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A VERY HIGH AFFINITY OPIOID BINDING SITE IN RAT BRAIN: DEMONSTRATION BY COMPUTER MODELING

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We present substantial new evidence for at least four distinct types of opioid receptors in rat brain, using quantitative ligand binding studies and mathematical modeling. Three of these binding sites are consistent with the well established "mu", "delta" and "kappa" receptors. The fourth has two distinctive features: 1) extremely high affinity (dissociation constant < 1 nM); 2) almost complete lack of specificity for the classical "delta" or "mu" selective ligands. These properties are consistent with the putative "mu_1" receptor described by Pasternak and coworkers.

A high affinity opiate receptor in brain, distinct from the generally accepted "mu", "delta" "kappa" receptors, has been reported by Pasternak (1) and investigated extensively in his laboratory on the basis of pharmacological, physiological, anatomical, and ontological studies (2-9). Pasternak's studies imply that this "high affinity" site does not show selectivity for ligands which are otherwise selective for mu, delta, kappa or [illegible] receptors. These "high affinity opioid binding sites" have not yet been confirmed independently from Pasternak. Indeed, several carefully performed and executed studies, some employing refined data analysis and mathematical modeling, were able to detect mu, delta and kappa sites, but failed to detect

ABBREVIATIONS: K_d, Dissociation constant; DADL, [D-Ala^2-D-Leu^5]-enkephalin; DAGO, [D-Ala^2-MePhe^4-Gly-ol^5]-enkephalin.

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or demonstrate the mu_1 site. The existence of the mu_1 site carries major implications for opiate drug design since it appears to mediate supraspinal analgesia, control of prolactin release and several other functions, while devoid of respiratory depression (6). Also, the apparent absence of mu_1 sites in guinea pig ileum suggests that it may be possible to provide analgesia with reduced gastrointestinal side effects. In the present study, we demonstrate the simultaneous existence of at least four classes of sites in rat brain interacting with DADL, DAGO, and Naloxone.

MATERIALS AND METHODS

Sprague-Dawley rats were decapitated, the brain was dissected and the cerebellum removed. Homogenization was performed in 50 mM Tris buffer, pH 7.0, at 0° Centigrade using a Polytron homogenizer, Brinkmann Instruments, Westbury, N.Y. at a half-maximal setting for 15 s followed by centrifugation at 36000 g for 15 min. The pellet was washed three times with the same buffer and then resuspended in 50 mM Tris buffer, pH 7.5 at room temperature shortly before the binding assay to 20 mg wet tissue weight per assay tube (2 ml). The assay tubes were incubated for 1 hour at room temperature in the presence of 25 µM Bestatin (Sigma B-8385) and 50 mg/L Bacitracin (Sigma B-0125). The unlabeled ligands, DADL, DAGO, and Naloxone were diluted using a Hamilton Micro Lab M programmable microprocessor controlled diluter/dispenser (Hamilton, Bonaduz, Switzerland) to final concentrations of 10^{-10} to 10^{-4} M. Each concentration was assayed in quadruplicate. [^{3}H]DADL (43.6 Ci/mmol, NET-648), [^{3}H]Naloxone (46 Ci/mmol, NET-719) from New England Nuclear, Boston, Massachusetts and [^{3}H]DAGO (32 Ci/mmol, TRK.681) from Amersham, Arlington Heights, Illinois were present at approximatly 0.1 nM for the tubes containing unlabeled ligand and at 0.01 nM for the tubes containing no labeled ligand. Measurement of "tracer" binding was assayed 12 times for each ligand. We therefore have a total of 216 data points. The incubation mixture was filtered through Whatman GF/B filters (Beckman, Mountainside, New Jersey) using the M-24R Cell-Harvester (Brandel, Gaithersburg, Maryland). Radioactivity retained on the filters was measured by liquid scintillation spectrometry 24 h after addition of 5 ml Ready-Solv HP (Beckman).

The displacement curves were fitted simultaneously by the computer with different models of increasing complexity using weighted nonlinear least-squares curve fitting. The mean of quadruplicate measurements were analyzed assuming constant percentage error in bound ligand concentration. Modeling was performed as described (10). Selection among models was based on the root-mean-square (RMS) error of each fit using the extra sum-of-squares F-test and on the randomness of residuals around the fitted curves (10).

RESULTS AND DISCUSSION

The displacement curves of [^{3}H]DADL, [^{3}H]DAGO and [^{3}H]Naloxone by the corresponding unlabeled ligands are shown in Figure 1. First a two site (mu-delta) model was fit (RMS = 10.27). The quality of the fit improved considerably when a third site was introduced (RMS=7.67). Introducing a fourth high affinity binding site (the mu_1 site) improved the goodness of fit

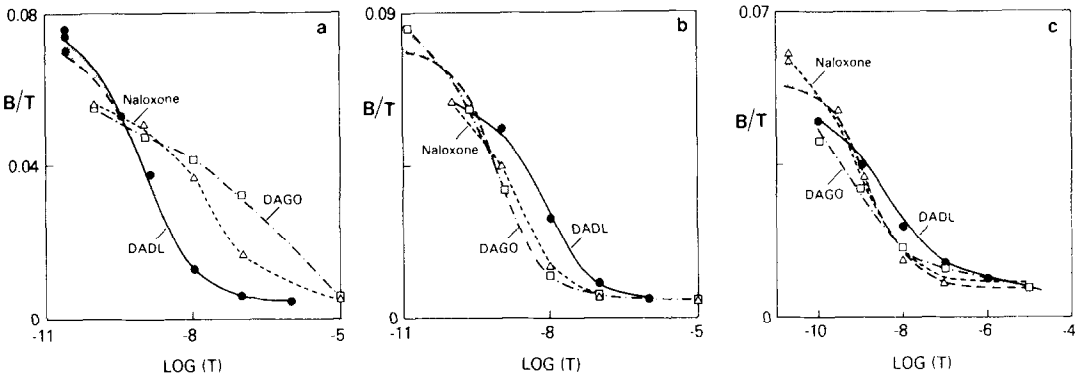

Figure 1: Displacement curves for unlabelled DADL, DAGO and Naloxone. The shaded area represents the difference in the predicted curves of the three-site and four-site models. B/T= bound divided by total radioactivity; Log(T) = logarithm of the total concentration for each ligand. Labeled ligands: a) [^{3}H]DADL; b) [^{3}H]DAGO; c) [^{3}H]Naloxone. Each point represent the mean of quadruplicates (superimposed points are not shown).

further (RMS = 5.38). Each of these improvements in the fit were highly statistically significant ($P<0.001$). The improvement of the fit of the displacement curves obtained by adding mu_1 sites to the mu-delta-kappa model is most evident in the predicted response (bound/total) for tracer only (Figure 1). These "tracer-only" values are based on twelve replicates containing unusually low tracer concentrations (0.01 nM). The customary "tracer" concentrations of 0.1 - 1.0 nM would effectively self-displace at a binding site with the high affinity of the putative mu_1 site. The binding constants for each of the four binding sites are presented in Table 1. A schematic representation of the four-site model is shown in Figure 2.

The failure of numerous other laboratories to detect the mu_1 binding sites is apparently because 1) of its very low concentration (<0.6 pM/g tissue wet weight), 2) it is easily obliterated by conventional labeled ligand concentration (0.1-1 nM), and 3) its demonstration requires optimized conditions for this purpose, including a) use of several labeled ligands, b) multiple displacement curves analyzed simultaneously, c) reduction of analytical errors with high degree of replication (4 or 12), d) use of only a few dose levels which have been selected by computer simulation and optimization (P.J. Munson et al., in preparation), and e) rigorous statistical

TABLE 1. BINDING PARAMETERS

	Receptor concentration	DADL K_d (nM)	DAGO K_d (nM)	Naloxone K_d (nM)
R_1 (mu)	6.5	9.4	1.2	2.6
R_2 (delta)	6.0	1.3	200	38
R_3 (mu_1)	0.54	0.22	0.15	0.20
R_4	0.53	820	290	1.2

Legend for Table 1: Receptor concentrations are expressed in pmoles/g tissue wet weight.

analysis and modeling. The results shown here have been confirmed in more than ten similar experiments without and with naloxonazine and further highly optimized experimental design (manuscripts in preparation). The methods used here place a lower but not an upper limit on the number of classes of sites in rat brain.

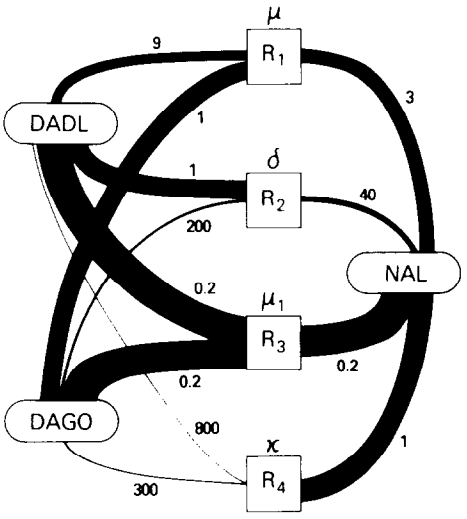

Figure 2: Schematic representation of the best-fitting model: The receptors are represented as squares, where R_1 = mu, R_2 = delta, R_3 = mu_1 and R_4 (kappa, epsilon and others). The binding affinities of the three ligands DADL, DAGO and Naloxone (NAL) are schematically represented by the thickness of the line connecting the ligands with the receptors. The numbers on each line indicate the K_d's (cf Table 1).

Conclusions: 1) The two site (mu/delta) model of opiate binding can now be unequivocally rejected in favor of a 3 site model. DADL and DAGO are shown to bind in a manner more complex than can be explained by the two site (mu/delta) model. 2) A third binding site (mu_1) with high affinity for DADL, DAGO and Naloxone has been demonstrated for the first time by refined, quantitative binding studies assisted by appropriate computer modeling. 3) At least one additional class of sites is necessary when ^{3}H-Naloxone is used as labeled ligand. Quantitative estimates of the binding capacities and of the equilibrium dissociation constants of three ligands for these sites are reported.

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