

Binding Profile of the Endogenous Novel Heptapeptide Met-Enkephalin-Gly-Tyr in Zebrafish and Rat Brain

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ABSTRACT

Zebrafish is considered a model organism, not only for the study of the biological functions of vertebrates but also as a tool to analyze the effects of some drugs or toxic agents. Five opioid precursor genes homologous to the mammalian opioid propeptide genes have recently been identified; one of these, the zebrafish proenkephalin, codes a novel heptapeptide, the Met-enkephalin-Gly-Tyr (MEGY). To analyze the pharmacological properties of this novel ligand, we have labeled it with tritium (^3H MEGY). In addition, we have also synthesized two analogs: (D-Ala²)-MEGY (Y-D-Ala-GFMGY) and (D-Ala², Val⁶)-MEGY (Y-D-Ala-GFVGY). The binding profile of these three agents has been studied in zebrafish and rat brain membranes. ^3H MEGY presents one binding site in zebrafish, as well as in rat brain

membranes, although it shows a slight higher affinity in zebrafish brain. The observed saturable binding is displaced by naloxone, thus confirming the opioid nature of the binding sites. Competition binding assays indicate that the methionine residue is essential for high-affinity binding of MEGY and probably of other peptidic agonists in zebrafish, whereas the change of a Gly for a D-Ala does not dramatically affect the ligand affinity. Our results show that the percentage of ^3H MEGY displaced by all the ligands studied is higher than 100%, thus inferring that naloxone (used to determine nonspecific binding) does not bind to all the sites labeled by ^3H MEGY. Therefore, we can deduct that some of the MEGY binding sites should not be considered classical opioid sites.

The endogenous opioid peptides such as enkephalins, β -endorphins, and dynorphins exert their actions mainly by acting on the opioid receptors present in the nervous system. These peptides are generated by the processing of larger precursors that are encoded in the genome: proenkephalin (PENK) (Comb et al., 1982; Gubler et al., 1982; Noda et al., 1982), proopiomelanocortin (POMC) (Takahashi et al., 1981), and prodynorphin (Horikawa et al., 1983). These precursors and the corresponding encoded peptides have been mainly identified in vertebrates, and the evolution of this gene fam-

ily has been widely analyzed (Dores et al., 2002). However, little is known about the pharmacological activity of newly discovered endogenous peptides from lower vertebrates.

The zebrafish has been considered a model organism in molecular biology and development (Golling et al., 2002), but nowadays it is also being used to analyze the genetic basis of human diseases, and it has been proposed as a candidate organism for the study of interactions between the genome and the environment (Ingham, 1997; Dooley and Zon, 2000; Fishman, 2001). Since drug addiction presents not only a genetic component but also is affected by outer influences, the zebrafish can be considered a valid model to unravel the molecular basis of drug tolerance and dependence (Darland and Dowling, 2001; Dlugos and Rabin, 2003; Pichler et al., 2003). For this reason, we are studying the zebrafish opioid system, and up to now we have characterized five opioid precursors from the zebrafish: zfPENK, zfPENK-like (a duplicate PENK gene) (González-Nuñez et al., 2003a), zfPOMC,

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ABBREVIATIONS: PENK, proenkephalin; POMC, proopiomelanocortin; MERF, Met-enkephalin-Arg-Phe; MEGY, Met-enkephalin-Gly-Tyr (YGG-FMGY); (D-Ala²)-MEGY, D-Ala-Met-enkephalin-Gly-Tyr (Y-D-Ala-GFMGY); (D-Ala², Val⁶)-MEGY, D-Ala-Val-enkephalin-Gly-Tyr (Y-D-Ala-GFVGY); HPLC, high-performance liquid chromatography.

zFPOMC-like (González-Núñez et al., 2003c), and zFPNOC (González-Núñez et al., 2003b). These precursors contain novel opioid peptides that can display different pharmacological properties than their counterparts in mammals. In particular, mammals present an enlarged form of Met-enkephalin [Met-enkephalin-Arg-Phe (MERF)] that is different from its homolog in zebrafish, the Met-enkephalin-Gly-Tyr (MEGY). It is important to note that the change of Arg by Gly implies not only the substitution of a bulky amino acid by a small residue but also a net loss of a positive charge in the peptide that can be significant for its binding properties.

The mammalian MERF was first isolated from bovine adrenal medulla (Stern et al., 1979), and it has been shown that this peptide displays important biological functions (Sharma et al., 1993) as, for example, antinociception (Inturrisi et al., 1980) and antitussive properties (Kamei et al., 1994). The binding properties of MERF and some of its analogs (Bozo et al., 2000; Toth et al., 2003) have been widely studied in amphibian (Wollemann et al., 1994) and mammalian brain (Benyhe et al., 1997, 1999; Kim et al., 2000), and it has been shown that MERF binds to opioid receptors with high affinity.

Taking the above into consideration, we aimed to characterize the binding profile of MEGY peptide in zebrafish, the organism in which this peptide is naturally present as an endogenous opioid ligand, and in rat brain membranes, as a representative model of the mammalian opioid system. In this way, we can compare the affinities of this peptide in these two species.

To achieve this objective, we have synthesized and labeled the MEGY peptide (^3H MEGY; 20 Ci/mmol) and performed binding assays. Two MEGY analogs were also synthesized: (D-Ala²)-MEGY (Y-D-Ala-GFMGY) and (D-Ala², Val⁵)-MEGY (Y-D-Ala-GFVGY). The change of a Gly by a D-Ala confers resistance against proteases such as dipeptidyl-aminopeptidases, which remove the N-terminal dipeptide Tyr₁-Gly₂ (Hazato et al., 1982; van Amsterdam et al., 1983). In addition, the substitution of Met by Val may help to determine the importance of the methionine residue for the specific opioid binding.

Materials and Methods

Drugs and Radioligands. Morphine was obtained from the Spanish Ministry of Health (Madrid, Spain), and naloxone and Met-enkephalin were obtained from Sigma-Aldrich (St. Louis, MO). ^3H Diprenorphine (50 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). G. Arsequell and G. Valencia (Consejo Superior de Investigaciones Científicas, Barcelona, Spain) originally synthesized MEGY. ^3H MEGY (20 Ci/mmol), unlabeled MEGY, and its two analogs (D-Ala²)-MEGY (Tyr-D-Ala-Gly-Phe-Met-Gly-Tyr) and (D-Ala², Val⁵)-MEGY (Tyr-D-Ala-Gly-Phe-Val-Gly-Tyr) were synthesized by G. Toth at the Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences. All other reagents used were of analytical grade.

Peptide Synthesis. The endogenous MEGY, its two analogs, (D-Ala²)-MEGY and (D-Ala², Val⁵)-MEGY, and the precursor peptide for tritiation (3'5'-I-Tyr-Gly-Gly-Phe-Met-Gly-Tyr) were synthesized by the solid-phase peptide synthesis method using the published Boc chemistry procedure (Tóth et al., 1997a). The crude peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) using a Vydac 218TP1010 column (W. R. Grace & Co., Columbia, MD). The peptide was eluted by the acetonitrile/0.1%

trifluoroacetic acid solvent system using a linear gradient. The structure of the peptides was confirmed by mass spectrometry.

[3'5'- ^3H]MEGY was prepared by catalytic dehalogenation of the precursor peptide using $^3\text{H}_2$ gas and PdO/BaSO₄ (Merck, Darmstadt, Germany) as the catalyst in the presence of triethylamine (Tóth et al., 1997b). The crude tritiated peptide was purified by HPLC as previously described. The purity of the final product was established by analytical HPLC using a Vydac 218TP46 column (W. R. Grace & Co.), and the degree of purity observed was >95%. The radioactivity was measured using a Packard liquid scintillation counter (Canberra Industries, Meriden, CT). The specific radioactivity was 20 Ci/mmol. The purified peptide was stored in ethanol under liquid nitrogen at a concentration of 1 mCi/ml.

Animals. Adult zebrafish *Danio rerio* were obtained from a local pet supplier, maintained at 25 to 28°C, and fed once a day. In all the experiments, fish from both sexes were used. Adult Sprague-Dawley rats ranging from 200 to 280 g were kept in cages with a 12-h light/dark cycle and free access to food and water. Animals were handled according to the guidelines of the European Communities Council directive of November 24, 1986 (86/609/EEC) and in all cases were treated in accordance with the Declaration of Helsinki and/or with *The Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health and the Spanish Ethical Committee.

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

Saturation Binding Assays. Either 30 (zebrafish) or 100 μg (rat) of protein was incubated with different concentrations of the radioligand ^3H MEGY for 1.5 h at 25°C in a final volume of 500 μl. Naloxone (10 μM) was used to determine nonspecific binding. After incubation, the reaction was stopped by adding 4 ml of ice-cold assay buffer; the mixture was rapidly filtrated using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD) and washed two times onto 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 EcoScint A scintillation liquid (National Diagnostics, Atlanta, GA). Radioactivity was counted using a Beckman Coulter, Inc. (Fullerton, CA) scintillation counter. All experiments were performed in duplicate and repeated at least three times.

Competition Binding Assays. Reactions were performed as described under "Saturation Binding Assays", except that the radioligand ^3H diprenorphine was used at 0.96 nM in the case of the zebrafish and 0.45 nM in the case of the rat brain homogenates, and ^3H MEGY was used at 1.65 nM in the case of the zebrafish and 3.85 nM in the case of the rat brain homogenates. The labeled ligands were displaced by several unlabeled compounds at a concentration range of 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 the presence of 10 μM naloxone). Data were analyzed using Prism software (GraphPad Software Inc., San Diego, CA), and affinity constant (K_D), receptor density (B_{max}), and inhibition constant (K_i) values for each ligand were obtained. K_i values were calculated using GraphPad software using the Cheng and Prusoff correction, which corrects for the concentration of radioligand used in each experiment and the affinity of the radioligand for its binding site (K_D). In all cases, data were fit to the one- or two-site binding model. In saturation binding assays, data were fit to either nonlinear function or the linear trans-

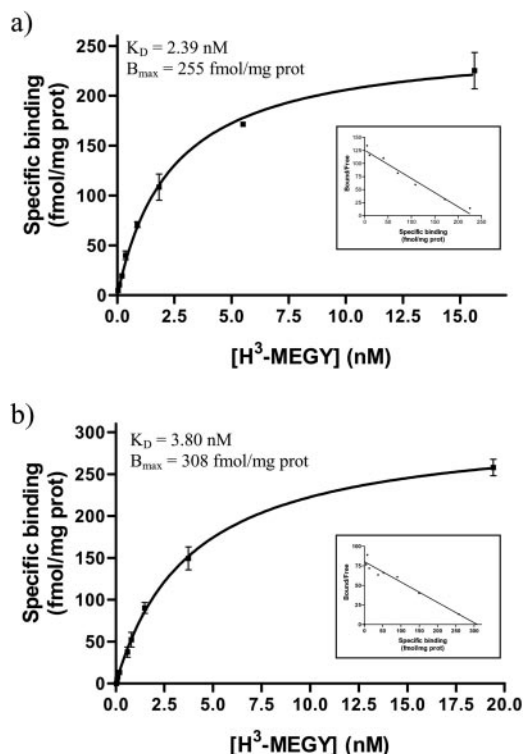


Fig. 1. Saturation binding analysis of [^3H]MEGY in (a) zebrafish and (b) rat brain membranes. Insets, Scatchard plots. Results represent the means \pm S.E.M. of three independent experiments performed in duplicate. In both cases, the results fit better to the one single-site binding model. Either 30 μg (zebrafish) or 100 μg (rat) of protein was incubated with different concentrations of [^3H]MEGY for 1.5 h at 25°C in a final volume of 500 μl . Nonspecific binding was determined with 10 μM naloxone.

formation (Scatchard plot, bound/free versus bound), whereas in competition binding assays, data were fit to the one- and two-site competition models and compared using the nonlinear least-squares curve-fitting, which is based upon a statistical F test.

Results

Saturation Binding Assays of [^3H]MEGY in Zebrafish and Rat Brain Membranes. The ability of [^3H]MEGY to bind opioid receptors from zebrafish, an organism in which this peptide is naturally present (González-Nuñez et al., 2003a), and from mammals, in which MERF is the corresponding endogenous peptide, was measured using increasing concentrations of this radioligand in zebrafish and rat brain membrane homogenates. In both cases, binding was displaced by naloxone, thus confirming the opioid nature of these sites. In Fig. 1a, the saturation curve of [^3H]MEGY in zebrafish brain membranes is shown, and the Scatchard transformation is presented in the inset. The data were better fit to the one single-binding site model, with $K_D = 2.39 \pm 0.29$ nM and $B_{\text{max}} = 255.1 \pm 10.84$ fmol/mg protein. The same analysis is presented for rat brain membranes (Fig. 1b), where data also better fit to the one single-binding site model, with $K_D = 3.8 \pm 0.34$ nM and $B_{\text{max}} = 308.1 \pm 11.45$ fmol/mg protein.

Competition Binding Assays Using [^3H]Diprenorphine. To analyze the ability of MEGY to displace other conventional opioid compounds, competition binding assays were performed using [^3H]diprenorphine as the radioligand and MEGY and its two analogs, (D-Ala 2)-MEGY and (D-Ala 2 , Val 5)-MEGY, as unlabeled ligands at a concentration range of 0.3 nM to 10 μM . Figure 2 shows the competition binding plots of these ligands in zebrafish and rat brain membranes,

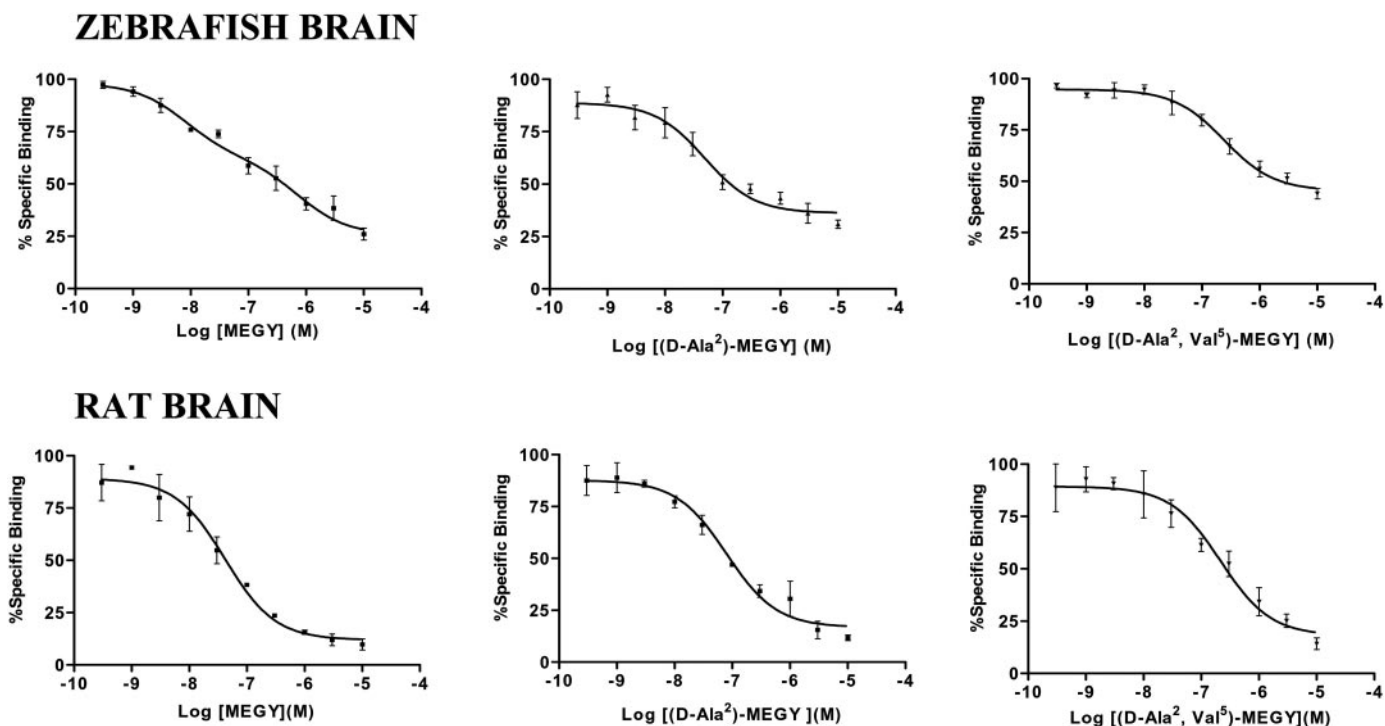


Fig. 2. Competition binding assays of [^3H]diprenorphine with unlabeled MEGY, (D-Ala 2)-MEGY, and (D-Ala 2 , Val 5)-MEGY in zebrafish and rat brain membranes. Results represent the means \pm S.E.M. of at least two independent experiments performed in duplicate. Brains were extracted, and membrane homogenates were obtained as described under *Materials and Methods*. Thirty (zebrafish) or 100 μg (rat) of protein was incubated with [^3H]diprenorphine and increasing concentrations of the MEGY analogs (from 0.3 nM to 10 μM) for 1 h at 25°C in a final volume of 500 μl . Nonspecific binding was determined with 10 μM naloxone.

TABLE 1

K_i values of MEGY and its two analogs in zebrafish and rat brain membranes obtained from competition binding assays using [3 H]diprenorphine. Percentage of displacement of [3 H]diprenorphine is shown at a concentration of 10 μ M of the unlabeled ligand.

Ligand	K_i , Zebrafish Brain	Hill Coefficient	Displacement in Zebrafish Brain	K_i , Rat Brain	Hill Coefficient	Displacement in Rat Brain
	<i>nM</i>			<i>nM</i>		
MEGY	$K_{i1} = 1.17 \pm 0.27$ $K_{i2} = 673 \pm 136$	-0.31	74.28 ± 4.20	$K_i = 22.10 \pm 1.57$	-0.90	89.09 ± 0.80
(D-Ala ²)-MEGY	$K_i = 12.40 \pm 2.67$	-0.65	63.89 ± 2.69	$K_i = 29.90 \pm 7.97$	-0.62	85.45 ± 2.23
(D-Ala ² , Val ⁵)-MEGY	$K_i = 137 \pm 26.45$	-0.85	54.64 ± 2.36	$K_i = 72.20 \pm 7.15$	-0.81	79.39 ± 3.30

and Table 1 summarizes the K_i values, the percentage of displacement, and the Hill coefficient shown by each peptide. It is important to note that in zebrafish brain membranes the native peptide MEGY fits better to the two-site displacement model ($F = 7.414$; $p = 0.0037$), whereas all the other displacement curves fit better to the one-site competition model. This result is also supported by the fact that the Hill coefficient for MEGY displacement of [3 H]diprenorphine in zebrafish brain is -0.30 , whereas for the other ligands this coefficient is closer to -1 (between -0.65 and -0.90). In zebrafish, the percentage of binding displaced by the native peptide MEGY is higher than that observed for the analogs, where (D-Ala², Val⁵)-MEGY shows the lowest ability to displace [3 H]diprenorphine binding.

Competition Binding Assays Using [3 H]MEGY. [3 H]MEGY was also used as the radioligand in competition binding assays using the unlabeled peptide MEGY and its two analogs as unlabeled ligands so that it was possible to determine the influence of the structural changes in the binding ability. Heterologous displacements with morphine (nonpeptidic opiate) and Met-enkephalin (an endogenous ligand for both species) were also performed. Results are summarized in Fig. 3, where the displacement curves in zebrafish and in rat brain membranes are shown, and in Table 2, which summarizes the K_i obtained for each ligand. In all cases, the one-site displacement model fits better to the experimental data, although the displacement of [3 H]MEGY by morphine in rat brain can also be adjusted to the two-site displacement model, with a Hill coefficient of -0.40 . The other ligands studied show a Hill coefficient between -0.86 and -1.61 for both species. Interestingly, the five ligands tested here prove to be able to displace more than 100% of the binding in zebrafish brain membranes (and also to a lower extent in rat brain), which means that part of the [3 H]MEGY binding cannot be displaced by naloxone. However, these binding sites are recognized by other peptidic ligands (e.g., MEGY and Met-enkephalin) and alkaloids such as morphine.

Discussion

Previous work has proved that the zebrafish can be a good vertebrate model to study the actions of different drugs (Darland and Dowling, 2001; Dlugos and Rabin, 2003; Pichler et al., 2003). In this work, we show that the zebrafish endogenous opioid peptide MEGY can bind to the opioid receptors present in zebrafish and rat brain with high affinity and that this binding is reversed by naloxone. The binding assays of [3 H]MEGY obtained by us give similar saturation curves for zebrafish and rat brain homogenates, although this peptide presents a significant higher affinity in zebrafish brain. K_D values obtained for this ligand are in the same range than what previous studies have found for MERF in mammalian

(6.7 ± 1.9 nM, Benyhe et al., 1997; 10.2 ± 0.5 nM, Kim et al., 2000) and in amphibian brain (3.6 ± 1.7 nM, Wollemann et al., 1994). However, the values obtained in mammalian brains for MERF are higher than the value for MEGY in rat brain ($K_D = 3.8 \pm 0.34$ nM); this difference may be because different methods for preparing the brain membrane homogenates have been used. Nevertheless, it should also be considered that the change of an Arg residue present in MERF for a Gly residue reduces the molecular volume of the peptide; thus, it can adjust better to the receptor binding pocket.

Given that the use of the nonspecific antagonist [3 H]diprenorphine as the labeled compound to determine opioid binding is well documented (Richards and Sadee, 1985; Wood and Traynor, 1989; Wood et al., 1989; Rodriguez et al., 1992) and that in our hands [3 H]diprenorphine presents two different binding sites in zebrafish brain (K_D values 0.08 and 17 nM) (V. González-Nuñez, R. E. Rodríguez, A. Barrallo, and J. Traynor, unpublished results), we have used it in competition binding assays to analyze the relative efficiency of

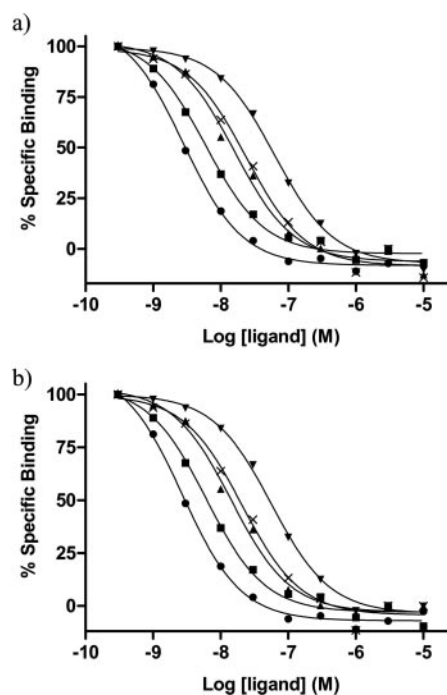


Fig. 3. Competition binding assays of [3 H]MEGY and several unlabeled ligands in (a) zebrafish and (b) rat brain membranes. Results are from a representative experiment performed in duplicate and repeated twice. Note that in zebrafish brain, the ligands used here displace more than 100%. ■, MEGY; ▲, (D-Ala²)-MEGY; ▼, (D-Ala², Val⁵)-MEGY; ●, Met-enkephalin; ×, morphine. Brains were extracted as previously described, and either 30 (zebrafish) or 100 μ g (rat) of protein was incubated with [3 H]MEGY and increasing concentrations of the unlabeled ligands ranging from 0.3 nM to 10 μ M for 1.5 h at 25°C in a final volume of 500 μ l. Nonspecific binding was determined with 10 μ M naloxone.

TABLE 2

K_i values of several ligands in zebrafish and rat brain membranes obtained from competition binding assays using [^3H]MEGY. Percentage of displacement of [^3H]MEGY is shown at a concentration of 10 μM of the unlabeled ligand.

Ligand	K_i , Zebrafish Brain	Hill Coefficient	Displacement in Zebrafish Brain	K_i , Rat Brain	Hill Coefficient	Displacement in Rat Brain
	<i>nM</i>		%	<i>nM</i>		%
MEGY	$K_i = 2.89 \pm 0.85$	-0.9598	106.85 ± 1.40	$K_i = 4.21 \pm 1.41$	-1.063	109.51 ± 1.19
(D-Ala ²)-MEGY	$K_i = 7.65 \pm 1.99$	-1.616	112.72 ± 4.90	$K_i = 4.45 \pm 1.21$	-0.9634	100.16 ± 1.48
(D-Ala ² , Val ⁵)-MEGY	$K_i = 25.93 \pm 3.47$	-0.8978	110.25 ± 2.46	$K_i = 21.06 \pm 2.71$	-0.9655	100.15 ± 0.92
Met-enkephalin	$K_i = 2.01 \pm 0.59$	-0.8618	108.43 ± 1.65	$K_i = 2.40 \pm 0.29$	-0.8680	102.55 ± 1.06
Morphine	$K_i = 14.15 \pm 2.61$	-0.9744	114.12 ± 2.69	$K_i = 7.23 \pm 1.57$	-0.4062	97.44 ± 3.78

MEGY and its two analogs to bind to the opioid sites and to compete for the opioid binding sites with other conventional ligands. These assays have established that the peptidic agents tested here can effectively displace the opioid binding shown by diprenorphine, not only in zebrafish brain homogenates but also in membrane preparations from rat brain, a mammalian model. MEGY shows a two-site displacement in zebrafish brain, with one high-affinity site and one site with lower affinity, which suggests that this peptide may act on two or more different receptors with different affinities. Preliminary work from our laboratory suggests that MEGY binds to the δ receptors from zebrafish (V. González-Núñez, R. E. Rodríguez, A. Barrallo, and J. Traynor, unpublished results). The fact that a ligand shows a biphasic curve in competition binding assays has been previously reported for other ligands, such as morphiceptin (Chang et al., 1981). In the rat, the analog (D-Ala²)-MEGY shows a similar K_i and percentage of displacement at 10 μM than MEGY, thus indicating that the change of Gly by a D-Ala does not affect the ability of the ligand to bind to opioid sites. However, the (D-Ala², Val⁵)-MEGY analog displays a higher K_i , revealing that the change of Met by Val entails a loss in binding affinity. These results can be taken into consideration for future opioid ligand design because an effective peptidic ligand should have the methionine in the fifth position, whereas the Gly can be replaced by another small residue that might confer resistance against protease degradation. As seen in Fig. 2, the peptides analyzed here can displace almost all the diprenorphine binding in rat brain, whereas in zebrafish brain, the native peptide displaces only up to 74%. This difference can be explained if we postulate that diprenorphine may bind to the opioid sites present in zebrafish brain in a different manner than to those present in mammalian brain. These results are interesting because it may be inferred that diprenorphine can be considered as a good and selective ligand to label opioid sites in mammalian brain, whereas in zebrafish diprenorphine does not seem to show such selectivity and thus, other opioid ligands are not able to displace up to 100% of its binding.

In competition binding assays with [^3H]MEGY, we have used this same peptide as an internal control and then the two MEGY analogs to establish the change in affinity triggered by the change in structure. In addition, we have also used Met-enkephalin since this peptide is present as an endogenous opioid ligand in both species and morphine because of its great medicinal importance. Besides, the use of morphine led us to examine the ability of a nonpeptidic opiate to compete for peptidic-labeled opioid sites. As seen in Fig. 3 and Table 2, the changes in the peptide structure comprise losses in binding affinity in zebrafish brain, the

MEGY-Val analog being the one with the highest K_i . However, in rat brain, the substitution of Gly by a D-Ala does not entail a change in the inhibition constant K_i , probably because this modification does not cause a change in size or in charge of the ligand. Met-enkephalin shows a similar pattern to the one observed for MEGY in zebrafish brain, whereas in rat brain it displays a lower K_i value, possibly because it is an endogenous ligand for mammalian opioid receptors, although to our knowledge the MEGY peptide is not naturally present in tetrapods. The alkaloid opiate morphine can displace [^3H]MEGY binding in a very similar manner in both brain membranes tested in this work, thus confirming our previous results in which we have shown that morphine also acts on zebrafish opioid receptors (Rodríguez et al., 2000). Finally, as seen in Fig. 3 and Table 2, all the ligands studied are able to present a higher displacement than naloxone, especially when acting on zebrafish brain. This observation can be explained if we consider that the [^3H]MEGY peptide, apart from binding to naloxone-sensitive opioid sites, also binds to some sites that are not recognized by naloxone. Hence, the sites recognized by naloxone should be considered as classical opioid sites, and the naloxone-insensitive sites should be named as nonclassical opioid sites. Therefore, the expression "nonopioid" could be used accurately when an opioid ligand acts on a different receptor than the opioid receptors (e.g., the Met-enkephalin and its derivatives on the cytosolic opioid growth factor receptor) (Zagon et al., 2002). Under this consideration, the endogenous peptides, as well as the peptidic opioid analogs and morphine, bind to the putative nonclassical opioid sites; hence, we believe that they are acting on the opioid receptors present in zebrafish brain. This can be explained if we consider that some parts of the opioid binding pocket may allow distinct agents, such as peptidic ligands and synthetic agonists and antagonists, to recognize different motifs when they bind to the opioid receptors; thus, not all sites are recognized by some ligands. This hypothesis has previously been raised by other authors (Befort et al., 1996). Our data point to the possibility that the antagonist naloxone does not bind to some sites that are recognized by the agonists; hence, the MEGY peptide presents a higher selectivity for recognizing the opioid binding sites in zebrafish.

In conclusion, our results prove that the MEGY peptide acts as a highly specific endogenous ligand for the zebrafish opioid receptors and also binds with high affinity to their mammalian counterparts. Our work reveals that the zebrafish opioid peptide MEGY presents a different binding profile than other opioid agonists; therefore, this ligand can be used as a new tool to investigate the ligand-receptor in-

teractions in relation to the modulation of pain and drug addiction.

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