British Journal of Pharmacology (1998) 125, 1485–1490
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Nociceptin receptor binding in mouse forebrain membranes: thermodynamic characteristics and structure activity relationships

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1 The present study describes the labelling of the nociceptin (NC) receptor, ORL_1, in mouse forebrain membranes with a new ligand partially protected from metabolic degradation at the C-terminal; the ligand, [^{3}H]-NC-NH_2, has a specific activity of 24.5 Ci mmol^{-1}

2 Saturation experiments revealed a single class of binding sites with a K_D value of 0.55 nM and B_{max} of 94 fmol mg^{-1} of protein. Non specific binding was 30% of total binding. Kinetic binding studies yielded the following rate constants: $K_{obs} = 0.104$ min^{-1}; $K_1 = 0.034$ min^{-1}; $T_{1/2} = 20$ min; $K_{+1} = 0.07$ min nM^{-1}.

3 Thermodynamic analyses indicated that [^{3}H]-NC-NH_2 binding to the mouse ORL_1 is totally entropy driven, similar to what has been observed for the labelled agonists to the opioid receptors $OP_1(\delta)$, $OP_2(\kappa)$ and $OP_3(\mu)$.

4 Receptor affinities of several NC fragments and analogues, including the newly discovered ORL-1 receptor antagonist [Phe$^1\psi$(CH_2-NH)Gly^2]NC(1–13)-NH_2 ([F/G]NC(1–13)-NH_2), were also evaluated in displacement experiments. The competition curves for these compounds were found to be parallel to that of NC and the following order of potency was determined for NC fragments: NC-OH = NC-NH_2 = NC(1–13)-NH_2 >> NC(1–12)-NH_2 > NC(1–13)-OH >> NC(1–11)-NH_2, and for NC and NC(1–13)-NH_2 analogues: [Tyr^1]NC-NH_2 ⩾ [Leu^1]NC(1–13)-NH_2 ⩾ [Tyr^1]NC(1–13)-NH_2 ⩾ [F/G]NC(1–13)-NH_2 >> [Phe^3]NC(1–13)-NH_2 > [DF/G]NC(1–13)-NH_2.

5 Standard opioid receptor ligands (either agonists or antagonists) were unable to displace [^{3}H]-NC-NH_2 binding when applied at concentrations up to 10 μM indicating that this new radioligand interacts with a non opioid site, probably the ORL_1 receptor.

Keywords: Nociceptin; mouse; brain; ORL_1; binding

Introduction

Nociceptin (NC), a recently identified heptadecapeptide, was reported to act as an endogenous ligand of the orphan receptor ORL_1 (Meunier *et al*., 1995; Reinscheid *et al*., 1995). In spite of the structural similarities between NC and opioid peptides (especially dynorphin A) and between ORL_1 and the opioid receptors of the δ, κ and μ types, NC does not interact with classical opioid receptors and, *vice-versa*, opioid peptides do not interact with the ORL_1 receptor (Henderson & McKnight, 1997; Meunier, 1997). Thus, NC and its receptor represent a new peptide receptor system (Meunier, 1997). After the identification of NC, a large number of papers were published in which the interaction of NC with its receptor was studied with different techniques (e.g. receptor binding, (Adapa & Toll, 1997; Dooley & Houghten, 1996; Makman *et al*., 1997; Mathis *et al*., 1997), second messenger studies (Cheng *et al*., 1997; Fawzi *et al*., 1997; Ma *et al*., 1997), bioassays (Berzetei-Gurske *et al*., 1996; Calo' *et al*., 1996, 1997; Giuliani & Maggi, 1996, 1997), see Meunier (1997) for a review). In some articles, structure-activity studies were performed to identify the most important residues of NC that may be responsible for receptor occupation and activation (Butour *et al*., 1997; Calo' *et al*., 1998a; Dooley & Houghten, 1996; Guerrini *et al*., 1997; Reinscheid *et al*., 1996).

In the present study, we used [^{3}H]-NC-NH_2 (a new ligand for the ORL_1 receptor) to (i) set up a binding assay in mouse forebrain membranes, (ii) study the thermodynamic characteristics of this binding assay, and (iii) determine the pharmacological spectrum of this binding site, using standard opioid ligands, as well as several NC related peptides (NC fragments and NC(1–13)-NH_2 analogues) which have been previously shown to act as agonists at the ORL_1 receptor (Calo' *et al*., 1997; Guerrini *et al*., 1997). We also tested the new peptide [Phe$^1\psi$(CH_2-NH)Gly^2]NC(1–13)-NH_2 ([F/G] NC(1–13)-NH_2) which has previously been shown to act as antagonist of the ORL_1 receptor in peripheral tissues (Guerrini *et al*., 1998).

Methods

Membrane preparation

Mice were decapitated and the forebrain was dissected on ice. The tissue was disrupted in a Polytron homogenizer (setting 5) in 20 volumes of 50 mM Tris HCl, 2 mM EDTA, 100 μM phenylmethylsulphonylfluoride (PMSF) at pH 7.4. The homogenate was centrifuged at 40,000 × *g* for 10 min and the pellet was resuspended in the same buffer. After 30 min of incubation at 37°C, the membranes were centrifuged and the resulting pellets were stored at −80°C. Prior to freezing, an aliquot of the homogenate was removed for protein assay according to a Bio Rad method (Bradford, 1976), using bovine albumin as reference standard. The final pellet was resuspended in the same incubation buffer at a concentration of 200 μg protein/100 μl and this homogenate was used for the binding assay.

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[^{3}H]-NC(1–17)-NH$_2$ binding to mouse forebrain membranes

Binding assays were carried out according to Dooley & Houghten (1996). In saturation studies, mouse brain membranes were incubated with 8 to 10 different concentrations of [^{3}H]-NC-NH$_2$, ranging from 0.05–5 nM. Inhibition experiments were carried out in duplicate in a final volume of 250 μl in test tubes containing 0.5 nM [^{3}H]-NC-NH$_2$, 50 mM Tris HCl buffer, 2 mM EDTA, 100 μM PMSF at pH 7.4, mouse forebrain membranes (200 μg protein/assay), and different concentrations of the ligand under study. Moreover, the inhibitory binding constant, K_i, was calculated from the IC_{50} value according to the Cheng & Prusoff equation (Cheng & Prusoff, 1973), $K_i = IC_{50} / (1 + [L]/K_D)$, where [L] is the concentration of the radioligand and K_D the dissociation constant. Non-specific binding was defined as the binding observed in the presence of 10 μM NC-NH$_2$ and was about 30% of the total binding. Incubation time was 120 min at 25°C, based on the results of previous time-course experiments. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass-fibre filters using a Brandel cell harvester. The incubation mixture was diluted with 3 ml of ice-cold incubation buffer; then, vacuum filtered rapidly and the filters were washed three times with 3 ml of incubation buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 Spectrometer (efficiency 55%). A weighted non linear least-squares curve fitting program LIGAND (Munson & Rodbard, 1980), was used for computer analysis of saturation and inhibition experiments.

Thermodynamic analysis

For the generic binding equilibrium [L]+[R]=[LR] (L=ligand, R=receptor), the affinity constant (K_A) is directly related to the standard free energy ΔG° ($\Delta G^\circ = -RT \ln K_A$) which can be divided in enthalpic and entropic contribution, according to the Gibbs equation: $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. ΔG° is calculated as $-RT \ln K_A$ at 25°C, while the determination of the other thermodynamic parameters (ΔH° and ΔS°) is made by measuring K_A at different temperatures ($K_A = 1/K_D$, where K_D is the dissociation constant at equilibrium).

Two general cases can be distinguished: (1) ΔCp° (the difference in standard specific heats at constant pressure and at equilibrium) is nearly zero. In this case, the equation $(\delta \ln K_A)/\delta(1/T) = -\Delta H^\circ/R$ gives a linear van't Hoff plot $\ln K_A$ versus (1/T) and standard enthalpy can be calculated from its slope, $-\Delta H^\circ/R$, while standard entropy is calculated as $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$ with T=298.15 K and R=8.314 J/mol/K; (2) ΔCp° is different from zero. The plot ΔG° versus T is often parabolic and other mathematical methods (Osborne *et al*., 1976) for calculating the thermodynamic parameters at the equilibrium are available.

Saturation experiments of [^{3}H]-NC-NH$_2$ binding to the mouse forebrain membranes were carried out at 5, 10, 20, 25 and 30°C, in a thermostatic bath, using concentrations of radioligand ranging from 0.5–5 nM. Since the van't Hoff plot was found to be linear, thermodynamic parameters were calculated with the first method.

Data analysis

The data are expressed as mean ± s.e.mean. For K_i, K_D and B_{max} values, the 95% confidence limits are given. Data have been statistically analysed *via* a software package (Tallarida & Murray, 1987): *P* values lower than 0.05 have been considered to be significant. The pharmacological terminology adopted in this paper is in line with the recent IUPHAR recommendations (Jenkinson *et al*., 1995; Vanhoutte *et al*., 1996).

Materials

All peptides used in this study were prepared and purified as described before (Guerrini *et al*., 1997, 1998). The synthesis of [^{3}H]-NC-NH$_2$ was as follows: 3 mg 1.4 μmol [pIPhe^1]-NC-NH$_2$ was dissolved in 1 ml of DMF, 8 μmol triethylamine and 10 mg of PdO/BaSO$_4$ (10% Pd) catalyst was added. After connecting the reaction vessel to the tritium manifold, the solution was frozen in liquid N_2 and evacuated. Tritium gas (555 GBq, 15 Ci) was introduced, liquid nitrogen was removed and the reaction mixture was stirred for 65 min. The tritiation was controlled by following the pressure with a manometer. After the reaction was complete, the reaction mixture was frozen in liquid N_2 and the unreactive tritium was absorbed on pyrophoric uranium. The liquid N_2 was removed and the reaction vessel was taken off from tritium manifold to work up the crude mixture. The catalyst was filtered on a Whatman GF/C filter and washed three times with ethanol. The solvent was evaporated and the labile tritium was removed by repeated evaporation from EtOH-H_2O 1:1. The total radioactivity was 865.8 MBq (23.4 mCi). The crude peptide was purified by HPLC on LiChrospher^R 100 RP-18 (5 μm) Merck column by elution (linear gradient of CH_3CN rising from 5 to 25% in 20 min). Retention time was 18.2 min. Unlabelled peptide was used as standard to determine the specific activity of [^{3}H]-NC-NH$_2$ from the radioactivity and peptide concentration: 0.906 TBq/mmol (24.5 Ci mmol^{-1}). U69593 (5α,7α,8β-(−)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxasipro(4,5)dec-8-yl)benzene acetamide)) was purchased from Amersham International (Buckinghamshire, U.K.), and CTOP (DPhe-Cys-Tyr-DTrp-Orn-Thr-Pen-Thr-NH$_2$) from Bachem (Bubendorf, Switzerland). Morphine was from Salars (Como, Italy), Norbinaltorphimine (Nor-BNI) and Naloxone were from Tocris Cookson (Bristol, U.K.). Other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or E. Merck (Darmstadt, Germany). Stock solutions (1 mM) were made in distilled water and kept at −20°C until use.

Results

Binding of [^{3}H]-NC-NH$_2$ to mouse forebrain membranes

The presence and binding capacity of the NC receptor in mouse forebrain membranes was evaluated by determining the saturation curve of the new ligand. Results obtained in three separate experiments, performed in triplicate, and the corresponding Scatchard plot are shown in Figure 1. The linearity of the Scatchard plot indicates that the mouse forebrain membranes possess a single class of binding sites with a K_D value of 0.55 (0.43–0.69) nM, and a B_{max} value of 94 (90–98) fmol mg^{-1} of protein. The kinetics of the binding of [^{3}H]-NC-NH$_2$ to mouse forebrain membranes was analysed by determining the time-course of the specific binding. Binding of the new ligand (0.5 nM) reached equilibrium after approximately 120 min at 25°C. Binding remained stable at least another 2 h (Figure 2A). Binding of [^{3}H]-NC-NH$_2$ was rapidly reversible after addition of cold ligand (10 μM NC-NH$_2$) (Figure 2B). Association and dissociation kinetic rate constants, as determined according to Burt (1986), were:

$K_{obs}=0.104$ (0.097–0.111)/min, $K_{-1}=0.034$ (0.030–0.038)/min from a $T_{1/2}=20$ min (19–22), and $K_{+1}=0.07$ (0.061–0.080)/min nM^{-1} A radioligand affinity constant (K_D) value of 0.50 (0.41–0.60) nM was estimated from these experiments.

Thermodynamic binding assay

K_D and B_{max} values derived from the saturation experiments of [^{3}H]-NC-NH_2 binding to mouse forebrain membranes performed at five chosen temperatures were found to fall within the following range: $K_D=0.45-2.34$ nM and $B_{max}=92-98$ fmol mg^{-1} of protein. While dissociation constants (K_D) changed with temperature, B_{max} values obtained from [^{3}H]-NC-NH_2 saturation experiments appear to be largely independent of temperature. Scatchard plots were linear at all temperatures investigated and computer analyses of the data (Munson & Rodbard, 1980) failed to show any significantly better fit with a two-site than with a one-site binding model, indicating that only one class of binding sites is present under the present experimental conditions. Figure 3, reports the van't Hoff plot $\ln K_A$ versus 1/T of the [^{3}H]-NC-NH_2 binding to the NC receptor. The final equilibrium thermodynamic parameters (expressed as means values ±s.e.mean of 3 independent determinations) were: $\Delta G^\circ=-52.38\pm0.12$ kJ mol^{-1}; $\Delta H^\circ=45.98\pm3.42$ kJ mol^{-1}; $\Delta S^\circ=330.0\pm12.5$ J mol K^{-1}. The linearity of the plot is statistically significant ($r=0.98$; $\Delta Cp^\circ \approx 0$) and its slope ($-\Delta H^\circ/R$) is negative.

Displacement studies

The binding site was characterized pharmacologically by displacing the binding of [^{3}H]-NC-NH_2 with a series of NC related peptides, namely fragments and analogues of NC, and analogues of NC(1–13)-NH_2. Figure 4 shows that displacement curves obtained with NC-NH_2, NC-OH, and NC(1–13)-NH_2 were superimposed, while NC(1–12)-NH_2, NC(1–13)-OH, and NC(1–11)-NH_2. showed a progressive reduction of affinity. All displacement curves were found to be parallel to that of the standard NC-NH_2, and reached the maximum. NC(1–12)-OH, NC(1–9)-NH_2, NC(1–5)-NH_2, NC(1–4)-NH_2, and NC(13–17)-OH were found to be inactive up to 10–50 μM (Table 1). The results (K_i values) obtained with a series of NC(1–13)-NH_2 analogues are shown in Table 1. The first three compounds in which Phe^1 of NC or of NC(1–13)-NH_2 was replaced with a Tyr or Leu residue did not show any important change of affinity. The new compound [F/G]NC(1–13)-NH_2 which has been shown to act as NC receptor antagonist in pheripheral tissues, showed a 13 fold reduced affinity as compared with NC(1–13)-NH_2. Several analogues of NC(1–13)-NH_2 in which Phe^1 or Phe^4 were replaced with DPhe or Leu, or in which Gly^2 or Gly^3 were replaced with Phe or DPhe were also tested. K_i values indicate that all these substitutions reduced the affinity of the compounds by at least 3 log units.

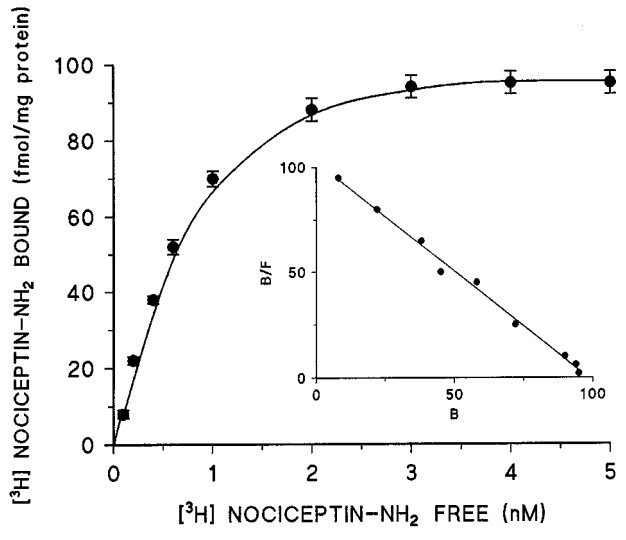

Figure 1 Saturation curve of [^{3}H]-NC-NH_2 binding to mouse forebrain membranes. Experiments were performed by incubating for 120 min at 25°C mouse forebrain membranes (200 μg protein 100 μl^{-1}) with 8–10 different concentrations of [^{3}H]-NC-NH_2 ranging from 0.05 to 5 nM. Values are the means±s.e.mean of 4 separate experiments performed in triplicate. In the inset, the Scatchard plot of the same data is shown (B bound; F free). K_D value (nM) was 0.55 (0.43–0.69) and B_{max} value (fmol mg^{-1} of protein) was 94 (90–98). Non-specific binding was determined in the presence of 10 μM NC-NH_2 and corresponded to 30% of the total binding.

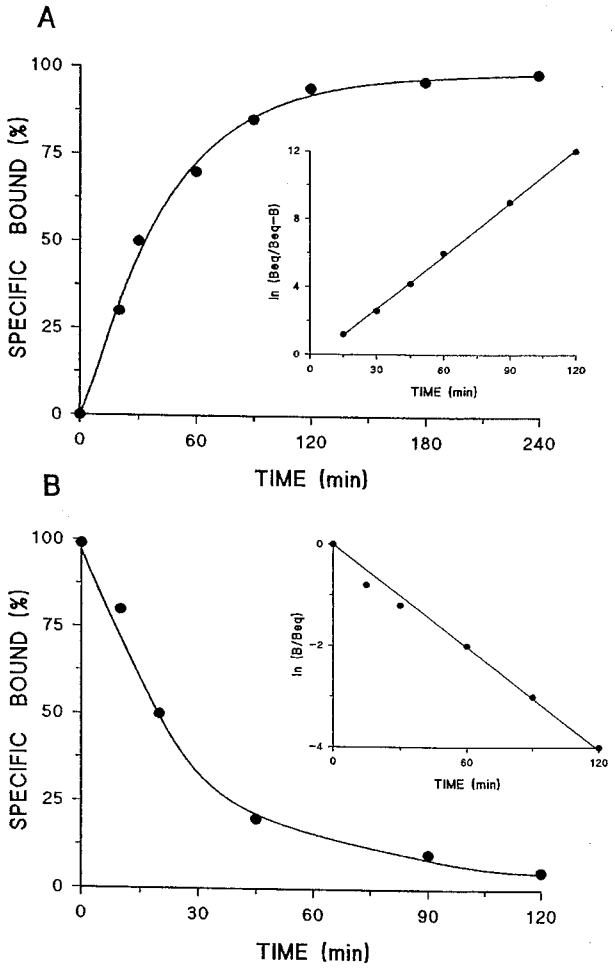

Figure 2 (A) Kinetics of 0.5 nM [^{3}H]-NC-NH_2 binding to mouse forebrain membranes with association curves representative of a single experiment. Inset, first-order plots of [^{3}H]-NC-NH_2 binding. Beq, amount of [^{3}H]-NC-NH_2 bound at equilibrium; B, amount of [^{3}H]-NC-NH_2 bound at each time. Association rate constant was: $K_{+1}=0.07$ (0.061–0.080) min nM^{-1} (B) Kinetics of 0.5 nM [^{3}H]-NC-NH_2 binding to mouse brain membranes with dissociation curves representative of a single experiment. Inset, first-order plots of [^{3}H]-NC-NH_2 binding. Beq, amount of [^{3}H]-NC-NH_2 bound to equilibrium; B, amount of [^{3}H]-NC-NH_2 bound to each time. Dissociation rate constant was: $K_1=0.034$ (0.030–0.038) min^{-1}. These experiments were performed at 25°C using a membrane concentration of 200 μg 100 μl^{-1}.

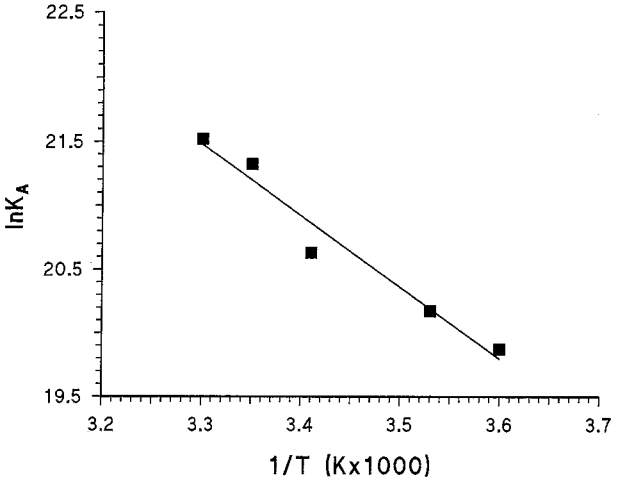

Figure 3 Van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_D$, of [^{3}H]-NC-NH_2. The plot is essentially linear in the temperature range investigated (5–30°C).

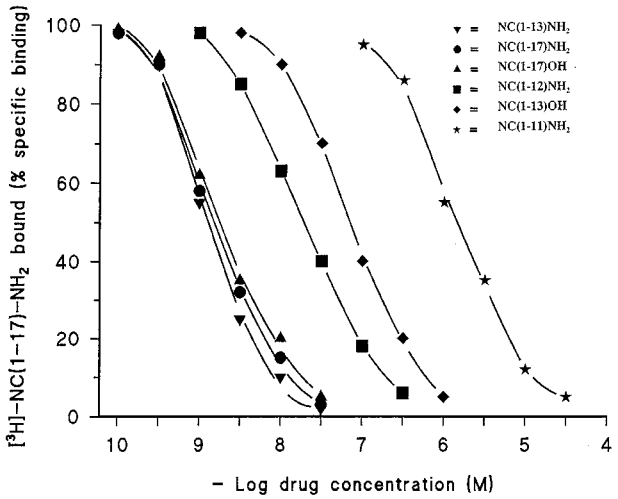

Figure 4 Inhibition curves of specific [^{3}H]-NC-NH_2 (0.5 nM) binding to mouse brain membranes by NC and NC fragments. Curves are representative of a single experiment from a group of 4 independent experiments. Non-specific binding was determined in the presence of 10 μM NC-NH_2 and corresponded to 30% of the total binding.

Some opioid receptor ligands (either selective or non selective agonists and antagonists) were also tested (see third group of compounds reported in Table 1) and they were found to be inactive at concentrations up to 10–50 μM.

Discussion

[^{3}H]-NC-NH_2 binding

Basic parameters (K_D and B_{max}) describing the binding of NC to ORL_1 receptors of the mouse brain have been reported by Mathis *et al.* (1997) (K_D 0.896 nM; B_{max} 233 fmol mg^{-1} of protein) using as a ligand [^{125}I]-[Tyr^{14}]NC and by Adapa & Toll (1997) (K_D 0.38 nM; B_{max} 64 fmol mg^{-1} of protein) with [^{3}H]-[Tyr^{14}]NC. The results obtained in the present study (K_D 0.55 nM; B_{max} 94 fmol mg^{-1} of protein) with the new ligand [^{3}H]-NC-NH_2 confirm the findings of the other authors. However, while Mathis *et al.* (1997) were also able to detect another site with extremely high affinity (K_D 0.004 nM) but very low mass (B_{max} 31 fmol mg^{-1} of protein), Adapa & Toll (1997) also found a low affinity (K_D 8.3 nM) but very abundant (B_{max} 1340 fmol mg^{-1} of protein) site. A single (see linearity of the Scatchard plot), homogeneous site was demonstrated in the present study. It is therefore suggested that the natural sequence [^{3}H]-NC-NH_2, which is partially protected from degradation because of the amidation of the C-terminal acidic group and maintains full biological activity both *in vitro* (Guerrini *et al.*, 1997) and *in vivo* (Calo' *et al.*, 1998b), is an appropriate ligand for binding assay of the ORL_1 receptor. This ligand is proposed for the use in other species (for instance in man) where great discrepancies have been reported by the use of [^{3}H]-NC on one side (Butour *et al.*, 1997; Ma *et al.*, 1997) and [^{125}I]-[Tyr^{14}]NC (Fawzi *et al.*, 1997; Makman *et al.*, 1997) on the other. Differences in affinities (by 30–100 fold) measured with the two ligands may not be compatible with the same biological entity.

The interaction of [^{3}H]-NC-NH_2 with the ORL_1 receptor of the mouse brain was also studied with a thermodynamic approach whose basic principles have been discussed by Gilli *et al.* (1994). In short, while a large number of values of the standard free energy of the binding equilibrium are available as $\Delta G^\circ = -RT\ln K_A = RT\ln K_D$, relatively few measurements of its

Table 1 K_i values for nociceptin, NC fragments, NC analogues, and for opioid compounds, obtained by competition with [^{3}H]-NC-NH_2 binding to mouse forebrain membranes

Compound	K_i (nM)
NC fragments	
NC-OH	1.97 (1.41–2.75)
NC-NH_2	0.80 (0.68–0.94)
NC(1–13)-NH_2	0.75 (0.66–0.85)
NC(1–13)-OH	120 (107–133)
NC(1–12)-NH_2	27 (24–31)
NC(1–12)-OH	>10.000
NC(1–11)-NH_2	2199 (2073–2333)
NC(1–9)-NH_2	>50.000
NC(1–5)-NH_2	>50.000
NC(1–4)-NH_2	>50.000
NC(13–17)-OH	>50.000
NC analogues	
[Tyr^1]NC-NH_2	1.80 (1.59–2.03)
[Tyr^1]NC(1–13)-NH_2	4.51 (3.90–5.22)
[Leu^1]NC(1–13)NH_2	2.80 (2.58–3.03)
[DPhe^1]NC(1–13)-NH_2	2499 (1913–3264)
[Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1–13)-NH_2	10.10 (8.81–11.58)
[DPhe$^1\Psi$(CH_2-NH)Gly^2]NC(1–13)-NH_2	150 (129–174)
[Phe^2]NC(1–13)-NH_2	8020 (7509–8566)
[DPhe^2]NC(1–13)-NH_2	>10.000
[Phe^3]NC(1–13)-NH_2	90 (85–97)
[DPhe^3]NC(1–13)-NH_2	>10.000
[Leu^4]NC(1–13)-NH_2	8993 (8366–9667)
[DPhe^4]NC(1–13)-NH_2	7099 (6918–7285)
Opioid compounds	
Naloxone	>50.000
Morphine	>50.000
CTOP	>50.000
nor-BNI	>10.000
Dmt-Tic-OH	>50.000
Dermorphine	>50.000
U69593	>50.000
Deltorphin I	>50.000

The data are mean of at least 4 experiments done in duplicate; the 95% confidence limits are given in parenthesis.

thermodynamic components ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$) are known in spite of their remarkable physical importance. In fact, the standard enthalpy ΔH° can be considered as a quantitative indicator of the changes in intermolecular bond energies (hydrogen bonding and van der Waals interactions) occurring during the binding, while the standard entropy ΔS° is most likely a good indicator of the rearrangements undergone by the solvent (water) molecules during the same process.

The main purpose of the thermodynamic experiments described above, was to characterize, at the molecular level, the mechanism of the interaction of [^{3}H]-NC-NH_2 with the ORL_1 receptor. The linearity of the van't Hoff plot for [^{3}H]-NC-NH_2 binding indicates that the ΔCp° values of the drug interaction is nearly zero. This means that ΔH° and ΔS° values are not significantly affected by changes of temperature, at least over the range investigated (5–30°C). On the other hand, such a linearity of the van't Hoff plot in a defined range of temperatures (usually 0–25/30°C) appears to be a common feature of non-solubilized membrane receptors so far studied from a thermodynamic point of view (Gilli *et al*., 1994). Thermodynamic data indicate that the binding of [^{3}H]-NC-NH_2 is totally entropy driven ($\Delta S^\circ = 330$ J mol K^{-1}; $\Delta H^\circ = 45{,}980$ J mol K^{-1}). This result is in qualitative agreement with that previously reported by other authors (Hitzemann *et al*., 1985) and by our group (Borea *et al*., 1988) for the binding of agonist ligands to opioid receptors of the μ, δ and κ types, which also show an entropy driven binding. Some quantitative differences (in particular a stronger entropy drive) between the present results and the findings mentioned above can be possibly attributed to differences of receptor types (NC versus opioid receptors) and/or animal species (mouse versus rat or guinea pig).

Displacement studies

Various peptides, fragments of NC or analogues of the shortest fully active sequence NC(1–13)-NH_2 were used in competition with [^{3}H]-NC-NH_2 in order to estimate their affinities and then correlate these data with those measured in biological assays. As shown by the displacement curves, NC-NH_2 and NC(1–13)-NH_2 were more potent than the corresponding free acids, suggesting that the system contains peptidases which may interfere with the assay by reducing the peptide concentrations. The use of a C-terminal protected ligand ([^{3}H]-NC-NH_2) appears therefore to be fully justified. The minimum active sequence is NC(1–13)-NH_2 since NC(1–12)-NH_2 loses affinity and NC(1–9)-NH_2 does not compete any more. These findings are in full agreement with the data of the biological assays recently published (Calo' *et al*., 1997, Guerrini *et al*., 1997). Indeed, the order of affinity that has been established with the various NC fragments utilized in the present experiment is exactly the same as that found in biological assays in the electrically stimulated mouse *vas deferens* (Guerrini *et al*., 1997) and in the mouse tail withdrawal assay (Calo' *et al*., 1998b). The same applies to data with a series of peptides in which modifications were made at the N-terminal. Thus, the replacement of Phe^1 with Tyr does not affect the binding properties of both NC-NH_2 and NC(1–13)-NH_2 to ORL_1 receptors. However, this modification confers to these molecules the ability to activate the μ-receptor, as reported by us (Calo' *et al*., 1997) and other groups (Shimohigashi *et al*., 1996). Substitution of Phe^1 with Leu did not modify binding affinity while the same substitution caused a dramatic decrease of affinity when applied to Phe^4. Again, this is in accord to biological assay findings (Guerrini *et al*., 1997) and point to the functional importance of Phe^4 in nociceptin (Dooley & Houghten, 1996; Guerrini *et al*., 1997). The replacement of Gly^2 or Gly^3 by L or DPhe induced an important decrease or total elimination of binding affinity. The addition of an aromatic residue of L or D chirality between Phe^1 and Phe^4 eliminated the ability of the peptide to occupy the NC receptor. These results are in line with what we found testing the same compounds in the mouse *vas deferens* (Guerrini *et al*., 1997). The comparison of the two sets of data (receptor binding on brain membranes and bioassay in the *vas deferens*) indicates that the structural requirements of NC for receptor occupation by agonists are very similar in the peripheral and the central receptor sites. As expected, none of the standard opioid receptor ligands (selective and non selective opioid receptor agonists and antagonists) compete with the binding of [^{3}H]-NC-NH_2 in the mouse forebrain membranes, suggesting that the site labelled by [^{3}H]-NC-NH_2 differs from classical opioid receptors.

The use of a pseudopeptide bond between Phe^1 and Gly^2 in NC(1–13)-NH_2 allowed us to obtain the first ORL_1 antagonist (Guerrini *et al*., 1998). This molecule showed a 10 fold decrease of binding affinity as compared to NC(1–13)-NH_2. These results are in line with the findings obtained in the mouse *vas deferens* where a similar difference of potency can be detected between NC(1–13)-NH_2 (EC_{50} 18 nM) (Calo' *et al*., 1997) and [F/G]NC(1–13)-NH_2 (EC_{50} 177 nM) (Guerrini *et al*., 1998). Although this latter molecule has the same relative potency on central and peripheral NC receptors, it acts as ORL_1 antagonist in the periphery (Guerrini *et al*., 1998) and as ORL_1 agonist in the central nervous system (Calo' *et al*., 1998b, Xu *et al*., 1998). Studies are under way to verify if the different activities of [F/G]NC(1–13)-NH_2 in the central nervous system and in the periphery may be due to the existence of multiple receptors for NC.

This work was supported by 60% and 40% grants, from the Italian Ministry of the University (MURST).

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(Received March 17, 1998
Revised July 21, 1998
Accepted September 14, 1998)