nucleus [38] stimulates CRH secretion. This, in turn, leads to ACTH secretion and also modulates, although in different manner than other PQRFamides, the accompanying behavioral phenomena related to the stress response [39,40]. Further studies are necessary, however, to clarify the signal transduction processes of individual PQRFamides, separate their distinct activities and outline the physiological profile of NPFF and NPVF on the HPA axis and behavior.

Acknowledgments

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