Characterization of Opioid-Binding Sites in Zebrafish Brain

Verónica González-Núñez, Alejandro Barrallo,1 John R. Traynor, and Raquel E. Rodríguez

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Salamanca, Salamanca, Spain (V.G.-N., A.B., R.E.R.); Instituto de Neurociencias de Castilla y León, Salamanca, Spain (V.G.-N., R.E.R.); and Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan (J.R.T.)

Received July 29, 2005; accepted October 4, 2005

ABSTRACT

The pharmacological profile of opioid-binding sites in zebrafish brain homogenates has been studied using radiolabeled binding techniques. The nonselective antagonist [3H]diprenorphine binds with high affinity (K D = 0.27 ± 0.08 nM and B max = 212 ± 14.3 fmol/mg protein), displaying two different binding sites with affinities of K D1 = 0.08 ± 0.02 nM and K D2 = 17.8 ± 9.18 nM. The nonselective agonist [3H]bremazocine also binds with high affinity to zebrafish brain membranes but only displays one single binding site with a K D = 1.1 ± 0.9 nM and a B max = 705 ± 19.3 fmol/mg protein. Competition binding assays using [3H]diprenorphine and several unlabeled ligands were performed. The synthetic selective agonists for mammalian opioid receptors DPDPE ([DPen²,D-Pen⁵]-enkephalin), DAMGO ([D-Ala²,NMe-Phe⁴,Gly⁵-ol]-enkephalin), and U69,593 [(6α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneacetamide] failed to effectively displace [3H]diprenorphine binding, whereas nonselective ligands and the endogenous opioid peptides such as dynorphin A showed good affinities in the nanomolar range, although several of the endogenous peptides only displaced approximately 50% of the specifically bound [3H]diprenorphine. Our results provide evidence that, although the selective synthetic compounds for mammalian receptors do not fully recognize the opioid-binding sites in zebrafish brain, the activity of the endogenous zebrafish opioid system might not significantly differ from that displayed by the mammalian opioid system. Hence, the study of zebrafish opioid activity may contribute to an understanding of endogenous opioid systems in higher vertebrates.

The study of the molecular mechanisms of the opioid system related to pain and drug addiction has been analyzed on countless occasions for more than 40 years, but there are some features that still remain unclear. An approach to the problem of understanding why opioid drugs can cause tolerance and dependence is to develop and use new animal models that would provide findings that could be extrapolated to higher vertebrates and ultimately to humans. The zebrafish has been widely and successfully used in molecular and developmental biology (Ingham, 1997; Fishman, 2001; Golling et al., 2002; Pichler et al., 2003), and it also has been proved to be a valid model to study the effects of some drugs of abuse, such as cocaine (Darland and Dowling, 2001) and ethanol (Dlugos and Rabin, 2003). Our group has studied the zebrafish opioid system, and we have characterized several receptors (Barrallo et al., 2000; Rodriguez et al., 2000) and five opioid propeptide genes (González-Núñez et al., 2003a,b,c) so far. Our previous results indicate that the zebrafish receptors as well as the endogenous peptides are similar to their mammalian homologues, thus indicating that the opioid system has been well conserved throughout the evolution of vertebrates. Hence, we propose the zebrafish as an organism where the study of the opioid system and its interactions with different drugs can be easily evaluated in basic research and that the results can be applied to the mammalian opioid system. However, to standardize the use of the zebrafish in opioid research, it is necessary to study the binding characteristics of different opioid ligands in this organism. Given that the action of a drug on the brain is usually due to its interactions with different tissue-specific structures, we believe that it is important to analyze the pharmacological profile of opioids using zebrafish brain homogenates in addition to opioid receptors in heterologous expression systems.

ABBREVIATIONS: DPDPE, [DPen²,D-Pen⁵]-enkephalin; DAMGO, [D-Ala²,NMe-Phe⁴,Gly⁵-ol]-enkephalin; U69,593, [(6α,7α,8β)-(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneacetamide; BW373U86, ((±)-4-[(R')-25]-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl][3-hydroxyphenyl[methyl]-N,N-diethylbenzamide hydrochloride.

900
In this work, we present a pharmacological characterization of the opioid-binding sites in zebrafish brain homogenates using radioligand binding techniques with drugs that are commonly used to label opioid-binding sites in mammalian brain, such as the nonselective antagonist diprenorphine and the nonselective agonist/antagonist bremazocine. The binding ability of other nonlabeled drugs has been evaluated by means of competition binding assays using \([3H]\)diprenorphine as the tritiated ligand. The results that we present here give new insight on the pharmacological profile of the opioid system in zebrafish.

Materials and Methods

Drugs and Radioligands. \([3H]\)Diprenorphine (50 Ci/mmol) and \([3H]\)bremazocine (30 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston MA). Morphine was obtained from the Spanish Ministry of Health, and the other unlabeled ligands were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

Animals. Adult zebrafish *Danio rerio* from both sexes were obtained from a local pet supplier, maintained at 25 to 28°C, and fed once a day. In all experiments, adequate measures were taken to minimize pain or discomfort, and animals were handled according to the guidelines of the European Communities Council directive of 24 November 1986 (86/609/EEC), the Spanish Ethical Committee, the National Institutes of Health, and the University of Michigan Committee on the Use and care of Animals.

Membrane Preparation. Zebrafish brains were extracted and kept on dry ice and homogenized with a Kinematika Polytron (Lucern, Switzerland) in assay buffer: 50 mM Tris-HCl, pH 7.4, with protease inhibitors (0.1 mg/ml bacitracin, 3.3 μM captopril, and 0.33 μM thiorphan; Sigma-Aldrich). The homogenates were centrifuged at 6000g for 15 min at 4°C, and the pellet was washed once in 50 mM Tris-HCl, pH 7.4, with protease inhibitors (assay buffer), homogenized, and centrifuged. Membranes were resuspended in ice-cold assay buffer, and protein concentration was determined by the Lowry method (Onishi and Barr Modification).

Saturation Binding Assays. For saturation binding assays, 30 μg of membrane homogenate were incubated with increasing concentrations of \([3H]\)diprenorphine (50 Ci/mmol) (PerkinElmer Life and Analytical Sciences) or \([3H]\)bremazocine (30 Ci/mmol) for 1 h at 25°C in a final volume of 250 μl, using 10 μM naltroxone (Sigma-Aldrich) to determine nonspecific binding. After incubation, 4 ml of ice-cold assay buffer was added, and the mixture was rapidly filtered using a cell harvester (Brandel Inc., Gaithersburg, MD) and washed twice on GF/B glass-fiber filters that were presoaked with 0.2% polyethylenimine for at least 1 h. The filters were placed in scintillation vials and incubated overnight at room temperature in scintillation liquid (EcoScint A, London, UK), and radioactivity was counted using a Beckman Coulter scintillation counter (Fullerton, CA). All experiments were performed in duplicate and repeated several times.

Competition Binding Assays. Reactions were performed as described above, with the exception that the radioligand \([3H]\)diprenorphine was used at a working concentration similar to the *Ks* (in the nanomolar range) and that the experiments were incubated for 1 h at 25°C in a final volume of 500 μl. \([3H]\)Diprenorphine was displaced by several unlabeled compounds at a concentration range from 0.3 nM to 10 μM. All experiments were performed in duplicate and repeated several times.

Data Analysis. Specific binding was defined as the difference between total binding and nonspecific binding (measured in presence of 10 μM naltroxone). Data were analyzed using the Prism software (GraphPad Software Inc., San Diego, CA), and affinity constant (*Ks*), receptor density (*Bmax*), and inhibition constant (*Ki*) values for each ligand were obtained. In the case of the *Ks* values, they were calculated by means of the GraphPad software, using the correction of Cheng-Prusoff, which corrects for the concentration of radioligand used in each experiment as well as the affinity of the radioligand for its binding site (*Ks*). In all cases, data were fit to the one-site or two-site binding model. In saturation binding assays, data were fit to either nonlinear function and to the linear transformation (Scatchard plot: bound/free versus bound), whereas in competition binding assays, data were fit to the one-site and two-site competition models and compared by using the nonlinear least-squares curve fitting, which is based upon a statistical *F*-test.

Results

Saturation Binding Assays in Zebrafish Brain Membranes. The ability of \([3H]\)diprenorphine to bind opioid receptors was measured by using increasing concentrations of this radioligand in zebrafish brain homogenates. The experimental data show specific and saturable binding with a *Kd* = 0.26 ± 0.08 nM and a *Bmax* = 212 ± 14.3 fmol/mg protein (Fig. 1a) that is displaced by naltroxone, thus confirming the opioid nature of these sites. Nevertheless, analysis done with the curve-fitting program GraphPad reveals that our experimental data fit better to a two-site binding model, displaying one high-affinity site with a *K1* = 0.08 ± 0.02 nM and a *Bmax1* = 120 ± 11 fmol/mg protein and one low-affinity site with a *K2* = 17.8 ± 9.18 nM and a *Bmax2* = 370 ± 138 fmol/mg protein (*F value* = 7.158, and *p* = 0.0045). The nonspecific *κ*-agonist/μ antagonist \([3H]\)bremazocine, with a benzomorphan structure, was also used in saturation binding assays (Fig. 1b), where it presents one single binding site with a *Kd* = 1.06 ± 0.09 nM and a *Bmax* = 705 ± 19.3 fmol/mg protein.

The selective ligands \([3H]\)DPDPE (\((\text{D-Pen}^2,\text{L-Pen}^5)\)-enkephalin) (*δ*), \([3H]\)DAMGO (\((\text{D-Ala}^2,\text{N-Me-Phe}^4,\text{Gly}^5\text{-ol})\)-enkephalin) (*μ*), and \([3H]\)U69,593 [(5α,7α,8β)-(+)-N-methyl-N-(7-1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneaceticamide] (*κ*) were also used in saturation binding assays on zebrafish brain homogenates. However, none of them displayed saturable binding (data not shown), hence indicating that the highly selective ligands for mammalian receptors have reduced affinity for opioid receptors in this lower vertebrate.

Competition Binding Assays of \([3H]\)Diprenorphine in Zebrafish Brain Membranes. To analyze the binding ability of nonlabeled opioid ligands, competition binding assays with \([3H]\)diprenorphine were performed in zebrafish brain homogenates (Fig. 2, a and b; Table 1). The synthetic antagonist naltroxone, the exogenous agonist morphine, the *δ*-agonist BW373U86, and the endogenous opioid peptide dynorphin A were able to displace all of the specifically bound \([3H]\)diprenorphine, with a Hill slope not statistically different from −1. In contrast, other peptic ligands such as the enkephalins, *β*-endorphin, and the endomorphins could only displace up to 50%, and for this part of the displacement curve, the Hill slope was also not statistically different from −1. The synthetic *δ*-peptide DPDPE, the synthetic *κ*-ligand U69,593, and nociceptin failed to show an effective displacement. In the case of the ligands that were unable to displace all of the specifically bound \([3H]\)diprenorphine, we have calculated the *Ks* value for the displaced part of the curve. Our results suggest that, in our system, Met-enkephalin, *β* -endorphin, endomorphin-1, and DAMGO displace that portion of the curve with relatively high affinity, whereas Leu-enkephalin and endomorphin-2 are still poorer displacers.
Discussion

To characterize the opioid-binding sites in zebrafish brain, we have performed saturation binding assays with [3H]diprenorphine (Fig. 1a), an oripavine, nonspecific antagonist that binds to μ, δ, and κ sites with high affinity (Chang et al., 1981; Gillan and Kosterlitz, 1982; Richards and Sadee, 1985). The $K_D$ value obtained for zebrafish brain (0.26 nM) is in the same range as those reported in mammalian and amphibian brain (Chang et al., 1981; Wood and Traynor 1989; Newman et al., 1999), thus indicating that this ligand labels opioid sites in a very similar manner in different organisms among the evolutionary scale. Nevertheless, the Scatchard transformation obtained by us reveals that our results fit better to a two-site binding model, one high-affinity site and one low-affinity site, which likely represent the ability of [3H]diprenorphine to recognize different receptor populations in the zebrafish brain, unlike the mammalian system where diprenorphine is considered a nonselective opioid antagonist ligand. Besides, previous reports have described the presence of two binding sites for opioid ligands in frog brain using [3H]naloxone (Simon et al., 1984 using Rana esculenta) or [3H]diprenorphine and [3H]etorphine (Mollereau et al., 1988 using Rana ridibunda), where the authors have found similar $K_D$ values than the ones we present here for [3H]diprenorphine.

Prior studies (Gillan and Kosterlitz, 1982; Tiberi and Magnan, 1989; Simonin et al., 2001) have shown that [3H]bremazocine binds to the three types of opioid receptors present in mammalian brain. Here, we have shown that there is also [3H]bremazocine binding in zebrafish brain, although the affinity is slightly lower than in mammals ($K_D = 0.3$ nM in rodent and 1 nM in zebrafish brain). The apparent discrepancy observed between the $B_{max}$ values for [3H]diprenorphine- and [3H]bremazocine-labeled sites suggests that [3H]bremazocine recognizes a group of binding sites that do not bind [3H]diprenorphine. However, all of the sites labeled by [3H]bremazocine are opioids because they are displaced by naloxone. Moreover, [3H]bremazocine seems to have similar affinity for all of the sites to which it binds, whereas [3H]diprenorphine may show differential binding. This was unexpected, because both of these ligands are nonselective at mammalian opioid receptors and label a similar number of receptors in mammalian tissues. Nevertheless, it has been previously pointed out that bremazocine labels additional opioid-binding sites (Tiberi and Magnan, 1990) that cannot be explained in the terms of classical pharmacological μ-, δ-, or κ-sites or opioid receptor-like-binding sites (Clarke et al., 2002). Furthermore, some authors maintain that different types of ligands can label differentially the opioid-binding sites, such as arylacetamides and benzomorphans on the κ-receptor (Benyhe et al., 1992; Rusovici et al., 2004), and that these differences in binding can stand for different affinity states of the same receptor (Rusovici et al., 2004). This may relate to the differences observed here, because the
Similarly, in a study of a rabbit, 
and the teleost zebrafish, [3H]DAMGO, and [3H]U69,593 were unable to display saturable binding in zebrafish brain can be explained if we assume that the highly selective agonists for mammalian receptors might not be effective in lower vertebrates. Previous studies of our group have shown that DPDPE binds very selectively to the cloned δ-like zebrafish opioid receptor ZFOR1 (Rodriguez et al., 2000). Similarly, in a study of a μ-receptor cloned from the teleost Catostomus commersoni, the μ-agonist DAMGO was only able to poorly and incompletely displace the antagonist [3H]naloxone, although some degree of saturable binding with [3H]DAMGO was observed (Darlington et al., 1997). In a prior study by Newman et al. (2002) in Rana pipiens, a vertebrate situated between mammals and the teleost zebrafish, [3H]DPDPE, [3H]DAMGO, and [3H]U69,593 could bind to the receptors present in frog brain but with lower affinities than those reported for mammalian receptors. However, nanomolar saturable binding was still obtained for these ligands, hence suggesting differences between the amphibian and evolutionary earlier fish receptors. Some authors have described previously that there are mainly κ- and δ-sites in guinea pig cerebellum (Tiberi and Magnan, 1990) and in frog brain (Rana esculenta) because of the weak competition ability of DAMGO and the high-affinity bremazocine (Benyhe et al., 1992). This can also be the case in zebrafish brain. Nevertheless, it also should be considered that [3H]bremazocine-binding sites can be a mixture of μ-, δ-, and κ-sites that cannot be resolved in the classical pharmacological terms.

To analyze the binding ability of nonlabeled opioid ligands, competition binding assays with [3H]diprenorphine were performed. The fact that naloxone (a synthetic antagonist), dynorphin A (an endogenous opioid peptide), and morphine (an exogenous agonist) were able to displace all of the specifically bound [3H]diprenorphine indicates that the sites recognized by [3H]diprenorphine are truly opioid sites as defined by studies in mammalian brain. Furthermore, taking into consideration that dynorphin A can act as an endogenous ligand of the three cloned opioid receptors in mammals (Zhang et al., 1998), the fact that this peptide is an effective displacer in zebrafish brain homogenates may indicate that this property has been conserved throughout the vertebrate evolution. Besides, the δ-selective nonpeptidic ligand BW373U86 also proved to be an effective displacer of [3H]diprenorphine binding, in agreement with our previous work on the δ-receptor cloned from zebrafish (Rodriguez et al., 2000). The fact that nociceptin does not displace [3H]diprenorphine binding indicates that there is no neurotransmitter cross-talk between opioid and nociceptin systems.

On the other hand, the synthetic, selective compounds for mammalian opioid receptors DPDPE and U69,593 failed to show an effective displacement, thus confirming the saturation binding results. In the case of DAMGO, this compound was able to poorly displace the [3H]diprenorphine binding, only reaching 40% of effective displacement at 10 μM of the

![Fig. 2. Competition binding assays of [3H]diprenorphine and several unlabeled ligands using zebrafish brain homogenates. a, competition assays with ligands that are good displacers and thus the displacement curve covers from 100 to 0% of the specific binding. b, competition binding assays with ligands that cannot effectively displace all of the specifically bound [3H]diprenorphine; note that the y-axis only reaches 50% of the specific binding. Data points are from a single representative experiment of three independent experiments performed in duplicate. ■ naloxone, △ morphine; ▽ dynorphin A; ◊ Met-enkephalin; ● Leu-enkephalin; + DAMGO.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Ki in Zebrafish Brain</th>
<th>% Displacement at 10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naloxone</td>
<td>2.31 ± 0.10</td>
<td>100 ± 1.64</td>
</tr>
<tr>
<td>Morphine</td>
<td>20.11 ± 5.19</td>
<td>100 ± 3.41</td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>1.53 ± 0.65</td>
<td>94.41 ± 2.58</td>
</tr>
<tr>
<td>BW373U86</td>
<td>7.13 ± 1.80</td>
<td>95.94 ± 5.69</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>0.48 ± 0.05</td>
<td>54.01 ± 2.68</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>60.66 ± 55.94</td>
<td>47.37 ± 1.91</td>
</tr>
<tr>
<td>β-Endorphin</td>
<td>0.65 ± 0.50</td>
<td>42.87 ± 7.91</td>
</tr>
<tr>
<td>Endorphin 1</td>
<td>1.84 ± 1.77</td>
<td>33.83 ± 3.40</td>
</tr>
<tr>
<td>Endorphin 2</td>
<td>112 ± 75.40</td>
<td>36.37 ± 8.55</td>
</tr>
<tr>
<td>DAMGO</td>
<td>5.78 ± 2.40</td>
<td>40.48 ± 3.96</td>
</tr>
<tr>
<td>U69,593</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>DPDPE</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Nociceptin</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

a In the case of the ligands that are unable to displace all of the specifically bound [3H]diprenorphine, the Ki is calculated using only the portion of the curve that shows [3H]diprenorphine displacement, this is, taking as "bottom" the maximal displacement at a concentration of 10 μM of the unlabeled ligand.

b Percentage of displacement of [3H]diprenorphine shown at a concentration of 10 μM of the unlabeled ligand.
unlabeled DAMGO. Thus, this explains why it is very difficult to see any binding with saturation binding assays, where typically concentrations of only up to 20 nM are commonly used. In contrast, sites labeled by [3H]diprenorphine are able to be recognized by ligands with reduced selectivity (approximately 10-fold or less) for mammalian opioid receptors such as naloxone ($\mu > \kappa > \delta$), diprenorphine ($\mu = \kappa = \delta$), bremazocine ($\kappa = \mu = \delta$), morphine ($\mu > \kappa = \delta$), and dynorphin 1 to 17 ($\kappa > \mu = \delta$) (Toll et al., 1998).

Although the endogenous ligand dynorphin A displaced all of the [3H]diprenorphine binding with good affinity, the other endogenous opioid peptides, Met- and Leu-enkephalin ($\mu = \delta \gg \kappa$), proved to displace $\sim50\%$ [3H]diprenorphine binding, a fact that may relate to their very poor recognition of the mammalian $\kappa$-opioid receptor. Similar results were obtained with the endogenous peptidic agents $\beta$-endorphin and the endorphins. It was also notable the differences in the $K_i$ values for Met- and Leu-enkephalin, two similar peptidic ligands that only differ from each other in one residue.

In conclusion, our study of the opioid-binding sites in zebrafish brain shows that, in effect, there are binding sites in zebrafish brain that can be classified as opioid, because the main characteristics of the opioid binding, such as saturation binding using nonspecific ligands, naloxone displacement, and the action of morphine and endogenous opioid peptides, are present in zebrafish brain homogenates. However, selective opioid ligands for mammalian opioid receptors are not well recognized. In fact, Stevens and Newman (1999) showed that highly selective opioid antagonists did not exhibit selectivity in blocking the analgesic effects of selective opioids in a behavioral assay in frogs. These findings support the idea that the mammalian opioid binding pocket is not fully conserved throughout evolution. Furthermore, bioinformatic analysis comparing the $\mu$, $\delta$, and $\kappa$-like opioid receptors clones in zebrafish and amphibians to mammalian orthologs shows that the similarity among these receptors is greater in earlier evolved vertebrates compared with humans (Stevens, 2004). Hence, before the zebrafish can be used as a straightforward model to study the complex activity of opioids in higher vertebrates, as humans and other mammalian systems, further work is necessary to accurately characterize how the individual zebrafish opioid receptors differ from their mammalian counterparts.

References

Address correspondence to: Dr. Raquel E. Rodriguez, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Salamanca, Avenida Alfonso X “El Sabio” s/n, 37007 Salamanca, Spain. E-mail: requelmi@usal.es