

Comparison of the structure-activity relationships of nociceptin and dynorphin A using chimeric peptides

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Abstract The aim of the present study was to delineate the functional domains of nociceptin (noc), a neuropeptide which is structurally related to dynorphin A (dyn). The binding and biological potencies towards the nociceptin (ORL1) and dynorphin A (κ -opioid) receptors of twenty dyn/noc and noc/dyn hybrid peptides were compared with those of the parent heptadecapeptides. Replacement of as many as eleven residues in the C-terminus of dynorphin by the corresponding nociceptin sequence has no significant effect on binding and biological activity towards the κ -opioid receptor. In marked contrast, replacement of as few as six residues (RKLANQ) in the C-terminus of nociceptin by the corresponding dynorphin sequence (LKWDNQ) dramatically impairs both affinity and activity towards the ORL1 receptor. This clearly indicates that the two neuropeptides have different functional architectures, despite the dual structural homology of both ligands and receptors. Moreover, the recombinant peptide approach led us to identify hybrids whose sequences differ only at positions 5 and 6 and displaying opposite or no receptor selectivity. One contains the dynorphin Leu⁵-Arg⁶ sequence and prefers the κ -opioid receptor, whereas the other comprises the nociceptin Thr⁵-Gly⁶ sequence and prefers the ORL1 receptor. A third, containing the mixed dynorphin/nociceptin Leu⁵-Gly⁶ sequence, does not discriminate between the two types of receptor.

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Key words: Structure-activity relationship; Dynorphin A; Nociceptin/orphanin FQ; Chimeric neuropeptide; Opioid and opioid receptor-like receptor

1. Introduction

Nociceptin [1] or orphanin FQ [2] is the endogenous agonist of the ORL1 (opioid receptor-like 1) receptor, an orphan G protein-coupled receptor whose human [3] and murine [4–10] cDNAs had been identified previously.

The ORL1 receptor is a member of the structural family of opioid receptors. Multiple sequence alignment places the ORL1 receptor equally distant ($\approx 60\%$ homology) from the μ -, δ - and κ -opioid receptor types. However, ORL1 and the κ -opioid receptors display a common physicochemical feature, not found in the two other types of opioid receptor, a highly acidic second exofacial loop. This loop has been shown to be critical for the κ -opioid receptor to bind dynorphin A, its

putative natural agonist, a highly basic heptadecapeptide, with high affinity and selectivity [11,12].

Nociceptin/orphanin FQ, the endogenous ligand of the ORL1 receptor was recently isolated from brain extracts as a component that inhibits adenyl cyclase in recombinant CHO cells expressing the orphan receptor [1,2]. Nociceptin, FGGFTGARKSARKLANQ, is a peptide which resembles dynorphin A, YGGFLRRIRPKLKWDNQ, in several respects: it has the same length (17 amino acids), six identical amino acids (underlined), and is highly cationic. Based upon the dual homology, of receptors and of peptide ligands, it has been proposed [1] that the structural requirements for nociceptin and dynorphin A to bind the ORL1 and κ -opioid receptors, respectively, are very similar, if not identical. This hypothesis has been investigated here using the 'recombinant' ligand approach. Twenty nociceptin/dynorphin (nd) and dynorphin/nociceptin (dn) hybrid heptadecapeptides were synthesized and compared with the two parent peptides in terms of their ability to bind and stimulate the ORL1 and κ -opioid receptors. The results clearly indicate that nociceptin and dynorphin A have different functional architectures. They also identify amino acid residues in the two neuropeptides, that are important in bestowing ORL1/ κ -opioid receptor selectivity.

2. Materials and methods

2.1. Recombinant cell lines and membrane preparations

Recombinant CHO cell lines expressing the human ORL1 and κ -opioid receptors and membrane preparations derived therefrom were obtained as previously described [13]. The κ -opioid receptor cDNA was kindly provided by Dr. Brigitte Kieffer (E.S.B.S., Illkirch).

2.2. Binding and competition studies

[³H]diprenorphine (1.52 TBq/mmol, Amersham) was used to probe the κ -opioid receptor site, and [³H]nociceptin (0.85 TBq/mmol, Amersham) to probe the ORL1 binding site. To minimize peptide loss due to nonspecific adsorption and/or proteolytic degradation, stock solutions and intermediate dilutions of peptide for use in the competition studies were made up in 0.1 mg/ml proteinase-free BSA (fraction V, Sigma) in polypropylene tubes, and glass fiber disks (Whatman GF/B) pre-soaked in polyethyleneimine (0.33%, v/v) used. Under these conditions, we verified that the presence of peptidase and proteinase inhibitors in the assay was unnecessary. Each incubation mixture (0.5 ml, in triplicate) contained ≈ 30 μ g membrane protein, [³H]diprenorphine or [³H]nociceptin at the fixed concentration of 1 nM, and the unlabelled peptide at the desired concentration, in 50 mM Tris-HCl, pH 7.5. Nonspecific binding ($< 20\%$ of total binding for the two radioligands) was determined in the presence of 1 μ M unlabelled diprenorphine or nociceptin. Following a 1 h incubation at 25°C, unbound radioligand was removed by rapid suction through glass fiber filters (see above) and rinsing three times with 4 ml 10 mM Tris-HCl buffer (pH 7.5). Filter-bound radioactivity was counted in 3 ml of Beckman Ready Protein⁺ cocktail using a Kontron MR 300 liquid scintillation counter.

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Abbreviations: ORL1, opioid receptor-like 1; noc, nociceptin; dyn, dynorphin A; nd, nociceptin/dynorphin A hybrid peptide; dn, dynorphin A/nociceptin hybrid peptide

2.3. Assay for intracellular cyclic AMP

Sterile hemolysis tubes were seeded with $\approx 200\,000$ recombinant CHO cells in culture medium and incubated overnight at 37°C . The culture medium was then removed and replaced with $200\ \mu\text{l}$ of HEPES-buffered Krebs-Ringer saline (KRH: 124 mM NaCl, 5 mM KCl, 1.25 mM MgSO_4 , 1.5 mM CaCl_2 , 1.25 mM KH_2PO_4 , 25 mM HEPES, 8 mM glucose, 0.5 mg/ml BSA; pH 7.4) containing $0.1\ \mu\text{M}$ adenosine and $0.6\ \mu\text{Ci}$ [^3H]adenine ($0.85\ \text{TBq/mmol}$, Amersham). After 1 h at 37°C , the cells were rinsed with $400\ \mu\text{l}$ of KRH and $180\ \mu\text{l}$ of fresh KRH was added to each tube. Intracellular accumulation of cAMP was initiated by the addition of $20\ \mu\text{l}$ of KRH containing $100\ \mu\text{M}$ forskolin (Sigma), $1\ \text{mM}$ 3-isobutyl-1-methylxanthine (Sigma), $1\ \text{mM}$ Ro20-1724 (Biomol. Res.), and the appropriate ligand(s) at 10-fold the desired final concentration. After exactly 10 min at 37°C , the reaction was stopped by addition of $20\ \mu\text{l}$ HCl 2.2 N and rapid mixing (Vortex). The [^3H]cAMP content of each tube was determined by selective batch elution on columns of $0.65\ \text{g}$ alumina (activity grade 1, type WA-1: acid, Sigma) essentially as described by Alvarez and Daniels [14].

2.4. Unlabelled ligands

Dynorphin A, nociceptin and the 20 'recombinant' peptides were synthesized using an automated peptide synthesizer (Applied Biosystems model 433A). All the peptides had the expected molecular mass, as determined by mass spectrometry (Finnigan Mat model TSQ 700), and were $>90\%$ pure, as judged by high performance liquid chromatography (Waters Instruments).

2.5. Analysis of the data

Unweighed, nonlinear regression analysis of the data was performed using InPlot ver 4.03 from GraphPad Software Inc., San Diego, CA, USA.

3. Results

The dynorphin A/nociceptin hybrid peptides (hereafter denoted dn1–dn10) were generated by stepwise replacement of dynorphin C-terminus with the corresponding nociceptin sequence. Likewise, the nociceptin/dynorphin A chimeric peptides (hereafter denoted nd1–nd10) were obtained by stepwise replacement of the nociceptin C-terminus by the corresponding dynorphin A sequence. The two parent and 20 hybrid peptides were tested both for affinity and activity towards the ORL1 and κ -opioid receptors, stably expressed in CHO cells. Peptide affinity was calculated from the capacity of the peptides to compete for equilibrium binding of $1\ \text{nM}$ [^3H]nociceptin ($K_D = 0.13\ \text{nM}$, [13,15]) (ORL1 receptor), and

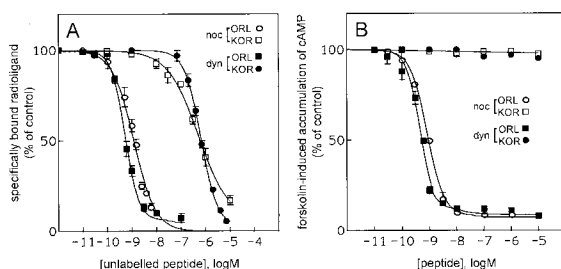


Fig. 1. Comparison of the binding and biological potencies of nociceptin and dynorphin A towards the ORL1 and κ -opioid receptors. A: Inhibition by nociceptin (noc) and dynorphin A (dyn) of equilibrium binding of [^3H]nociceptin ($1\ \text{nM}$) or [^3H]diprenorphine ($1\ \text{nM}$), in a crude membrane fraction from recombinant CHO cells expressing the ORL1 or κ -opioid (KOR) receptor, respectively. Each data point is the mean \pm s.e.m. of triplicate determinations. B: Inhibition by nociceptin (noc) and dynorphin A (dyn) of forskolin-induced accumulation of cAMP in intact recombinant CHO cells expressing the ORL1 and κ -opioid (KOR) receptors. Each data point is the mean \pm s.e.m. of triplicate determinations.

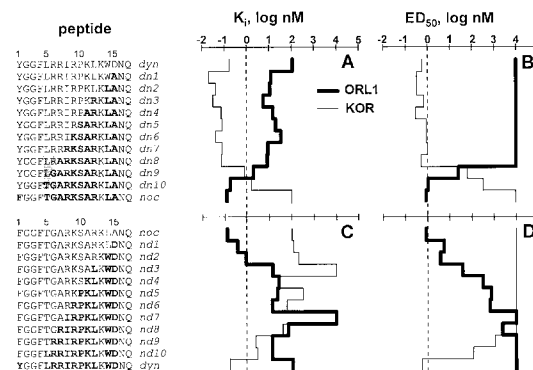


Fig. 2. Affinity (K_1 , A and C) and activity (ED_{50} , B and D) of the dn and nd series of chimeric peptides, towards the ORL1 (thick staircase line) and κ -opioid (KOR, thin staircase line) receptors. Each step represents the mean of at least three values differing by less than 20%. K_1 values were calculated from IC_{50} values obtained from competition curves such as those shown in Fig. 1A, using the Cheng and Prusoff equation [22]. EC_{50} values were obtained from inhibition experiments such as those shown in Fig. 1B. The nociceptin- and dynorphin-specific amino acid residues in the dn and hybrid peptides are shown in bold. Amino acid residues considered to be important for receptor-type selection are shadowed.

of $1\ \text{nM}$ [^3H]diprenorphine ($K_D = 0.3\ \text{nM}$) (κ -opioid receptor). Activity was estimated from the ability of the peptides to half maximally inhibit forskolin-induced accumulation of cyclic AMP in recombinant CHO cells expressing the ORL1 or κ -opioid receptors. Fig. 1A shows that the ORL1 receptor binds nociceptin, its natural ligand, almost 1000-fold tighter (K_1 : 0.13 vs. $104\ \text{nM}$) than it does dynorphin A. Conversely, the κ -opioid receptor binds dynorphin A, its natural ligand, with 500–1000-fold the affinity (K_1 : 0.17 vs. $110\ \text{nM}$) it does nociceptin. Likewise, Fig. 1B shows that nociceptin is a potent ORL1 receptor agonist (ED_{50} : $0.8\ \text{nM}$) and is inactive towards the κ -opioid receptor ($\text{ED}_{50} \geq 10\ \mu\text{M}$) while, conversely, dynorphin A is a potent κ -opioid receptor agonist (ED_{50} : $0.56\ \text{nM}$) and is inactive at the ORL1 receptor ($\text{ED}_{50} \geq 10\ \mu\text{M}$).

3.1. Binding properties and biological activity of the dynorphin/nociceptin (dn) chimeric peptides

In comparison with dynorphin A, the dn1 hybrid peptide ([Ala¹⁵]dynorphin A) showed a nearly 10-fold increased affinity for both the ORL1 and κ -opioid receptors (Fig. 2A and C). Further substitution of the C-terminal dynorphin sequence by that of nociceptin, such as in the dn2 through dn8 chimeras, resulted in no further change in binding affinity for either the ORL1 or the κ -opioid receptors. In terms of biological activity, dynorphin A and the dn1 through dn8 chimeras were equally highly potent κ -opioid receptor agonists, with E_{50} s in the range 0.3 – $1\ \text{nM}$, whereas they were inactive at the ORL1 receptor (ED_{50} s $\geq 10\ \mu\text{M}$). The first dn hybrid peptide to exhibit activity at the ORL1 receptor was dn9, i.e. dn8[Arg6-Gly]. In comparison with dn8, dn9 displayed a dramatically increased (ED_{50} : 25 vs. $>10\,000\ \text{nM}$) potency in the ORL1 receptor-mediated cyclase inhibition assay. Moreover, the dn9 chimera exhibited similarly high (nanomolar) affinities, and comparable biological activities towards the ORL1 ($K_1 = 2\ \text{nM}$; $\text{ED}_{50} = 25\ \text{nM}$) and κ -opioid ($K_1 = 0.8\ \text{nM}$; $\text{ED}_{50} = 63\ \text{nM}$) receptors. In this sense, the dn9 hybrid peptide behaved as a nonselective ('universal') agonist of the two types of receptor. Further substitution in dn9 of Leu⁵ by the threonyl

residue of nociceptin resulted in a chimeric peptide (dn10 or [Tyr¹]nociceptin) that showed reversed ORL1 vs. κ -opioid receptor selectivity in comparison with dynorphin A and the dn1–8 chimeras. Overall, evolution of binding affinity with stepwise exchange of the C-terminus of dynorphin with that of nociceptin, paralleled well the change in biological activity, with a marked transition at the dn9 hybrid peptide, corresponding to a reversal of receptor-type selectivity.

3.2. Binding properties and biological activity of the nociceptin/dynorphin (nd) chimeric peptides

Progressive substitution of the nociceptin C-terminal amino acid residues by the corresponding residues of dynorphin A yielded hybrid peptides with progressively decreased yet in absolute terms high ($K_1 < 1$ nM; nd1 and nd2) to moderate (K_1 s in the range 10–70 nM; all others, nd7 excepted) affinities for the ORL1 receptor (Fig. 2B and D). Interestingly, the nd7 chimera, the Arg8Gly ‘mutant’ of nd6, did not bind ($K_1 > 10$ μ M) the ORL1 receptor. The decreased ORL1 receptor affinity in the nd series of peptides was broadly paralleled by a progressively diminished biological potency. The correlation was however less than ideal since nd3, nd9 and nd10 had comparable affinities for the ORL1 receptor, yet nd3 showed substantial biological activity ($ED_{50} = 38$ nM) whereas nd9 and nd10 were inactive ($ED_{50} > 10$ μ M).

Nociceptin and the nd1, nd2, nd4, nd5, nd6 and nd8 chimeras had similar, although low (K_1 s in the range 30–200 nM) affinities for the κ -opioid receptor, whereas, nd3 (nd2[Arg12Leu]) and nd7 (nd6[Arg8Ile]) did not bind this receptor. None of the nd1–nd8 hybrid peptides displayed measurable activity in the κ -opioid receptor-mediated cyclase inhibition assay. The first nd chimera to show nanomolar ($K_1 = 2.5$ nM) affinity albeit with low biological potency for the κ -opioid receptor was nd9 (nd8[Gly6Arg]). Interestingly, κ -opioid receptor activity, but not affinity, was further augmented (≈ 10 -fold) in nd10 ([Phe¹]dynorphin A). No ‘universal’ (nonselective) and potent agonist of the ORL1 and κ -opioid receptors could be identified in the nd series of hybrid peptides.

4. Discussion

Dynorphin A-(1–13), which is equipotent with dynorphin A-(1–17) in the guinea pig ileum bioassay [16], has been suggested to consist of two distinct domains: a ‘message’ domain, Tyr¹-Gly-Gly-Phe, the shortest fragment with naloxone-reversible opioid activity, and a potency-enhancing, ‘address’ domain, Leu⁵-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, in which Arg⁷ makes the greatest contribution [17]. Recently, it was proposed that a necessary and sufficient requirement for a peptide to bind the κ -opioid receptor with high specificity is the presence of the N-terminal Tyr-Gly-Gly-Phe-Met or Leu opioid core plus an Arg-X extension [18]. In comparison with dynorphin A, dynorphin A-(1–7) displays the same affinity for the κ -opioid receptor and the same opioid receptor-type selectivity [18], and only moderately (< 10 -fold) reduced biological potency [17], indicating that the C-terminal decapeptide section does not play a too critical role in dynorphin occupancy and activation of the receptor. Consistent with this conclusion is the present finding that exchange of the C-terminal 11-mer of dynorphin by the corresponding nociceptin sequence comprising just three common residues (Lys¹³, Asn¹⁶ and Gln¹⁷), re-

sults in a chimeric peptide, dn8 (YGGFLR/ARKSAR-CLANQ), whose binding and activity towards the κ -opioid receptor are identical with those of dynorphin A.

With regard to nociceptin, the necessary and sufficient requirements for selective and potent peptide binding to the ORL1 receptor are yet to be identified. It has been found that nociceptin-(1–13)-NH₂ (which is somewhat longer than the smallest fully active fragment of dynorphin A, see above) has the same binding [19] and biological [20] potencies as nociceptin towards the ORL1 receptor. Furthermore, evidence has now accumulated that its N-terminal residues play an important role in nociceptin occupation and stimulation of the ORL1 receptor: deleting Phe¹ [15], or replacing Phe¹, Gly² or Phe⁴ by an alanyl residue [19,21], inactivates the peptide. However, mutations of more internal residues, particularly Arg⁸ and Arg¹², also adversely affect nociceptin affinity and activity towards the ORL1 receptor ([19,21], and the present data). Indeed, comparison of the structure-activity relationships of nociceptin and dynorphin A has led most investigators to suggest that the two peptides have different functional architectures [15,20,21]. Our data obtained with the nd series of hybrid peptides lend considerable weight to this conclusion. Had the functional architecture of nociceptin been equivalent to that of dynorphin A, the binding and activity profiles of the nd series of chimeric peptides should have been exactly mirrored by the dn series. This is clearly not the case since progressive replacement of the dynorphin C-terminus by that of nociceptin (dn series) adversely affected affinity for the κ -opioid receptor only ‘late’ (dn9) in the series, whereas exchange of the nociceptin C-terminus with the analogous section in dynorphin (nd series) adversely affected affinity for the ORL1 receptor at a much ‘earlier’ point (nd3) in the series. This suggests that the (T)GARKSAR(K) nociceptin sequence delimited approximately by the dn9 and nd3 hybrid peptides endows nociceptin with affinity and activity towards the ORL1 receptor. Particularly important in this sequence is the Arg⁸ residue since moving from nd6 to nd7 (nd6[Arg8Ile]) effectively abolishes binding to the ORL1 receptor. This is in keeping with the earlier demonstration that [Ala⁸]nociceptin has only 0.3% the affinity of nociceptin for ORL1 [19]. An intriguing observation is that the nd3–nd6 and the dn3–dn8 chimeras display comparable apparent affinities for the ORL1 receptor, yet only the former are biologically active. A plausible explanation is that the nd3–nd6, but not the dn3–dn8 chimeras have access to the ORL1 receptor signal transduction apparatus, implying that the two series of hybrid peptides bind the ORL1 receptor in a different way.

Another interesting observation here, using the ‘recombinant’ peptide approach, is that identified nociceptin and dynorphin A residues are critical not only for potency but also for receptor-type selection. Indeed, the dn8 and dn10 hybrid peptides, whose primary structures differ only at positions 5 and 6, show reciprocal ORL1 and κ -opioid receptor selectivity. Dn8 contains the dynorphin Leu⁵-Arg⁶ sequence and prefers the κ -opioid receptor, whereas dn10 contains the nociceptin Thr⁵-Gly⁶ sequence and prefers the ORL1 receptor. Likewise, dn9 (YGGFL/GARKSARKLANQ) contains the mixed Leu⁵-Gly⁶ sequence and binds and activates the two types of receptor equally well. The Leu⁵-Arg⁶ and Thr⁵-Gly⁶ ‘hinge’ sequences may therefore be important in specifying dynorphin κ -opioid/ORL1, and nociceptin ORL1/ κ -opioid receptor selectivity, respectively. Whether or not the ORL1 re-

ceptor will accept peptides lacking a glycine at position 6 remains speculative, although a similar requirement for an arginine at the same position in opioid peptides has been advanced for the κ -opioid receptor [18].

Based upon the present and previous [15–21] data, the N-terminal heptapeptide appears to be both necessary and sufficient for dynorphin A to selectively bind and activate the κ -opioid receptor. This requirement is not however sufficient for nociceptin to selectively bind and activate the ORL1 receptor. Nociceptin needs an additional, highly positively charged (Arg⁺-Lys⁺-Ser-Ala-Arg⁺-Lys⁺) extension, in order to display biological activity. The κ -opioid receptor site may thus be viewed as comprising two functional domains: a trans-membrane opioid binding pocket that recognizes the Tyr¹-Gly-Gly-Phe opioid 'message', and an acidic second extracellular loop, not present in the μ and δ receptors, that interacts with the positively charged amino acid residues at positions 6 and 7 (the potency- and selectivity-enhancing 'address'), of dynorphin [18]. Likewise, the ORL1 receptor can be functionally compartmentalized in terms of a structurally related binding pocket [15] able to recognize the nociceptin Phe¹-Gly-Gly-Phe 'message', and a highly acidic second extracellular loop interacting with the positively charged residues at positions 8, 9, 12 and 13 ('address') of nociceptin. According to this view, the two neuropeptides possess very similar functional architectures, differing only in the length of their 'address' sequences. This notion is however untenable since several of our nd hybrid peptides, whilst they contain the Phe¹-Gly-Gly-Phe sequence, are totally inactive at the ORL1 receptor. In marked contrast, the dn chimeras, which all contain the Tyr¹-Gly-Gly-Phe opioid 'message' sequence, are all active at the κ -opioid receptor. Clearly, the 'message' and 'address' partition of dynorphin does not apply to nociceptin. Notwithstanding the dual structural homology of peptide ligands and receptors, these findings imply that either dynorphin A and nociceptin bind the κ -opioid and ORL1 receptors in a different way, respectively, or that the dynorphin A (κ -opioid) and nociceptin (ORL1) receptor sites are not equivalent. Determination of the bioactive (receptor-bound) conformation of the two neuropeptides will be necessary to answer this fundamental question.

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References

- [1] Meunier, J.-Cl., Mollereau, C., Toll, L., Suaudeau, Ch., Moissand, Ch., Alvinerie, P., Butour, J.-L., Guillemot, J.-Cl., Ferrara, P., Monsarrat, B., Mazarguil, H., Vassart, G., Parmentier, M. and Costentin, J. (1995) *Nature* 377, 532–535.
- [2] Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J. and Civelli, O. (1995) *Science* 270, 792–794.
- [3] Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.-L., Moissand, Ch., Chalon, P., Caput, D., Vassart, G. and Meunier, J.-Cl. (1994) *FEBS Lett.* 341, 33–38.
- [4] Fukuda, K., Kato, S., Mori, K., Nishi, M., Takeshima, H., Iwabe, N., Miyata, T., Houtani, T. and Sugimoto, T. (1994) *FEBS Lett.* 343, 42–46.
- [5] Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C.A. and Yu, L. (1994) *FEBS Lett.* 347, 279–283.
- [6] Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M. and Grandy, D.K. (1994) *FEBS Lett.* 347, 284–288.
- [7] Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M. and Uhl, G.R. (1994) *FEBS Lett.* 348, 75–79.
- [8] Nishi, M., Takeshima, H., Mori, M., Nakagawara, K.I. and Takeuchi, T. (1994) *Biochem. Biophys. Res. Commun.* 205, 1353–1357.
- [9] Lachowicz, J.E., Shen, Y., Monsma, F.J. and Sibley, D.R. (1995) *J. Neurochem.* 64, 34–40.
- [10] Wick, M.J., Minnerath, S.R., Roy, S., Ramakrishnan, S. and Loh, H.H. (1995) *Mol. Brain Res.* 32, 342–347.
- [11] Wang, J.B., Johnson, P.S., Wu, J.M., Wang, W.F. and Uhl, G.R. (1994) *J. Biol. Chem.* 269, 25966–25969.
- [12] Xue, J.-C., Chen, C., Zhu, J., Kunapuli, S., DeRiel, J.K., Yu, L. and Liu-Chen, L.-Y. (1994) *J. Biol. Chem.* 269, 30195–30199.
- [13] Mollereau, C., Moissand, C., Butour, J.-L., Parmentier, M. and Meunier, J.-Cl. (1996) *FEBS Lett.* 395, 17–21.
- [14] Alvarez, R. and Daniels, D.V. (1992) *Anal. Biochem.* 203, 76–82.
- [15] Butour, J.-L., Moissand, C., Mazarguil, H., Mollereau, C. and Meunier, J.-Cl. (1997) *Eur. J. Pharmacol.* 321, 97–103.
- [16] Goldstein, A., Fischli, W., Lowney, L.I., Hunkapiller, M. and Hood, L. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7219–7223.
- [17] Chavkin, C. and Goldstein, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6543–6547.
- [18] Mansour, A., Hoversten, M., Taylor, L.P., Watson, S.J. and Akil, H. (1995) *Brain Res.* 700, 89–98.
- [19] Dooley, C.T. and Houghten, R.A. (1996) *Life Sci.* 59, PL23–29.
- [20] Guerrini, R., Calo, G., Rizzi, A., Bianchi, C., Lazarus, L.H., Salvadori, S., Temussi, P.A. and Regoli, D. (1997) *J. Med. Chem.* 40, 1789–1793.
- [21] Reinscheid, R.K., Ardati, A., Monsma Jr., F.J. and Civelli, O. (1996) *J. Biol. Chem.* 271, 14163–14168.
- [22] Cheng, Y.-C. and Prusoff, W.H. (1973) *Biochem. Pharmacol.* 22, 3099–3108.