Behavioral effects of 26RFamide and related peptides

Jean-Claude do Rego [a], Jérôme Leprince [b], Nicolas Chartrel [b],
Hubert Vaudry [b], Jean Costentin [a,*]

[a] CNRS FRE 2735, Laboratoire de Neuropsychopharmacologie Expérimentale, Institut Fédératif de Recherches Multidisciplinaires sur les Peptides (IFRMP 23), U.F.R. de Médecine et Pharmacie, Université de Rouen, 22 Boulevard Gambetta, 76183 Rouen, France
[b] INSERM U 413, Laboratoire de Neuroendocrinologie Cellulaire et Moléculaire, IFRMP 23, Université de Rouen, 76821 Mont-Saint-Aignan, France

ABSTRACT

A novel 26-amino acid peptide possessing the Arg–Phe–NH$_2$ motif at its C-terminal extremity has been recently characterized and named 26RFamide (26RFa). The 26RFa precursor encompasses several potential cleavage sites and thus may generate various mature peptides including an N-terminally extended form of 26RFa (termed 43RFa), two fragments of 26RFa (26RFa$_{1-16}$ and 26RFa$_{20-26}$), and a 9-amino acid peptide (9RFa) located in tandem in the human 26RFa precursor. In the present study, we have investigated the central effects of 26RFa and related peptides on food intake and locomotor activity in mice. We observed that i.c.v. injection of 26RFa, 43RFa, 26RFa$_{20-26}$ and 9RFa stimulated food consumption while 26RFa$_{1-16}$ and 26RFa$_{8-16}$ had no effect. A dose-dependent stimulation of locomotor activity was observed after i.c.v. administration of 26RFa, 43RFa and 26RFa$_{1-16}$, but not 26RFa$_{20-26}$, 26RFa$_{8-16}$ or 9RFa. These data indicate that the novel neuropeptides 26RFa and 43RFa act centrally to stimulate feeding and locomotor activities but the domains of the peptide involved in each of these responses are different suggesting that the two behavioral effects may be mediated through distinct receptors.

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1. Introduction

The term RFamide–related peptides (RFRPs) designates a family of biologically active peptides that possess the motif Arg–Phe–NH$_2$ at their C-terminal extremity [6]. The first member of this family, the tetrapeptide H–Phe–Met–Arg–Phe–NH$_2$, was originally isolated from the ganglia of the venus clam on the basis of its cardiovascular activity [21]. Since then, a number of RFRPs have been characterized in both invertebrates [7,16] and vertebrates [4,6], and shown to exert a large array of biological activities [7]. For instance, neuropeptide FF (NPFF), the first mammalian RFRP that has been characterized [30], attenuates morphine-induced analgesia [13,18,30], inhibits food intake [8,28], reduces locomotor activity [3,22] increases arterial blood pressure

[24], stimulates prolactin release [1] and inhibits aldosterone secretion [14].

A 26-amino acid peptide exhibiting the Arg–Phe–NH$_2$ signature at its C-terminus has been recently isolated from a frog brain extract, and termed 26RFa [5]. This peptide does not show any appreciable sequence similarity with the other RFRPs identified so far, indicating that 26RFa is a brand-new member of this family [6]. The cDNA encoding the 26RFa precursor has been characterized in rat [5,9,11], mouse [9,11], ox [9] and human [5,9,11], and it appears that the primary structure of 26RFa has been strongly preserved across vertebrates. The 26RFa precursor possesses several basic amino acids that may serve as cleavage-recognition signals for prohormone-convertases [27]. As shown in Fig. 1, processing at a single Arg located upstream of 26RFa may generate an
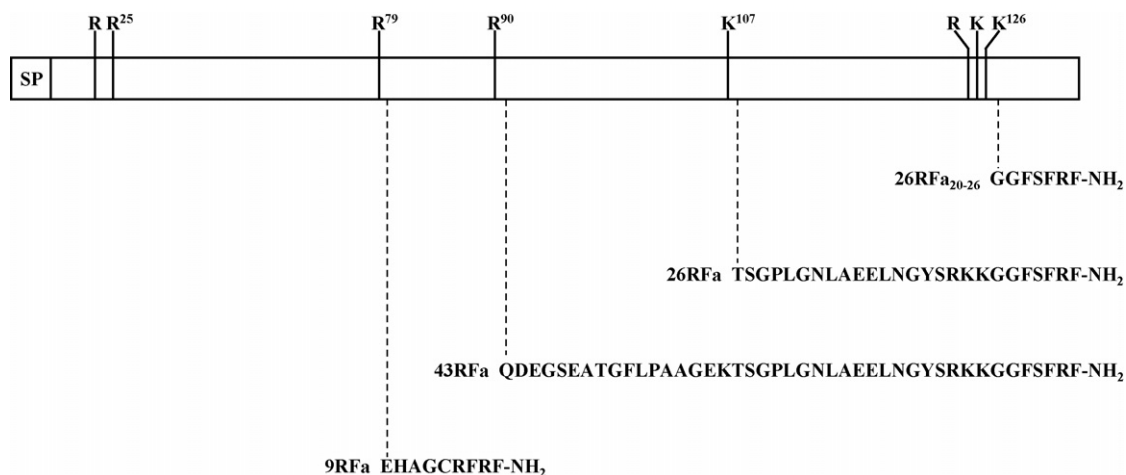
Fig. 1 – Schematic representation of the human 26RFa precursor. Potential mono/polybasic cleavage sites are indicated by vertical bars. The sequences of the four RFamide peptides that may be generated by processing at these cleavage sites are indicated. SP, signal peptide.

N-terminally extended form of 43 amino acids (43RFa). Indeed, it has been shown that 43RFa is produced in CHO cells transfected with the pre-pro26RFa cDNA [9]. The existence of a tribasic endoproteolytic signal within the 26RFa sequence (Fig. 1) suggests that the peptide may be cleaved by prohormone-convertases to produce two fragments, i.e. $26RFa_{1-16}$ and $26RFa_{20-26}$. In addition, the human precursor encompasses another RFamide peptide of 9-amino acids (termed 9RFa) that is delimited by single Arg residues at its N- and C-terminal extremities (Fig. 1). The three-dimensional structure of 26RFa consists of a well-defined central amphipathic helix flanked by two N- and C-terminal disordered regions [29].

26RFa and 43RFa act as endogenous ligands of the G protein-coupled receptor GPR103 [9,11]. The genes encoding the 26RFa precursor and GPR103 are both actively expressed in hypothalamic nuclei involved in the control of energy homeostasis [5], suggesting that 26RFa and/or 43RFa may regulate feeding behavior. In fact, intracerebroventricular (i.c.v.) administration of 26RFa in mice was found to stimulate food consumption in a dose-dependent manner [5].

Structure–activity relationship studies conducted on transfected cells have shown that both 26RFa and 43RFa are potent ligands of GPR103 while 9RFa has a very low affinity [9]. However, besides the orexigenic activity of 26RFa [5], nothing is known regarding the behavioral effects of pro26RFa-derived peptides. In the present study, we have investigated the effect of i.c.v. administration of 26RFa and various related peptides on food consumption and locomotor activity in mice.

2. Materials and methods

2.1. Animals

Male Swiss albinos CD1 mice (IFFA-CREDO/Charles River, Saint-Germain sur l'Arbresle, France), weighting 22–25 g were housed 20 in Makrolon cages (L: 40 cm, W: 25 cm, H: 18 cm), with free access to standard semi-synthetic laboratory diet and tap water, under controlled temperature (22 ± 1 °C) and lighting (light on from 7:00 a.m. to 7:00 p.m.). Each animal was used once. Animal manipulations were performed according to the European Community Council Directive of 24 November 1986 (86/609/EEC), and were approved by the local Ethical Committee (authorization numbers: N/10-04-04-12, N/12-04-04-14 and N/13-04-04-15).

2.2. Peptide synthesis

Human 43RFa, 26RFa, $26RFa_{1-16}$, $26RFa_{8-16}$, $26RFa_{20-26}$, and 9RFa, were synthesized (0.1-mmol scale) by the solid-phase methodology on a Rink amide 4-methylbenzhydrylamine resin (Biochem, Meudon, France) using a 433A peptide synthesizer (Applied Biosystems, Foster City, CA) and the standard Fmoc procedure as previously described [15]. The synthetic peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC) on a 2.2 cm × 25 cm Vydac 218TP1022 C_{18} column (Alltech, Templemars, France), using a linear gradient (10–50% over 50 min) of acetonitrile/TFA (99.9:0.1, v/v) in water, at a flow rate of 10 ml/min. Analytical RP-HPLC, performed on a Vydac 218TP54 C_{18} column (0.46 cm × 25 cm), showed that the purity of the peptides was greater than 99%. The molecular mass of each peptide was verified by mass spectrometry on a MALDI-TOF Voyager DE-PRO instrument (Applied Biosystems). Bovine neuropeptide FF (NPFF) was purchased from NeoMPS (Strasbourg, France). Peptides were dissolved in saline (0.9% NaCl), just before i.c.v. injection.

2.3. Intracerebroventricular injection

Intracerebroventricular (i.c.v.) injections (10 μl) were performed in the left ventricle of manually immobilized mice, within about 3 s, according to the procedure of Haley and McCormick [10], by using a Hamilton microsyringe (50 μl) connected to a needle (diameter 0.5 mm) equipped with a guard at 3.5 mm from the tip in order to limit its penetration into the brain. Peptides were injected at doses 10, 100 and

1000 ng/mouse corresponding, respectively, to 3.53, 35.3 and 353 pmol for 26RFa; 2.22, 22.2 and 222 pmol for 43RFa; 6.16, 61.6 and 616 pmol for 26RFa$_{1-16}$; 10, 100 and 1000 pmol for 26RFa$_{8-16}$; 12.25, 122.5 and 1225 pmol for 26RFa$_{20-26}$; and 8.92, 89.2 and 892 pmol for 9RFa. NPFF was administered at a dose of 1000 ng/mouse corresponding to 925 pmol/mouse.

2.4. Food intake

Mice were isolated in individual cages (24 cm × 10 cm × 7 cm), 2 days before the experiments, with free access to water and the pellets of food laid down on the floor of the cages, in order to make them accustomed to the test conditions. Eighteen hours before the experiments (3:00 p.m. to 9:00 a.m.), each mouse had access to tap water ad libitum, but only 3 g of food (that represented approximately half of their daily consumption). The day of testing, at 8:00 a.m., unconsumed food was removed and the test was performed at 9:00 a.m. After i.c.v. administration (10 min before testing) mice had access to a weighed food pellet (5 g) deposited on the floor of the cage. During the test, every 30-min, the pellet was briefly (<20 s) removed with forceps and weighed.

2.5. Locomotor activity

Locomotor activity was measured using a Digiscan actimeter (Omnitech Electronics, Colombus, OH) which monitored horizontal displacements and vertical movements, including rearing, leaning and jumping. Ten minutes after i.c.v. injection, the animals were placed individually in 20 cm × 20 cm × 30 cm compartments, in a dimly lit, sound-attenuated room. Locomotor activities, expressed as number of beams crossed by mice, were recorded during three consecutive 10-min periods.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by Student Newman–Keuls test for multiple comparisons. A probability level of 0.05 or less was considered as statistically significant.

3. Results

3.1. Effects of 26RFa and related peptides on food consumption

In food-restricted mice, i.c.v. injection of 1 μg of 26RFa, 43RFa, 26RFa$_{20-26}$ or 9RFa per mouse caused an increase in food intake which was statistically significant during the first and second period of testing. In contrast, at the same 1-μg dose, 26RFa$_{1-16}$, 26RFa$_{8-16}$ and NPFF had no effect on food intake (Fig. 2).

3.2. Effects of 26RFa and related peptides on locomotor activity

i.c.v. administration of graded doses (10, 100 or 1000 ng/mouse) of 26RFa, 43RFa or 26RFa$_{1-16}$ elicited a dose-dependent increase in both horizontal (Fig. 3) and vertical (Fig. 4) locomotor activities, during each of the three 10-min
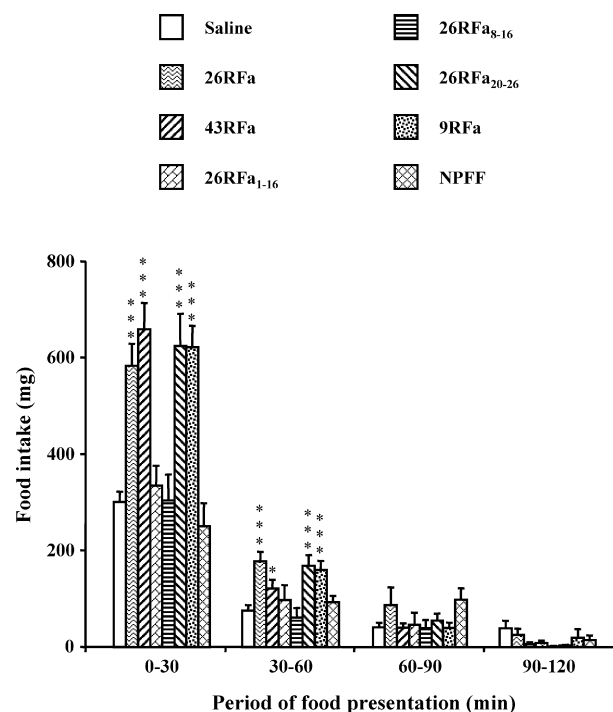

Fig. 2 – Effect of 26RFa and related peptides on food intake. Mice, partially food-deprived by presenting only about half of their daily consumption during the 18 h preceding testing, were injected i.c.v. either with saline, 26RFa or related peptides (1 μg/mouse). Ten minutes after injection, the animals were given a weighed food pellet and their food consumption was measured during the periods indicated. Mean ± S.E.M. of data from 12 mice per group. Student Newman–Keuls test: $^{*}p < 0.05$; $^{***}p < 0.001$.

consecutive periods of testing, i.e. during the total duration of the test. In contrast, i.c.v. injection of the same doses of 26RFa$_{8-16}$, 26RFa$_{20-26}$ or 9RFa had no effect on horizontal (Fig. 3) and vertical (Fig. 4) locomotor activities.

Pre-treatment of mice with naloxone (3 mg/kg, i.p.) did not modify the stimulatory effect of 26RFa (1000 ng/mouse, i.c.v.) on horizontal (Fig. 5(A)) and vertical (Fig. 5(B)) locomotor activities.

4. Discussion

In the rat brain, the gene encoding the novel neuropeptide 26RFa is almost exclusively expressed in the ventromedial hypothalamic nucleus and the lateral hypothalamic area [5], two hypothalamic nuclei that control appetite and energy homeostasis. Consistent with this observation, we have previously shown that 26RFa induces a dose-dependent stimulation of food intake in mice [5]. The human 26RFa precursor has the potential to generate several mature peptides including 26RFa, 43RFa, 26RFa$_{20-26}$ and 9RFa that all possess the Phe–Arg–Phe–NH$_2$ motif at their C-terminal extremity (Fig. 1). Here, we show that i.c.v. injection of any of these four peptides stimulated food intake in partially starved mice, whereas the N-terminal peptide 26RFa$_{1-16}$ and
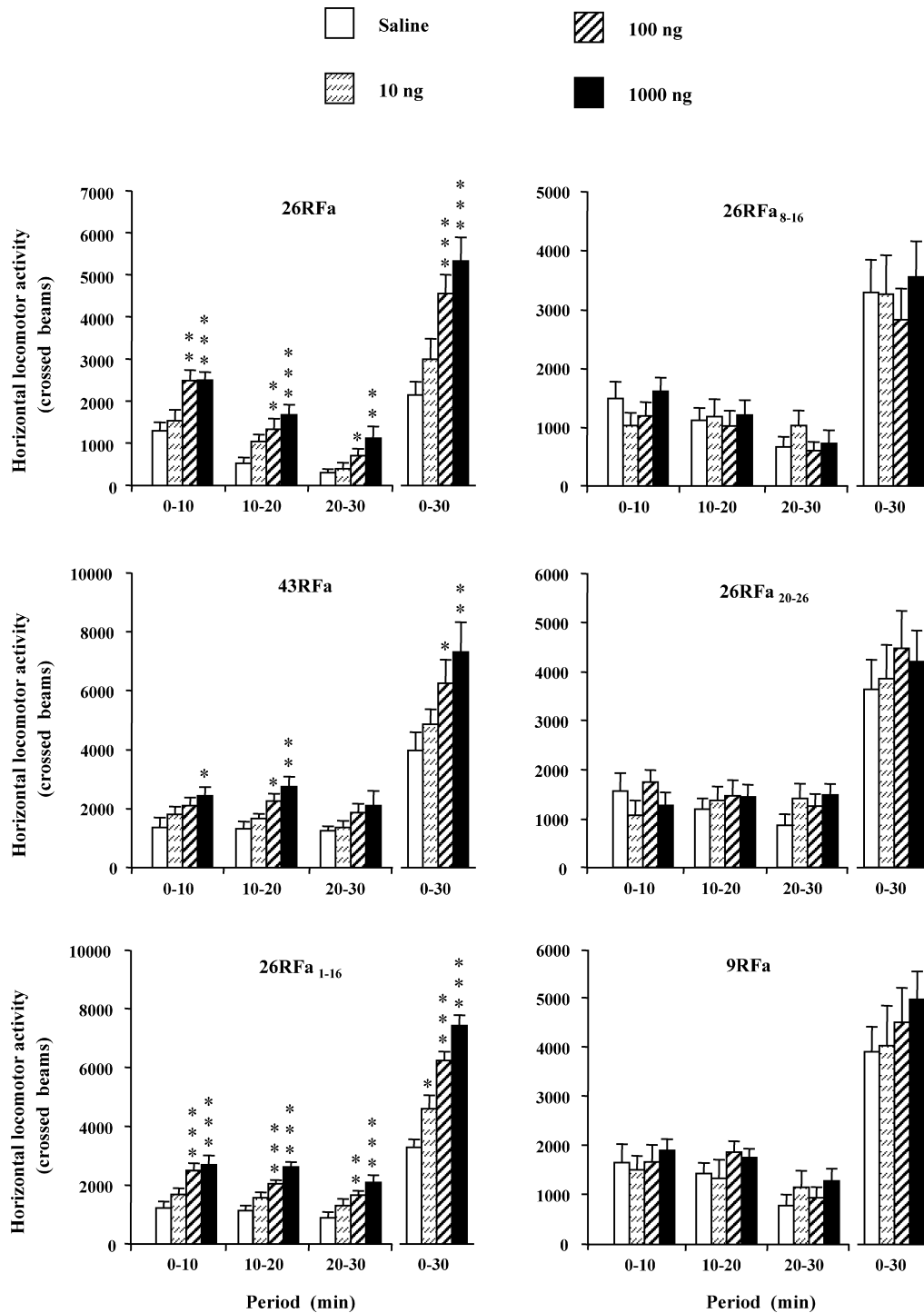
Fig. 3 – Effect of graded doses of 26RFa and related peptides on horizontal motor activity. Mice were injected i.c.v. with saline or graded doses of 26RFa or related peptides (10–1000 ng/mouse). The animals were placed into the actimeter 10 min after the injection. The horizontal motor activity was measured for three consecutive periods of 10 min. Means ± S.E.M. of data from 10 mice per group. Student Newman–Keuls test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the central domain of the peptide 26RFa$_{8-16}$ were totally inactive, supporting the notion that the orexigenic activity of 26RFa, 43RFa and 9RFa resides in their common C-terminal domain.

Since 26RFa belongs to the RFRP family, a large group of peptides that exhibit a C-terminal Arg–Phe–NH$_2$ sequence, we have investigated whether other peptides of this family would mimic the orexigenic action of 26RFa. We found that NPFF, whose C-terminal sequence is Gln–Arg–Phe–NH$_2$, does not affect food consumption, indicating that the Arg–Phe–NH$_2$ motif is not sufficient to determine the appetite-stimulating activity of 26RFa. Interestingly, the C-terminal heptapeptide of
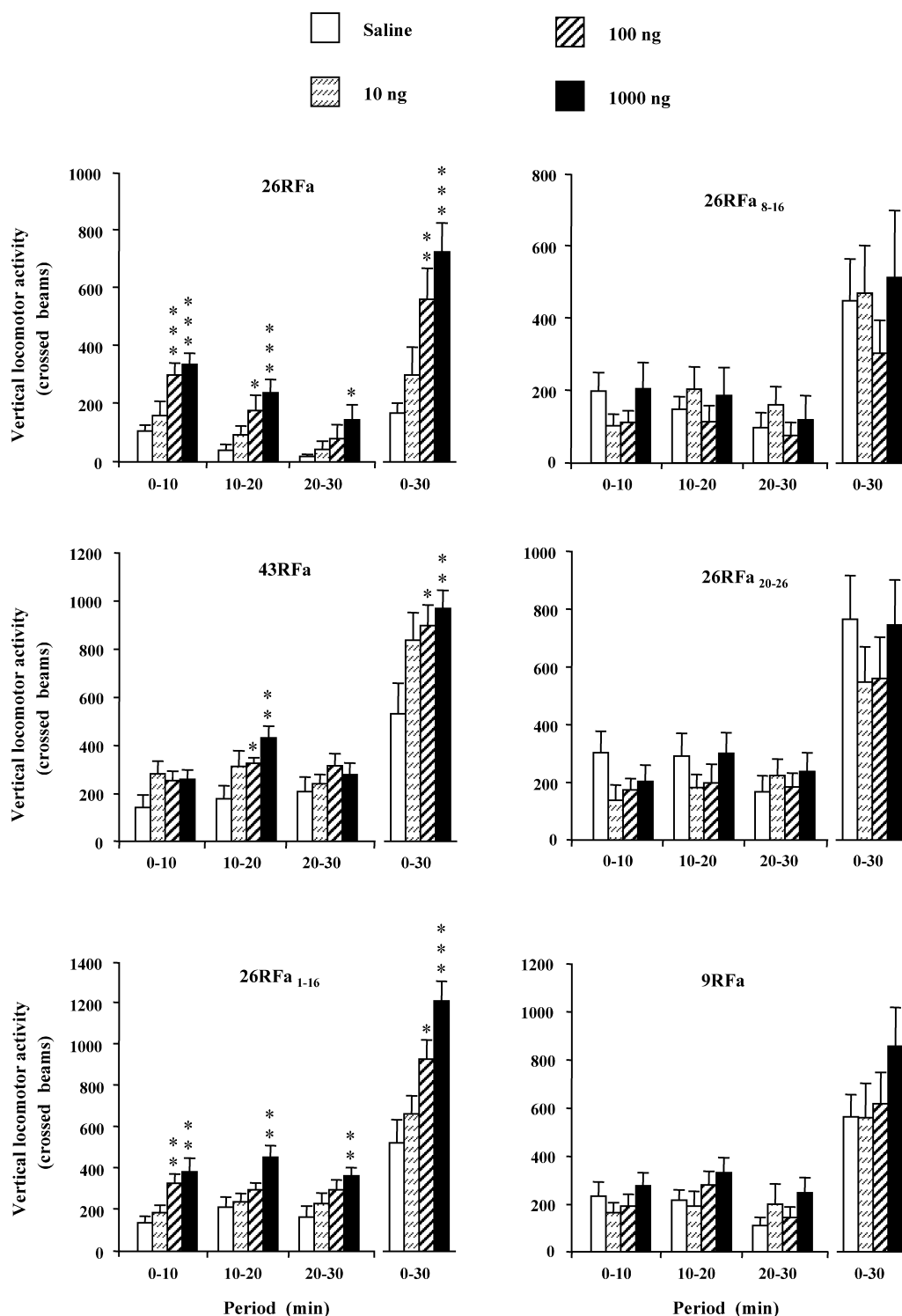
Fig. 4 – Effect of graded doses of 26RFa and related peptides on vertical motor activity. Mice were injected i.c.v. with saline or graded doses of 26RFa or related peptides (10–1000 ng/mouse). The animals were placed into the actimeter 10 min after the injection. The vertical motor activity was measured for three consecutive periods of 10 min. Means ± S.E.M. of data from 10 mice per group. Student Newman–Keuls test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

26RFa has been fully conserved from amphibians to mammals [5], indicating that a strong evolutionary pressure has acted to preserve the sequence of the orexigenic domain of the peptide. A recent structural study has shown that, in methanol solution, 26RFa adopts a well-defined conformation consisting of an amphipathic α-helical structure, flanked by two N- and C-terminal disordered regions [29]. The present data indicate that the C-terminal flexible domain plays a crucial role in the appetite-regulating activity of 26RFa, while the N-terminal domain and the central helical region are devoid of activity.
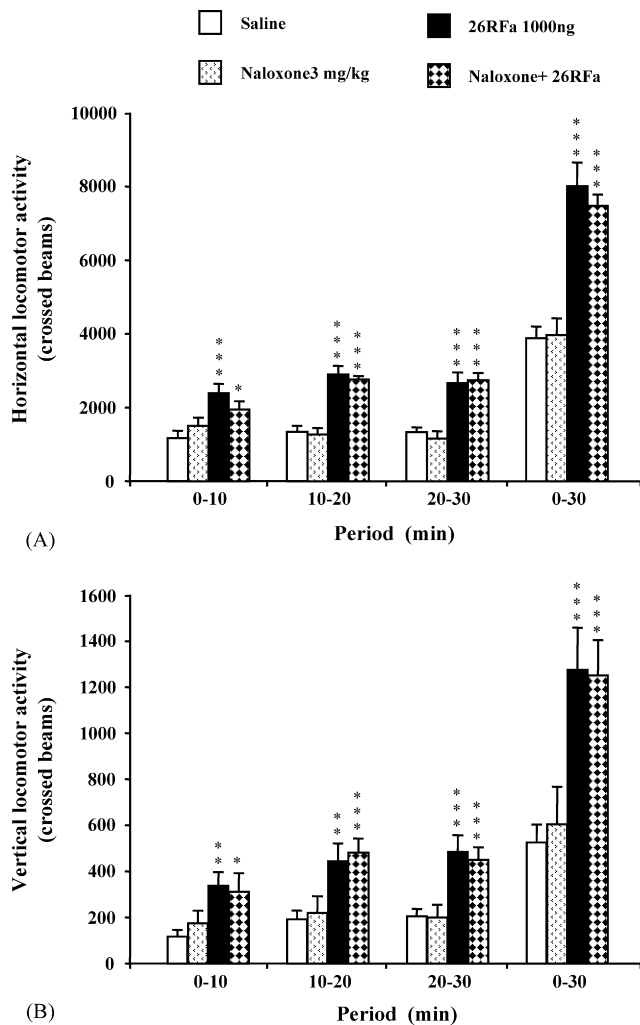
Fig. 5 – Effect of naloxone on 26RFa-induced stimulation in locomotor activity. Mice were pretreated with saline or naloxone (3 mg/kg, i.p.) 30 min before i.c.v. administration of either saline or 26RFa (1000 ng/mouse). The animals were placed into the actimeter 10 min after the i.c.v. injection. The horizontal (A) and vertical (B) components of motor activity were measured during three consecutive periods of 10 min. Means ± S.E.M. of data from 10 mice per group. Student Newman–Keuls test: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Structure–activity relationship studies have previously shown that the C-terminal region of 26RFa is also essential for eliciting calcium mobilization in GPR103-transfected cells [9,11]. These studies have shown that 43RFa and 26RFa are the most potent peptides to stimulate cAMP formation, and that $26RFa_{20–26}$, $26RFa_{19–26}$ and $26RFa_{18–26}$, although less potent than 26RFa, are still active on GPR103-expressing cells. These observations, together with the present report, strongly suggest that the orexigenic effect of 26RFa is mediated through activation of GPR103. Consistent with this notion, it has recently been reported that GPR103 mRNA is particularly abundant in the ventromedial nucleus of the hypothalamus [2]. The human 26RFa precursor encompasses a nonapeptide,

termed 9RFa, that possesses the same C-terminal FRF–NH_2 motif as 26RFa (Fig. 1). While 9RFa mimicked the stimulatory effect of 26RFa on food intake (this study), 9RFa is considerably less active than 26RFa on calcium mobilization and cAMP formation in GPR103-transfected cells [9,11], suggesting that the orexigenic effect of 9RFa may be mediated through a distinct receptor. Alternatively, although much less potent than 26RFa, at the relatively high dose injected (892 pmol/ mouse), 9RFa may induce maximal response via activation of GPR103. While this study was submitted, a report has shown that chronic administration of 43RFa in mice causes obesity by stimulating food intake and reducing energy expenditure [20]. In agreement with this report, the present data indicate that 26RFa and 43RFa may play an important role in the control of body weight and energy homeostasis.

In situ hybridization studies indicate that the GPR103 gene is widely expressed in the central nervous system, and binding experiments using ^{125}I-labeled 26RFa as a tracer have shown the existence of functional receptors in various regions of the rat brain (unpublished observations), suggesting that 26RFa, like other RFRPs, may exert multiple central activities. It has been previously reported that i.c.v. administration of FMRFa-mide [23] and microinjection of NPFF into the ventral tegmental area [3,17] inhibit morphine-induced locomotor hyperactivity in mice and rat. Similarly, systemic adminis-tration of FMRFamide [12] or [D-Tyr^1, (NMe)Phe^3]NPFF (an NPFF analog resistant to degradation) [22] decreases locomotor and cerebral activities in mice. These observations prompted us to investigate the effects of 26RFa on locomotion. Here, we show that i.c.v. injection of 26RFa dose-dependently stimulates horizontal and vertical locomotor activity in mice. The hyperlocomotor effect of 26RFa was mimicked by the N-terminally elongated form 43RFa and by the C-terminally truncated peptide $26RFa_{1–16}$, but not by $26RFa_{8–16}$ or $26RFa_{20–26}$, nor by 9RFa, indicating that the N-terminal domain of 26RFa is required for the regulation of locomotion. These data suggest that the stimulatory effects of 26RFa and 43RFa on horizontal and vertical locomotor activities, in contrast to the orexigenic effects of these peptides, are not mediated via an interaction with GPR103. Previous studies have shown that the N-terminal region of RFRPs is involved in receptor recognition and biological functions [9,19,26]. In particular, it has been proposed that the N-terminal domain of NPFF is required for the high affinity of the peptide for its receptor [19].

It is now firmly established that RFRPs exert their biological effects through modulation of opioid neurotransmission [25]. In particular, as indicated above, NPFF counteracts the effect of morphine on locomotor activity [3,17]. Since i.c.v. injection in immobilized mice induces stress, we looked for a possible involvement of endogenous opioid peptides in the hyperlo-comotor response to 26RFa. The data presented herein show that pre-treatment of mice with the opiate receptor antagonist naloxone did not affect the locomotor response to 26RFa. This observation confirms that the hyperlocomotor effect of 26RFa is mediated through mechanisms that are totally distinct from those of other RFRPs.

In conclusion, the present study has shown that central injection of the novel neuropeptides 26RFa and 43RFa stimu-lates feeding and locomotor activities in mice. The two types of responses elicited by 26RFa and 43RFa require different

domains of the peptides, suggesting that these two behavioral effects may be mediated through distinct receptors.

Acknowledgments

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