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Presence of nociceptin (orphanin FQ) receptors in rat retina: Comparison with receptors in striatum

Maynard H. Makman *, Bernyce Dvorkin

Departments of Biochemistry and Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA

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Abstract

Nociceptin (orphanin FQ), a heptadecapeptide with some sequence homology to dynorphin A, has been proposed as an endogenous ligand for a previously cloned orphan receptor with significant homology to opioid receptors. Utilizing [^{125}I][Tyr^{14}]nociceptin as ligand, saturable and high affinity nociceptin binding sites were detected and characterized in rat retina and striatum. For retina, $B_{max} = 44.0 \pm 4.5$ fmol/mg and $K_d = 32.4 \pm 2.7$ pM; for striatum, $B_{max} = 51.6 \pm 7.7$ fmol/mg and $K_d = 98.6 \pm 11.3$ pM. In competition studies, nociceptin bound with picomolar affinity, dynorphin A with nanomolar affinity, naloxone and dynorphan A-(1–8) with micromolar affinity, while [des-Tyr^1]dynorphin (dynorphin A-(2–17)), several other opioids, morphine and benzomorphans failed to compete for binding at 1–10 μM. Gpp(NH)p plus NaCl markedly decreased binding, consistent with involvement of a G protein-linked receptor. It is concluded that rat retina contains nociceptin receptors similar in concentration to those present in striatum. Properties of both the retinal and the striatal receptors are similar to those previously found for rat hypothalamus. © 1997 Elsevier Science B.V.

Keywords: Nociceptin; Orphanin FQ; Retina; Striatum; Dynorphin A; Opioid

1. Introduction

Nociceptin (orphanin FQ), a heptadecapeptide isolated from rat and porcine brain, has been proposed to be an endogenously occurring agonist of the orphan ORL_1 receptor, a G protein-coupled receptor (Meunier et al., 1995; Reinscheid et al., 1995; Mollereau et al., 1996; Nothacker et al., 1996). Sequences of rat, mouse and human cDNAs indicate significant sequence homology of ORL_1 to opioid receptors (Bunzow et al., 1994; Chen et al., 1994; Fukada et al., 1994; Mollereau et al., 1994; Wang et al., 1994; Lachowicz et al., 1995; Wick et al., 1995). However, potent radioligands to μ-, δ- and κ-opioid receptors failed to bind to ORL_1 receptors of transfected cells. Also, etorphine, a potent opioid agonist, inhibited adenylyl cyclase of cells expressing ORL_1 only at micromolar concentration (Mollereau et al., 1994). In contrast, nociceptin at extremely low (picomolar) concentration inhibited adenylyl cyclase of transfected cells expressing ORL_1 receptors (Meunier et al., 1995; Reinscheid et al., 1995). High affinity radioligand binding of [^{125}I][Tyr^{14}-nociceptin to receptors of transfected cells was previously reported (Reinscheid et al., 1995), as was the binding of a tritiated analog of nociceptin to whole brain (Dooley and Houghton, 1996). In situ hybridization in rat and mouse brain has revealed a relatively high abundance of ORL_1 transcripts in hypothalamic areas, as well as in limbic and certain other regions and also a relatively low abundance in striatum (caudate-putamen) (Bunzow et al., 1994; Chen et al., 1994; Fukada et al., 1994; Mollereau et al., 1994). Radioligand binding studies of nociceptin receptors in specific brain regions was reported previously for adult rat and fetal human hypothalamus, utilizing [^{125}I][Tyr^{14}]nociceptin as ligand (Makman et al., 1997b). The present study further utilizes [^{125}I][Tyr^{14}]nociceptin to demonstrate the presence of receptors with picomolar affinity for nociceptin in rat retina as well as in rat striatum.

2. Materials and methods

2.1. Materials

[D-Ala^2, D-Leu^5]enkephalin and [D-Ala^2, *N*-Me-Phe^4, Gly-ol]enkephalin were from Sigma, other opioid peptides and nociceptin were from Peninsula, naloxone was from

* Corresponding author. Tel.: (1-718) 430-3032; Fax: (1-718) 430-8565.

Endo, ethylketocyclazocine and cyclazocine were from Sterling-Winthrop and *N*-allylnormetaocine was from Research Biochemicals. [^{125}I][Tyr^{14}]nociceptin (2200 Ci/mmol; NEX-338) was from New England Nuclear.

2.2. Membrane preparation

Binding studies utilized retinal and striatal membranes of both male and female Sprague–Dawley rats (150–200 g). Tissue was homogenized in 50 mM Tris buffer (pH 7.5) at 4°C with use of a Brinkmann Polytron (15 s, setting 5), the homogenate kept on ice for 15 min, followed by centrifugation at $50\,000 \times g$ for 10 min. Membranes were then washed two times by resuspension in Tris buffer and centrifugation (Cruciani et al., 1993; Dobrenis et al., 1995; Makman et al., 1997b). Membranes were routinely freshly prepared for each assay, since freezing and storage of membranes at −70°C led to appreciable loss of binding sites, as previously reported (Makman et al., 1997b).

2.3. Receptor binding assay

Binding assays were kept at 22°C for 60 min in 0.25 ml containing 50 mM Tris buffer (pH 7.5), 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% bovine serum albumin, 40–150 μg membrane protein and [^{125}I][Tyr^{14}]nociceptin (present at 70–80 pM for displacement studies and at 10–400 pM for saturation studies). Incubation was terminated by chilling on ice, rapid filtration through Whatman GF/B filters, followed by three 5 ml rinses with ice-cold solution containing 50 mM Tris buffer (pH 7.5), 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 0.1% bovine serum albumin. The filters were presoaked in 0.5% polyethylenimine for 60 min, an essential step for obtaining low non-specific binding. In separate experiments (not shown), with the assay conditions used, binding equilibrium was readily attained and the amount of radioligand bound was proportional to the membrane protein present in the incubation. Specific binding was defined as total binding minus that with 10^{-7} M unlabeled nociceptin (Peninsula Laboratories). Data were subjected to iterative non linear analysis with a PC software program (Graphpad Prism, Graphpad Software), as previously described and the results also confirmed by the program Ligand (Makman et al., 1997b). Data were pooled for male and female rats, since no significant sex difference was found. Values are means ± S.E.M. (number of separate experiments in parentheses).

3. Results

3.1. Saturation binding studies

For both retinal and striatal membranes, with [^{125}I][Tyr^{14}]nociceptin as ligand, direct analysis of the data obtained in saturation experiments, as well as Scatchard analysis of those data, indicated a single distinct saturable binding site of very high affinity (representative data and analyses shown in Fig. 1). In each case, including the additional experiments not shown, the best fit for a two site analysis of the saturation data was not significantly better than that for a one site analysis (Fig. 1, upper panel). Scatchard analyses (Fig. 1, middle panel) and Hill plots (Fig. 1, lower panel) of the saturation data supported the presence of a single site with a Hill coefficient (n_H) close

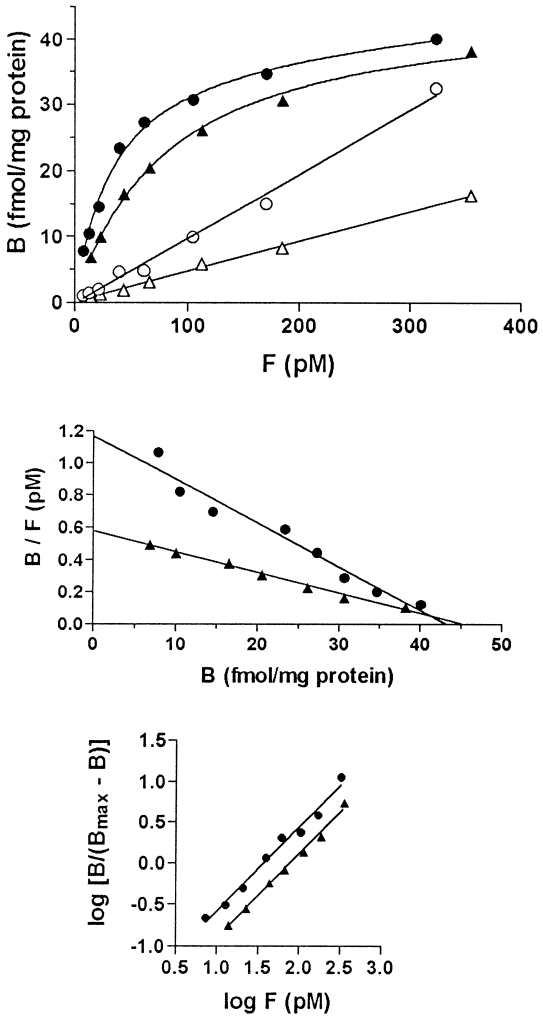

Fig. 1. Upper panel: Saturation analysis of specific [^{125}I][Tyr^{14}]nociceptin binding sites of rat retina (circles) and striatum (triangles). Values shown are for a representative experiment for each tissue (mean values for all experiments given in the text). Analysis (closed symbols, specific binding; open symbols, nonspecific binding) indicated very good fits for both retina ($R^2 = 0.9929$) and striatum ($R^2 = 0.9962$) to a single site (solid lines). B_{max} values were 43.7 fmol/mg protein for retina and 45.9 fmol/mg for striatum; K_d values were 38.6 pM for retina and 82.7 pM for striatum. A two site analysis did not afford a significantly better fit for either retina ($P = 0.554$) or striatum ($P = 0.095$). Each fmol/mg protein bound represented 100 cpm (50 μg protein/assay incubation) for retina and 200 cpm (100 μg protein/assay incubation) for striatum. Middle panel: Scatchard plots of saturation data. Lower panel: Hill plots of saturation data ($n_H = 1.017 \pm 0.053$ for retina and 1.017 ± 0.035 for striatum).

Table 1
B_{max} and K_d values for rat CNS [^{125}I][Tyr^{14}]nociceptin binding sites

Tissue	B_{max} (fmol/mg protein)	K_d (pM)
Retina	44.0 ± 4.5	32.4 ± 2.7
Striatum	51.6 ± 7.7	98.6 ± 11.3
Hypothalamus [a]	127 ± 28	103 ± 20

Each value represents the mean ± S.E.M. for 3 separate experiments such as shown in Fig. 1. Additional details are given in the text.
[a] Data from Makman et al. (1997b).

to 1 for both retina and striatum. Based on one site analysis of saturation data of independent experiments, mean K_d values were 32.4 pM for retina and 98.6 pM for striatum; mean B_{max} values were 44.0 ± 4.5 fmol/mg for retina and 51.6 ± 7.7 fmol/mg for striatum (Table 1). These results indicate that the concentrations of receptor sites in retina and striatum are appreciable, approximately comparable to one another and approximately 40% that reported previously for hypothalamus in assays carried out under the same conditions (Table 1; Makman et al., 1997b; see Section 4).

3.2. Competitive binding studies

Competition studies indicated the specificity and selectivity of nociceptin binding, as well as the similar affinities of compounds for receptors in retina and striatum. The results of these studies are summarized in Fig. 2; also, the mean K_i values for nociceptin, dynorphin A, dynorphin A-(1–8) and naloxone binding are given in Table 2. For both retina and striatum, nociceptin bound with picomolar affinity, dynorphin A with nanomolar affinity, and naloxone and dynorphan A-(1–8) with micromolar affinity. Morphine, ethylketocyclazocine, cyclazocine, *N*-allylnormetazocine, as well as a number of opioid peptides including [D-Ala^2, D-Leu^5]enkephalin, [D-Ala^2, *N*-Me-Phe^4, Gly-ol]enkephalin and [D-Ala^2]deltorphin 1 failed to compete for binding when present at 1–10 μM (not shown). [Des-Tyr^1]dynorphin (dynorphin A-(2–17)) and dynorphin A-(6–17) also failed to compete (Fig. 2), further indicating a high degree of specificity for binding (see also below).

For the majority of competition experiments, sigmoidal analysis with variable slope (variable Hill coefficient, n_H) did not result in a significantly better fit than did an analysis with a fixed slope with $n_H = 1$ (Fig. 2). For all competition experiments with nociceptin, dynorphin A, dynorphin A-(1–8) and naloxone, for both retina and striatum, the average n_H was 0.79, raising the possibility of some degree of cooperative binding. In all instances variable slope sigmoidal analysis resulted in a better fit than did a two-site analysis. Thus, neither the saturation studies nor the competition studies provided evidence for the presence of more than one binding site.

Dynorphin A-(2–17) ([des-Tyr^1]dynorphin A) not only failed to compete for binding, but at higher concentrations also caused a moderate but significant increase in specific binding (Fig. 2), without change in non-specific binding (not shown). Dynorphin A-(6–17) also failed to compete for binding as indicated above and caused a more marked

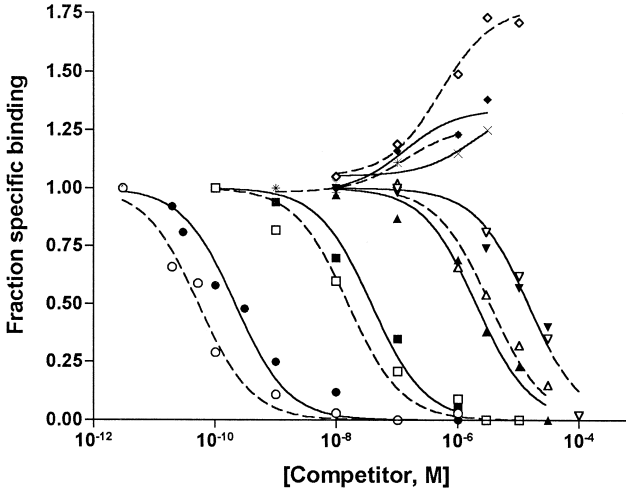

Fig. 2. Competition for specific [^{125}I][Tyr^{14}]nociceptin binding sites of rat retina (solid lines, closed symbols and asterisks) and striatum (dashed lines, open symbols and crosses). Circles, nociceptin; squares, dynorphin A; upright triangles, dynorphin A(1–8); inverted triangles, naloxone; crosses and asterisks, dynorphin A(2–17); diamonds, dynorphin A(6–17). Each value for fraction of specific binding is the mean of 3–5 separate experiments. Each line represents the best fit for a sigmoidal dose response with fixed slope, $n_H = 1$. Log EC_{50} values ± S.E.M.: For retina: nociceptin, −9.59 ± 0.11; dynorphin A, −7.15 ± 0.18; dynorphin A(1–8), −5.69 ± 0.07; naloxone, −4.84 ± 0.07. For striatum: nociceptin, −10.27 ± 0.06; dynorphin A, −7.62 ± 0.17; dynorphin A(1–8), −5.46 ± 0.09; naloxone, −4.88 ± 0.06. Mean K_i values ± S.E.M. are given in the text. The n_H values obtained from sigmoidal analysis with variable slope (Variable slope analysis yielded a significantly better fit ($P < 0.05$) than analysis with a fixed $n_H = 1$, only for nociceptin and dynorphin A(1–8) with retinal membranes, and for dynorphin A with striatal membranes.) (lines not shown) were as follows: For retina: nociceptin, 0.63 ± 0.08; dynorphin A, 0.64 ± 0.17; dynorphin A(1–8), 0.91 ± 0.15; naloxone, 0.65 ± 0.03. For striatum: nociceptin, 0.94 ± 0.18; dynorphin A, 0.57 ± 0.06; dynorphin A(1–8), 0.77 ± 0.12; naloxone, 1.19 ± 0.17.

Table 2
Affinities of drugs and peptides for rat central nervous system [^{125}I][Tyr^{14}]nociceptin binding sites

Competitor	K_i (nM)		
	retina	striatum	hypothalamus [a]
Nociceptin	0.062 ± 0.007	0.029 ± 0.005	0.031 ± 0.008
Dynorphin A	10.8 ± 1.4	8.5 ± 1.0	43 ± 7
Dynorphin A(1–8)	582 ± 39	1830 ± 110	1820 ± 380
Naloxone	4090 ± 420	7110 ± 550	12100 ± 2900

Each value represents the mean ± S.E.M. for 3–5 separate experiments. Additional details are given in the text and in the legend of Fig. 2. Morphine, ethylketocyclazocine, cyclazocine, *N*-allylnormetazocine, [D-Ala^2, D-Leu^5]enkephalin, [D-Ala^2, *N*-Me-Phe^4, Gly-ol]enkephalin and [D-Ala^2]deltorphin 1, [des-Tyr^1]dynorphin (dynorphin A-(2–17)) and dynorphin A-(6–17) failed to compete for binding at concentrations of 1–10 μM.
[a] Data from Makman et al. (1997b).

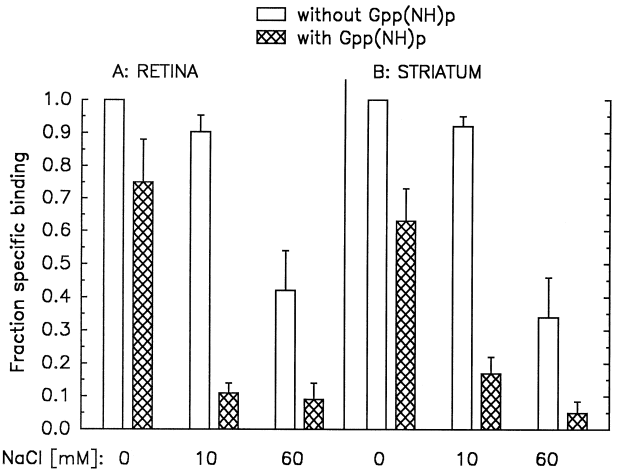

Fig. 3. Influence of sodium ion and Gpp(NH)p on specific [^{125}I][Tyr^{14}]nociceptin binding to rat retinal and striatal membranes. Open bars indicate incubations without Gpp(NH)p and cross-hatched bars indicate incubations with 100 μM Gpp(NH)p. Each value represents the fraction of control specific binding and is the mean ± S.E.M. of 3–5 separate experiments.

increase in specific binding, particularly in striatum (Fig. 2). Thus, dynorphin A-(6–17) increased striatal binding by $19 \pm 4\%$ at 10^{-7} M, by $49 \pm 8\%$ at 10^{-6} M, by $76 \pm 7\%$ at 3×10^{-6} M and by $66 \pm 11\%$ at 10^{-5} M (mean % increase ± S.E.M.; $n = 3–5$) (see Section 4).

3.3. Influence of Gpp(NH)p and NaCl on binding

Agonist binding to G_i-linked receptors and the subsequent inhibition of adenylyl cyclase are well known to be sensitive to Na^+ as well as to GTP or GTP analogues such as Gpp(NH)p (Birnbaumer, 1990; Cruciani et al., 1993). Na^+ is generally required to maximally increase the rate of dissociation of agonist from G_i-linked receptors in the presence of guanine nucleotide. As shown in Fig. 3, for both retina and striatum, specific nociceptin binding was decreased by approximately 40% in the presence of either Gpp(NH)p or 60 mM NaCl alone. The presence of 10 mM NaCl had no effect by itself but greatly potentiated the inhibitory effect of Gpp(NH)p (less than 10% specific binding remaining). Gpp(NH)p and NaCl had no effect on nonspecific binding; also ATP was without effect (not shown).

4. Discussion

The present studies represent an extension of our previous studies of hypothalamus. Evidence is provided for the presence of nociceptin (orphanin FQ) receptor sites in both retina and striatum. Furthermore, the experiments involving Gpp(NH)p and sodium ion indicate that nociceptin binds to the receptor as an agonist and that the receptor is linked to a G protein other than the Na^+-insensitive G_s, most likely to G_i. Nociceptin receptor binding was previously reported to be influenced by GppNHp and Na^+ both for receptors expressed in stably transfected chinese hamster ovary cells ((Butour et al., 1997) and for receptors in rat and human hypothalamus (Makman et al., 1997b). The results presented here for the relative affinities of compounds for receptors in retina and striatum are also essentially in agreement with previous results for nociceptin binding in hypothalamus (Makman et al., 1997b). Thus, retinal and striatal receptors were found to differ from hypothalamic receptors only with respect to receptor density.

The retina does not appear to have been examined in previous studies with respect to the possible presence of ORL_1 (nociceptin) mRNA or receptor, or of nociceptin mRNA or protein. The present examination of nociceptin receptors in retina arose from our recent studies of opiate alkaloid-selective μ_3 receptors in retina (Makman et al., 1996), as well as further studies indicating the presence of classical peptide-sensitive μ and κ opioid receptor subtypes in rat retina (M.H. Makman, unpublished results). We initially investigated the possibility that nociceptin might be an endogenous ligand for the μ_3 receptor, a possibility which proved not to be the case (Makman et al., 1997a). Based on the present study, rat retina contains nociceptin receptors at concentrations essentially the same as those found in striatum. It will be of interest to investigate further the nociceptin system in retina, including the possible presence and localization of nociceptin itself, as well as the function of this system in retina. With respect to function, it has recently been reported that in rabbit retina, nociceptin inhibits the light-evoked neuronal release of acetylcholine (Neal et al., 1997).

The receptor density in striatum, about 40% that found in hypothalamus (Table 1), was somewhat higher than predicted based on mRNA levels in these two regions as indicated by in situ hybridization (see Section 1). The localization of the nociceptin system within striatum, as well as the possible function of this system in striatum, at present are essentially unknown.

Prior to the discovery of nociceptin and its affinity for the ORL_1 receptor, Zhang and Yu (1995) proposed that the structurally related dynorphins might be endogenous ligands for ORL_1. Upon expression in *Xenopus* oocytes, ORL_1 coupled to a G protein-linked K^+ channel that was activated by dynorphins in a dose-dependent manner, with dynorphan A exhibiting an EC_{50} value of 45 nM and naloxone competitively inhibiting the activation with an apparent K_e of 1.4 μM; dynorphin A-(1–8) had a weak effect at 1 μM and Met- and Leu-enkephalin were inactive (Zhang and Yu, 1995). Those results are in close agreement with the present results for binding affinities of these opioid peptides and naloxone to rat retinal and striatal receptors. While endogenous concentrations of dynorphins probably would be insufficient to activate nociceptin receptors in vivo, activation might well occur upon exoge-

nous application of dynorphins at nanomolar concentrations, either in vivo or in vitro. Thus, possible activation of nociceptin receptors should be considered in any studies involving dynorphins in the nanomolar or higher concentration range.

Dynorphin A-(6–17) and, to a lesser extent, dynorphin A-(2–17) not only failed to compete for binding, but in fact brought about a significant increase in specific binding. The reason for this increase is not known. One possible explanation is that the increase might be due to a protective or stabilizing effect of the dynorphin A-(6–17) sequence on the radioligand. If that were the case, then the actual concentration of receptors and/or affinity for radioligand measured (in the absence of dynorphin A-(6–17)) might have been underestimated. However, that possibility seems unlikely, since the presence of nociceptin A-(6–17) was not found to change the affinity for nociceptin (data not shown). Another possible explanation for the increase in specific binding is that the dynorphin A-(6–17) sequence might bind (with relatively low affinity) to the nociceptin receptor and thereby render that component of the nociceptin binding site more exposed or available to nociceptin itself. It may be noted that the dynorphin A-(6–17) sequence is believed not to be primary but nevertheless to contribute to binding of the complete dynorphin A sequence to opioid receptors.

Dynorphin A-(2–17), shown here to be inactive at nociceptin receptors, has previously been reported to be inactive at opioid receptors as well (Friederich et al., 1987). However, dynorphin A-(2–17), through non-opiate mechanisms, has been found to exert potent effects on the nigrostriatal motor system (Friederich et al., 1987), as well as on opiate analgesia, tolerance and withdrawal (Walker et al., 1982a,b; Takemori et al., 1993). At relatively high concentrations dynorphin A-(2–17) also was found to inhibit rat striatal adenylyl cyclase in a naloxone-independent manner Claye et al., 1996). Based on the present studies, those nonopioid effects of dynorphin A-(2–17) would also appear to be independent of the nociceptin system.

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