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Identification and Characterization of Possible Multiple Binding Sites for ((3)H)8-OH-DPAT in the Hippocampus

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IDENTIFICATION AND CHARACTERIZATION OF POSSIBLE MULTIPLE BINDING SITES FOR [^{3}H]8-OH-DPAT IN THE HIPPOCAMPUS

by

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Dawna Lea Evans, M.S.

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The purpose of this project was to determine if there were subsets to the 5HT1A type receptor located in the brain. This was accomplished using both traditional homogenate receptor binding experiments and autoradiographic studies. [^{3}H]8-OH-DPAT was chosen for both approaches based on its 5HT1A binding capability as shown by others. Since this ligand binds to the 5HT1A receptor, blocking studies were used to explore the possibility of subsets. It was suggested, through a non-linear analysis of saturation data, that more than one site was present. This was confirmed with inhibition data which showed a high affinity site to which buspirone, a known 5HT1A agonist, bound readily and a low affinity site to which it did not. Further studies demonstrated that the low affinity site could be bound by ritanserin which had only a low affinity for the buspirone sensitive subsite. Based on these results it is evident that two subsites exist as seen with [^{3}H]8-OH-DPAT binding.

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CHAPTER I

INTRODUCTION

Until 1948, researchers knew of serotonin's action as a vasocontrictor but referred to it as vasotonin. At that time Rapport, Green, and Page isolated vasotonin and renamed it serotonin. By 1951, serotonin (5-hydroxytryptamine or 5HT) had become synthetically available and the total research effort increased tremendously. Two years later, Page and Twarog (1953) demonstrated the presence of 5HT in the central nervous system (CNS). They showed that the raphe nuclei were the main 5HT containing cell bodies in the CNS. Characterization of storage, release, and synthesis mechanisms led to the acceptance of 5HT as a neurotransmitter (Peroutka, 1986).

A neurotransmitter is a chemical messenger released by neurons into a synapse for cell to cell communication. Once released, the messenger binds to specific receptors located on cells (neurons, muscle cells, or secretory cells). The binding action between the messenger and the receptor activates the second messenger system which in turn elicits a physiological response (Berridge, 1985).

Research has shown that more than one type of 5HT

receptor exists and the response is dependent on the type of receptor to which 5HT binds. Brain homogenate binding experiments conducted in 1979 by Peroutka and Snyder showed the existence of two types of 5HT receptors. This was supported anatomically by Young and Kuhar in 1980 and neurophysiologically by Rogawski and Aghajanian in 1981.

Radiolabeled binding experiments give information on the density of the receptors and the affinity of drugs for those receptors (Peroutka, 1986). Several equations describing the state of equilibrium may be applied to receptor binding theory, e.g., the law of mass action which states that the concentration of the products divided by the concentration of the reactants is constant. In the case of a radioligand and receptor interaction this is denoted as:

$$L^* + R \underset{k_2}{\overset{k_1}{\leftrightarrow}} L^*R \qquad \text{and} \quad k_2 / k_1 = Kd$$

where L^* = unbound radiolabeled ligand
R = unbound receptor
L^*R = radiolabeled ligand - receptor complex
k_1 = association rate
k_2 = dissociation rate
Kd = equilibrium dissociation constant.

If Bmax denotes the total concentration of receptors, then at equilibrium:

$$R = Bmax - L^*R.$$

From these equations the Langmuir adsorption isotherm was

derived (1918):

$$L^*R = L^*(Bmax) / (L^* + Kd).$$

Linear transformation of the Langmuir isotherm allows for the direct estimation of the binding parameters, receptor density (Bmax) and binding affinity (Kd). One such transformation, the Scatchard analysis, states that:

$$L^*R / L^* = (Bmax/Kd) - (1/Kd)(L^*R).$$

When plotted, the Kd is estimated by the negative inverse of the slope while Bmax is estimated by the x-intercept (Ruppel, 1984). Simple binding systems often employ the Scatchard analysis for evaluation. A curvilinear Scatchard plot may indicate the presence of more than one receptor site, subsequently a Scatchard analysis is not sufficient thus making a nonlinear analysis necessary. Nonlinear analyses allow the data to be fit to models with varying numbers of additional sites until a model of best fit is reached.

Blocking experiments aid in characterization of subsites in multiple site systems. Blocking study procedures include the use of a compound that is selective for an individual subsite followed by analysis of the interactions which involve the unblocked subsite. The same basic equations apply for the analysis, but the blocking agent is taken into account.

The 5HT receptor contains more than one subtype, as mentioned above, and Peroukta and Snyder (1979) classified these subtypes as 5HT1 and 5HT2. Further studies by many investigators showed the 5HT1 class to be subdivided further into 5HT1A, 5HT1B, 5HT1C, and 5HT1D types. These subtypes exist in different anatomical locations and possess different physiological functions.

Compounds which are agonists at the 5HT1A receptor cause an anxiolytic effect. At the present time, most marketed anxiolytics are of the benzodiazepine chemical structure which bind to the GABA/benzodiazepine receptor and produce negative side effects such as memory loss, drowsiness, euphoria, and ataxia. Compounds which bind to the 5HT1A receptor appear to possess the anxiolytic activity without side effects and therefore may be more desirable than the benzodiazepines. The present study evaluates the 5HT1A binding site to determine if in fact there are additional subsites present. Identification of additional subsites could lead to development of more specific anxiolytics.

CHAPTER II

REVIEW OF THE LITERATURE

Serotonin (5HT) was first isolated from bovine serum and characterized by Rapport et al. (1948). In 1951, Hamlin and Fisher first synthesized 5HT thus allowing for a tremendous research effort and the wealth of information available today. Although the distribution of 5HT in the body is known to be vast and its functions are numerous, it was Page and Twarog (1953) who demonstrated the presence of 5HT in the brain.

Before the availability of radiolabeled ligand-receptor binding experimentation, researchers relied on physiological responses as indicators of drug activity. Using gut bath experimentation, Gaddum and Picarelli (1957) showed that there were two types of tryptamine receptors present in the guinea pig ileum. These experiments aided in the identification of two classes of tryptamine receptors: M receptors, blocked by morphine and D receptors, blocked by dibenzyline. Testing the activity of various compounds on this system, they found that the compounds which were active on the D receptors were also active in various muscle studies. Conversely, 5HT and other compounds active on M receptors appeared to affect neuronal

structures.

When radiolabeled compounds became available, a new field of receptor studies began. By assuming a direct relationship between receptor occupancy and physiological response, it became more efficacious to measure drug activity by receptor binding techniques.

The discovery that tritiated d-LSD ([^{3}H]d-LSD) bound to sites in both cortical and subcortical regions of the rat brain evoked further interest in the field of 5HT since these were areas of known serotonergic input (Bennett & Aghajanian, 1974). Areas without serotonergic input, such as the cerebellum, did not show [^{3}H]d-LSD binding. Additionally, Bennett and Aghajanian showed that 5HT, a compound structurally related to LSD, supressed LSD binding leading to the belief that the receptor to which LSD binds was a serotonergic receptor. A comparison of whole cells versus lysed cells by Bennett and Snyder (1975) indicated that [^{3}H]d-LSD binding occurred on the membrane surface and did not involve transport into intact cells. In addition, Bennett and Snyder discovered that d-LSD binding was reversible and 5HT inhibition was competitive. Studies comparing the regional distribution of [^{3}H]d-LSD binding in rat and monkey brains indicated a great amount of similarity, with the highest binding area being the hippocampus and the lowest being the cerebellum.

In 1979, Peroutka and Snyder showed that [^{3}H]5HT and [^{3}H]spiroperidol ([^{3}H]spiperone) each labeled distinct classes of 5HT receptors and that [^{3}H]d-LSD labeled both. They reasoned that if 5HT, spiperone, and LSD bound to the same receptor, then the potencies and displacement curves for each of the three drugs should be similar. The testing of this theory showed that 5HT was one hundred times more potent in decreasing [^{3}H]5HT binding than [^{3}H]spiperone binding, and intermediate in [^{3}H]LSD binding. LSD had identical potencies in decreasing binding with [^{3}H]5HT, [^{3}H]spiperone, and [^{3}H]d-LSD. Further studies indicated that in the presence of 30 nM of spiperone, the Bmax and Kd for [^{3}H]5HT binding were essentially unchanged. Likewise, 300 nM of 5HT did not affect [^{3}H]spiperone's binding parameters. Yet, both 5HT and spiperone decreased the Bmax of [^{3}H]d-LSD by fifty percent. These results led to the conclusion that 5HT and spiperone bind selectively to different sites but LSD binds to both. Based on these experiments, receptors labeled by [^{3}H]5HT became known as 5HT1 and those labeled by [^{3}H]spiperone, 5HT2.

Peroutka's two site classification of 5HT receptors stimulated interest into the characteristics associated with each site. Early investigation suggested that: (a) guanine nucleotides regulate the 5HT1 site but not the 5HT2 site, (b) synaptic inhibition is associated with the 5HT1

site and synaptic excitation with the 5HT2 site, and (c) the 5HT2 site is involved in the Serotonin Behavioral Syndrome but the 5HT1 site is not (Peroutka, Lebovitz, & Snyder, 1981). As studies in the area continued, Peroutka and Snyder reported that tryptamines were more potent 5HT1 inhibitors and neuroleptics were more potent 5HT2 inhibitors. Further evaluations proved the existence of differences in anatomical receptor site locations for rat, calf, and guinea pig brains. The Bmax for 5HT1 sites were dominant over the 5HT2 sites in all regions, except the cortex where they were similar (Peroutka & Snyder, 1983). Pedigo, Yamamura, and Nelson (1981) conducted [^{3}H]5HT binding experiments and discovered that the addition of spiperone decreased the Bmax without changing the Kd, suggesting the non-competitive binding of spiperone to a high affinity site of [^{3}H]5HT. Further investigation led to the discovery of differing regional variations in the concentration of this high affinity [^{3}H]5HT site. The data obtained by Pedigo et al. did not conform to Peroutka's classification of the 5HT receptors. Autoradiographic studies showed that the [^{3}H]5HT site displaceable by spiperone was the high affinity site of a two site system. Subsequently, the two sites became identified as 5HT1A and 5HT1B and were shown to have different distributions in the brain (Deshmukh, Nelson, & Yamamura, 1982).

Middlemiss and Fozard (1982) found the centrally active agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) to be selective for the 5HT1A subclass in the rat frontal cortex. No appreciable activity for 8-OH-DPAT at the 5HT2 or the 5HT1B sites could be observed. However, the selectivity for the 5HT1A site was almost 1000 fold greater than any other 5HT receptor. Based on these results, Middlemiss and Fozard concluded that 8-OH-DPAT would be a good compound to use for more definitive studies. Comparison of [^{3}H]8-OH-DPAT and [^{3}H]5HT binding in the rat brain displayed significant differences. Most of the [^{3}H]8-OH-DPAT sites correlated well with the [^{3}H]5HT sites; however, some areas of high [^{3}H]5HT binding showed only low [^{3}H]8-OH-DPAT binding. Results such as this helped to substantiate the identification of 8-OH-DPAT as a selective 5HT1A ligand (Marcinkiewicz, Verge, Gozlan, Pichat, & Hamon, 1984). The hippocampus expressed the highest [^{3}H]8-OH-DPAT binding while progressively lower binding existed in the cerebral cortex and striatum. Scatchard anaylsis of these areas revealed single binding sites in all areas except for the cortex, which suggested multiple receptor sites for [^{3}H]8-OH-DPAT (Hall et al., 1985).

Various ions and nucleotides affect [^{3}H]8-OH-DPAT binding. The ion and nucleotide effects were different in the hippocampus and striatum. In the hippocampus, divalent

cations enhanced binding and nucleotides reduced binding, similiar to that seen in [^{3}H]5HT binding (Peroutka et al., 1979). In the striatum, divalent cations inhibited binding and nucleotides did not. The cortex appeared to contain a mixture of the receptor sites typical of the hippocampus and striata. These differences suggest that [^{3}H]8-OH-DPAT may be binding to two types of sites, one a pre- and the other a post-synaptic 5HT1 site (Hall et al., 1985).

In 1984, Pazos, Hoyer, and Palacios proposed the existence of yet another 5HT1 receptor, 5HT1C, which is present in the choroid plexus. Comparisons of the 5HT1 receptor subtypes in the rat and pig revealed the existence of both 5HT1A and 5HT1C sites in both species. However, autoradiographic studies have shown that the porcine brain is devoid of the 5HT1B site (Hoyer, Engel, & Kalkman, 1985). Further investigation of the 5HT1 site showed that all three subtypes exist in rat frontal cortex (Peroutka, 1986).

Heuring and Peroutka characterized yet another 5HT1 receptor (5HT1D) in 1987. Studies of drugs which interact with this receptor suggest that it may be involved in the pathogenesis of migraine. Sumatriptan binds with high affinity to the 5HT1D receptor and has clinically been shown to be effective in the treatment of migraine (Doenicke, Brand, & Perrin, 1988; and Ferrari, Bayliss, Ludlow, & Pilgrim, 1989). The most potent compounds at

these 5HT1D sites are tryptamines and ergots (Heuring & Peroutka, 1987).

The M receptor identified by Gaddum and Picarelli (1957) has been renamed the 5HT3 receptor. This receptor, unlike the 5HT1 and 5HT2 receptors functions via ligand gated ion channels (Gothert, 1992). It was first found in the PNS and later found in the CNS by Kilpatrick, Jones, and Tyers (1987). Antagonism of the 5HT3 receptor exhibits antiemetic properties in patients who are undergoing chemotherapy (Fozard, 1987). Additionally, behavioral studies have suggested possible roles in antipsychotic, and anxiolytic activity as well as the treatment of drug addiction (Peroutka, 1991; & Tricklebank, 1989).

With the recent research tool of molecular biology and specifically receptor cloning, many of these 5HT receptors have been sequenced and cloned into stable cell lines such as chinese hamster ovary (CHO) cells (Frazer, Maayani, & Wolfe, 1990). This has added a new dimension to the classification of receptor subtypes by providing a means of evaluating specific drug/receptor interactions and is quickly becoming an important tool for the identification of new subtypes. Additionally, with the amino acid sequence known it will be possible to identify brain localization at an ultrastructural level using immunocytochemistry and in situ hybridization, determine receptor gene

expression, and study the role of 5HT receptors in normal as well as pathological states (Shih, 1990).

The use of animal models for the study of anxiety revealed that a decrease in 5HT neurotransmission produced an anxiolytic effect. Prior to this discovery, diazepam, which acts via the GABA-benzodiazepine receptor, was the leading anxiolytic. The lack of tolerance and long duration of action supported the use of diazepam but its side effects which include sedation, euphoria, ataxia, and dependence could not be ignored. The quest for a new anxiolytic which would not express the undesirable side effects common to diazepam, placed 5HT in the pharmacological research arena. In 1979, Goldberg and Finnerty compared the anxiolytic effects of buspirone and diazepam. Buspirone did not produce the ataxia and hypoactivity common with diazepam treatment but in terms of efficacy it was indistinguishable from diazepam. In 1982, Hjorth and Carlsson, hypothesized that buspirone acted via the 5HT1 receptor. Buspirone displaced [^{3}H]5HT binding with a Ki of 20 nM, indicating that it was only twenty times less potent than 5HT (Glaser & Traber, 1983). The buspirone displacement results gave hope for a new type of anxiolytic and research on the 5HT1 site increased. The 5HT1A agonist, 8-OH-DPAT, produced an anxiolytic effect in some, but not all, of the anxiolytic animal models. Considering the number of

serotonergic pathways and the fact that most anxiolytic tests were set up for benzodiazepines, it is difficult to determine the potential use of 5HT1A type anxiolytics. Buspirone and other azapirones such as gepirone and ipsapirone, which display high affinity for the 5HT1A site, have been shown in the clinic to lack the side effect profile of the benzodiazepines (Taylor & Moon, 1991). The various pathways of 5HT, anxiolytic tests for benzodiazepines, and unreported anxiolytic side effects of non-anxiolytic drugs have made it difficult to identify the 5HT1A receptor and its role in producing anxiolytic effects (Chopin & Briley, 1987).

Clearly it can be seen that although the pharmacology of 5HT has been widely researched, there are still several untouched areas. To date at least six different 5HT subtypes exist in the central nervous system: the 5HT1A, 5HT1B, 5HT1C, 5HT1D, 5HT2, and 5HT3 (Peroutka, 1988). There are several implications for serotonergic activity in human disorders such as anxiety, depression, epilepsy, etc. The exact role of 5HT and its extensive multiplicity have as of yet to be fully understood.

The present research further characterizes the 5HT1A receptor as classified by Peroutka. Saturation studies provide evidence suggesting the existence of two subtypes for the 5HT1A receptor. Inhibition curves revealing two

plateaus for buspirone further support the explanation of a multiple site system.

CHAPTER III

MATERIALS AND METHODS

Tissue Homogenate Studies

Dividing tissue homogenate studies into two categories, saturation and inhibition studies, allows for the characterization of receptors. Scatchard and/or nonlinear analyses of saturation studies reveal information pertaining to the density of a particular receptor in the tissue and the affinity of the radiolabeled ligand for that receptor. Inhibition studies examine the competition between a radiolabeled ligand and a drug for the receptor. Probit-log and/or nonlinear analysis serve as methods of evaluation for this competition.

Materials

The high concentration of 5HT1A receptors in the hippocampus made it the tissue of choice for the following homogenate studies. A local slaughter house furnished the bovine hippocampi and The Upjohn Company (Kalamazoo, Michigan) provided the rat hippocampi. Amersham supplied the tritiated 8-OH-DPAT and the drugs used in the study came from various commercial suppliers.

Methods

Tissue Preparation

Tissue preparation requires homogenization of approximately 1 g of hippocampus in 10 ml of 50 mM TRIS-HCl buffer, pH 7.4, using a Brinkmann Polytron at a setting of 7 for 30 seconds. Following homogenization, the tissue undergoes centrifugation in a Sorval RC5C refrigerated centrifuge at 27000 x g for 10 minutes after which the supernatant is decanted and the pellet resuspended with 10 ml of buffer. Incubation of the homogenate at 37° C for 15 minutes allows for dissociation of endogenous 5HT from receptor. After incubation, the homogenate undergoes another centrifugation for 10 minutes. The supernatant is discarded, buffer is added, and the tissue resuspended using the Polytron. The tissue homogenate is diluted to a final concentration of 0.5 g / 100 ml.

Assay Procedure

Homogenate assays, whether saturation or inhibition studies, involve incubation of the radiolabeled ligand with the receptor. Depending on the type of study, the incubation mixture (IM) includes other components such as buffer, cold (unlabeled) ligand, or varying concentrations of a competing drug. To determine the amount of specific

radiolabeled ligand which binds to the receptor, known as the total bound (TB), the tissue homogenate is incubated with the radiolabel and buffer. Non-receptor entities, such as filters, also bind ligand and is referred to as non-specific bound (NSB). The amount of radiolabel ligand bound to these entities is determined by evaluating the amount of radiolabel bound in the presence of a very high concentration of cold ligand, the radiolabeled ligand, and the homogenate. The cold ligand binds all the receptor sites, allowing for the binding of radiolabeled ligand to non-receptor entities only. The NSB accounts for some experimental noise and is either subtracted from all values or incorporated into the analysis. Triplicate tubes of the various incubation mixtures provide an average value to best estimate experimental error.

Addition of the homogenate starts the 30 minute incubation period which is carried out in a 30° C shaking water bath. Upon completion of the incubation, the IM undergoes filtration through Schleicher and Schuell #24 glass fiber filters using a Cambridge PHD cell harvester. After filtration, the cell harvester drops the filters into vials containing scintillation fluid. A Packard beta particle counter with a counting efficiency of fifty-four percent, measures the radioactivity trapped by the filters.

Saturation Studies. A saturation experiment allows

for the estimation of ligand binding parameters. Analysis of TB and NSB at several concentrations of ligand reveals the amount of label specifically bound to the receptor. Specific binding (SB) refers to the value obtained by subtracting NSB from TB. The SB value and amount of free ligand constitute the information necessary for compiling a Scatchard, the regression plot of the ratio of SB/Free vs SB. The x-intercept depicts the maximum number of binding sites (Bmax) and the negative reciprocal of the slope represents the affinity of the ligand for the receptor (Kd). Estimation of the binding parameters (Bmax and Kd) improves with an increased number of ligand concentrations within the selected range (Hrdina, Pappas, Roberts, Bialik, & Ryan 1986).

The linearity of a Scatchard plot may indicate the existence of only one receptor site whereas a non-linear plot can be used to suggest the presence of more than one site. Saturation experiments run with bovine and rat tissue, using eighteen concentrations of [^{3}H]8-OH-DPAT ranging from approximately 0.01 nanomolar (nM) to 3 nM, both revealed non-linearity of the Scatchard plots. The non-linearity suggested the existence of multiple sites and led to the evaluation of [^{3}H]8-OH-DPAT binding via inhibition studies.

Inhibition Studies. Inhibition studies measure the

ability of a drug to compete with a ligand for a particular receptor site. This is accomplished by running several concentrations of the drug with a constant concentration of the radiolabeled ligand. Analysis requires measurements of TB and NSB. Values for the amount of label bound in the presence of the drug, TB, and NSB provide sufficient information for identifying the concentration at which fifty percent of the label is displaced by the drug (IC50). Probit-log or nonlinear analysis of the data allow for the determination of a drug's IC50. The affinity of the inhibiting drug for the receptor (Ki) can be calculated from the Cheng Prusoff equation:

$$Ki = IC50 / (1 + [L]/Kd)$$

where [L] is the concentration of radiolabeled ligand in the assay and Kd is the radiolabel equilibrium dissociation constant determined from saturation analysis.

By running various drugs through this procedure it became possible to identify the types of compounds that bound to the different 5HT1A subtypes. Reviewing the results from previous inhibition studies disclosed compounds of high affinity for the 5HT1A receptor and others with a lower affinity. Inhibition studies used approximately 0.5 nM [^{3}H]8-OH-DPAT. Characterization of receptor subtypes required the use of drugs which expressed binding to only

one subtype. Blocking studies served as the experimental procedure for evaluation of the different subtypes.

Blocking Studies. A blocking study allows for the evaluation of the site of interest by suppressing the other sites to which the radioligand binds. Data gathered from this type of analysis provide information pertaining to the number of receptors, ligand affinity, and inhibiting drug affinity for the various subtypes.

A Scatchard analysis of saturation studies using rat and bovine tissue, with the high affinity site of [^{3}H]8-OH-DPAT blocked by buspirone, revealed the binding parameters of the second subtype. Further inhibition studies in the presence of buspirone allowed for the identification of compounds which bound to the low affinity [^{3}H]8-OH-DPAT subtype.

A complex inhibition study, run after identification of the different subtypes, verified that compounds could be found which worked at different subtypes of the 5HT1A receptor. Inhibition studies such as this require measuring the inhibition of the radiolabel binding by one of the selective blocking drugs (A) in the presence of the second selective drug (B) at increasing concentrations. As the first blocking drug (A) binds to its respective site it blocks the radiolabel from binding to this site, thus the only sites labeled are those to which drug (B) has high

affinity. As a result, the concentration of the second drug that inhibits the label by 50 percent decreases, i.e. the IC50 decreases. Addition of the first drug to a point of excess for the drug's most active site results in spill-over blocking of the second site. The IC50s for the second drug increase in such a situation. Changes in the IC50s for the different drugs indicate differences in binding affinities for the two subsites. Compounds having high affinities for the same site would exhibit unchanged or constantly increased IC50 values. The displacement of [^{3}H]8-OH-DPAT by altanserin in the presence of increasing buspirone concentrations revealed unique and distinct drug affinities for each subsite.

Autoradiographic Studies

Autoradiographic studies provide the advantage of looking at numerous brain structures to determine if there are regional differences in receptor binding. This requires measurements of TB (total bound) and NSB (non-specific bound). Tissue slices, 10 - 12 μm, undergo incubation in a solution of radiolabel and buffer or radiolabel and cold drug to determine TB and NSB, respectively. Addition of blocking agents specific to the different subtypes, allows for the measurement of specific bound (SB) for each subtype, making comparisons between blocked and unblocked

groups possible.

Materials

The autoradiographic studies require the use of radiolabeled 8-OH-DPAT, blocking drugs, tritium sensitive film, and microscope slides. The film used in this study is Hyperfilm-3H provided by Amersham.

Methods

Tissue preparation includes saggital slicing of frozen rat brains and thaw mounting them onto microscope slides. Storage at -80° C until the time of experimentation prevents tissue decomposition.

Autoradiographic studies involve incubation of tissue sections in a TB solution containing [^{3}H]8-OH-DPAT and a NSB solution containing radiolabeled ligand ([^{3}H]8-OH-DPAT) in combination with unlabeled 8-OH-DPAT. Total bound and NSB values allow for the calculation of the SB for the radiolabeled ligand. To determine the competition between a drug (e.g., buspirone or altanserin) and the radiolabeled ligand for a particular receptor subsite, tissue samples undergo exposure to an additional set of TB and NSB which include the blocking drug. A comparison of the SB values with and without the blocking drug demonstrates the localization of the receptor subtypes.

CHAPTER IV

RESULTS

Homogenate Studies

Multiple saturation and inhibition studies in bovine hippocampal tissue revealed the existence of two subtypes of the 5HT1A receptor. Saturation in rat hippocampi supported the existence of two subtypes but was less defined. Results reported herein are based on two or more experimental trials. Additionally, experimentation was conducted in triplicate providing a statistically valid estimate of binding activity thereby reducing the possibility of abberant data.

Bovine Saturation Study

A Scatchard analysis of the data gave a Bmax of 3.4 pmol/g tissue and a Kd of 0.3 nM. Statistically, in a test for a linear vs a quadratic model, the data did not fit a linear model (p=0.0001*). A nonlinear analysis of the data suggested that a two-site model represented the best statistical fit with a high affinity site having a Bmax of 2.2 pmol/g tissue and a Kd of 0.2 nM and a low affinity site with a Bmax of 3.1 pmol/g tissue and a Kd of 3.9 nM. (Table

1, Figure 1).

Table 1

[^{3}H]8-OH-DPAT Saturation in Bovine Hippocampus

	1 site	2 site
Bm_1	3.4 ± 0.1	2.2 ± 0.5
Kd_1	0.3 ± 0.02	0.2 ± 0.05
Bm_2		3.1 ± 2.3
Kd_2		3.9 ± 6.6
SSE	0.246	0.160
AIC	-61.225	-77.888
p 1 vs 2	0.0001*	

Based on the saturation study, the amount of [^{3}H]8-OH-DPAT binding at each site for an inhibition study can be calculated with the following equation:

$$B = [L](Bmax) / ([L] + Kd)$$

where B is the number of sites, [L] is the concentration of the radiolabeled ligand in the inhibition study, and the Bmax and Kd values are taken from the saturation analysis. Using this equation for an inhibition study, which uses 0.5 nM of [^{3}H]8-OH-DPAT, approximately 80% of the radiolabeled ligand would bind to the high affinity site (0.2 nM) and 20% would bind to the low affinity site (3.9 nM).

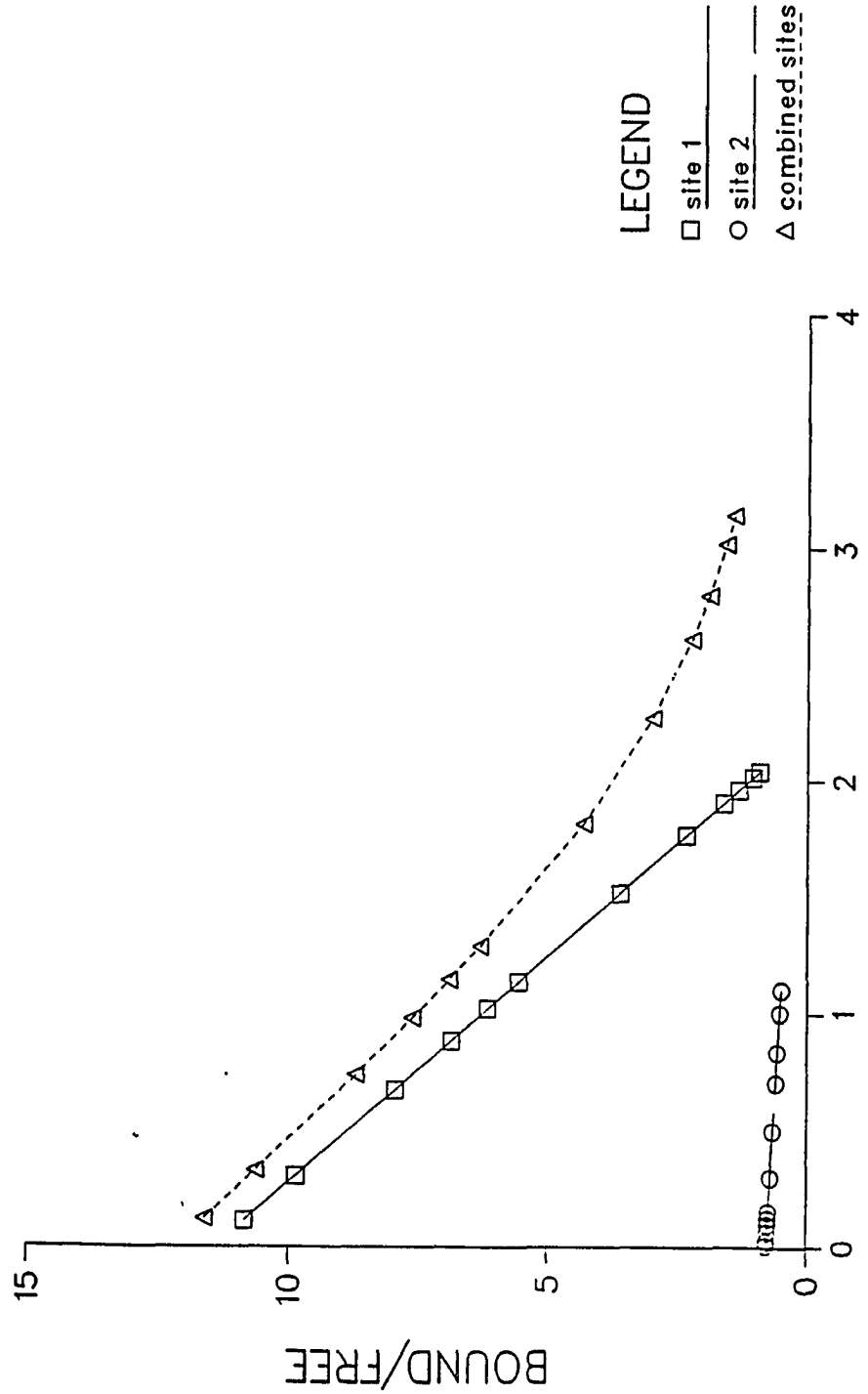

Figure 1. [^{3}H]8-OH-DPAT Saturation In Bovine Hippocampus Nonlinear Analysis.

Inhibition Screen

Several compounds which were known to be active on the 5HT1A receptor were tested for their [^{3}H]8-OH-DPAT inhibition and analyzed for a one site or two site fit (Table 2, Figure 2). Of the compounds tested, buspirone and ipsapirone demonstrated a better fit to a two site model, as can be seen by the decrease in the AIC (Akaike Information Criterion) value (Kamikubo, Murase, Murayama, & Miura, 1986). Buspirone bound with an $IC50_1$ of 13.6 nM and an $IC50_2$ of 798 nM, while ipsapirone bound with an $IC50_1$ of 0.7 nM and an $IC50_2$ of 237 nM.

The high affinity site for these compounds ($IC50_1$) comprised 82 and 71 percent of the [^{3}H]8-OH-DPAT sites. According to the previous equation this would suggest that the buspirone and ipsapirone high affinity site is competing for the [^{3}H]8-OH-DPAT high affinity site (80%).

Saturation Study in the Presence of 30 nM Buspirone

When the saturation study was done in the presence of buspirone it was found that a two site model no longer fit the data (Table 3, Figure 3). The buspirone appears to have eliminated the high affinity binding site of [^{3}H]8-OH-DPAT. The remaining site was found to have a Bmax of 4.5 pmol/g tissue and a Kd of 2.6 nM in the presence of 30 nM buspirone. These values are close to that of the lower

Table 2

[^{3}H]8-OH-DPAT Inhibition by Selected Agents

Compound	1 site IC50	AIC	2 site			
			$IC50_1$	$IC50_2$	% of site 1	AIC
Buspirone*	26.7 ± 4.9	66.3	13.6 ± 1.2	797.8 ± 323.6	82	36.7
Ipsapirone*	4.4 ± 1.7	81.9	0.7 ± 0.18	237 ± 63.5	71	45.0
Pindolol	25.3 ± 1.1	35.2	46.5 ± 299	24.0 ± 11.3	7	37.0
Ketanserin	4940 ± 549	60.8	5010 ± 521	>10000	100	62.5
Mianserin	1030 ± 54	42.5	394 ± 258	805 ± 141	-	43.1
Quipazine	6557 ± 416	49.0	414 ± 1742	>10000	13	95.5
Clozapine	485 ± 30	46.6	9.1 ± 22.0	456 ± 43	-	46.5

Nonlinear least squares fitting (NLLSF) analysis

*Statistically better 2 site fit

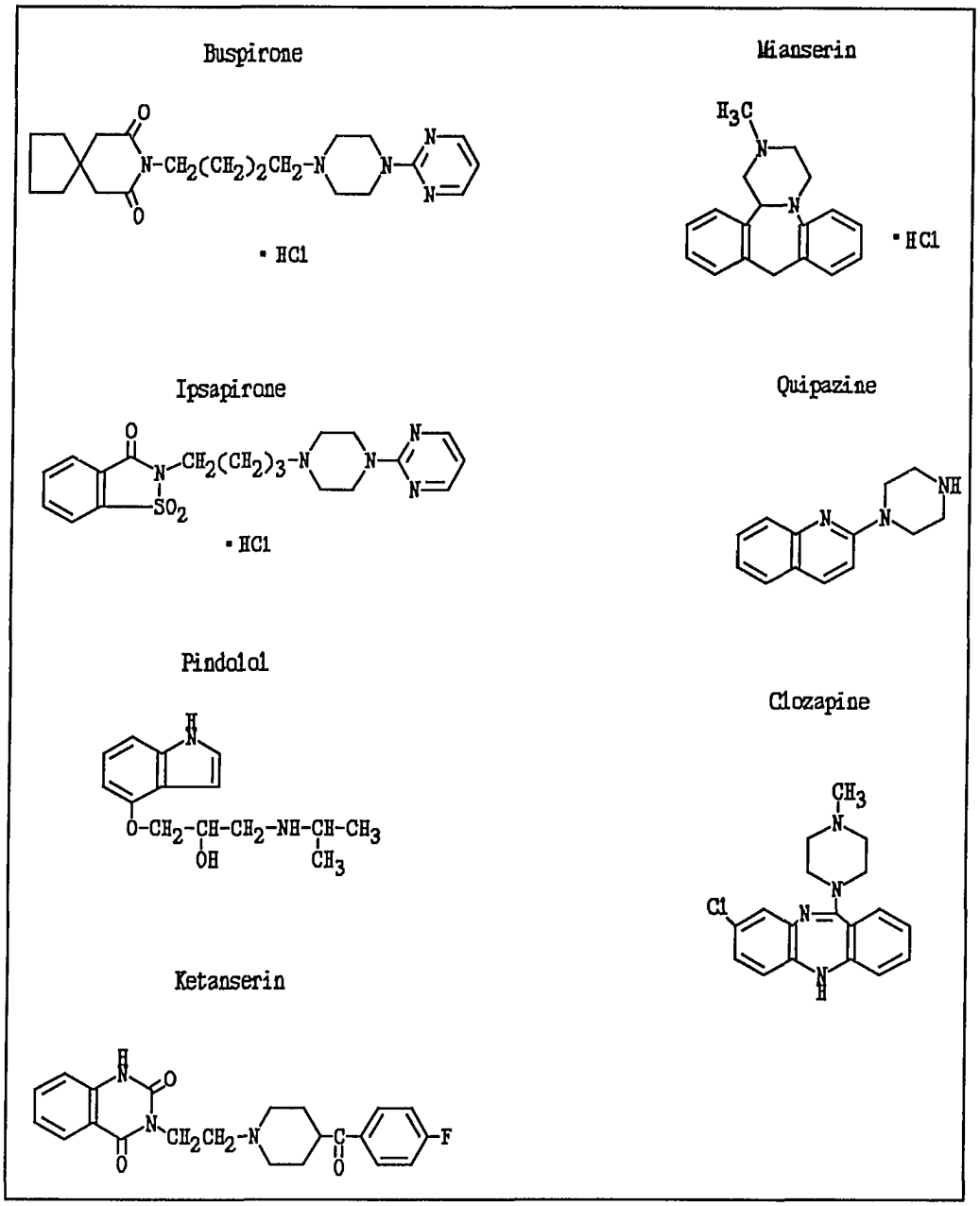

Figure 2. Chemical Structure of Compounds Tested. I.

Table 3

[^{3}H]8-OH-DPAT Saturation in Bovine Hippocampus in the presence of 30 nM Buspirone

	1 site	2 site
Bm_1	4.5 ± 0.4	1.6 ± >10000
Kd_1	2.6 ± 0.3	2.6 ± >10000
Bm_2		2.9 ± >10000
Kd_2		2.6 ± 9242
SSE	0.188	0.188
AIC	-78.49	-68.49
p 1 vs 2	NO IMPROVEMENT	

affinity [^{3}H]8-OH-DPAT site obtained in the first saturation study (Bm_2=3.1, Kd_2=3.9) (Table 1). This supports that buspirone binds to the high affinity site of [^{3}H]8-OH-DPAT.

Inhibition Study

To identify compounds which expressed preferential binding to one subsite over the other, several drugs underwent an inhibition study in the presence and absence of a buspirone block (30 or 300 nM). The concentration of [^{3}H]8-OH-DPAT was approximately 0.5 nM. Compounds were chosen from various pharmacological classes to investigate variations due to structure. This data was then analyzed for one and two site models using a nonlinear least squares fitting program provided by F. Kedzy (TUC) (Table 4).

AJ-76, spiperone, pindolol, and (-)3PPP (see Figure 4

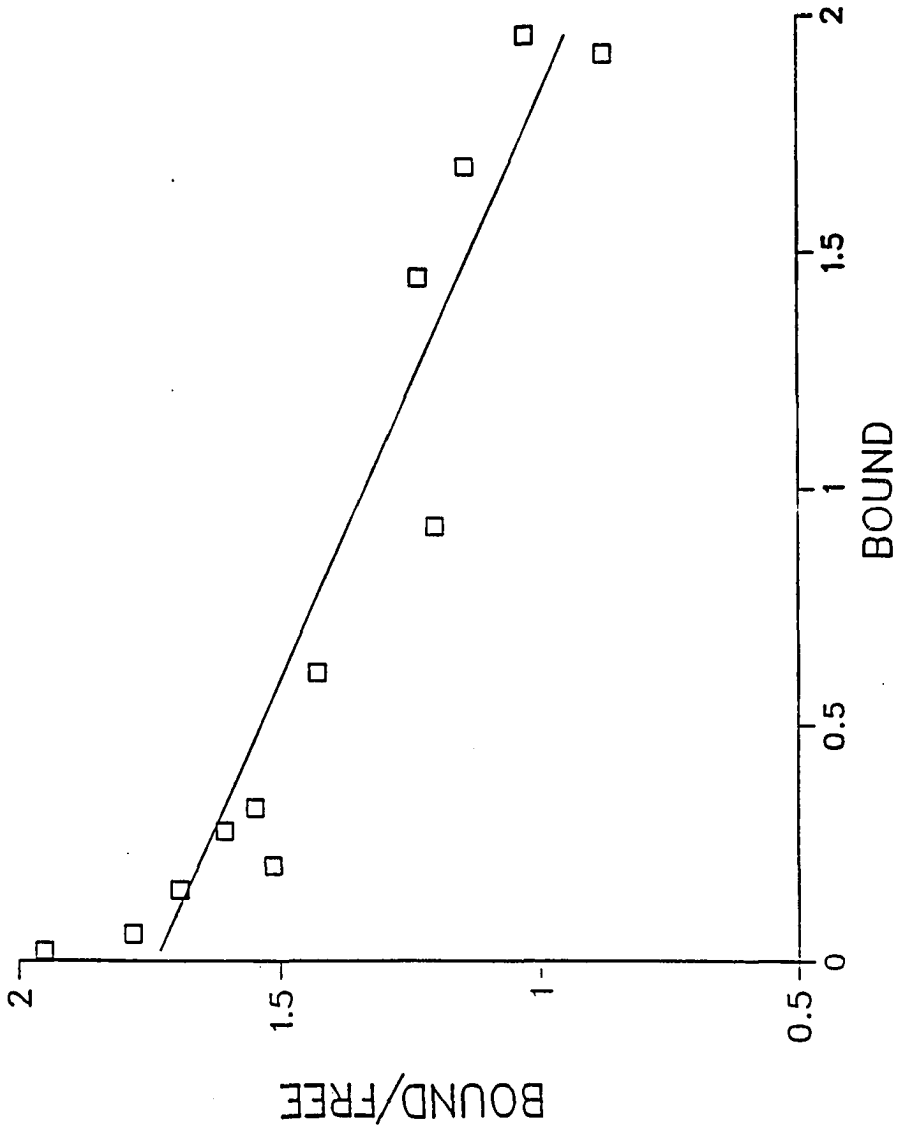

Figure 3. [^{3}H]8-OH-DPAT Saturation in Bovine Hippocampus With 30 nM Buspirone.

for structures) showed a statistically significant increase in their IC50's in the presence of buspirone and ritanserin was marginally significant ($p = 0.07$) when analyzed using a one site model. This demonstrates that these compounds are active at the same site upon which buspirone is acting.

Compounds such as ketanserin and Br-LSD did not significantly shift in the presence of buspirone but they demonstrated a two site fit in the presence of buspirone while having only a one site fit without it. This suggests that these compounds may be binding to both of the [^{3}H]8-OH-DPAT sites with equal affinity. When buspirone is present there is more competition at one site and therefore ketanserin and Br-LSD demonstrate different affinities for the two sites.

Ritanserin, spiperone, altanserin, and (-)3PPP show a statistically better fit to a two site model without buspirone than a one site model as represented by the decrease in AIC value. Buspirone eliminated one of the binding sites for altanserin and (-)3PPP thereby making it impossible to calculate a two site model. Ritanserin in the presence of buspirone had a statisticaly better fit to a one site model, however a two site model could still be calculated. When looking at the two site model, it appears that buspirone is working at the low affinity site of ritanserin as there is a statistically significant decrease in the

Table 4

[^{3}H]8-OH-DPAT Inhibition by Various Compounds

Compd	[Busp] nM	IC50	*p*	AIC	2 site			
					$IC50_1$	$IC50_2$	% 1	AIC
AJ-76	0	802 ± 96	.0384*	19.8	Couldn't Calculate			
	30	6658 ±1584		34.0	7.2 ± 10.2	7941 ± 843	14	28.9
Spip	0	51.9 ±17.5	.0012*	22.4	12.8± 20.3	157± 279	50	18.0
	300	>10-000		29.6	4 ± 4254	115± 29843	27	42.7
Ketan	0	2573 ±330	.4596	38.6	Couldn't Calculate			
	300	4565 ±691		44.2	463± 728	6930± 1302	20	42.4
Pind	0	32.6 ± 2.1	.0019*	22.9	Couldn't Calculate			
	300	376 ± 82		41.4	134± 138	2643± 5060	64	34.2
Ritan	0	1642 ± 436	.0735#	59.5	2.6 ± 5	1858± 524	15	57.7
	30	6633± 1177		54.2	22.5± 83.5	6846± 1238	8	54.3
Altan	0	1049 ± 436	.0366*	57.4	199± 126	6176± 3444	54	49.8
	300	264 ± 60		40.9	Couldn't Calculate			
(-)3PPP	0	968 ± 125	.0000*	47.0	1.3± 1.3	1111± 121	11	40
	30	3985 ± 376		20.4	Couldn't Calculate			
Br-LSD	0	18.9 ± 3.1	.3479	54.8	Couldn't Calculate			
	30	148 ± 8.4		26.1	134 ± 19	1016± 1760	95	26.5
Meterg	0	7.6 ± 1.4	.6643	58.1	Couldn't Calculate			
	30	66.1 ± 9.1		47.6	Couldn't Calculate			

*significant #marginally significant

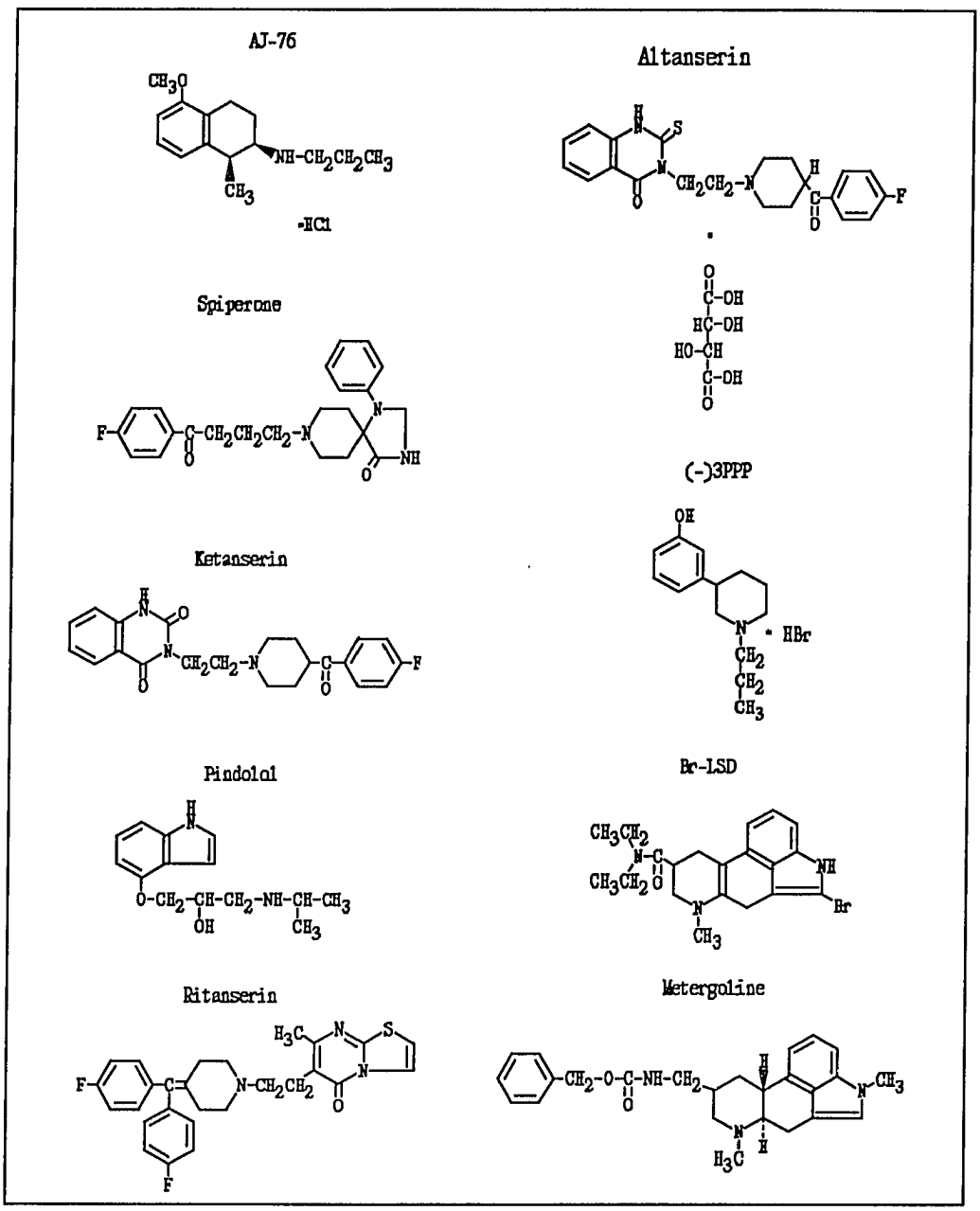

Figure 4. Chemical Structure of Compounds Tested. II.

$IC50_2$ (p=0.003) but not in $IC50_1$ (p=0.7014).

Complex Inhibition Study

In order to better clarify the separation of these two binding sites, the inhibition of [^{3}H]8-OH-DPAT by various compounds in the presence of increasing amounts of buspirone was conducted.

Ipsapirone

Table 2 demonstrated that ipsapirone was better fit by a two site model and therefore several inhibition curves in the presence of various concentrations of buspirone were conducted to more clearly identify the subsites of [^{3}H]8-OH-DPAT binding (Table 5, Figure 5).

This ipsapirone study did not show a two site fit without buspirone, however, at low concentrations of buspirone (0.5, 1, and 5 nM) a two site model gave a better fit as demonstrated by a decrease in the AIC value. The major portion of these sites (>90%) were of a high affinity (5-7 nM) type with the smaller portion (<10%) of a lower affinity (1-4 μM).

At 10 nM of buspirone the data fit a one site model which may imply that buspirone has filled one of the sites thereby forcing ipsapirone to compete with [^{3}H]8-OH-DPAT at only one of its binding sites.

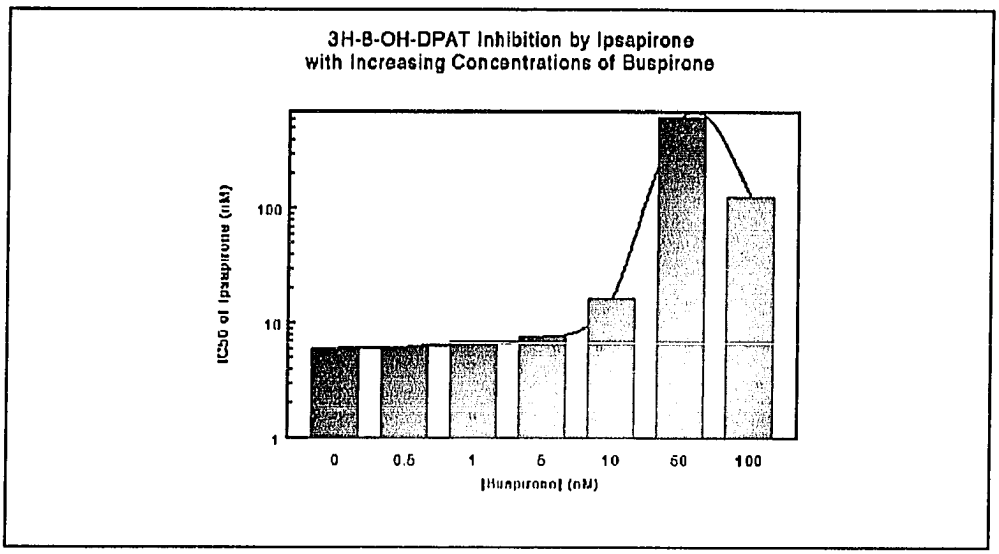

Figure 5. [^{3}H]8-OH-DPAT Inhibition by Ipsapirone with Increasing Concentrations of Buspirone.

At 50 nM of buspirone a two site fit is once again the statistically best fit for this inhibition curve, but with a much reduced number of sites (<40% vs >90%) being of the high affinity type. This demonstrates the competition of buspirone with ipsapirone for its high affinity site with little effect at the lower affinity site.

At the higher concentrations of buspirone there is less than 20% of the [^{3}H]8-OH-DPAT specifically bound to the receptors and therefore analysis of this binding is questionable. Buspirone is competing for both the high and low affinity [^{3}H]8-OH-DPAT sites.

Table 5

[^{3}H]8-OH-DPAT Inhibition by Ipsapirone With Increasing Concentrations of Buspirone

Busp (nM)	1 site IC50 (nM)	AIC	2 site		% of site 1	AIC	%SB
			$IC50_1$	$IC50_2$			
0	6.0 ± 0.6	54.4	Couldn't Calculate			55.4	69
0.5	6.2 ± 1	70.2	4.7 ± 0.6	4277 ± 6215	94	65.8	85
1	6.8 ± 0.9	65.0	4.8 ± 0.4	1000 ± 688	91	44.4	70
5	7.6. ± 0.6	47.5	6.9 ± 0.5	3528 ± 1150	97	40.9	63
10	16.1 ± 3.3	62.3	Couldn't Calculate			64.1	56
50	616 ± 348	74.2	1.1 ± 0.5	1677 ± 406	38	56.0	34
100	126 ± 23	44.8	161 ± 1672	143 ± 216	13	46.8	19
500	8593 ± 3560	41.2	Couldn't Calculate			42.2	13
5000	11.8 ± 3.7	44.6	10.4 ± 4.3	9294 ± 2351	95	46.0	8

Ritanserin

From Table 4 it was evident that ritanserin displaced [^{3}H]8-OH-DPAT binding with different affinities for the various subtypes. This study demonstrated that as the concentration of buspirone increased from 0 to 10 nM, ritanserin's IC50 gradually increased suggesting that buspirone was displacing it from the [^{3}H]8-OH-DPAT sites. At

50 and 100 nM of buspirone the binding could be separated into two binding sites where ritanserin demonstrated differing affinities for these two sites (Table 6, Figure 6).

This ritanserin study was unable to fit a two site model with 0 to 5 nM of buspirone. However at 50 and 100 nM of buspirone a two site model fit the data best. As the concentration of buspirone increased, the percent of ritanserin high affinity sites increased (7 to 30%). Taken together with the fact that the total percentage of available [^{3}H]8-OH-DPAT sites decreased (64 to 27%) it seems likely that buspirone and ritanserin were working at different [^{3}H]8-OH-DPAT sites.

Rat Saturation Study With and Without Buspirone

A linear analysis of [^{3}H]8-OH-DPAT saturation in rat tissue had a Bmax of 6.6 ± 0.3 pmol/g tissue and a Kd of 0.7 ± 0.05 nM without buspirone and a Bmax of 10.1 ± 1.9 pmol/g tissue and a Kd of 5.3 ± 1.2 nM in the presence of 100 nM buspirone, however, neither curve fit a linear model (Figure 7). Using a non-linear analysis, although the two site model suggested a better fit, the Bmax and Kd values for one of the two sites was extremely high with large standard errors (Table 7). This was consistent even in the presence of 100 nM buspirone.

As can be seen, according to the analysis, a two site

Table 6

[^{3}H]8-OH-DPAT Inhibition by Ritanserin With Increasing Concentrations of Buspirone

Busp (nM)	1 site IC50	AIC	$IC50_1$	$IC50_2$	% of site 1	AIC	% SB
0	211 ± 55	59.5	Couldn't Calculate			61.5	67
0.5	284 ± 60	648.6	Couldn't Calculate			48.6	63
1	312 ± 75	57.9	Couldn't Calculate			58.7	66
5	433 ± 161	49.0	Couldn't Calculate			51.0	60
10	631 ± 167	59.8	8 ± 52	697 ± 299	7	61.4	64
50	566 ± 95	51.5	4 ± 9	660 ± 161	10	50.7	38
100	703 ± 207	53.1	19 ± 33	1220 ± 617	24	48.8	27
500	1497 ± 457	47.5	289 ± 786	2824 ±1544	30	48.8	10
5000	605 ± 650	43.3	Couldn't Calculate			45.2	9

model best fits the data. However the values would suggest that the low affinity site could not be analyzed with much precision since the standard deviations are so large.

Autoradiographic Study

Based on the homogenate binding studies, [^{3}H]8-OH-DPAT binding in the absence and presence of buspirone or altanserin was chosen to be tested in rat whole brain autoradiography.

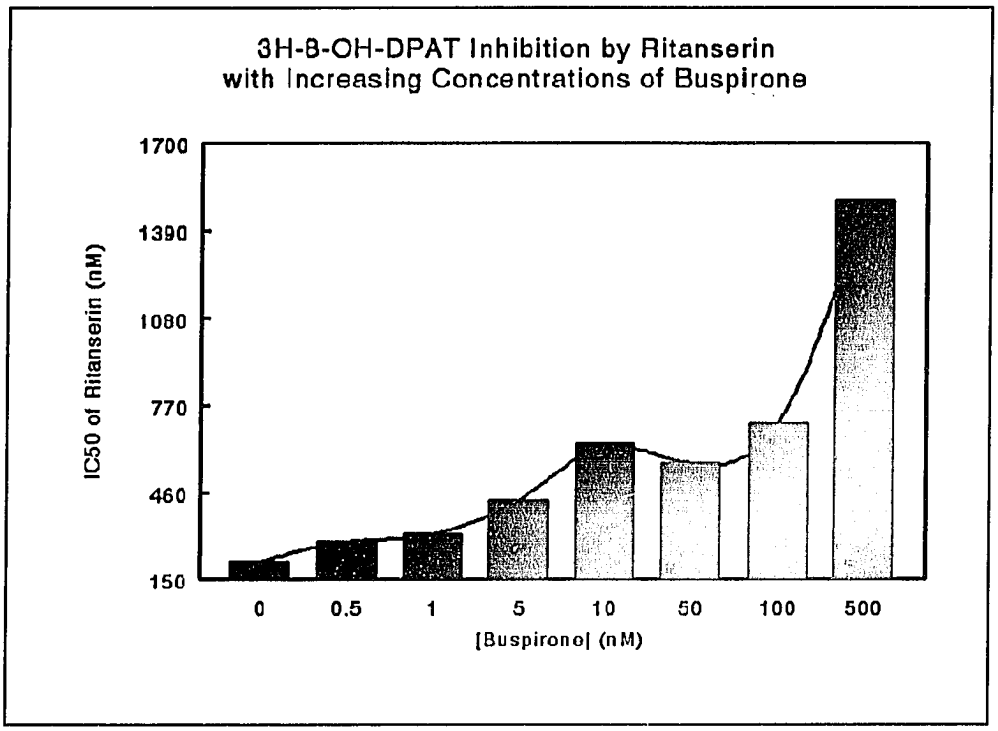

Figure 6. [^{3}H]8-OH-DPAT Inhibition by Ritanserin with Increasing Concentrations of Buspirone.

Despite the lack of precision for the second site of [^{3}H]8-OH-DPAT in rat hippocampus, rat tissue was chosen for autoradiographic study.

As can be seen in Figures 8a and 9a, [^{3}H]8-OH-DPAT binds to various regions of the hippocampus, entorhinal cortex, frontal and occipital cortex, and amygdala. As can be seen in Table 8 and Figures 8b, 8c, 9b, and 9c, in the presence of 200 nM buspirone or 20 μM altanserin, there is a decrease in [^{3}H]8-OH-DPAT binding in all areas. This was expected since buspirone and altanserin bind to both [^{3}H]8-OH-DPAT sites.

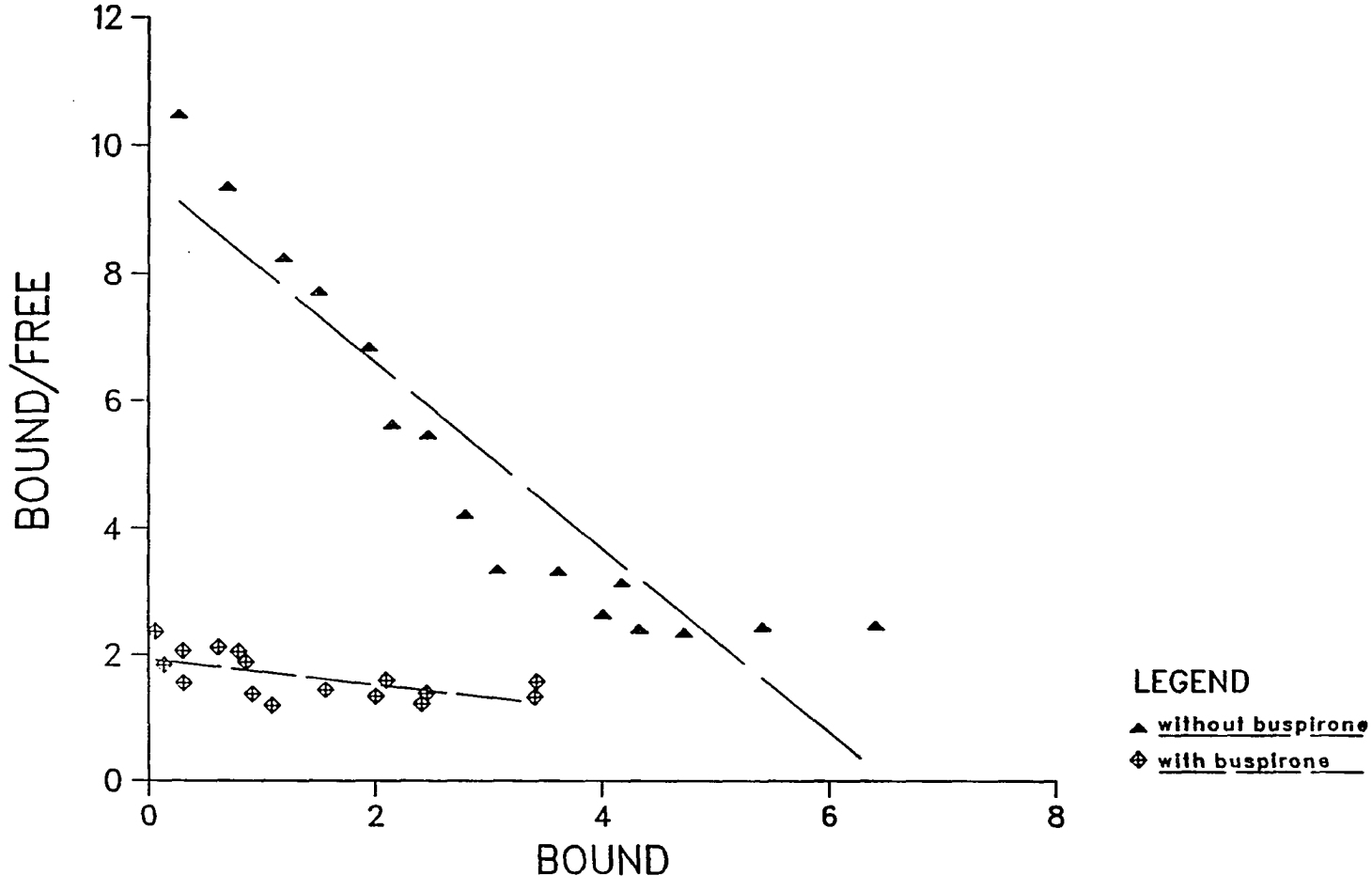

Figure 7. [^{3}H]8-OH-DPAT Saturation in Rat Hippocampus With and Without 100 nM Buspirone.

Table 7

Nonlinear Analysis of [^{3}H] 8-OH-DPAT Binding in Rat Hippocampus

Parameter	without Buspirone		with 100 nM Buspirone	
	1 site	2 site	1 site	2 site
Bm_1	6.4 ± 0.3	2.4 ± 0.6	8.1 ± 1.5	0.38 ± 0.37
Kd_1	0.7 ± 0.1	0.2 ± 0.06	4.0 ± 1.0	0.16 ± 0.20
Bm_2		41878 ± >10000		24440 ± >10000
Kd_2		29795 ± >10000		20866 ± >10000
SSE	2.3932	1.1163	1.6614	1.309
AIC	61.8500	17.041	38.4921	27.241
p 1 vs 2	0.0000*		0.0009*	

Table 8

Optical Densities of Selected Regions

Lateral 4.2			
Region	w/o block	w/ Buspirone	w/ Altanserin
Dentate Gyrus	408.5 ± 44.5	89.5 ± 3.5	48.0 ± 1.0
CA1 (hippocampus)	274.0 ± 65.0	60.5 ± 1.5	31.0 ± 0.0
CA2 (hippocampus)	242.0 ± 11.0	47.5 ± 12.5	19.0 ± 0.0
CA3 (hippocampus)	302.0 ± 12.0	55.0 ± 3.0	19.5 ± 0.5
Entorhinal Cortex	368.5 ± 20.5	71.0 ± 6.0	40.5 ± 6.5
Frontal/Occip Cx	202.0 ± 4.0	47.0 ± 9.0	21.0 ± 2.0
Orbital Cortex	170.5 ± 7.5	31.5 ± 1.5	18.5 ± 0.5
Lateral 2.9			
Region	w/o block	w/ Buspirone	w/ Altanserin
Dentate Gyrus	256.0 ± 1.0	69.0 ± 1.0	18.5 ± 0.5
Ammon's horn	166.5 ± 4.5	46.5 ± 3.5	18.0 ± 0.0
Cortex	141.5 ± 3.5	35.0 ± 3.0	19.0 ± 0.0
Amygdala	152.0 ± 2.0	44.0 ± 4.0	18.5 ± 0.5

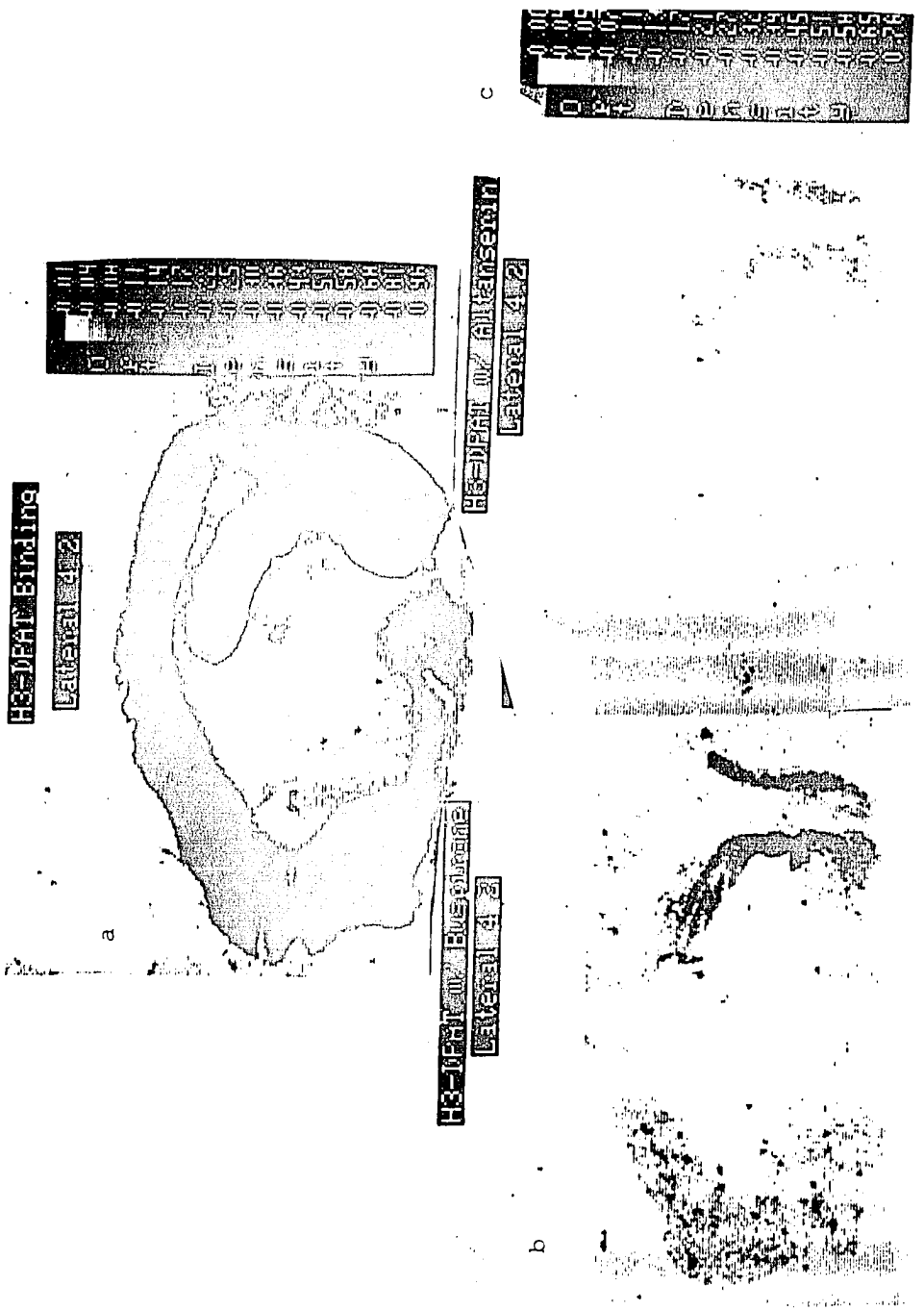

Figure 8. [^{3}H] 8-OH-DPAT Autoradiography in Rat Whole Brain. Lateral 4.2.

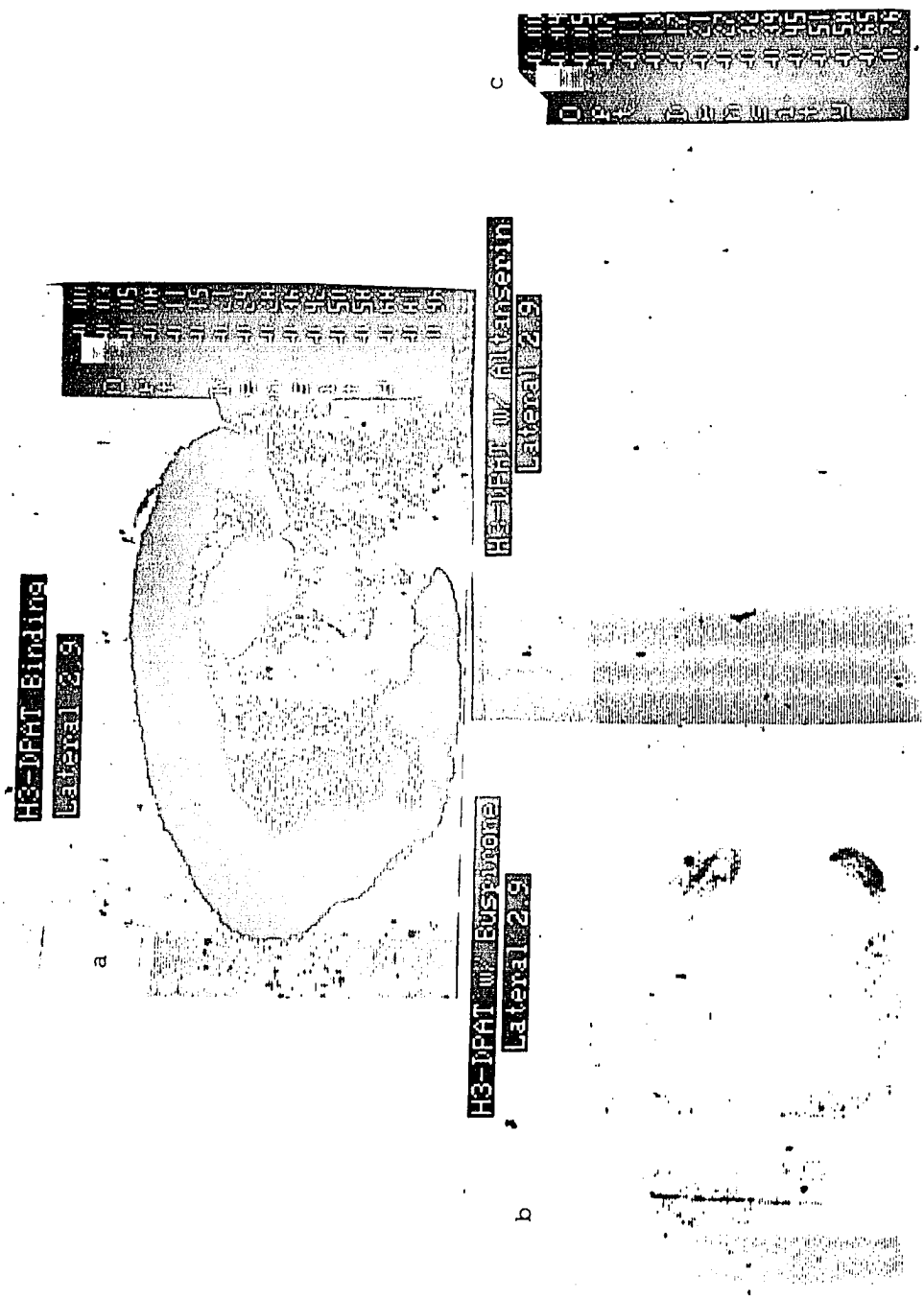

Figure 9. [^{3}H] 8-OH-DPAT Autoradiography in Rat Whole Brain. Lateral 2.9.

CHAPTER V

DISCUSSION

The results of this study demonstrated that [^{3}H]8-OH-DPAT binds to two sites with different affinities in bovine hippocampus. Likewise, a two-site model gave a better fit for rat hippocampus although saturation analysis could not clearly calculate the lower affinity site.

An attempt was made to identify a compound which would bind preferentially to one site over the other. Initially, buspirone and ipsapirone demonstrated a lower affinity for the low affinity (Kd = 3.9 nM) [^{3}H]8-OH-DPAT site with approximately 10 fold selectivity. As a result of these findings, additional compounds were looked at in the absence and presence of buspirone at a sufficient concentration to block the high affinity [^{3}H]8-OH-DPAT (Kd=0.2 nM) site in an attempt to identify a compound which would show a preference for the low affinity (Kd = 3.9 nM) [^{3}H]8-OH-DPAT site. Of the compounds tested, ritanserin appeared to show a preference for the low affinity [^{3}H]8-OH-DPAT site.

The complex inhibition studies confirmed that ipsapirone was working at the same site as that of buspirone and that ritanserin showed a greater affinity for the low affinity [^{3}H]8-OH-DPAT site. However both ipsapirone and

ritanserin demonstrated reasonable affinities for the second [^{3}H]8-OH-DPAT site.

Overall, although a complete characterization of the low affinity [^{3}H]8-OH-DPAT site was not possible due to the lack of more selective compounds, it was demonstrated that this 5HT1A selective ligand, [^{3}H]8-OH-DPAT, clearly binds to two sites with differing affinities in the hippocampus. And it can be theorized that if a more selective compound becomes available a cleaner separation between the anxiolytic effects of 5HT1A selective drugs and the 5HT1A associated side effects (i.e., cardiovascular effects) may be possible.

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