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Anti-analgesia of a selective $NPFF_2$ agonist depends on opioid activity

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Abstract

NPFF agonists designed to be selective $NPFF_2$ receptor probes were synthesized. D.Asn-Pro-(*N*-Me)Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 (dNPA) displays a very high affinity (0.027 nM) for $NPFF_2$ receptors transfected in CHO cells, and a very high selectivity with a discrimination ratio greater than 100 versus $NPFF_1$ receptors. dNPA acts as a potent and selective agonist in [^{35}S]GTPγS binding experiments and inhibits intracellular cAMP production with the same efficacy as NPA-NPFF. In SH-SY5Y cells expressing $NPFF_2$ receptors dNPA, in the presence of carbachol, stimulates Ca^{2+} release from the intracellular stores. In vivo, after intracerebroventricular injection dNPA increases body temperature in mice and reverses the morphine-induced analgesia. Also, dNPA displays anti-opioid activity after systemic administration. So far, dNPA exhibits the highest affinity and selectivity for $NPFF_2$ receptors and reveals that its behavioral anti-opioid activity depends on the degree of opioid-induced analgesia.

Keywords: NPFF receptor; Agonist; Morphine; Analgesia

NPFF (NPFF, FLFQPQRF-NH_2) may act as a modulator of endogenous opioid functions [1–3]. Two precursors, which could maturate peptides with a C-terminal PQRF-NH_2 sequence [4], have been cloned in mammals [5,6], a proNPFF$_A$ containing especially NPFF and a proNPFF$_B$ [7] generating another peptide with a LPLRF-NH_2 sequence. Several peptides processed from these precursors have been isolated in the rodent central nervous system [8,9]. Several data argue that the longest form of peptides corresponding to the classical processing rules of precursor maturation are likely to be the physiologically active neurotransmitters in rat brain [10].

The pharmacological effects of NPFF, which does not bind to opioid receptors [11], result from their interactions with two Gi/o-protein coupled receptors, $NPFF_1$ and $NPFF_2$ [12–14,7]. Mammalian neuropeptides derived from NPFF$_A$ precursors bind preferentially to $NPFF_2$ whereas peptides derived from NPFF$_B$ precursors have a higher affinity for $NPFF_1$ receptors [15]. In isolated neurones, $NPFF_1$ as well as $NPFF_2$ receptors participate in an anti-opioid activity by attenuating the magnitude of the inhibitory effect of opioid receptor agonists on the $[Ca^{2+}]_i$ transient induced by depolarization [16]. Similarly in a transfected cellular model, $NPFF_2$ receptor exerts a non-reciprocal antagonism on opioid receptors in two different paradigms [17].

This cellular activity has a physiological significance since several pharmacological data have evidenced that NPFF influences the actions of opioid peptides within the spinal cord and brain. In rodents, NPFF exhibits either anti-opioid activities or opioid-like effects when injected intracerebroventricularly or intrathecally, respectively [1]. After intracerebroventricular administration, NPFF reverses morphine antinociception in the tail-flick test, both in rat and mouse. Conversely, in rats, intrathecal injection of NPFF produces a strong

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and long-lasting antinociception and potentiates the morphine-induced analgesia [18]. These antinociceptive effects are related to an increased release of endogenous opioid peptides in the spinal cord and, in fact, NPFF acts in this model as a functional δ-opioid autoreceptor antagonist [19].

The question remains however among the different pharmacological activities induced by NPFF receptor agonists, whether each receptor type is particularly associated with one neuronal function since $NPFF_1$ and $NPFF_2$ receptors are distributed very differently in the central nervous system [20,21].

We have recently demonstrated that both $NPFF_1$ and $NPFF_2$ receptors participate in the anti-opioid actions observed at the cellular level [16], suggesting that the pro-opioid effects in functional pharmacology reflect a circuitry-induced control of opioid functions rather than a specific effect mediated by one NPFF receptor type. Furthermore, we have recently demonstrated that pro- and anti-opioid actions in mice are not strictly related to the selectivity towards $NPFF_2$ or $NPFF_1$ receptors [22]. It should be observed that at high doses, EFW-NPSF, a specific $NPFF_2$ ligand, loses its effect, suggesting the existence of a bell-shaped dose–response curve corresponding to two different effects of $NPFF_1$ and $NPFF_2$ receptors only observed for analogues with a high selectivity.

In order to investigate the exact activity mediated by $NPFF_2$ receptors towards opioid analgesia, we have synthesized and screened various analogues of NPFF for selectivity. We now describe the biochemical and pharmacological properties of these peptides which will be useful probes to investigate the precise role of $NPFF_2$ receptors.

Materials and methods

Chemicals. 1DMe ([D.Tyr^1,(*N*-Me)Phe^3]NPFF), Asn-Pro-Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 (NPA-NPFF), D.Asn-Pro-(*N*-Me)Ala-Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 (dNPA), Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH_2 (VPNLPQRF-NH_2), D.Val-Pro-(*N*-Me)Asn-Leu-Pro-Gln-Arg-Phe-NH_2 (D.VP(*N*-Me)NLPQRF-NH_2), D.Pro-Asn-(*N*-Me)Leu-Pro-Gln-Arg-Phe-NH_2 (D.PN(*N*-Me)LPQRF-NH_2), D.Glu-Tyr-(*N*-Me)Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2 (D.EY(*N*-Me)FSLAAPQRF-NH_2), Glu-Tyr-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2 (EYWSLAAPQRF-NH_2) were synthesized using an automated peptide synthesizer (Applied Biosystems model 433A) as previously described [23]. Morphine hydrochloride was obtained from Francopia (France).

Binding experiments. [^{125}I]YVP and [^{125}I]EYF were obtained by iodination of YVPNLPQRF-NH_2 (YVP) and EYWSLAAPQRF-NH_2 (EYF), respectively, as previously described [20].

Binding of [^{125}I]YVP and [^{125}I]EYF on membranes of CHO-K1 cells was measured by rapid filtration [15]. $NPFF_1$ receptors were labelled with 0.1 nM [^{125}I]YVP using 1–2 μg protein and 1 μM YVP for non-specific binding. $NPFF_2$ receptors were labelled with 0.05 nM [^{125}I]EYF using 0.9–1.5 μg protein and 1 μM EYF to label non-specific binding.

K_i values were calculated by non-linear regression analysis using GraphPad Prism software. K_D values of 0.14 nM for iodinated [^{125}I]YVP and 0.07 nM for iodinated [^{125}I]EYF have been used.

Intracellular cAMP assay. Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (4.5 g/L glucose, GlutaMAXI) containing 10% fetal calf serum and 50 μg/ml gentamicin (Invitrogen, France), in a humidified atmosphere containing 5% CO_2. cAMP was measured as previously described [17].

Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$). The $[Ca^{2+}]_i$ of SH-SY5Y neuroblastoma cells transfected with the $NPFF_2$ receptor was monitored with the fluorescent probe Fluo4 as already described [17]. The capacity of dNPA (1 μM) to raise $[Ca^{2+}]_i$ in the presence of carbachol was measured.

[^{35}S]GTPγS binding assay. Membranes of CHO cells, transfected and prepared as described [15], expressing $hNPFF_1$ (1.5–3 μg protein per assay) or $hNPFF_2$ (1.0–1.4 μg protein per assay) were incubated in 500 μl final volume containing Hepes 20 mM, pH 7.4, BSA 0.1%, $MgCl_2$ 3 mM, NaCl 150 mM, 5 μg saponin, [^{35}S]GTPγ 0.1 nM (1086 Ci/mmol, Amersham) 0.1 ($NPFF_1$) or 1 μM GDP ($NPFF_2$), and various concentrations of agonists at 30 °C for 60 min. The membranes were collected by rapid vacuum filtration through glass fiber filters, pre-incubated in the buffer at room temperature during 1 h, and washed three times with 4 ml ice-cold buffer. Radioactivity retained on the filters was determined in a scintillation Packard counter with 61% efficiency.

Experimental results were analyzed by non-linear regression curve fitting with Prismsoftware.

Animals. All experiments were performed with male Swiss mice (22–32 g) obtained from Depré (Saint-Doulchard, France). Mice were maintained at 21 ± 1 °C under 12/12 h light–dark cycle with food and water available ad libitum. Animals were tested according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US. National Institutes of Health. This study was approved by the Local Committee of Biomedical Ethics (Comité Régional d'Ethique Midi-Pyrénées, France).

Drugs injections. The procedure for intracerebroventricular (i.c.v.) injection was previously described [22]. Five microliters of the solution was injected into the third ventricle (thermoregulation) or the lateral ventricle (antinociception) over a period of 5 s. The stereotaxic coordinates for the injection into the third ventricle were 2 mm anterior to the interaural line, on the interhemispheric line, to a depth of 2.5 mm. For the injection into the lateral ventricle, the coordinates were 2 mm posterior to the bregma and 2.5 mm from the interhemispheric line, to a depth of 3 mm.

Intraperitoneal (i.p.) injection volumes were 20 μl/g of mouse.

Temperature measurement. Body temperature was measured with a thermistor probe (Ellab Instrument, Copenhagen) before injection and then at 10-min intervals for 60 min after injection for each animal restrained in a cylindrical plastic holder. The experiments were performed between 10:00 and 14:00 h. Statistical analysis was performed on the time course of changes in body temperature before injection (baseline) and every 10 min until 60 min after injection of NaCl or peptide for each animal. Data were analyzed by two-way analysis of variance (ANOVA), the factors being treatment (peptide versus NaCl) and time.

Nociceptive test. The nociceptive response was assessed by the tail-flick test. Mice were restrained in a cylindrical plastic holder and the activity of the drugs was measured by changes in the tail-flick latency time. The lamp intensity was adjusted to obtain a pre-drug latency time of 2–3 s. A cut-off time was set at 8 s to minimize tissue damage and was taken as criterion for complete analgesia. Data are expressed as the maximum percentage effect (MPE) calculated as:

$$\mathrm{MPE} = 100 * [(\text{post-drug response} - \text{baseline response})/(\text{cut-off response} - \text{baseline response})].$$

Statistical analysis was performed on the time course of MPE after injection of morphine or peptide during 120 min. Data were analyzed by two-way analysis of variance (ANOVA), the factors being treatment (peptide plus morphine versus morphine) and time. Post hoc comparisons were made with Bonferroni's test.

Results and discussion

Affinity and selectivity selection of $NPFF_1$ and $NPFF_2$ receptor agonists

The ability of several synthetic peptides to displace the specific binding of [^{125}I]YVP or [^{125}I]EYF on human $NPFF_1$ and $NPFF_2$ receptors, respectively, has been compared. The selectivity and affinity for $NPFF_1$ and $NPFF_2$ receptors of the different ligands are presented in Table 1. Considering the ratio between the apparent affinities for $NPFF_1$ receptors over $NPFF_2$ receptors, NPA-NPFF and EYWSLAAPQRF-NH_2 ($S_{1/2}$ about 75) exhibited a high selectivity towards $NPFF_2$ receptors, whereas NPFF analogues generated from the proNPFF$_B$ precursor and containing the LPQRF-NH_2 sequence were selective towards $NPFF_1$ receptors. The chemical modifications of the N-terminal part of these peptides, designed to resist aminopeptidase activities [24], induced structural constraints. The N-terminally modified peptide D.VP(*N*-Me)NLPQRF-NH_2 exhibited a sixfold loss of affinity for both $NPFF_1$ and $NPFF_2$ sites without modification of the selectivity index. In contrast, the shorter peptide, D.PN(*N*-Me)LPQRF-NH_2, was 10 times ($S_{1/2} = 0.2$) less selective. Similarly, the substitution of the first residue with the D-enantiomer and the methylation of the second peptidic bond of EYFSLAAPQRF-NH_2 decreased both the affinity and the selectivity for $NPFF_2$ receptors by 8.75 and 10 times, as compared to EYWSLAAPQRF-NH_2. The substitution of L-Asn^1 with D-Asn and the methylation of the second peptidic bond of NPA increased slightly the affinity of the analogue and enhanced the selectivity index from 77 to 107.

Taking into account the absolute affinity towards $NPPF_2$ receptors of these ligands, it appears that dNPA and NPA-NPFF exhibit the highest affinity and selectivity towards $NPFF_2$ among ligands so far available [15]; 10-fold larger affinity than EFW-NPSF and 2-fold greater selectivity than SPAFLFQPQRF-NH_2 [22].

Characterization of dNPA and NPA-NPFF with functional assays

Since the binding affinity might not reflect completely the biological potency of the ligands used, we have compared the selective compounds dNPA and NPA-NPFF in functional assays. In SH-SY5Y cells transfected with $hNPFF_2$ receptors, dNPA and NPA-NPFF inhibited 80% of the forskolin-induced accumulation of cAMP. The IC_{50} values for NPA-NPFF and dNPA were 0.43 ± 0.09 nM ($n = 3$) and 0.29 ± 0.15 nM ($n = 3$), respectively.

Both peptides were tested in the GTPγS binding assay with membranes from $NPFF_2$ receptor transfected CHO cells. In the presence of 1 μM ($hNPFF_2$) or 0.1 μM ($hNPFF_1$) GDP and 150 mM NaCl, dNPA and NPA-NPFF dose-dependently increased the binding of [^{35}S]GTPγS with similar EC_{50} values for each receptor (Table 2). However, the potencies of NPA-NPFF and dNPA on $NPFF_2$ receptors were, respectively, 73- and 50-fold greater than on $NPFF_1$ receptors (Table 2). Both peptides induced a maximal stimulation 210% and 970% of basal values for $NPFF_1$ and $NPFF_2$ receptors, respectively, indicating that these analogues exhibit the same relative agonist efficacy.

Thus, dNPA and NPA-NPFF display the same ability to inhibit adenylyl cyclase in transfected cells as well as the same efficacy and potency to stimulate selectively G-protein coupling to $NPFF_2$ receptors.

Table 1
Apparent affinities (K_i, nM) of NPFF analogues on human $NPFF_1$ and $NPFF_2$ receptors expressed in CHO cell membranes

	$NPFF_1$	$NPFF_2$	$S_{1/2}$
1DMe, D.YL(*N*-Me)FQPQRF-NH_2	1.09 ± 0.03	0.18 ± 0.04	6
$NPFF_1$ analogues			
D.PN(*N*-Me)LPQRF-NH_2	0.30 ± 0.04	1.5 ± 0.3	0.2
D.VP(*N*-Me)NLPQRF-NH_2	3.6 ± 0.6	142.0 ± 12	0.025
VPNLPQRF-NH_2	0.6 ± 0.1	23.0 ± 2	0.026
$NPFF_2$ analogues			
EFWSLAAPQRF-NH_2	20.8 ± 0.8	0.21 ± 0.01	99
EYWSLAAPQRF-NH_2	18 ± 3	0.24 ± 0.03	75
D.EY(*N*-Me)FSLAAPQRF-NH_2	16 ± 3	2.1 ± 0.3	7.6
NPAFLFQPQRF-NH_2	3.4 ± 0.2	0.044 ± 0.006	77
D.NP(*N*-Me)AFLFQPQRF-NH_2	2.9 ± 0.5	0.027 ± 0.001	107

Data represent mean K_i values in nM ± SEM of three to seven independent experiments, each performed in duplicate. $S_{1/2} = K_i$ $NPFF_1/K_i$ $NPFF_2$ for the selectivity of the ligand.

Table 2
Effects of dNPA and NPA-NPFF at the human $NPFF_1$ and $NPFF_2$ receptors, on the [^{35}S]GTPγS binding assay

	EC_{50} (nM)	Maximal effect (%)
NPA (NPAFLFQPQRF-NH_2)		
$NPFF_1$	897 ± 219	216 ± 8
$NPFF_2$	12.3 ± 1.4	997 ± 12
dNPA (D.NP(N-Me)AFLFQPQRF-NH_2)		
$NPFF_1$	645 ± 145	214 ± 24
$NPFF_2$	12.8 ± 2.1	968 ± 18

Agonist potencies and efficacies for the stimulation of [^{35}S]GTPγS binding were determined by agonist concentration–effect curves in CHO cell membranes. Values are means ± SEM from three to four experiments. Maximal effect is expressed as % of basal values.

Stability of dNPA and NPA-NPFF in brain tissue

The comparison of degradation kinetics of dNPA and NPA-NPFF (data not shown) by mouse brain sections investigated by capillary electrophoresis for the separation showed that the half time of disappearance of both peptides was similar (about 20 min), despite the protection against aminopeptidase degradation afforded by the D-amino acid substitution in dNPA. This effect could be due to endopeptidase activities in the tissue largely compensating for the protection against aminopeptidase activity obtained with N-terminal protection of dNPA as observed with SPA-FLFQPQRF-NH_2 the degradation of which is insensitive to aminopeptidase inhibition [25].

Interactions with muscarinic receptor signalling

In SH-SY5Y cells expressing NPFF receptors, NPFF agonists stimulate Ca^{2+} release from the intracellular stores only if muscarinic receptors are concomitantly activated by carbachol [17]. In SH-SY5Y cells transfected with $NPFF_2$ receptors, perfusion with 5 μM carbachol produced an immediate increase of intracellular Ca^{2+} concentration (Fig. 1). In the continuing presence of 5 μM carbachol, dNPA (1 μM, 40 s) also induced an increase in $[Ca^{2+}]_i$ (Fig. 1). The increase in fluorescence was the same ($p > 0.05$, unpaired t test) as with 1DMe (1 μM, 40 s) the classical NPFF agonist [15]: DF/$F_0 = 1.84 \pm 0.08$ ($n = 101$) and 2.15 ± 0.16 ($n = 84$), respectively.

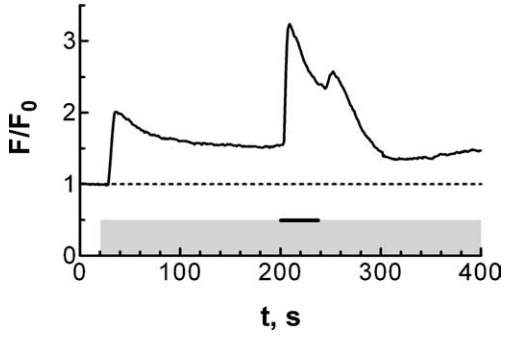

Fig. 1. dNPA increase $[Ca^{2+}]_i$ in the presence of carbachol. Mean (black line) F/F_0, calculated every second for the 53 cells present in the microscope field during application of 1 μM dNPA (horizontal line) in the continuing presence of 5 μM carbachol (gray area).

Pharmacological tests on dNPA used as referent $NPFF_2$ agonist

To further investigate the pharmacological potency associated to $NPFF_2$ receptors, dNPA, which possesses the highest selectivity and affinity, was used.

Effects of i.c.v. injection on mouse body temperature

As previously described [26,27] 1DMe (25 nmol) administered into the third ventricle induced a significant hypothermia compared to NaCl. A $NPFF_1$ receptor agonist (VPNLPQRF-NH_2) induced hypothermia while $NPPF_2$ agonists significantly increased the body temperature [22]. In this test, 5 nmol dNPA significantly increased body temperature as compared to NaCl (Fig. 2). The effect was maximal between 25 and 60 min after injection. A dose of 20 nmol dNPA did not significantly modify the body temperature as compared to NaCl (Fig. 2). Thus, dNPA injected into the third ventricle induces hyperthermia in contrast to the non-selective agonist 1DMe which decreases body temperature [26].

Other ligands, selective for $NPFF_2$ receptors as EFWSLAAPQRF-NH_2 which is as selective as dNPA, also induced an hyperthermic response at 10 nmol while its affinity for $NPFF_2$ is only 10-fold lower than dNPA, revealing the ability of $NPFF_2$ receptors to increase body temperature [22]. Accordingly, SPAFLFQPQRF-NH_2 which is only half selective than NPA (56 versus 107 for dNPA) produced two opposite effects which tend to cancel each other [22].

Effects on morphine-induced analgesia

In the mouse tail-flick test, after injection into the lateral ventricle, the effects of dNPA were analyzed by comparison to co-injected morphine responses (1.5, 2.5, and 5 nmol) corresponding to 50% or 65% of MPE (Fig. 3).

I.c.v. administration of dNPA (0.1–20 nmol) alone did not modify antinociceptive threshold. When mor-

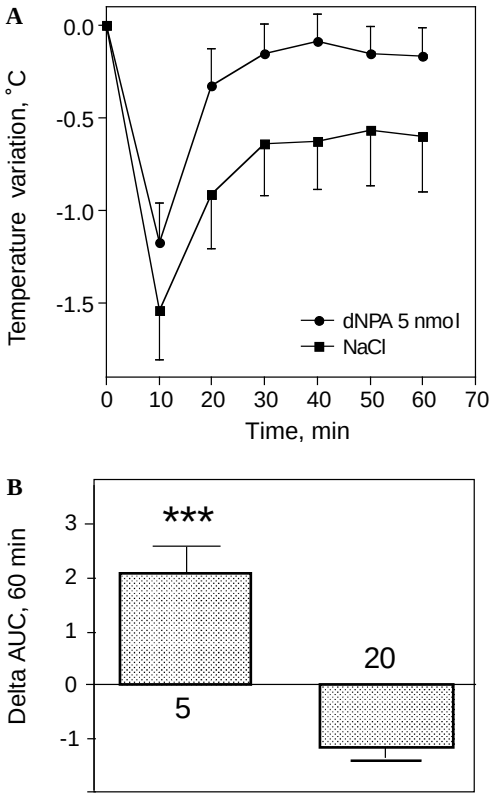

Fig. 2. Effects of dNPA injected in the third ventricle on changes in body temperature in mouse. (A) Time course of the change in body temperature induced by 5 nmol dNPA. (B) Data are expressed as differences in area under the curve (AUC) between peptide at the doses indicated (nmol) and NaCl (±SEM) during 60 min (***$P < 0.001$, significant differences from the action of NaCl with two-way ANOVA, followed by Bonferroni's post hoc test).

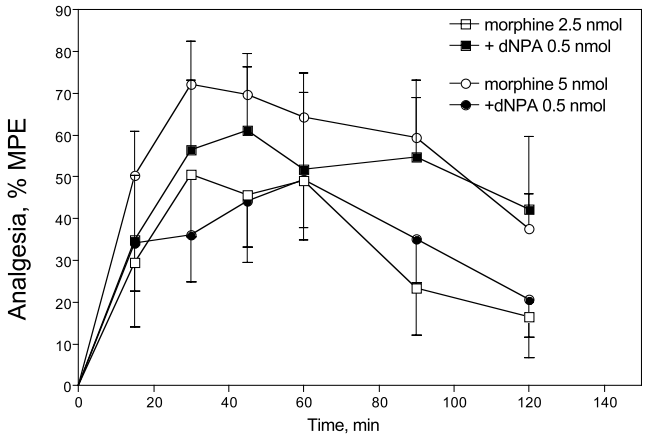

Fig. 3. Time course of the effects of intracerebroventricular injection dNPA on the antinociception induced by morphine in the tail-flick test. 0.5 nmol dNPA ($n = 10$) was co-injected with 2.5 or 5 nmol morphine ($n = 8$). Data are means ± SEM of maximum percentage effect (MPE). The corresponding statistical analysis is given in Fig. 4.

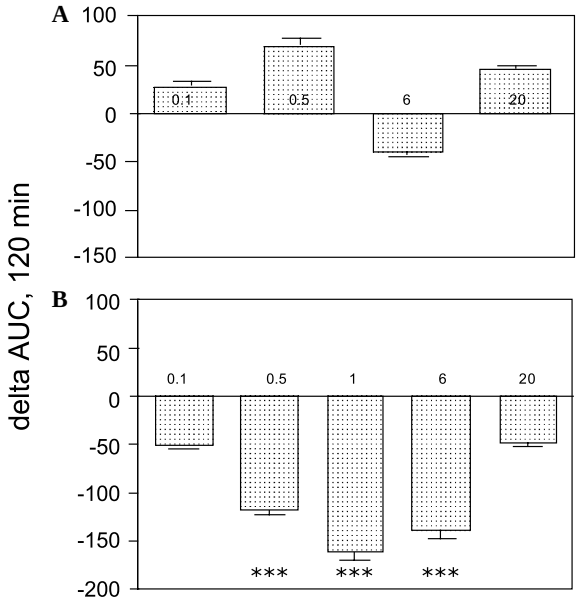

Fig. 4. Effects of different doses of dNPA (nmol) injected i.c.v. on the antinociception induced by morphine in the mouse tail flick. (A) morphine (1.5 and 2.5 nmol) induced a 50% analgesia at the peak effect. (B) Morphine (5 nmol) induced 65% analgesia. Data are means (±SEM) differences in area under the curve (AUC) during 120 min between morphine alone and co-injected with the peptide. ***$P < 0.001$ significant differences from the action of morphine alone with two-way ANOVA, followed by Bonferroni's post hoc test.

phine induced 50% analgesia, dNPA slightly, but not significantly, increased morphine analgesia (Fig. 4). In contrast, when morphine induced 65% analgesia at the peak effect, 0.5, 1, and 6 nmol dNPA significantly decreased morphine effects (Fig. 4). The effect of dNPA was maximal at 60 min. The doses 0.1 and 20 nmol were inactive in this test, revealing a bell-shaped dose–response curve for dNPA.

After an intra-peritoneal injection, dNPA 8 μmol/kg reversed the analgesia induced by a morphine dose of 13.3 μmol/kg (17.2% versus 34.6% analgesia for morphine at 60 min). dNPA exerted its effect in an homogeneous fashion from 15 to 120 min. A dose of dNPA 2 μmol/kg was inactive in this test.

At the cellular level, both $NPFF_2$ and $NPFF_1$ receptor activation produces an anti-opioid effect [16]. This seems coherent with the fact that after supraspinal administration, 1DMe, a poorly selective $NPFF_2$ agonist, exerts an anti-opioid effect [1]. However, our recent study [22] demonstrates that selective $NPFF_2$ agonists do not reproduce the anti-opioid effects of 1DMe raising the question of the role of $NPFF_1$ sites in this anti-analgesia. By using a highly selective ligand, we observed two important phenomena; the anti-opioid effect of dNPA depends on the degree of morphine analgesia; this highly selective $NPFF_2$ agonist is anti-opioid in a supraspinal test. This effect is reminiscent of that of EFWSLAAPQRF-NH_2 (affinity of 0.21 nM versus 0.027 nM for dNPA on $NPFF_2$ receptors) which reverses morphine actions only when morphine induces 75 % analgesia [22]. Such dependence of opioid actions is

already reported in rats since the hyperalgesic effect of NPFF was followed, during nighttime, by a clear analgesic effect [28]. It has been proposed that the analgesic rebound could result from the triggering of endogenous opiate systems which are more active during the night in rodents.

Conclusion

A NPFF receptors agonist, characterized in vitro as potent and selective $NPFF_2$ agonist with a very high affinity, exerts anti-opioid actions depending on the level of morphine-induced analgesia in mice.

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References

[1] M. Roumy, J.M. Zajac, Neuropeptide FF, pain and analgesia, Eur. J. Pharmacol. 345 (1998) 1–11.
[2] P. Panula, E. Kalso, M. Nieminen, V.K. Kontinen, A. Brandt, A. Pertovaara, Neuropeptide FF and modulation of pain, Brain Res. 848 (1999) 191–196.
[3] C. Mollereau, M. Roumy, J.M. Zajac, Opioid-modulating peptides: mechanisms of action, Curr. Top. Med. Chem. 5 (2005) 341–355.
[4] J.M. Zajac, Neuropeptide FF: new molecular insights, Trends Pharmacol. Sci. 22 (2001) 63.
[5] F.S. Vilim, E. Ziff, Cloning of the neuropeptide NPFF and NPAF precursor from bovine, rat, mouse and human, Abstract for the Society of Neuroscience USA, congress San Diego, November 1995 (1995).
[6] S.J. Perry, E.Y. Huang, D. Cronk, J. Bagust, R. Sharma, R.J. Walker, S. Wilson, J.F. Burke, A human gene encoding morphine modulating peptides related to NPFF and FMRFamide, FEBS Lett. 409 (1997) 426–430.
[7] S. Hinuma, Y. Shintani, S. Fukusumi, N. Iijima, Y. Matsumoto, M. Hosoya, R. Fujii, T. Watanabe, K. Kikuchi, Y. Terao, T. Yano, T. Yamamoto, Y. Kawamata, Y. Habata, M. Asada, C. Kitada, T. Kurokawa, H. Onda, O. Nishimura, M. Tanaka, Y. Ibata, M. Fujino, New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals, Nat. Cell Biol. 2 (2000) 703–708.
[8] E. Bonnard, O. Burlet-Schiltz, B. Frances, H. Mazarguil, B. Monsarrat, J.M. Zajac, A. Roussin, Identification of neuropeptide FF-related peptides in rodent spinal cords, Peptides 22 (2001) 1085–1092.
[9] E. Bonnard, O. Burlet-Schiltz, B. Monsarrat, J.-P. Girard, J.-M. Zajac, Identification of proNeuropeptide FFA peptides processed in neuronal and non-neuronal cells and in nervous tissue, Eur. J. Biochem. 270 (2003) 4187–4199.
[10] M. Roumy, C. Gouarderes, H. Mazarguil, J.M. Zajac, Are neuropeptides FF and SF neurotransmitters in the rat? Biochem. Biophys. Res. Commun. 275 (2000) 821–824.
[11] C. Gouarderes, J.A. Tafani, J.M. Zajac, Affinity of neuropeptide FF analogs to opioid receptors in the rat spinal cord, Peptides 19 (1998) 727–730.
[12] J.A. Bonini, K.A. Jones, N. Adham, C. Forray, R. Artymyshyn, M.M. Durkin, K.E. Smith, J.A. Tamm, L.W. Boteju, P.P. Lakhlani, R. Raddatz, W.J. Yao, K.L. Ogozalek, N. Boyle, E.V. Kouranova, Y. Quan, P.J. Vaysse, J.M. Wetzel, T.A. Branchek, C. Gerald, B. Borowsky, Identification and characterization of two G protein-coupled receptors for neuropeptide FF, J. Biol. Chem. 275 (2000) 39324–39331.
[13] N.A. Elshourbagy, R.S. Ames, L.R. Fitzgerald, J.J. Foley, J.K. Chambers, P.G. Szekeres, N.A. Evans, D.B. Schmidt, P.T. Buckley, G.M. Dytko, P.R. Murdock, G. Milligan, D.A. Groarke, K.B. Tan, U. Shabon, P. Nuthulaganti, D.Y. Wang, S. Wilson, D.J. Bergsma, H.M. Sarau, Receptor for the pain modulatory neuropeptides FF and AF is an orphan G protein-coupled receptor, J. Biol. Chem. 275 (2000) 25965–25971.
[14] M. Kotani, C. Mollereau, M. Detheux, E. Le Poul, S. Brezillon, J. Vakili, H. Mazarguil, G. Vassart, J.M. Zajac, M. Parmentier, Functional characterization of a human receptor for neuropeptide FF and related peptides, Br. J. Pharmacol. 133 (2001) 138–144.
[15] C. Mollereau, H. Mazarguil, D. Marcus, I. Quelven, M. Kotani, V. Lannoy, Y. Dumont, R. Quirion, M. Detheux, M. Parmentier, J.M. Zajac, Pharmacological characterization of human NPFF(1) and NPFF(2) receptors expressed in CHO cells by using NPY Y(1) receptor antagonists, Eur. J. Pharmacol. 451 (2002) 245–256.
[16] M. Roumy, M. Garnier, J.-M. Zajac, Neuropeptide FF receptors 1 and 2 exert an anti-opioid activity in acutely dissociated rat dorsal raphe and periventricular hypothalamic neurons, Neurosci. Lett. 348 (2003) 159–162.
[17] C. Mollereau, H. Mazarguil, J.M. Zajac, M. Roumy, Neuropeptide FF (NPFF) analogs functionally antagonize opioid activities in NPFF2 receptor-transfected SH-SY5Y neuroblastoma cells, Mol. Pharmacol. 67 (2005) 965–975.
[18] C. Gouardères, K. Jhamandas, M. Sutak, J.-M. Zajac, Role of opioid receptors in the spinal antinociceptive effects of neuropeptide FF analogues, Br. J. Pharmacol. 117 (1996) 493–501.
[19] A. Mauborgne, S. Bourgoin, H. Polienor, M. Roumy, G. Simonnet, J.M. Zajac, F. Cesselin, The neuropeptide FF analogue, 1DMe, acts as a functional opioid autoreceptor antagonist in the rat spinal cord, Eur. J. Pharmacol. 430 (2001) 273–276.
[20] C. Gouarderes, I. Quelven, C. Mollereau, H. Mazarguil, S.Q. Rice, J.M. Zajac, Quantitative autoradiographic distribution of NPFF1 neuropeptide FF receptor in the rat brain and comparison with NPFF2 receptor by using [^{125}I]YVP and [^{125}I]EYF as selective radioligands, Neuroscience 115 (2002) 349–361.
[21] C. Gouardères, A. Puget, J.M. Zajac, Detailed distribution of neuropeptide FF receptors (NPFF1 and NPFF2) in the rat, mouse, octodon, rabbit, guinea pig, and marmoset monkey brains: a comparative autoradiographic study, Synapse 51 (2004) 249–269.
[22] I. Quelven, A. Roussin, J.M. Zajac, Comparison of pharmacological activities of Neuropeptide FF1 and Neuropeptide FF2 receptor agonists, Eur. J. Pharmacol. 508 (2005) 107–114.
[23] H. Mazarguil, C. Gouarderes, J.A. Tafani, D. Marcus, M. Kotani, C. Mollereau, M. Roumy, J.M. Zajac, Structure–activity relationships of neuropeptide FF: role of C-terminal regions, Peptides 22 (2001) 1471–1478.
[24] S. Gicquel, H. Mazarguil, M. Allard, G. Simonnet, J.-M. Zajac, Analogs of F8Famide resistant to degradation, with high affinity and in vivo effects, Eur. J. Pharmacol. 222 (1992) 61–67.

[25] J.C. Sol, A. Roussin, S. Proto, H. Mazarguil, J.M. Zajac, Enzymatic degradation of neuropeptide FF and SQA-neuropeptide FF in the mouse brain, Peptides 20 (1999) 1219–1227.
[26] C. Desprat, J.-M. Zajac, Hypothermic effects of Neuropeptide FF analogues in mice, Pharmacol. Biochem. Behav. 58 (1997) 559–563.
[27] B. Frances, H. Lahlou, J.M. Zajac, Cholera and pertussis toxins inhibit differently hypothermic and anti- opioid effects of neuropeptide FF, Regul. Pept. 98 (2001) 13–18.
[28] P. Oberling, L. Stinus, M. Le Moal, G. Simonnet, Biphasic effect on nociception and antiopiate activity of the neuropeptide FF (FLFQPQRFamide) in the rat, Peptides 14 (1993) 919–924.