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BINDING PROFILE OF THE ENDOGENOUS NOVEL HEPTAPEPTIDE Met-ENKEPHALIN-Gly-Tyr IN ZEBRAFISH AND RAT BRAIN.

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Nonstandard abbreviations: 1. MEGY – Met-Enkephalin-Gly-Tyr (YGGFMGY); (D-Ala^2)-MEGY – D-Ala-Met-Enkephalin-Gly-Tyr (Y-D-Ala-GFMGY); (D-Ala^2, Val^5)-MEGY – D-Ala-Val-Enkephalin-Gly-Tyr (Y-D-Ala-GFVGY); PENK – proenkephalin; POMC – proopiomelanocortin; PDYN – prodynorphin; MERF – Met-Enkephalin-Arg-Phe.

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ABSTRACT

Zebrafish is considered as 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 been identified recently and one of these, the zebrafish proenkephalin (zfPENK), codes a novel heptapeptide, the Met-Enkephalin-Gly-Tyr (MEGY). In order to analyze the pharmacological properties of this novel ligand, we have labelled it with tritium ([^{3}H]-MEGY). Besides, we have also synthesized two analogues: (D-Ala^2)-MEGY (Y-D-Ala-GFMGY) and (D-Ala^2, Val^5)-MEGY (Y-D-Ala-GFVGY). The binding profile of these three agents has been studied in zebrafish and rat brain membranes. [^{3}H]-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, while the change of a Gly for a D-Ala does not affect dramatically the ligand affinity. Our results show that the percentage of [^{3}H]-MEGY displaced by all the ligands studied is higher than 100%, thus inferring that naloxone (used to determine non-specific binding) is not binding to all the sites labelled by [^{3}H]-MEGY. We can hence deduct that some of the MEGY-binding sites should not be considered as classical opioid sites.

The endogenous opioid peptides like 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 (PDYN) (Horikawa et al., 1983). These precursors and the corresponding encoded peptides have been mainly identified in vertebrates and the evolution of this gene family 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 as 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 environment (Dooley and Zon, 2000; Fishman, 2001; Ingham, 1997). Since drug addiction presents not only a genetic component, but is also affected by outer influences, the zebrafish can be considered as 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 proenkephalin gene) (Gonzalez-Nuñez et al., 2003a), zfPOMC, zfPOMC-like (Gonzalez-Nuñez et al., 2003c) and zfPNOC (Gonzalez-Nuñez et al., 2003b). These precursors contain novel opioid peptides that can display different pharmacological properties than their counterparts in mammals. Particularly, mammals present an enlarged form of Met-Enkephalin (Met-Enkephalin-Arg-Phe, MERF) that is different from its homologue 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 (Inturrissi et al., 1980) and antitusive properties (Kamei et al., 1994).

The binding properties of MERF and some of its analogues (Bozo et al., 2000; Toth et al., 2003) have been widely studied in amphibian (Wollemann et al., 1994) and in mammalian brain (Benyhe et al., 1997; Benyhe et al., 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 labelled the MEGY peptide ([^{3}H]-MEGY 20Ci/mmol) and performed binding assays. Two MEGY analogues were also synthesized: (D-Ala^2)-MEGY (Y-D-Ala-GFMGY) and (D-Ala^2, Val^5)-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 Tyr_1-Gly_2 (Hazato et al., 1982; van Amsterdam et al., 1983). Besides, the substitution of Met by Val may help to determine the importance of the Methionine residue for the specific opioid binding.

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METHODS

Drugs and Radioligands. Morphine was obtained from the Spanish Ministry of Health and naloxone and Met-Enkephalin from Sigma-Aldrich (St Louis, MO). [^{3}H]-Diprenorphine (50Ci/mmol) was purchased from Perkin-Elmer (Boston MA). MEGY was originally synthetized by G. Arsequell and G. Valencia at the Consejo Superior de Investigaciones Científicas, Barcelona. [^{3}H]-MEGY (Met-Enkephalin-Gly-Tyr) (20 Ci/mmol), unlabelled MEGY and its two analogues (D-Ala^2)-MEGY (Tyr-D-Ala-Gly-Phe-Met-Gly-Tyr) and (D-Ala^2, Val^5)-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, Szeged Hungary. All other reagents used were from analytical grade.

Peptide synthesis. The endogenous MEGY, its two analogues, (D-Ala^2)-MEGY (Tyr-D-Ala-Gly-Phe-Met-Gly-Tyr) and (D-Ala^2, Val^5)-MEGY (Tyr-D-Ala-Gly- Phe-Val-Gly-Tyr) and the precursor peptide for tritiation (3'5'I-Tyr-Gly-Gly-Phe-Met-Gly-Tyr) were synthesized by solid phase peptide synthesis method using published Boc chemistry procedure (Tóth et al., 1997a). The crude peptides were purified by reverse-phase high performance liguid chromatography (HPLC) using Vydac 218TP1010 column (Separations Group, Hesperia, CA, USA). 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'-^{3}H]-MEGY was prepared by catalityc dehalogenation of the precursor peptide using $^3H_2$ gas and $PdO/BaSO_4$ (Merck, Darmsadt, Germany) as catalyst in the presence of triethylamine (Tóth et al., 1997b). The crude tritiated peptide was purified by HPLC as above. The purity of the final product was established by analytical HPLC using a Vydac 218TP46 column and the degree of purity observed was >95%. The radioactivity was measured by means of a Packard liquid scintillation counter (Camberra, Vienna, Austria). 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 25-28ºC and fed once a day. In all experiments fish from both sexes were used. Adult Sprague-Dawley rats

ranging from 200 to 280 grams were kept in cages with a 12 hour light-dark cycle and free access to food and water. Animals were handled according to the guidelines of the European Communities Council directive of 24 November 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 from rat were extracted and kept on dry ice and homogenized with a Kinematika Polytron (Luzern, Switerland) in Tris HCl 50 mM pH 7.4 (assay buffer) with protease inhibitors (0.1mg/ml bacitracin, 3.3μM captopril and 0.33μM tiorphan). The homogenates were centrifuged at 6000g for 15 min at 4ºC 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 Lowry method (Onishi and Barr Modification).

Saturation Binding Assays. 30μg (zebrafish) or 100μg (rat) protein were incubated with different concentrations of the radioligand [^{3}H]-MEGY for 1h 30min at 25ºC in a final volume of 500μl. 10μM naloxone 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 and washed two times onto GF/B glass-fiber filters that were presoaked with 0.2% polyethylenimine for at least 1h. The filters were placed in scintillation vials and incubated overnight at room temperature in EcoScint A scintillation liquid (London, England). Radioactivity was counted using a Beckman Coulter (Pasadena 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 in that the radioligands [^{3}H]-Diprenorphine was used at 0.96nM in the case of the zebrafish and 0.45nM in the case of the rat brain homogenates and and [^{3}H]-MEGY was used at 1.65nM in the case of the zebrafish and 3.85nM in the case of the rat brain homogenates. The labelled

ligands were displaced by several unlabelled compounds at a concentration range from 0.3nM to 10μM. 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 10μM naloxone). Data were analyzed using the Graph Pad Prism software (San Diego, CA) and affinity constant (K_D), receptor density (B_{max}) and inhibition constant (K_i) values for each ligand were obtained. In the case of the K_i values, they were calculated by the GraphPad software, using the correction of Cheng and Prusoff, which corrects for the concentration of radioligand used in each experiment as well as the affinity of the radioligand for its binding site (K_D). In all cases, data were fit to the one-site or two-site binding model. In saturation binding assays, data were fit to either non-linear function and to the linear transformation (Scatchard-plot: Bound/Free vs Bound), while 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 of [^{3}H]-MEGY in zebrafish and rat brain membranes. The ability of [^{3}H]-MEGY to bind opioid receptors from zebrafish, organism in which this peptide is naturally present (Gonzalez-Nunez et al, 2003a), and from mammals, where Met-Enkephalin-Arg-Phe -MERF- is the corresponding endogenous peptide, was measured by 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. 1.a the saturation curve of [^{3}H]-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 a K_D = 2.39±0.29 nM and a B_{max}=255.1±10.84 fmol/mg protein. The same analysis is presented for rat brain membranes (Fig 1.b), where data also better fit to the one single binding site model, with a K_D = 3.8±0.34 nM and a B_{max} = 308.1±11.45 fmol/mg protein.

Competition Binding Assays using [^{3}H]-Diprenorphine. To analyze to ability of MEGY to displace other conventional opioid compounds, competition binding assays were performed using [^{3}H]-Diprenorphine as radioligand and MEGY and its two analogues (D-Ala^2)-MEGY and (D-Ala^2, Val^5)-MEGY as unlabelled ligands at a concentration range from 0.3nM to 10μM. Fig. 2 shows the competition binding plots of these ligands in zebrafish and rat brain membranes 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-value = 0.0037), while all 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, while 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 analogues, where (D-Ala^2, Val^5)-MEGY shows the lowest ability to displace [^{3}H]-Diprenorphine binding.

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Competition Binding Assays using [^{3}H]-MEGY. [^{3}H]-MEGY was also used as radioligand in competition binding assays, using the unlabelled peptide MEGY and its two analogues as unlabelled ligands, so that it is possible to determine the influence of the structural changes in the binding ability. Heterologous displacement with morphine (non peptidic 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 be also 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 in 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 (MEGY, Met-Enkephalin) and alkaloids like 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 Met-Enkephalin-Gly-Tyr (MEGY) can bind to the opioid receptors present in zebrafish and in 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- and 10.2 ± 0.5 nM -Kim et al., 2000-) and in amphibian brain (3.6 ± 1.7 nM -Kim et al., 2000-). However, the values obtained in mammalian brains for MERF are higher than the value for MEGY in rat brain (K_D = 3.8 ± 0.34 nM); this difference might be due to the fact that 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 and thus it can adjust better to the receptor binding pocket.

Given that the use of the non-specific antagonist [^{3}H]-Diprenorphine as the labelled compound to determine opioid binding is well documented (Wood and Traynor, 1989; Richards and Sadee, 1985; 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 0.08nM and 17nM) (unpublished results) we have used it in competition binding assays to analyze the relative efficiency of MEGY and its two analogues 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 are able to 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.

Actually, preliminary work form our laboratory suggests that MEGY binds to the delta receptors from zebrafish (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 el at., 1981). In the rat, the analogue (D-Ala^2)-MEGY shows a similar K_i and also a similar percentage of displacement at 10μM than MEGY, thus indicating that the change of Gly by a D-Ala does not affect the ligand ability to bind to opioid sites. However, the (D-Ala^2, Val^5)-MEGY analogue 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, since an effective peptidic ligand should have the Methionine in the fifth position, while the Gly can be replaced by another small residue that might confer resistance against protease degradation. As it can be seen in Fig. 2, the peptides analyzed here are able to displace almost all diprenorphine binding in rat brain, while in zebrafish brain the native peptide displaces only up to 74%. This difference can be explained if we postulate that diprenorphine might bind to the opioid sites present in zebrafish brain in a different manner than to those present in mammalian brain. These results are interesting, since it might be inferred that diprenorphine can be considered as a good and selective ligand to label opioid sites in mammalian brain, while 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 [^{3}H]-MEGY we have used this same peptide as an internal control and then the two MEGY analogues to establish the change in affinity triggered by the change in the 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 non-peptidic opiate to compete for peptidic-labelled opioid sites. As can be seen in Fig. 3 and Table 2, the changes in the peptide structure comprise losses in binding affinity in zebrafish brain, being the MEGY-Val analogue 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, while in rat brain it displays a lower K_i value, possibly because it is an endogenous ligand for mammalian opioid receptors while, to our knowledge, the MEGY peptide is not naturally present in tetrapods. The alkaloid opiate morphine is able to displace [^{3}H]-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 (Rodriguez et al., 2000). Finally, as seen in Fig. 3 and Table 2, all 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 [^{3}H]-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 non-classical opioid sites. Therefore, the expression "non-opioid" could be used accurately when an opioid ligand acts on a different receptor than the opioid receptors (for example, 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 analogues and morphine, bind to the putative non-classical opioid sites; and 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, synthetic agonists and antagonists, to recognize different motifs when they bind to the opioid receptors and thus not all sites are recognized by some ligands. This hypothesis has previously been raised by other authors (Befort et al., 1996). Our data points to the possibility that the antagonist naloxone does not bind to some sites that are recognized by the agonists and hence the MEGY peptide presents a higher selectivity for recognizing the opioid-binding sites in zebrafish.

In conclusion, our results prove that the Met-Enkephalin-Gly-Tyr (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 and therefore this ligand can be used as a new tool to investigate the ligand-receptor interactions in relation to the modulation of pain and drug addiction.

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FOOTNOTES

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LEGENDS FOR FIGURES

FIGURE 1. Saturation Binding analysis of [^{3}H]-MEGY in a) zebrafish and b) rat brain membranes. Insets: Scatchard-plots. Results represent the means ± SEM of three independent experiments performed in duplicate. In both cases, the results fit better to the one single site binding model. 30µg (zebrafish) or 100µg (rat) protein were incubated with different concentrations of [^{3}H]-MEGY for 1h 30min at 25ºC in a final volume of 500µl. 10µM naloxone was used to determine nonspecific binding.

FIGURE 2. Competition binding assays of [^{3}H]-Diprenorphine with unlabelled MEGY, (D-Ala^2)-MEGY and (D-Ala^2, Val^5)-MEGY in zebrafish and rat brain membranes. Results represent the means ± SEM of at least two independent experiments performed in duplicate. Brains were extracted and membrane homogenates were obtained as described in the Methods section. 30µg (zebrafish) or 100µg (rat) protein were incubated with [^{3}H]-Diprenorphine and increasing concentrations of the MEGY analogues (from 0.3nM to 10µM) for 1h at 25ºC in a final volume of 500µl. 10µM naloxone was used to determine nonspecific binding.

FIGURE 3. Competition binding assays of [^{3}H]-MEGY and several unlabelled 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%. Legend: ■- MEGY; ▲ - (D-Ala^2)-MEGY; ▼ – (D-Ala^2, Val^5)-MEGY; ● – Met-Enkephalin; ✕ - Morphine. Brains were extracted as previously described and 30µg (zebrafish) or 100µg (rat) protein were incubated with [^{3}H]-MEGY and increasing concentrations of the unlabelled ligands ranging from 0.3nM to 10µM for 1h 30min at 25ºC in a final volume of 500µl. Nonspecific binding was determined with 10µM naloxone.

Table 1. Ki values of MEGY and its two analogues in zebrafish and rat brain membranes obtained from competition binding assays using [^{3}H]-Diprenorphine.

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

a) Percentage of displacement of [^{3}H]-Diprenorphine shown at a concentration of 10μM of the unlabelled ligand.

Table 2. Ki values of several ligands in zebrafish and rat brain membranes obtained from competition binding assays using [^{3}H]-MEGY.

Ligand	K_i (nM) Zebrafish Brain[a)]	Hill Coefficient	% Displacement in Zebrafish Brain	K_i Rat Brain (nM)	Hill Coefficient	% Displacement in Rat Brain [a)]
MEGY	K_i = 2.89 ± 0.85	-0,9598	106.85 ± 1.40 %	K_i = 4.21 ± 1.41	-1,063	109.51 ± 1.19 %
(D-Ala^2)-MEGY	K_i = 7.65 ± 1.99	-1,616	112.72 ± 4.90 %	K_i = 4.45 ± 1.21	-0,9634	100.16 ± 1.48 %
(D-Ala^2, Val^5)-MEGY	K_i = 25.93 ± 3.47	-0,8978	110.25 ± 2.46 %	K_i = 21.06 ± 2.71	-0,9655	100.15 ± 0.92 %
Met-Enkephalin	K_i = 2.01 ± 0.59	-0,8618	108.43 ± 1.65 %	K_i = 2.40 ± 0.29	-0,8680	102.55 ± 1.06 %
Morphine	K_i = 14.15 ± 2.61	-0,9744	114.12 ± 2.69 %	K_i = 7.23 ± 1.57	-0,4062	97.44 ± 3.78 %

a) Percentage of displacement of [^{3}H]-MEGY shown at a concentration of 10μM of the unlabelled ligand.

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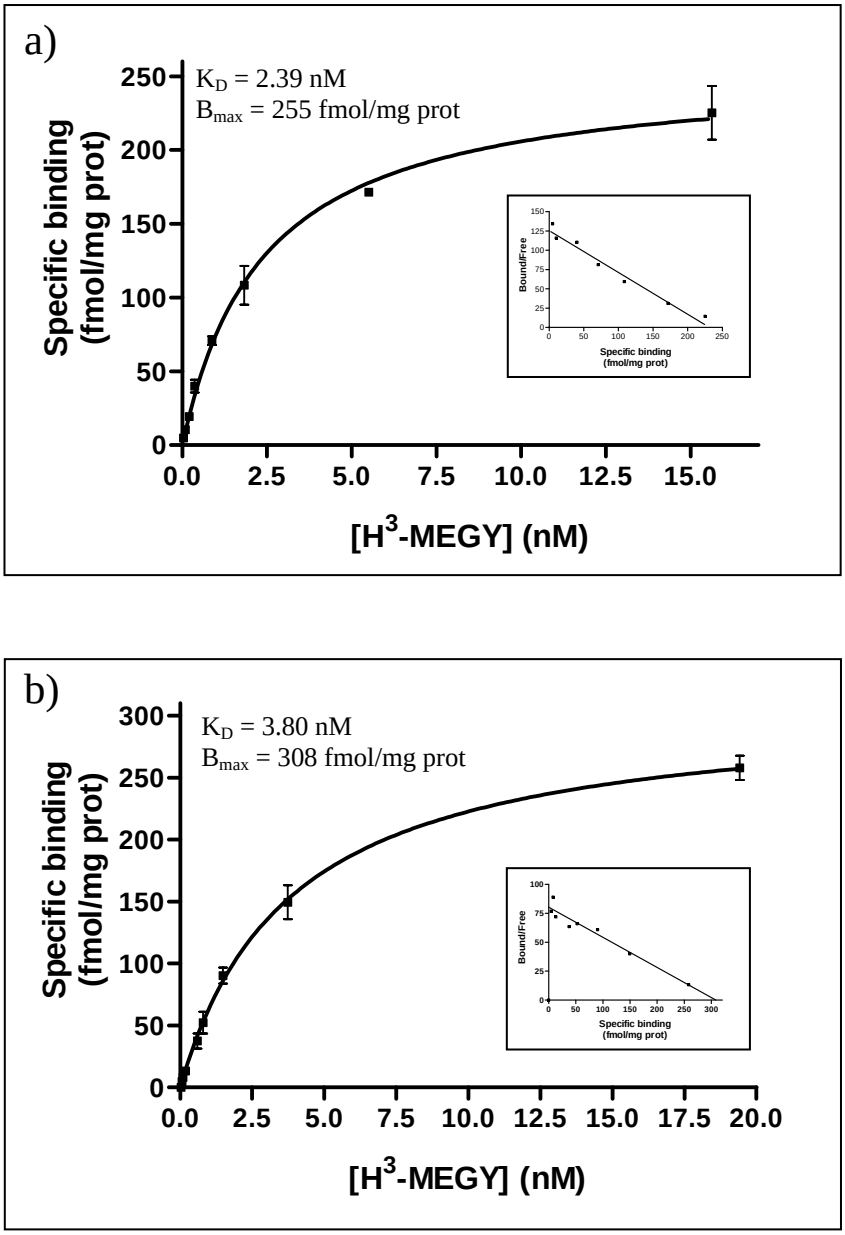

FIGURE 1

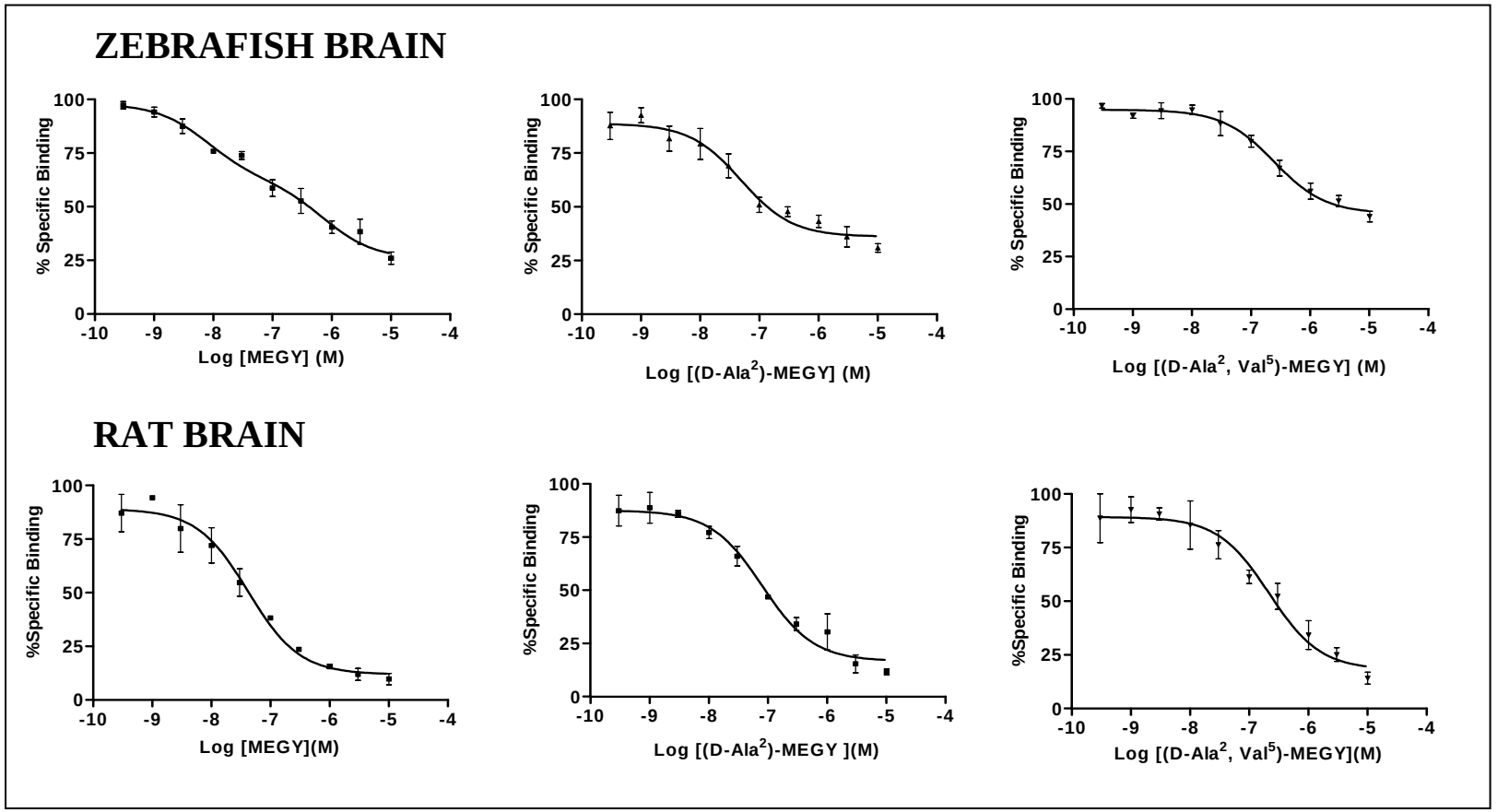

FIGURE 2

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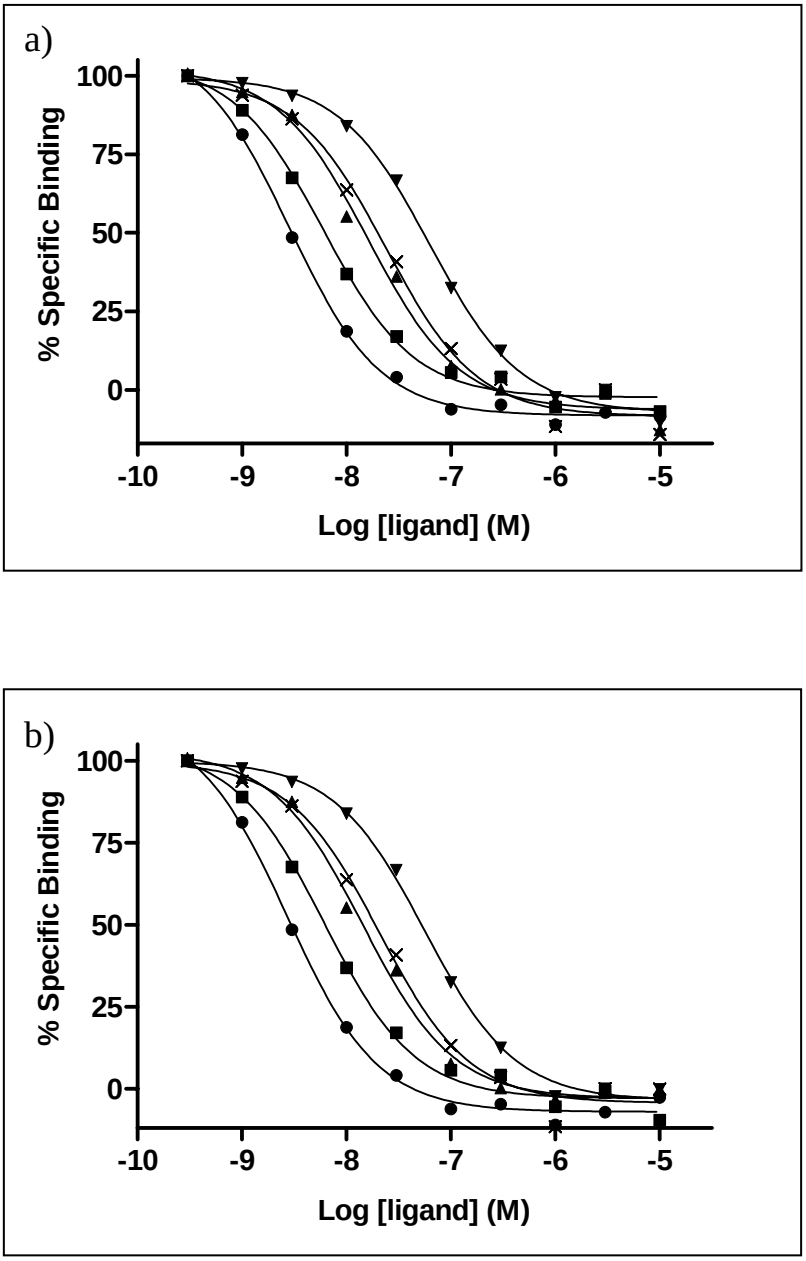

FIGURE 3